

**Evolution of
Nickel Hyperaccumulation
in *Alyssum* L.**

D.Phil. Thesis

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Abstract

Phylogenetic studies are providing powerful new insights into the evolution of complex traits. Metal hyperaccumulation is an unusual and complex physiological trait found in about 500 plant species and is associated with an exceptionally high degree of tolerance of metalliferous soils. *Alyssum* L. (Brassicaceae) is the largest known hyperaccumulator genus, comprising approximately 188 species distributed throughout the Mediterranean region and south-west Asia. Approximately one-quarter of these are largely restricted to areas of serpentine soils and have the ability to accumulate nickel to high concentrations in shoot tissue. This genus provides a good example in which to study the origins of a complex physiological trait, but its phylogeny is currently poorly understood.

To produce a well-resolved phylogenetic tree to investigate the number and timing of origins of nickel hyperaccumulation within *Alyssum*, DNA sequences were generated for four chloroplast regions (*matK*, *rps16-trnK*, *trnD-T* and *trnL-F*) from 170 of 255 species in the tribe Alysseae. Additional sequencing was carried out for the chloroplast genes *ndhF* and *rbcl* and the nuclear gene *PHYA*. A Bayesian analysis employing a relaxed uncorrelated lognormal molecular clock and multiple fossil-age calibration points was carried out to reconstruct a time-calibrated phylogeny of this tribe using appropriate outgroups.

Optimization of the nickel hyperaccumulation trait onto the resulting phylogenetic tree suggests that nickel hyperaccumulation arose twice in the Alysseae in the late Miocene/early Pliocene: 3.3–8.3 Mya in *Alyssum* and 6.3–8.8 Mya in *Bornmuellera*. The single origin in *Alyssum* is strongly associated with a significant acceleration in net species diversification rate, suggesting the ability to hyperaccumulate nickel could have provided a key evolutionary innovation facilitating rapid range expansion and subsequent species diversification. The scattered distribution of nickel hyperaccumulators across small island-like patches of serpentine soil suggests that allopatric speciation may have driven rapid diversification in this clade.

Chapter 1: Introduction

Evolution of Complex Physiological Traits in Plants

Phylogenetic studies are providing powerful new insights into the evolution of complex adaptive plant traits, and especially physiological traits such as C4 photosynthesis (Sage, 2001; Christin *et al.*, 2008; Edwards & Smith, 2010), crassulacean acid metabolism (CAM) (Crayn *et al.*, 2004; Givnish *et al.*, 2011), nitrogen fixation (Doyle, 2001), cold and frost tolerance (Breeman 2002, Sandve *et al.*, 2008; Donoghue, 2008; Karpinets *et al.*, 2010), succulence (Arakaki *et al.*, 2011), drought tolerance more generally (Pennington *et al.*, 2009), metal hyperaccumulation (Reeves & Baker, 2000; Verbruggen *et al.*, 2009), fire resistance (Simon *et al.*, 2009; Crisp *et al.*, 2011), and tolerance of salinity and anoxia (Ricklefs *et al.*, 2006). These studies can reveal how many times and in which plant lineages such traits evolved, and pinpoint the origins of these evolutionary innovations in time and space. They can also permit quantification of the relative extent of phylogenetic niche conservatism across the plant kingdom, resulting from adaptive barriers posed by environmental variation, especially extreme environments, across the globe. Such recent work has suggested that phylogenetic niche conservatism whereby lineages tend to maintain their ancestral ecologies and to switch geography more easily than ecology (Donoghue, 2008), has had a profound influence on geotemporal patterns of historical species assembly in several of the world's major biomes (Wiens and Donoghue, 2004; Lavin *et al.*, 2004; Schrire *et al.*, 2005; Donoghue, 2008; Crisp *et al.*, 2009; Edwards *et al.*, 2010). However, the extent to which this applies across different biomes and the tradeoffs between adaptive trait evolution and geographic dispersal in shaping and determining the distribution of diversity, remain very poorly understood. To address these questions I have investigated the evolution of one such complex physiological trait - nickel hyperaccumulation in the plant genus *Alyssum* to reveal one of the most startling and striking examples of phylogenetic niche conservatism, complex adaptive trait evolution and rapid dispersal across an archipelago of

serpentine soil islands, prompting extensive parallel geographical isolation and rapid species diversification.

Nickel Hyperaccumulation in Plants

Over 500 plant species have been reported to have the ability to hyperaccumulate metals (van der Ent *et al.*, 2012), to levels as high as 4.4% of above-ground dry biomass for zinc (Reeves and Baker, 2000), 6.7% for nickel (Kazakou *et al.*, 2010), and 1.4% for cadmium (Lombi *et al.*, 2000). Though relatively few in number, hyperaccumulating plants occur in a wide range of families, and are geographically widespread (Reeves & Baker, 2000). Hyperaccumulation of nickel was first reported in 1948 by Minguzzi and Vergnano (cited in Reeves, 1992), who found up to 1.2% nickel by dry mass in leaves of *Alyssum bertolonii* Desv. (Brassicaceae) on nickel-rich serpentine soils in Tuscany, Italy. However, the term '*hyperaccumulator*' was first used by Jaffré *et al.* (1976) in reference to the high concentration of nickel (up to 26% of dry mass) found in the sap of *Sebertia acuminata* Pierre ex Baill. (Sapotaceae), a concentration which led Lee *et al.* (1978) to refer to this sap as '*one of the most remarkable of all biological fluids*'. The term '*hyperaccumulation*' is usually only used with reference to metals (and sometimes also metalloids such as arsenic and selenium) which are phytotoxic at relatively low concentrations, although some of these (such as nickel, zinc and copper) are nevertheless essential plant nutrients.

Why is Hyperaccumulation of Interest?

Metal hyperaccumulation is a complex physiological trait which exhibits a distinct phenotype associated with specific habitats (i.e., metal-rich soils), some of which have been recently produced by humans and some of which are naturally occurring (e.g., serpentine-derived soils), providing a model system for investigating fundamental questions about the ecology and evolution of complex physiological traits in plants more generally. These questions might include whether complex traits of this kind can evolve repeatedly (i.e., in parallel); whether they restrict or enhance the survival or

speciation potential of lineages; and whether the assembly of plant communities in locally novel habitats occurs through the *de-novo* adaptation of species from adjacent but different habitats, as found for the evolution of serpentine endemism in *Halacsya* Dörfl. and *Paramoltkia* Greuter (Boraginaceae) by Cecchi and Selvi (2009), or through the dispersal, potentially over long distances, of species pre-adapted to such conditions. This study uses the evolution of nickel hyperaccumulation in the genus *Alyssum* L. and the tribe Alyseae, to address a series of fundamental questions about the evolution of complex physiological traits and the potential tradeoffs between trait evolution to overcome adaptive barriers, and dispersal to overcome geographic barriers. It also addresses questions relating to the origin of local endemism in plants, in particular that resulting from edaphic specialization. At the heart of this research is the reconstruction of a densely sampled species-level phylogeny, which can be used to investigate these questions, but also wider questions in the evolution, ecology, physiology and genetics of this important plant lineage.

The extreme phenotypes presented by metal hyperaccumulators also provide a means of experimentally investigating fundamental theoretical questions about metal homeostasis in plants. Research to elucidate the physiological and genetic basis of metal hyperaccumulation is needed to enable the manipulation and enhancement of this naturally-occurring trait for practical purposes. There is great interest in using hyperaccumulation for phytoextraction of useful quantities of metals from soils or water, both in phytomining (the extraction of valuable metals from low grade ore using plants), or phytoremediation (the use of plants to remove heavy metal pollution from contaminated soil or water) (Krämer, 2005; Marques *et al.*, 2009; Rascio & Navari-Izzo, 2011).

Apart from a small number of accumulators of zinc and cadmium (e.g., *Noccaea caerulea* (J.Presl & C.Presl) F.K.Mey. (Perronnet *et al.*, 2003)) and nickel and cobalt (e.g., *Alyssum bracteatum* Boiss. & Bushe (Ghaderian *et al.*, 2008)), hyperaccumulators only show

hyperaccumulation and tolerance of one metal, yet ores or contaminated soils often contain high levels of several metals together. Effective phytoextraction crops for these substrates will therefore need be able to hyperaccumulate (or at least tolerate) multiple metals. Hyperaccumulator species often form small plants with low biomass, probably reflecting their ability to grow in the shallow, infertile and free-draining soils that are often associated with metal-rich igneous rock. In order to maximize metal yields, phytoextraction crops will ideally need to couple high biomass with high levels of metal accumulation to be effective and commercially viable (Baker *et al.*, 2000; Li *et al.*, 2003). Phytoextraction of nickel using *Alyssum corsicum* Duby and *A. murale* Waldst. & Kit. has been demonstrated to be economically viable under field conditions, especially with the use of certain soil management techniques (e.g. to increase pH) (Li *et al.*, 2003).

Comparative genetics and physiology, and transcriptome and genome analysis of hyperaccumulator and non-accumulator species are at the forefront of hyperaccumulation research (Hammond *et al.*, 2006; Filatov *et al.* 2006; van de Mortel *et al.*, 2006, Talke *et al.*, 2006; Tuomainen *et al.*, 2010; Shanmugam, 2011). However, the selection of appropriate species for such comparative work requires both thorough documentation of the occurrence of hyperaccumulator species, and knowledge of the evolutionary history of hyperaccumulation. Nickel hyperaccumulation is of particular interest because it is both the most widespread form of hyperaccumulation (Reeves and Baker, 2000; Krämer, 2010), and because it can involve such high concentrations (Kazakou *et al.*, 2010)).

This introduction reviews what is known about the evolution of nickel hyperaccumulation in plants, and particularly in the genus *Alyssum*. It then outlines the specific questions to be addressed in this thesis, and the approach that was taken to investigate them.

Role of Nickel in Plants

Nickel is an essential plant nutrient, although the amount required is very low. Its stoichiometric abundance in plant biomass is joint-lowest (with molybdenum) of all trace elements, and typical plant nickel concentrations are of the order of 1–10 mg kg⁻¹ of dry matter (Marschner, 1995; Yusuf *et al.*, 2011). Nickel is a component of a number of metalloenzymes which are involved in important metabolic processes in bacteria and archaea (e.g., family I glyoxalases, peptide deformylases, methyl-CoM reductase and ureases, and some superoxide dismutases and hydrogenases (Chen *et al.*, 2009)). However, it is generally believed that urease is the only nickel-containing enzyme in plants (Witte, 2011). The required nickel is obtained from soils by plant roots most likely using both passive diffusion and active transport systems. Examples of the former might include uptake into cells via relatively non-selective divalent cation channels (e.g. in competition with Ca²⁺), while the latter may include uptake of Ni²⁺ ions via more specific cation transport systems (Cataldo and Wildung, 1978; Körner *et al.*, 1987). Many different cation transport systems have been identified at the molecular level in plants, but it is still not clear which of them may contribute to nickel uptake (Nishida *et al.*, 2011). There is also the possibility of the uptake of chelated Ni²⁺ via a high-affinity nickel transport protein in plants, as occurs (via the HoxN protein) in bacteria (Degen *et al.*, 1999; Eitinger *et al.*, 2005). Within plants, nickel moves from roots to shoots via the transpiration stream, and by active translocation from older leaves to regions of new growth (Neumann & Chamel, 1986; Page *et al.*, 2006). Within plant cells, nickel is chelated by a number of organic ligands such as nicotianamine, histidine, and organic acids (Schaumlöffel *et al.*, 2003). It is thought that metallochaperone proteins deliver nickel to metalloenzymes (Chen *et al.*, 2009; Yusuf *et al.*, 2011), as has been observed in bacteria (Hausinger, 1997). These specific controls on how nickel is taken up and transported are thought to maintain the concentration of free Ni²⁺ ions within healthy plant cytosol at extremely low levels, probably in the subnanomolar range (Smart *et al.*, 2010).

Nickel Toxicity

Tight control of free nickel ions within living plant tissue is important because this element is critically phytotoxic at relatively low concentrations (typically $>10 \text{ mg kg}^{-1}$ dry mass: Chen *et al.*, 2009; Yusuf *et al.*, 2011). Nickel toxicity symptoms include chlorosis, necrosis, and wilting; reduced germination, growth and crop yields, and damage to photosynthetic apparatus at both the cellular and molecular levels. Two key mechanisms of nickel toxicity have been proposed: (1) interference with the uptake or utilization of other essential metal ions in plants through competition (particularly Fe^{2+} , to which Ni^{2+} has rather similar chemical properties); and (2) induction of oxidative stress through interference with a number of antioxidant metalloenzymes (Pandolfini *et al.*, 1992; Baccouch *et al.* 1998; Schickler and Caspi, 1999; Boominathan and Doran, 2002; Pandey & Sharma, 2002; Ingle *et al.*, 2005b), whereby the proliferation of reactive oxygen species causes direct damage to various parts of living cells, such as membranes and macromolecules.

Mechanisms of Nickel Regulation

Under exposure to increased levels of nickel in the soil, non-hyperaccumulator plants exhibit various regulatory responses, which include nickel exclusion (Baker, 1981). Exclusion from shoot tissues, which due to their high photosynthetic and metabolic activity are likely to be more susceptible to oxidative stress than roots, is achieved through reduced metal mobility within the plant and enhanced storage in the roots (Kachenko *et al.*, 2007). Typically, over 50% of nickel that is taken-up by plants is sequestered in organic ligand complexes in the vacuole or in cell walls within the root system (Cataldo and Wildung, 1978). Metal detoxification is enhanced on exposure to nickel through the increased production of organic ligands (Kim *et al.*, 2005), and plants also exhibit responses which counter the downstream oxidative stress effects of high nickel concentrations, for example by increasing the synthesis of antioxidant enzymes (Ingle *et al.*, 2005b; Gomes *et al.*, 2006).

Nickel Tolerant Plants

When growing in normal soils, which typically have nickel concentrations of 7 to 50 mg kg⁻¹ (Reeves & Adigüzel, 2008), the mechanisms described above for controlling nickel uptake and movement usually allow plants to maintain their nickel concentrations at levels below critical toxicity, typically at 0.05 to 10 mg kg⁻¹ (Chen *et al.*, 2009)). However, some soils, both naturally occurring (typically ultramafic soils derived from serpentine rocks), and anthropogenically polluted ground, have much higher nickel concentrations (typically 700 to 5000 mg kg⁻¹ in serpentine soils (Reeves & Adigüzel, 2008) and up to 26 000 mg kg⁻¹ in polluted soils (McGrath, 1995)). Plants with a high degree of nickel tolerance, often having critical toxicity levels of >50 mg kg⁻¹ (Chen *et al.*, 2009) have evolved to survive on some of these soils with very high nickel contents.

Most nickel-tolerant species are metal excluders, whereby enhanced exclusion mechanisms allow them to maintain moderately elevated tissue nickel concentrations on soils with very high nickel content (Baker, 1981; Pollard *et al.*, 2002). For example, in their study of the serpentine vegetation of Lesbos (Greece), Kazakou *et al.* (2010) found that only three out of 21 plant species tested had nickel concentrations >60 mg kg⁻¹, despite growing on soils with nickel concentrations of 210–3330 mg kg⁻¹. However, a relatively small group of nickel tolerant plants are not excluders. These species actively accumulate nickel in their shoot tissues to extremely high concentrations (for example, >10 000 mg kg⁻¹ in a number of species of *Alyssum*: Brooks *et al.*, 1979), i.e., concentrations far in excess of critical toxicity levels for most plants.

Defining Nickel Hyperaccumulation

Of the *ca.* 500 plant species which hyperaccumulate heavy metals (van der Ent, 2012), nickel hyperaccumulators are by far the most numerous (*c.* 368 species; Burge and Barker, 2010). Other metals for which hyperaccumulation has been reported include lead, zinc, cadmium, copper, cobalt and chromium, as well as the metalloids selenium, arsenic, and thallium, although van der Ent *et al.*

(2012) suggested that some reports for lead and copper may be the result of surface contamination rather than hyperaccumulation *per se*. The prevalence of nickel hyperaccumulation is thought to reflect the relatively large area of the Earth's surface made up of nickel-enriched soils compared with soils enriched in other minerals (Reeves & Baker 2000; van der Ent *et al.*, 2012).

Brooks *et al.* (1977) first defined the threshold for nickel hyperaccumulation as 1000 mg kg^{-1} of above-ground dry mass, on the grounds that this value is 10 to 100 times higher than the level usually found in plants growing on ultramafic soils, and 100 to 1000 times higher than that found in plants growing on non-ultramafic soils. For at least some groups of plants, such as the genus *Alyssum*, it is clear that the spectrum of nickel concentrations in plants growing in their natural habitats is not normally distributed; in this instance, the distribution of average nickel contents of species is strongly bimodal (Fig. 1-1; Pollard *et al.*, 2002), with a minimum between the peaks of hyperaccumulators and non-accumulators in the region of 1000 mg kg^{-1} (i.e. 0.1% (w/w)). Reeves (1992) refined the definition of nickel hyperaccumulation to 'a plant in which a nickel concentration of at least 1000 mg kg^{-1} has been recorded in the dry matter of any above ground tissue in at least one specimen growing in its natural habitat', and this cutoff has subsequently been widely adopted. Although very useful for herbarium and field-based studies, a definition based on growth in natural habitats means that such measures of plant nickel content are likely to be confounded to some extent with variation in soil nickel content. Laboratory studies show a clear relationship between nickel exposure and plant nickel content in hyperaccumulators (e.g., Krämer *et al.*, 1996; Ingle *et al.*, 2005), thus, there may well be a strong environmental component underlying the bimodal distribution in Fig. 1-1. Van der Ent *et al.* (2012) reviewed the logic behind Reeves's definition and considered a number of alternative measures of hyperaccumulation such as shoot/root nickel ratio and bioconcentration factor, including measurements from plants grown under controlled experimental conditions of known soil nickel contents. They concluded that the 1000 mg kg^{-1} threshold represents a useful criterion, and that it is meaningful to compare healthy

plants growing in presumably viable populations in their respective natural habitats, even though these may differ in various ways. Given the lack of detailed analyses under controlled conditions for more than a few species, field measurements currently provide a pragmatic way to determine hyperaccumulation status.

[Image removed for copyright reasons.]

Figure 1-1. Bimodal distribution of nickel hyperaccumulation in the genus *Alyssum*. Histogram of average dry shoot nickel concentrations in 170 taxa of *Alyssum* collected in their natural habitats (from Pollard *et al.* (2002)). These data support the threshold of 0.1% (w/w) nickel proposed by Brooks *et al.* (1977) for hyperaccumulators in this genus.

Physiological Basis of Nickel Hyperaccumulation

In addition to their high absolute metal contents, an important characteristic of hyperaccumulator plants is their partitioning of a large component of their metal content into the above-ground parts, rather than (as tends to be the case in non-accumulators) its immobilization in the roots (Krämer *et al.*, 1996; Pollard *et al.*, 2002). This is thought to be achieved through a number of physiological alterations in hyperaccumulators, including increased metal uptake rates in the roots, reduced sequestration of metals in root vacuoles, enhanced rate of loading of metals into the transpiration stream in the roots, high mobility and efficient transport of metals within the shoot

tissue (Ingle *et al.*, 2008), and increased metal detoxification (*via* organic ligands) and (mostly vacuolar) sequestration in the leaves (Pollard *et al.*, 2002; Verbruggen *et al.*, 2009; Krämer, 2010). For example, some nickel hyperaccumulators show increased levels of the free amino acid histidine when exposed to nickel (Krämer *et al.*, 1996) and this molecule has been shown to be important for loading of nickel into the transpiration stream of *Alyssum lesbiacum* (Candargy) Rech.f. (Ingle *et al.*, 2005), and for reducing net transport of nickel into root vacuoles (Richau *et al.*, 2009).

Genetic Basis of Hyperaccumulation

Genetic mechanisms of nickel hyperaccumulation are not yet completely understood, but the phenotype seems unlikely to result from a small number of hyperaccumulator-specific genes or mutations. Though QTL-based studies have identified genes with major effects on zinc hypertolerance and uptake, such as *AhHMA4* (which encodes a heavy metal pump ATPase) (Courbot *et al.*, 2007; Willems *et al.*, 2007) the large number and wide range of physiological processes involved in hyperaccumulation suggest a multiplicity of controlling genetic factors. It therefore seems likely that hyperaccumulation results from the differential expression of a large number of genes (Macnair *et al.*, 2000; Verbruggen *et al.*, 2009; Rascio & Navari-Izzo, 2011). Transcriptome-based comparisons of the zinc hyperaccumulator *Arabidopsis halleri* (L.) O’Kane & Al-Shehbaz with the non-accumulator plant *A. thaliana* L. (Heynh.) using DNA microarrays have shown that a large number of genes are associated with hyperaccumulation of this metal (Becher *et al.*, 2004; Weber *et al.*, 2004; Filatov *et al.*, 2006). Unlike for nickel, a number of the molecular mechanisms involved in zinc hyperaccumulation are relatively well understood. For example, the gene *Heavy Metal ATPase 4* (*HMA4*, encoding a cellular metal export pump) affects various parts of the metal homeostasis network in *A. halleri*, and is required for cadmium and zinc tolerance and for zinc hyperaccumulation in this species (Hanikenne *et al.*, 2008), in which multiple copies of this gene can be found. Comparative genomics is likely to offer further important insight into the genetics of metal hyperaccumulation, particularly in species of the Brassicaceae, due to the wealth

of genomic information available for model species such as *Arabidopsis thaliana* and the hyperaccumulators *A. halleri* and *Noccaea caerulea* (J.Presl & C.Presl) F.K.Mey. (Krämer, 2010).

Because it involves numerous physiological processes and biochemical pathways, and is likely to be controlled by multiple genetic factors, nickel hyperaccumulation can be described as a complex physiological trait. This term can be applied to any distinctive plant phenotype that involves multiple diverse biochemical, genetic and developmental pathways, including C4 and CAM photosynthesis, succulence, nitrogen fixation, and tolerance of salinity, submergence, drought, fire and frost. The evolution of such traits has been investigated in some detail, including from a phylogenetic perspective (e.g., Doyle, 2001; Breeman 2002; Sage, 2001; Crayn *et al.*, 2004; Ricklefs *et al.*, 2006; Silvera *et al.*, 2009; Christin *et al.*, 2008; Donoghue, 2008; Sandve *et al.*, 2008; Simon *et al.*, 2009; Pennington *et al.*, 2009; Edwards & Smith, 2010; Karpinets *et al.*, 2010; Arakaki *et al.*, 2011; Crisp *et al.*, 2011; Givnish *et al.*, 2011). Such work can reveal the number of separate evolutionary origins of these traits, their relative ease of gain and loss, why they originated in some lineages and not others, and why they originated at particular times in Earth's history.

Such traits provide potentially useful model systems in which to also address biogeographic questions about niche filling in specialized habitats. For example, do complex adaptations to such habitats tend to evolve from local lineages *in situ*, or are lineages in which such adaptations are already present able to disperse widely and colonize specialized habitats from afar (Donoghue, 2008)? There is also growing evidence to suggest that the adaptive barriers that underlie the evolution of these complex physiological traits are reflected in larger-scale patterns of phylogenetic niche conservatism that have played a significant role in shaping broad-scale geotemporal patterns of diversification and the historical assembly of species diversity across the major biomes. The consequences of the evolution of complex physiological traits for the subsequent evolution and diversification of lineages can be far-reaching and profound. For example, apparently as a result of

the evolution of succulence in the Miocene in response to climate aridification, the Cactaceae now plays a major role in the New World desert biome (Arakaki *et al.*, 2011). Similarly the rise of extensive global C₄-dominated grasslands, also during the Miocene aridification, has profoundly shaped the face of global terrestrial vegetation (Christin *et al.*, 2008).

Succulence, salinity and frost tolerance are complex and variable syndromes of traits which are difficult to compare and equate between lineages. However, some complex physiological traits, such as C₄ and CAM photosynthesis, have a distinct and readily identifiable phenotype. Nickel hyperaccumulation is just such a trait. Although its underlying biochemistry and genetics may be complex, it has a simple and easily measured phenotype: extremely elevated nickel levels in shoot tissue. This trait is thus much more easily investigated than a syndrome such as serpentine or metal tolerance. Whilst the latter two can be identified and quantified only through extensive growth trials, and may result from various different underlying adaptations, nickel hyperaccumulation is a distinctive trait that can be easily measured in field (e.g., using dimethylglyoxime test papers), or in the laboratory (e.g., using atomic adsorption spectrophotometry). The high sensitivity of the latter method even allows reliable nickel measurements to be made from herbarium specimens that are several decades old (e.g., Brooks *et al.*, 1977, 1978, 1979).

Adaptive Value of Hyperaccumulation

A well-supported explanation of the adaptive value of metal hyperaccumulation is a key requirement for understanding the evolution of such a distinctive trait. The possible adaptive significance of metal hyperaccumulation has been much debated but remains controversial. Although nickel hyperaccumulation is generally considered to be an adaptation to serpentine soils (Boyd & Martens, 1998), a number of different mechanisms by which hyperaccumulation may increase fitness have been proposed. These include: (i) metal tolerance, e.g., through sequestration of metal within the vacuole, where it is less likely to have toxic effects than in the cytoplasm

(Krämer *et al.*, 1996); (ii) plant defence, e.g., through protection from herbivory or disease through toxic effects of the metal on herbivores or pathogens (Boyd and Martens, 1992); (iii) drought tolerance, e.g., through a decrease in plant water potential, and hence water loss, caused by accumulation of metal ions (Bhatia *et al.*, 2005); (iv) allelopathy, e.g., through the build-up of metal-rich leaf litter around perennial plants, reducing competition from less metal-tolerant plant species (Boyd and Martens, 1992; Boyd, 2007); and (v) protection against ultraviolet radiation, e.g., through the absorptive properties of nickel ions (de la Fuente *et al.*, 2007). Of these, the elemental defence hypothesis has perhaps the greatest level of empirical support (Boyd, 2007). For example, Ghaderian *et al.* (2000) found that increasing metal concentrations gave increased protection (against the fungus *Pythium*) to several hyperaccumulator species in *Alyssum*. Fones *et al.* (2011), in their studies with *Noccaea caerulescens*, showed that hyperaccumulation of nickel, as well as zinc or cadmium, could protect the plant from phytopathogenic bacteria and fungi. There are other reasons to suspect that nickel hyperaccumulation is more than merely a mechanism of metal or serpentine soil tolerance, not least the fact that tolerance of serpentine soils does not necessarily involve nickel hyperaccumulation, given that many serpentine tolerant and serpentine endemic species that do not hyperaccumulate this or any other metal.

Taxonomic Distribution of Nickel Hyperaccumulation

Except for one species of fern (*Azolla filiculoides* Lam.; Arora and Sood, 2004), as far as is known, nickel hyperaccumulation is restricted to the angiosperms. The most recent detailed review of the taxonomic distribution of nickel hyperaccumulators at the family level gave four monocots (from four families) and 363 Eudicots (from 39 families) (Burge and Barker, 2010). In this enumeration, the largest number of nickel hyperaccumulator species are in families Euphorbiaceae (83 species), Brassicaceae (87) and Asteraceae (46). Of the remaining families, three have between 16 and 19 species, 15 have between two and eight species, and 23 contain only one nickel hyperaccumulator

species. Reeves and Baker (2000) observed that (with the significant exception of *Alyssum*), many hyperaccumulator species are the only such species in their genera.

Such taxonomic patterns led both Pollard *et al.* (2002) and Verbruggen *et al.* (2009) to conclude that hyperaccumulation has probably had multiple evolutionary origins at the family level. The sporadic occurrence of nickel hyperaccumulation across disparate, unrelated families of flowering plants clearly points more parsimoniously to multiple independent evolutionary origins rather than a single gain and numerous losses at various times. More specifically, but based on the same criterion of parsimony, Krämer (2010) suggested that nickel hyperaccumulation evolved independently at least six times in the Brassicaceae. An up-to-date list of nickel hyperaccumulator species in the Brassicaceae is presented in Table 1-1.

Table 1-1. Numbers of nickel hyperaccumulator species and total species in each of the eight genera within Brassicaceae which contain nickel hyperaccumulators.

Genus (Tribe)	Ni hyperaccumulator species	Total Species
<i>Aethionema</i> W.T.Aiton (Aethionemeae)	1 ¹	c.55 ⁷
<i>Alyssum</i> L. (Alysseae)	51 ^{2*}	188 ⁵
<i>Bornmuellera</i> Hausskn. (Alysseae)	4 ²	7 ⁵
<i>Cardamine</i> L. (Cardaminae)	1 ³	c.200 ⁷
<i>Cochlearia</i> L. (Cochlearieae)	2 ⁴	16 ⁷
<i>Leptoplax</i> O.E.Schulz (Alysseae)	1 ²	1 ⁵
<i>Noccaea</i> Moench (Noccaeae)	16 ⁴	85 ⁷
<i>Streptanthus</i> Nutt. (Schizopetaleae)	1 ⁶	34 ⁷
Total	77	586
Sources: ¹ R. D. Reeves <i>et al.</i> , 2001; ² Appendix 1; ³ Vergnano <i>et al.</i> , 1982 Verger, 1992; ⁴ Reeves, 1988; ⁵ Appendix 3; ⁶ Reeves <i>et al.</i> , 1981; ⁷ Mabberley, 2008. * The 51 hyperaccumulators in <i>Alyssum</i> include 17 species known to be polymorphic for this trait.		

Despite being generally rare across the angiosperms as a whole, the presence of multiple origins of nickel hyperaccumulation within Brassicaceae and other families, and numerous nickel hyperaccumulators in the genus *Alyssum* itself led Broadly (2001), Burge and Barker (2010) and Cecchi *et al.* (2010) to speculate that the presence of some (as yet unknown) underlying genetic or

physiological architecture could make these taxa pre-adapted to nickel hyperaccumulation. In this case, the evolutionary changes required for the full phenotypic expression of nickel hyperaccumulation could possibly be relatively minor. Other complex physiological traits appear to show such deep homology (Shubin *et al.*, 2009); for example, nitrogen fixation, which involves a complex arrangement of root nodulation and symbioses between plants and nitrogen-fixing bacteria, shows multiple independent evolutionary origins located deep in the angiosperm phylogeny. However, these lineages are themselves closely related and the evolution of the trait involved the recruitment of some of the same genetic components (and perhaps even entire plant – bacteria signaling pathways from ancient symbioses) (Soltis *et al.*, 1995; Doyle, 2011). Models of character evolution that might be used to locate such evolutionary precursors that precede convergent evolution have only recently been developed (e.g., Marazzi *et al.*, 2012), and might provide a useful avenue for exploring the evolution of metal hyperaccumulation across the angiosperms as a whole.

***Alyssum*: a Hyperaccumulator-rich Genus**

Of the 77 nickel-hyperaccumulator taxa present within the family Brassicaceae, a large number, 51, are present within *Alyssum* L. (Table 1-1). This is a genus of 188 species distributed across the Mediterranean and south-west Asian region, with a particularly high number of species in Turkey (94; Davis 1965–1988, 1988; Güner *et al.*, 2000; Özhatay & Kültür, 2006; Özhatay *et al.*, 2009, 2011; Appendix 1). Many of the nickel hyperaccumulator species have very narrowly restricted endemic serpentine distributions (Reeves and Baker, 2000). Nickel Hyperaccumulators are present in seven other genera of Brassicaceae (Table 1-1), and of these, both *Bornmuellera* Hausskn. and *Leptoplax* O.E.Schultz are in the same tribe as *Alyssum*, the Alysseae.

Extensive surveys of nickel content in the genus *Alyssum* and other genera in the Alysseae were carried out over the period 1978–1983 (Brooks & Radford, 1978; Brooks *et al.*, 1979; Reeves *et al.*

1983), and a smaller number of species have been tested more recently (e.g., R. D. Reeves *et al.*, 2001; Reeves *et al.*, 2009; see Appendix 3 for a full list of references). With 51 of its 188 species (i.e., 27%) able to hyperaccumulate nickel, the genus *Alyssum* contains more hyperaccumulator species than any other genus of plants (Reeves & Baker, 2000; Burge and Barker, 2010). As described above, the distribution of average nickel concentrations of species in this genus is strongly bimodal (Fig. 1-1). However, some species appear to be polymorphic for this trait, having both hyperaccumulating and non-accumulating individuals or populations (*A. anatolicum* (R. D. Reeves *et al.*, 2001) the *A. serpyllifolium* species complex (Brooks *et al.*, 1981), *A. sibiricum* (Mengoni *et al.*, 2003), *A. murale* (Cecchi *et al.*, 2010) and *A. peltarioides* (Reeves *et al.*, 1983)). Ingle *et al.* (2005) demonstrated clear genetic differences in nickel hyperaccumulation between taxa in the *A. serpyllifolium* complex. However, for the other species such work has not yet been carried out, and the extent to which the variability in their nickel content is due to genetic or to environmental factors is unclear. Furthermore, the relatively small numbers of individual plants that have been tested for nickel hyperaccumulation in the majority of *Alyssum* species (e.g. Brooks *et al.*, 1979) suggests that polymorphism in this trait is likely to have been underestimated.

Brooks *et al.* (1979) made the interesting observation that nickel hyperaccumulation in *Alyssum* is almost entirely restricted to section *Odontarrhena* (C.A.Mey.). (The exception, *Alyssum lenense* Adams (sampled by Brooks and Radford (1978) under the synonym *A. fischerianum* DC.), is in section *Alyssum* (Griseb.) (Dudley, 1964a).) These authors also noted that 70% of species in section *Odontarrhena* are hyperaccumulators and made the intriguing and somewhat cryptic statement that there is a “definite relation between species diversity, proliferation and endemism, on the one hand, and extraordinarily high concentrations of nickel on the other”. Despite a considerable number of subsequent descriptive accounts of the geographic distribution of nickel hyperaccumulators in *Alyssum* and related genera, and their close associations with areas of serpentine soils (e.g., Brooks *et al.* 1981; Reeves & Adigüzel, 2004; 2008; Ghaderian *et al.* 2007a;

Reeves *et al.*, 2009), only two studies (Mengoni *et al.*, 2003; Cecchi *et al.*, 2010) have attempted to trace the evolution of hyperaccumulation across the tribe, and there have been no attempts to examine formally the biogeographic history of *Alyssum*, nor of the nickel hyperaccumulation trait. Questions such as how many times, when, where and under what circumstances nickel hyperaccumulation has evolved within the Alyseae have not yet been fully resolved.

The observations of Brooks *et al.* (1979) predate molecular phylogenetic approaches, molecular clocks, and lineage diversification birth–death models (e.g. Rabosky, 2007), which now provide more powerful methods to investigate the evolutionary origins of nickel hyperaccumulation. However, given the uncertainty about relationships within the Alyseae, including doubt about the monophyly of *Alyssum* (discussed in detail below), and the considerable historical and recent flux in the taxonomy of the group, it is essential that new phylogenetic studies sample appropriately across the tribe Alyseae as a whole, rather than focusing on only the genus *Alyssum* itself; this is especially important given the presence of nickel hyperaccumulators in two other genera in the tribe.

Taxonomy of *Alyssum* and Alyseae

The genus *Alyssum* is based on the type species *A. montanum* L. of Linnaeus (1753). Many species that were subsequently described have later been moved to other more recently described genera, many of which are members of the tribe Alyseae. The tribe Alyseae was first described by Schulz (1936), and was later expanded by Dudley and Cullen (1965). In the most recent published delimitation (hereafter referred to as Alyseae *s.l.*), the Alyseae is taken to include the following 17 genera: *Alyssoides* Tourn. ex Adans., *Alyssum*, *Asperuginoides* Rauschert, *Aurinia* Desv., *Berteroa* DC., *Bornmuellera*, *Clastopus* Bunge ex Boiss., *Clypeola* L., *Degenia* Hayek, *Didymophysa* Boiss., *Farsetia* Turra, *Fibigia* Medik., *Galitzkya* V.V.Botschantz, *Hormathophylla* Cullen & T.R.Dudley, *Lobularia* Desv., *Physoptychis* Boiss. and *Straussiella* Hausskn. (Al-Shehbaz *et al.*, 2006). However, a

number of recent phylogenetic studies (discussed more fully below) have shed light on the relationships between these genera, and led to the exclusion of some of them from the Alysseae and the inclusion of *Leptoplax* (Cecchi *et al.*, 2010) and *Physocardamum* (Warwick *et al.*, 2010). Despite extensive sampling within Alysseae *s.l.*, of all the genera listed in this paragraph, nickel hyperaccumulators have only been reported from *Alyssum*, *Bornmuellera* and *Leptoplax* (Table 1-1).

Dudley's (1964a) synopsis of the genus *Alyssum* recognized six sections (*Alyssum* (Griseb.) T.R.Dudley, *Meniocus* (Desv.) Hook., *Psilonema* (C.A.Mey.) A.P.Iljinsk., *Odontarrhena*, *Tetradenia* (Spach) T.R.Dudley and *Gamosepalum* (Griseb.) T.R.Dudley), some of which are subdivided into subsections and series. Species from sect. *Tetradenia* are now regarded as species within *Hormathophylla* (Dudley and Cullen 1965). The taxonomy of Alysseae is reviewed in detail in Appendix 1, which includes a list of the 255 species of the tribe that are accepted in this study.

The extent of natural hybridization in the Alysseae appears to be limited (possible examples include *A. chlorocarpum* Hausskn. × *A. heldreichii* Hausskn. (Strid & Tan, 2002), and *A. montanum* subsp. *pluscanescens* (Raim. ex Jos.Baumgartner) Trpin). However, extensive surveys of chromosome numbers have been carried out for species in the Brassicaceae (Warwick & Al-Shehbaz, 2006), revealing considerable variation within the Alysseae (e.g. $2n = 8, 14, 16, 24, 28, 30, 32, 48, 64$ in *Alyssum s.str.* (see *Phylogeny of Alysseae* below)), and therefore considerable scope for polyploidization and possibly also hybridisation events in the evolutionary history of the group. Although little detailed evolutionary analysis of this variation has been carried out, AFLP-based work by Španiel *et al.* (2011) on the *A. montanum* species complex of central-southern Europe indicates that both diploids and tetraploids are present within closely-related taxa.

Phylogeny of Alysseae

Thirteen phylogenetic studies (based on nuclear, chloroplast and mitochondrial markers) have included species from Alysseae (Table 1-2). Five included >20 Alysseae species (Mengoni *et al.* 2003; Warwick *et al.*, 2008; Khosravi *et al.*, 2009; Cecchi *et al.*, 2010; Cecchi 2011) and all of these were based on the 5.8S unit and flanking internal transcribed spacers *ITS1* and *ITS2* of nuclear ribosomal DNA, (hereafter referred to as *ITS*).

Mengoni *et al.* (2003)

Mengoni *et al.* (2003) explicitly investigated the evolution of nickel hyperaccumulation within *Alyssum*. However, this study has several important limitations in terms of taxon sampling, analytical methods and data quality. Only 28 *Alyssum* species were sampled, 22 of which were nickel hyperaccumulators, and the phylogeny is thus seriously under-sampled, particularly for non-accumulator species. A single outgroup, *Iberis umbellata* Georgi (Brassicaceae) was used to root the tree, thereby forfeiting the opportunity to test the monophyly of *Alyssum*. This study generated neighbour-joining, parsimony, and maximum likelihood trees and claimed that these trees showed the presence of four well supported 'groups' within *Alyssum*, and highlighted the fact that each of these groups contained both nickel hyperaccumulator and non-hyperaccumulator species. On this basis, Mengoni *et al.* (2003) suggested that 'nickel hyperaccumulation could have been lost or acquired independently more than once in *Alyssum*'. However, no formal character optimization was carried out, the groups (clades) were not consistent between trees derived from the same data using different tree search methods, and the high support values obtained in the parsimony tree could not be replicated in subsequent re-analyses of the same dataset (C.D. Bailey, unpubl. data, and Appendix 5). Furthermore, there is evidence to suggest that some *ITS* sequences included in the Mengoni *et al.* (2003) alignment represent potentially non-functional pseudogene copy types, and that for these accessions no functional copy types were recovered (Appendix 5), further

undermining the confidence in this analysis. For all the above reasons, the results of the Mengoni *et al.* (2003) study cannot be regarded as robust, and the dataset was therefore re-analyzed in some detail in the present work in Appendix 5.

Table 1-2. Summary of phylogenetic studies which have sampled species from *Alyssum* and Alyseae.

Scope of Study	Locus	No. of <i>Alyssum</i> species included	Total Alysseae <i>s.l.</i> species sampled	Reference
<i>Alyssum</i>	<i>ITS</i>	28	29*	Mengoni <i>et al.</i> (2003)
Alysseae		18	53*	Warwick <i>et al.</i> (2008)
		26	39*	Cecchi <i>et al.</i> (2010)
		6	29*	Cecchi (2011)
Brassicaceae		1	1	Heenan <i>et al.</i> (2002)
		0	2	Hong <i>et al.</i> (2003)
		1	3	Bailey <i>et al.</i> (2006)
		20	33*	Khosravi <i>et al.</i> (2009)
		6	17	German <i>et al.</i> (2009)
		<i>ndhF</i> & <i>PHYA</i>	2	5
	0		3	Beilstein <i>et al.</i> (2010)
	<i>matK</i> & <i>Chs</i>	3	6	Koch <i>et al.</i> (2001)
	<i>Nad4</i>	1	2	Franzke <i>et al.</i> (2009)
	*Indicates studies which included >20 Alysseae species and are the focus of discussion in the main text.			

Warwick *et al.* (2008)

Warwick *et al.* (2008) sampled 18 *Alyssum* species and 35 further Alyseae species which represented all other genera of Alyseae *s.l.* except *Straussiella*. An additional 26 outgroup taxa from other Brassicaceae tribes were used to test the monophyly of Alyseae. Resulting parsimony and maximum likelihood trees had similar topologies. The strict consensus parsimony tree is reproduced here as Figure 1-2. With the exception of *Alyssum klimesii* Al-Shehbaz (now regarded as a synonym of *Ladakiella klimesii* (Al-Shehbaz) D.A.German & Al-Shehbaz), all species of *Alyssoides*, *Alyssum*, *Aurinia*, *Clypeola*, *Fibigia*, *Degenia*, *Physoptychis*, *Clastopus*, *Hormathophylla*, *Bornmuellera*, *Berteroa*, *Galitzkya* and *Aurinia* formed a robustly supported monophyletic group; i.e., an Alyseae clade, referred to hereafter as Alyseae *s.str.* *Ladakiella klimesii* accessions formed

a monophyletic group within tribe Camelinae. Accessions from *Asperuginoides*, *Didymophysa*, *Farsetia*, and *Lobularia* were placed outside the Alysseae s.str. clade.

In the following descriptions, and throughout this thesis, branch support is referred to as follows: strong: bootstrap support (bs) values of 90 % or above or posterior probabilities (pp) of 0.95 or above; moderate: bs of 70–89 % or pp of 0.90–0.95; low: bs of 50–69 % or pp of 0.80–0.89. Branches with bootstrap values below 50 % or posterior probabilities below 0.80 are considered unsupported. Within the Alysseae s.str., Warwick *et al.* identified six clades labelled A1–A6. Of these, Clade A1 (which contained accessions from *Alyssum* sect. *Alyssum*, sect. *Meniocus*, and *Clypeola*) received low branch support; Clade A2: (*Alyssum* sect. *Alyssum*, sect. *Gamosepalum* and sect. *Psilonema*) received moderate support; the remainder received strong support and contained the following accessions: Clade A3: *Alyssoides*, *Clastopus*, *Degenia*, *Fibigia* and *Physoptychis*; Clade A4: *Hormathophylla*; Clade A5: *Bornmuellera*; and Clade A6: *Aurinia*, *Berteroa* and *Galitzkyia*. However, branches subtending relationships between these clades had low support. Within the A1 Clade, *Clypeola* was monophyletic and nested within *Alyssum*, prompting Warwick *et al.* (2008) to suggest that these two genera should be united. However, the branch support for the clade combining these elements was low.

Khosravi *et al.* (2009)

ITS sequences were also generated by Khosravi *et al.* (2009) for 57 species of mainly Iranian Brassicaceae, including eight previously unsequenced species from *Asperuginoides*, *Clastopus*, *Clypeola*, *Farsetia*, *Fibigia* and *Physoptychis*. Their analysis also included sequences of 100 species from GenBank, including sequences generated by both Mengoni *et al.* (2003) and Warwick *et al.* (2008). Parsimony and Bayesian analyses were carried out (the tree generated in the latter is reproduced here in Figure 1-3), recovering the same monophyletic Alysseae s.str. clade as Warwick

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Figure 1-2. Generic relationships of Alyseae and relationships of some other Brassicaceae tribes. Strict consensus of 1400 most parsimonious trees generated from 131 *ITS* sequences for 85 taxa, presented by Warwick *et al.* (2008). Tree length 2071 steps (332 parsimony informative characters); CI: 0.322; RI: 0.740; bootstrap values (where >50%) are noted above branches. Numbers in brackets refer to accession numbers where multiple accessions of a species were included. Clades are indicated at the right: A1-A5 from Warwick *et al.* (2008); Alyseae; Anchonieae (Anch); Descurainieae (Desc); Physarieae (Phys); Camelinaeae (Caml); Malcolmieae (Malc); Iberideae (Iber); Sisymbrieae (Sisy); Arabideae (Arab); Cochlearieae (Coch); outgroup (OG).

et al. (2008) (albeit with the recognition of the Iranian endemic genus *Irania* (the five species of which are here considered members of *Fibigia*; Appendix 1). The Alysseae *s.str.* clade received strong support. The single species of *Farsetia*, *Lobularia* and *Asperuginoides* included in the analysis were placed outside the Alysseae *s. str.* clade, nested within other Brassicaceae tribes.

Where they receive moderate or high branch support, the results of Khosravi *et al.* (2009) are consistent with Warwick *et al.* (2008), and add further information on the relationships between the clades within Alysseae *s.str.* Of the six Alysseae sub-clades identified by Warwick *et al.* (2008), four were present in the Khosravi *et al.* (2009) tree (A1, A2, A3 and A6) with strong support, but the A4 (*Hormathophylla*) and A5 (*Bornmuellera*) clades of Warwick *et al.* (2008) were not sampled in their study. In contrast to Warwick *et al.* (2008), some of the relationships between these clades were strongly supported. Clades A1 and A2 were sister with strong support. *Alyssum s.l.* is therefore monophyletic on this tree. Together, Clades A1, A2, A3 and A5 are monophyletic, albeit lacking support, making the position of Clade A6 as the most basally divergent clade within the Alysseae uncertain. The Alysseae *s.str.* clade was sister to a clade containing Chorisporaeae and Hesperideae species with moderate support (although only 18 of the 25 Brassicaceae tribes of Al-Shehbaz *et al.* (2006) were represented in this study).

Figure 1-3. Relationships of Brassicaceae tribes. 50% majority rule Bayesian consensus tree with posterior probability values (Khosravi *et al.*, 2009). Length: 3355 steps; CI: 0.325; HI: 0.675; RC: 0.226. Tribal concepts are those of Al-Shehbaz *et al.* (2006), and Alysseae sub-clades from Warwick *et al.* (2008) have been added where appropriate; note that taxa from clades A4 and A5 are not represented.

[Image removed for copyright reasons.]

Cecchi *et al.* (2010)

Cecchi *et al.* (2010) explicitly investigated the evolution of nickel hyperaccumulation in European Alyseae. Their study included 26 *Alyssum* species, nine of which were nickel hyperaccumulators, so sampling of the genus was even lower than in the Mengoni *et al.* (2003) study. None of the species from Turkey was sampled, despite the fact that there are more *Alyssum* species there than in any other country, and only four non-European *Alyssum* species were sampled. As in Warwick *et al.* (2008), representatives from all the other genera of Alyseae *s.l.* were sampled, except for *Straussiella*. The study also included the hyperaccumulator *Leptoplax emarginata* (Boiss.) O.E.Schulz (Brassicaceae), previously of uncertain affinity.

The resulting parsimony and Bayesian trees obtained by Cecchi *et al.* (2010) (the latter of which is reproduced here as Figure 1-4) gave strong support to clades A1, A3, A5, and A6 of Warwick *et al.* (2008), the Alyseae *s.str.* and Alyseae *s.l.* clades. The monophyly of clades A2 and A4 was not tested because these were each represented by only one accession. The single A2 accession (*Alyssum montanum*) was sister to Clade A1 with strong support. *Leptoplax emarginata* was sister to the three *Bornmuellera* species sampled, with strong support, and therefore is considered a member of Clade A5 here. Relationships between the other Alyseae clades were poorly supported, and there was very little resolution within Clade A1. Nevertheless, the authors identified three clades within the latter clade, each containing both hyperaccumulator and non-accumulator species. This, they concluded, provides evidence for ‘multiple origins or losses’ of hyperaccumulation in *Alyssum*, and in their abstract, they were more explicit, stating that hyperaccumulation had ‘multiple origins even within the three European clades recognized’.

However, no formal optimization of hyperaccumulation was carried out, and two of the three clades that Cecchi *et al.* (2010) identified receive poor support (posterior probabilities of 0.84–0.85). No justification was given for the inclusion of only European species, or any qualifications

regarding how the sparse sampling of *Alyssum* species might have influenced the results. The authors also identified a single origin of nickel hyperaccumulation in the *Bornmuellera* + *Leptoplax* clade, and this part of their tree received strong support; however, neither of the 2 non-accumulator species (Reeves *et al.*, 1983), nor the single untested species from *Bornmuellera* (*B. dieckii* Degen) were sampled.

Cecchi (2011)

In order to investigate the relationships of the two species in the genus *Phyllolepidum* Trinajstić (a genus which Cecchi (2011) resurrected from synonymy within *Aurinia*), Cecchi (2011) produced a further *ITS*-based phylogeny that included accessions of six *Alyssum* species and a further 23 species from 11 other Alysseae *s.str.* genera. Not surprisingly, given that these analyses were based largely on the same data, where clades were present with moderate or strong support, these were consistent with the results of Cecchi *et al.* (2010). Relationships between clades were unclear due to a large basal polytomy. The two *Phyllolepidum* accessions formed a clade sister to *Bornmuellera* + *Leptoplax emarginatum*, and not close to *Aurinia*, providing the key evidence for resurrecting *Phyllolepidum* as a distinct genus.

Other Studies

Phylogenetic studies of the Brassicaceae as a whole by Koch *et al.* (2001), Bailey *et al.* (2006), Beilstein *et al.* (2006), Beilstein *et al.* (2008), Franzke *et al.* (2009), German *et al.* (2009), Beilstein *et al.* (2010) and Warwick *et al.* (2010) also included small numbers of Alysseae *s.l.* taxa (Table 1-2). Where they received strong or moderate support, the results of these analyses were consistent with the more focused studies already discussed. In the *ITS*-based phylogeny of Warwick *et al.* (2010) the monospecific genus *Physocardamum* Hedge was nested within the Alysseae with weak support.

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Figure 1-4a. Generic relationships of Alysseae. Consensus Bayesian tree presented by Cecchi *et al.* (2010). Posterior probabilities of branches are indicated. Brassicaceae Tribes are indicated at the right. Alysseae Clades A1-A5 from Warwick *et al.* (2008) have been indicated. Nodes A–D indicate the ‘four main clades’ in Alysseae as identified by Cecchi *et al.* (2010). Scale indicates substitutions site⁻¹.

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Figure 1-4b. Species relationships of *Alyssum* sect. *Odontarrhena*. Consensus Bayesian tree presented by Cecchi *et al.* (2010). Posterior probabilities of branches are indicated. Brassicaceae Tribes are indicated at the right. Alysseae Clades A1-A5 from Warwick *et al.* (2008) have been indicated. Nodes O1–O3 indicate the three ‘main clades’ in section *Odontarrhena* as identified by Cecchi *et al.* (2010). Species names in bold type indicate nickel hyperaccumulator species. Scale indicates substitutions site⁻¹.

Reliance on *ITS* sequence data

Nine of the 13 published phylogenies that included species of *Alysseae s.l.* (Table 1-2) relied solely on internal transcribed spacer (*ITS*) nuclear ribosomal DNA sequences, and therefore do not provide independent phylogenetic estimates in any way, but rather a single gene tree with successive accretions of taxa. Furthermore, Álvarez and Wendel (2003) reported that, although the nuclear ribosomal *ITS* region had been used as the sole locus in 34% of published plant phylogenies, high levels of intra-individual *ITS* copy type diversity, which may include both distinct functional copy types (arising through polyploidy or hybridization events: Mishima *et al.* 2002) and/or potentially non-functional pseudogene copy types (e.g., in *Gossypium* L., *Nicotiana* L.: Buckler, 1997; *Quercus* L.: Mayol and Rosselló, 2001; and *Leucaena* Benth.: Hughes *et al.*, 2002), make this locus far from ideal for phylogeny reconstruction. The prevalence of such diversity means that there are real risks of including *ITS* copies that exhibit unsuspected deep paralogy (*sensu* Bailey *et al.*, 2003), and of under-sampling of *ITS* diversity when taxa are only represented by potentially non-functional pseudogene sequences, with no functional copies sampled (Bailey *et al.*, 2003). *ITS* gene trees may therefore not reflect species relationships. For this reason, Álvarez and Wendel (2003) recommended that *ITS* sequences should only be used in phylogenetic inference after extensive surveys of *ITS* sequence variation in the taxa under study, or preferably, that single-copy nuclear loci be used instead. Despite these issues, and given the very large reservoir of existing *ITS* sequence data for taxa lacking any other sequence data, this locus can still provide potentially sound data for phylogenetics, assuming appropriate methods are used to determine paralogy issues and apply rigorous methods to detect potentially non-functional copy types (Hughes *et al.*, 2002 and Bailey *et al.*, 2003; see Appendix 5).

Taken together, the phylogenetic studies described above suggest that whilst the *Alysseae s.l.* of Al Shehbaz *et al.* (2006) is paraphyletic, there is good evidence for a robustly supported monophyletic *Alysseae s.str.*, comprising the 13 genera *Alyssoides*, *Alyssum*, *Aurinia*, *Berteroa*, *Bornmuellera*,

Clastopus, *Clypeola*, *Degenia*, *Fibigia*, *Galitzkya*, *Hormathophylla*, *Leptoplax* and *Physoptychis*.

There is also good support for the six major clades within Alysseae *s.str.*, some of which correspond to existing genera (e.g., A4: *Hormathophylla*; A5: *Bornmuellera*). There is only limited support for relationships between these clades, and these require further testing. Perhaps due to its relatively large size, there has been relatively sparse taxon sampling from within *Alyssum* in all studies undertaken to date, and there is currently no well sampled and robust hypothesis of species relationships within the genus. The sister-group relationship of Alysseae *s.str.* within the Brassicaceae also remains uncertain, although Khosravi *et al.* (2009) suggested that Alysseae may be sister to Chorisporaeae.

Current knowledge of the phylogeny of *Alyssum* and the Alysseae is inadequate to make any inferences about the evolution of nickel hyperaccumulation in these taxa, and there is a real need for a new, much more densely sampled (in terms of taxa and genes), well-resolved and robustly supported phylogeny that can be used to investigate the evolution of nickel hyperaccumulation in detail. The complete lack of time-calibrated phylogenies for this tribe, mean that there is currently no evidence for the age of nickel hyperaccumulation in the Alysseae, even within very broad terms, the age of the trait could conceivably range from tens of thousands of years to tens of millions. It is clear that in order to obtain a robust phylogeny for *Alyssum*, it will be necessary to use multiple independent loci, such as single-copy nuclear genes or highly variable non-coding chloroplast regions (e.g., Slotte *et al.*, 2006; Song *et al.*, 2007; Shaw *et al.* 2007). The need for such additional DNA sequence data is underlined by the poor species-level resolution in *ITS*-based Alysseae trees.

Given uncertainties surrounding the relationships within Alysseae, and the presence of nickel hyperaccumulators in closely related genera, the scope of any new study needs to include as many taxa as possible from across the Alysseae as a whole. The monotypic genus *Straussiella* should also be included if material can be obtained, since this genus has not been sampled in any phylogeny so

far. Furthermore, closely related lineages from outside *Alysseae s.str.* should also be included in order to provide robust rooting and to determine the polarity of nickel hyperaccumulation character-state changes within the *Alysseae s.str.*, by confirming the ancestral condition for this taxon.

Evolution of Serpentine Endemism

The fact that nickel hyperaccumulators in *Alyssum* are largely restricted to serpentine was noted by Brooks *et al.* (1978, 1979), though surprisingly there have not been any formal geographical, or even basic statistical analyses of this association. It appears that species which are polymorphic for nickel hyperaccumulation occur both on and off serpentine soils (e.g., *Alyssum serpyllifolium* complex (Brooks *et al.*, 1981); *A. peltarioides*, Mengoni *et al.*, 2003). More generally for metal hyperaccumulators, it appears that hyperaccumulation rarely or never occurs on non-metaliferous soils (Reeves and Baker, 2000). However, although nickel hyperaccumulation is strongly associated with serpentine soil in *Alyssum*, the reverse is not true; there are many non-accumulator species that grow on or are restricted to serpentine soils. Hyperaccumulation therefore appears related to but distinct from the ability to tolerate serpentine soils. Indeed, the vast majority of plant species growing on a serpentine site will typically not be nickel hyperaccumulators (Brooks 1987, Kazakou *et al.*, 2010). Some plant species found growing on serpentine are common on a range of substrates (e.g., *Erica scoparia* L. (Messerli, 1936) *Cistus ladanifer* L. (Quintela-Sabarís, 2011), however, many are restricted to serpentine habitats (e.g., *Layia discoidea* D.D.Keck (Baldwin, 2005); many species of *Alyssum* and *Bornmuellera* (Appendix 3)). Plants of the latter group are usually referred to as serpentine endemic species. Such specialization is not surprising given the extreme nature of the serpentine environment in comparison with the majority of soils: it typically has a very high availability of iron, nickel, cobalt and magnesium, a low availability of calcium, is very rocky and free draining and is low in organic matter and nitrogen; Kazakou, 2008).

The geographic distribution of serpentine in many parts of the world takes the form of numerous relatively small areas, each on the order of kilometers or tens of km in diameter. This is certainly the case in the Mediterranean and south-west Asian regions (Figure 1-5). Because of the extreme soil environment of these serpentine sites compared with the surrounding matrix of non-serpentine soils, they have been regarded as edaphic islands (Rajakaruna, 2004; Cecchi and Selvi 2009; Cecchi *et al.*, 2010). Rajakaruna (2004) has suggested that variation in soil conditions, especially island-like distributions of extreme soil types such as serpentine, could be a key factor in plant speciation.

The specialized vegetation of serpentine was investigated in detail by Brooks (1987) and others (e.g., Proctor and Woodell, 1975; Roberts and Proctor, 1992), before the era of phylogenetics. More recent, phylogenetic, studies have investigated the evolution of serpentine endemism in various plant taxa (e.g., *Streptanthus* Nutt. and *Caulanthus* S.Watson (Pepper and Norwood, 2001); *Navarretia* Ruiz & Pav., *Collinsia* Nutt., *Erythronium* L. and *Allium* L. (Anacker *et al.*, 2011); *Paramoltkia* and *Halacsya* (Cecchi and Selvi, 2009); *Onosma* L. Cecchi *et al.*, 2011). Many such studies have reported multiple independent origins of serpentine endemism within closely-related species. However, few (e.g., Anacker and Harrison, 2012a,b) have generated time-calibrated phylogenies which are vital to properly investigate the geographic or climatic circumstances surrounding the evolution of this trait. It would be particularly interesting to compare the age of the naturally-evolved metal tolerance of serpentine tolerant or endemic species with the recently-evolved metal tolerance that has evolved in plants of anthropogenically polluted sites (e.g., Antonovics *et al.*, 1971; McNair and Baker, 1994; Pauwels *et al.*, 2005, 2012).

There are numerous examples of *Alyssum* species which are endemic not only to serpentine soil, but also to one small area of serpentine soil. For example, various species are limited to Greek islands (e.g., *A. akamasicum*, *A. tenium*, *A. troodi*, *A. lesbiacum*; Strid and Tan, 2002), and others

[Image removed for copyright reasons.]

Figure 1-5a (above) and 1-5b (below). Maps indicating the broad-scale distribution of serpentine formations (shown in black) in the Mediterranean region and Turkey. From Brooks *et al.* 1978 and Brooks *et al.* 1979 respectively. The numbers in Figure 1-5a relate to sample collection locations. The symbols in Figure A1-5b represent *Alyssum* specimens containing $>10\,000\text{ mg kg}^{-1}$ (black squares), $1000\text{--}10\,000\text{ mg kg}^{-1}$ (black circles), $100\text{--}999\text{ mg kg}^{-1}$ (white circles) and $<100\text{ mg kg}^{-1}$ (white squares) of nickel.

are restricted to very small geographic areas of the Turkish mainland (e.g., *A. crenulatum* Boiss., *A. dubertretii* Gomb., *Bornmuellera kiyakii* Aytaç & Aksoy; Adigüzel and Reeves, 2012). Furthermore, serpentine endemic *Alyssum* species appear to be largely allopatric across their distribution, *i.e.* on any given serpentine island only one or at most a few species will be present. This may well be related to the conspicuous lack of morphological (and hence presumably ecological) divergence within these plants, and suggests that allopatric mechanisms of speciation may have been important in the diversification of these plants.

Species endemic to small geographic regions have been divided conceptually into paleoendemics and neoendemics (Stebbins 1942; Brooks 1987). The former are species that have formerly had a wide geographic range which has been reduced (to what is also called a relictual distribution). Such species or are generally considered older than the specific geography which limits their current range. Neoendemics on the other hand are species that have evolved within their endemic range, and hence are considered to be younger than the geography which limits this. Given that serpentine soils are likely to always have had a patchy, island like distribution across Eurasia (*i.e.*, there is no evidence of widespread areas of serpentine rocks or soils ever linking together the current set of serpentine soil islands), it seems likely that *Alyssum* species and other serpentine specialists are neoendemics. However, there are other possibilities. For example, Cecchi *et al.*, (2011) have suggested that serpentine sites could (through their high levels of exposed rock and hence their capacity for absorbing thermal radiation) have acted as refugia for southern Eurasian species during glacial periods of the Pleistocene. In this case, such serpentine endemics would be considered paleoendemics. However, there is no evidence for such a process ever actually having operated.

For the majority of *Alyssum* species, there are relatively few herbarium collections, and the level of information available about their geographic distributions is highly variable (e.g., Jalas and

Suominen (1994) and Strid and Tan (2002) give detailed distribution maps for Alyseae species Europe and Greece respectively, but little detail is available for Turkey and Iran, both of which contain large numbers of Alyseae species, (Parsa, 1978; Davis 1965–1988; Ghaderian *et al.*, 2007a; Adigüzel and Reeves, 2012). The lack of geographic analyses in the Alyseae in this group is therefore unsurprising. However, because of its wide distribution and large number of serpentine endemic species, the Alyseae may well provide valuable insights in to the potential for edaphic allopatric speciation, and the origin of endemism.

Key Objectives

The central aim of my research is to investigate the evolution of complex plant physiological traits using nickel hyperaccumulation in *Alyssum* as the study system. Specific scientific questions to be addressed include:

- 1) How many origins of nickel hyperaccumulation were there in *Alyssum*?
- 2) When were they likely to have occurred?
- 3) Do hyperaccumulators evolve from locally distributed adjacent non-accumulators, or do pre-adapted hyperaccumulator lineages disperse widely across ultramafic sites?
- 4) What were the impacts of the evolution of nickel hyperaccumulation on species diversification within *Alyssum*, and specifically did hyperaccumulation act as a possible evolutionary innovation prompting accelerated species diversification?

Since these questions will be investigated using phylogenetic approaches, the reconstruction of an enhanced species-level phylogeny is a critical step for addressing them, and is central to this thesis. Specifically this will involve:

- 1) Reconstructing an enhanced phylogeny of the Alysseae with substantially greater species sampling than previous studies (which have included a maximum coverage of: 53/255, i.e., 21% of species), by generating new sequence data for DNA regions other than *ITS*.
- 2) Estimation of divergence times for key clades within the tribe Alysseae.
- 3) Assembly of data on nickel hyperaccumulation status and serpentine endemism data from the literature and optimization of these traits onto the new phylogeny
- 4) To use species birth-death models to estimate species diversification rates across the Alysseae, detect possible diversification rate shifts and test whether these are significantly associated with the evolution of nickel hyperaccumulation or species range size.

These new phylogenetic analyses will take advantage of increased taxon sampling combined with the use of highly variable genetic markers, and formal ancestral state reconstruction methods which will allow the number of origins of nickel hyperaccumulation in *Alyssum* to be investigated more comprehensively than in previous studies. They will also employ recently-developed Bayesian analysis methods such as relaxed log-normal molecular clocks (Drummond *et al.*, 2006) reversible-jump hyper-prior ancestral state reconstruction (Pagel & Meade, 2006), and lineage diversification models (e.g. Alfaro *et al.*, 2009).

Chapter 2: Materials and Methods

DNA Marker Screening Pilot Study

Choice of DNA regions

In order to select DNA regions of high phylogenetic utility within Alysseae, in terms of both sequence variation and ease of PCR amplification and sequencing, a screening trial was carried out between December 2008 and July 2009. A total of 21 DNA regions were chosen for screening, including 18 chloroplast and three nuclear regions (Table 2-1). The chloroplast regions included 15 tested by Shaw *et al.* (2005; 2007) which show both relatively high sequence variability, and typically include non-coding regions such as inter-genic spacers (IGSs) or introns combined with relatively conserved primer regions (many of which are within short tRNA or other coding regions). Three additional chloroplast regions that have been used in inter-specific studies in the Brassicaceae were included: *trnL-trnF* which includes an inter-genic spacer and the trnL transfer RNA intron (Dobeš *et al.*, 2004); *ndhF* (Beilstein *et al.*, 2006), which codes for the ND5 subunit of chloroplast NADH dehydrogenase (Shinozaki *et al.*, 1986); and *matK* (Koch *et al.*, 2001), which codes for a maturase located within the *trnK* intron (Neuhaus and Link, 1987). The three nuclear regions that were included are single-copy genes used previously in species-level phylogenetic studies in the Brassicaceae: *LUMINDEPENDENS* (*LD*) (Slotte *et al.*, 2006) which codes for the LD transcription factor (Aukerman *et al.*, 1999); *Dpa1* (also called *INCURVATA2*) (Song & Mitchell-Olds, 2007) which codes for the catalytic subunit of the DNA polymerase alpha (Barrero *et al.*, 2007); and *Nuo* (Song & Mitchell-Olds, 2007) which codes for a subunit of NADH ubiquinone oxidoreductase (Weidner *et al.*, 1993). Sequence lengths for these 21 DNA regions were in the range 560 to 1145 base pairs (bp) in previous studies (see references in Table 2-1).

Taxon Sampling

In order to assess DNA region variability and primer conservation at infra-specific and infra-generic levels within Alysseae, eight Alysseae accessions were selected for inclusion in the screening study. These are listed in Table 2-2, and include both putatively closely and more distantly related taxa. For taxonomic authorities of all Alysseae species mentioned, see Appendix 1.

Table 2-1. DNA marker regions included in screening study.

Marker Region	Source	Marker Region	Source
Chloroplast Genome		Chloroplast Genome	
<i>matK</i>	Koch <i>et al.</i> (2001)	<i>rps16</i> intron	Shaw <i>et al.</i> (2007)
<i>ndhF</i>	Beilstein <i>et al.</i> (2006)	<i>rps16-trnK</i>	Shaw <i>et al.</i> (2007)
<i>ndhF-rpl32</i>	Shaw <i>et al.</i> (2007)	<i>trnD-trnT</i>	Shaw <i>et al.</i> (2007)
<i>rpl16</i>	Shaw <i>et al.</i> (2007)	<i>trnL-F</i> (IGS & <i>trnL</i> Intron)	Dobeš <i>et al.</i> (2004)
<i>trnG-trnS</i>	Shaw <i>et al.</i> (2007)	<i>trnS-trnF</i>	Shaw <i>et al.</i> (2007)
<i>trnQ-rps16</i>	Shaw <i>et al.</i> (2007)	<i>trnS-trnL</i>	Shaw <i>et al.</i> (2007)
<i>trnH-psbA</i>	Shaw <i>et al.</i> (2007)	<i>trnV-ndhC</i>	Shaw <i>et al.</i> (2007)
<i>rps12-rpl20</i>	Shaw <i>et al.</i> (2007)	Nuclear Genome	
<i>rpl32-trnF</i>	Shaw <i>et al.</i> , (2007)	<i>LUMINIDEPENDENS</i> (LD)	Slotte <i>et al.</i> (2006)
<i>rpl32-trnL</i>	Shaw <i>et al.</i> (2007)	<i>Dpa1</i>	Song & Mitchell-Olds (2007)
<i>rpoB-trnC</i>	Shaw <i>et al.</i> (2007)	<i>Nuo</i>	Song & Mitchell-Olds (2007)

Plants were grown from seed under glasshouse conditions at the Department of Plant Sciences, University of Oxford (temperature: 18–25 °C, lighting 16 h light/8 h dark). Plant tissue was either harvested and used fresh for DNA extraction, or was dried for 48 h at room-temperature using silica gel, and subsequently stored at –20°C.

DNA Extraction

Genomic DNA was extracted using the Qiagen Plant Minikit (Qiagen UK, Crawley, UK) and NBS Biologicals Spin Column Genomic DNA Isolation Kit (for Plant) (NBS Biologicals, Cambs, UK) according to instructions provided by the manufacturers. Tissue sample masses of 0.5 mg were used for fresh tissue and 0.1 mg for silica-dried tissue. To confirm successful DNA extraction, 2 µl of

genomic DNA solution was visualized via agarose gel electrophoresis, using a 1.5% (w/v) agarose gel stained with ethidium bromide (1 µg ml⁻¹). Gels were run for 30 minutes at 100 V (max current 0.2 A), and then photographed on a UV illuminator.

Table 2-2. Alyseae species and accessions included in the screening study.

Genus	Infra-generic Section	Species	Seed Source/Collector & Accession Number
<i>Alyssum</i>	<i>Alyssum</i>	<i>Alyssum alyssoides</i>	USDA ¹ : PI633054
		<i>Alyssum montanum</i>	USDA ¹ : PI633061
	<i>Odontarrhena</i>	<i>Alyssum lesbiacum</i>	A.J.M. Baker: 00150n
		<i>Alyssum malacitanum</i>	A.J.M. Baker: 00207d
		<i>Alyssum murale</i>	A.J.M. Baker: 00067a
		<i>Alyssum pintodasilvae</i>	A.J.M. Baker: 00337a
<i>Aurinia</i>	N/A	<i>Aurinia saxatilis</i>	BGTH ² : 700
<i>Bornmuellera</i>	N/A	<i>Bornmuellera tymphaea</i>	M. Pavelka: 187
Notes to Accompany Table 2-2 ¹ United States Department for Agriculture North Central Regional Plant Introduction Station, Plant Introduction Unit, Iowa State University, Ames, Iowa, 50011, USA. ² Botanischer Garten der Technischen Hochschule, Aachen, Germany.			

Amplification

The DNA marker regions (Table 2-1) were separately amplified from each of the DNA samples by polymerase chain reaction (PCR) in volumes of 15 µl. The PCR reaction mixture was made up from the following reagents: 10.85 µl of distilled water, 1.5 µl of 10× Buffer N (Yorkshire Bioscience Ltd, York, UK), 0.5 µl of 50 mM magnesium chloride, 0.3 µl of mixed dNTPs (each at 10 mM) (Fermentas, UK), 0.25 µl of Taq DNA Polymerase (at 5 U µl⁻¹) (Fermentas UK, York, UK), 0.15 µl of each of two amplification primers (each at 10 pmol µl⁻¹), 0.3 µl of 100× BSA (at 10 mg ml⁻¹) and 1 µl of template DNA solution. Absolute reagent concentrations were: 1.67 mM of MgCl₂, 0.2 mM of each dNTP, 0.083 U µl⁻¹ of Taq DNA Polymerase, 0.67 pmol µl⁻¹ of each primer, and 20 mg ml⁻¹ of BSA. BSA was added in order to reduce the activity of potential Taq inhibitors present within plant tissue, such as Ni²⁺ and glucosinolates. Where the initial reactions gave weak or absent products,

the quantity of template DNA solution was decreased to 0.5 µl or increased to 2 µl; the quantity of distilled water was adjusted accordingly.

Table 2-3. Amplification and sequencing primers employed in this study.

*Asterisked regions were used in the pilot study only.

Marker Region	Primer Name	Primer Sequence (5'–3')
<i>Dpa1</i> *	Dpa1F ¹	AAT CAG ACG GTG GTA GG
	Dpa1R ¹	GCC TCC TCA TAA GCG TCA AG
<i>LD</i> *	LD-D1F ²	AGG CCA GGT GAA ACT AAT GG
	LD-XC4R ²	CAG AAG CAA TTG ACT CTG CG
<i>matK</i>	trnK710F ³	CGC ACT ATG TGT CAT TTC AGA ACT C
	matK1010R ³	CCA CTA AAG GAT TTA ATC GCA AAC
	matK495R ³	CGG ATT CGT AGG AGG GTT TGA ACC AAG
	matK495F ³	CTT GGT TCA AAC CCT ACG TTA CCG
<i>ndhF</i>	ndhF5F ⁴	ATG GAA CAT ACA TAT CAA TAT TCA TGG
	ndhF599R ⁴	TAG GTC TTT ATT GGA TAA C
	ndhF989R ⁴	CCC ATA CCT AGA GCT AAC ATC A
	ndhF989F ⁴	TGA TGT TAG CTC TAG GTA TGG G
	ndhF1410F ⁴	GCT CCT TVT ATT CAC TYT CTT TRT GGG
	ndhF1703R ⁴	GGC TCC AAT AAA YAA AGT
	ndhF2100R ⁴	CAA AGA AAC TYG TAA KAC STA CTC C
<i>ndhF-rpl32</i> *	ndhF ⁵	GAA AGG TAT KAT CCA YGM ATA TT
	rpl32-R ⁵	CCA ATA TCC CTT YYT TTT CCA A
<i>Nuo1</i> *	Nuo1F ¹	GAA GGG AAG CAA GGA AAA CC
	Nuo1R ¹	TTA AGC AGC AGC CTG TAG CA
<i>PHYA</i>	PHYA230F ⁶	GAC TTT GAR CCN GTB AAG CCT TAY G
	PHYA832R ⁶	RTT CCA YTC NGT RCA CCA NCC
	PHYA481F ⁶	GTT GTA GTW AAY GAG GAA GAT GG
	PHYA577R ⁶	GTA TGW GAA CGG AAC CAG AA
<i>rpl16</i> *	rpl16F71 ⁷	GCT ATG CTT AGT GTG TGA CTC GTT G
	rpl16R1516 ⁷	CCC TTC ATT CTT CCT CTA TGT TG
<i>rpl32-trnF</i> *	rpl32-F ⁵	CAG TTC CAA AA A AAC GTA CTT C
	trnF ⁸	CGA AAT CGG TAG ACG CTA CG
<i>rpl32-trnL</i> *	rpl32-F ⁵	CAG TTC CAA AA A AAC GTA CTT C
	trnL ^{(UAG)5}	CTG CTT CCT AAG AGC AGC GT
<i>trnG-trnS</i> *	trnS ^{(GCU)5}	AAC TCG TAC AAC GGA TTA GCA ATC
	trnG ^{(UUC)5}	GAA TCG AAC CCG CAT CGT TAG
<i>trnH-psbA</i> *	trnH ^{(GUG)7}	CGC GCA TGG TGG ATT CAC AAT CC
	psbA ⁷	GTT ATG CAT GAA CGT AAT GCT C
<i>trnL-trnF</i>	trnF ^{(GAA)8*}	ATT TGA ACT GGT GAC ACG AG
	3'trnL ^{(UAA)F} ¹⁰	GGT TCA AGT CCC TCT ATC CC
	3'trnL ^{(UAA)R} ¹⁰	GGG GAT AGA GGG ACT TGA AC

Table 2-3. Amplification and sequencing primers employed in this study.
*Asterisked regions were used in the pilot study only.

Marker Region	Primer Name	Primer Sequence (5'–3')
	5'trnL ^(UAA) F ^{10*}	CGA AAT CGG TAG ACG CTA CG
<i>trnQ–rps16*</i>	trnQ ^(UUG) J ⁵	GCG TGG CCA AGY GGT AAG GC
	rps16x1 ⁵	GTT GCT TTY TAC CAC ATC GTT T
<i>rbcl</i>	rbcl1F ⁹	ATG TCA CCA CAA ACA GAA AC
	rbcl1388R ¹¹	TTC CAT ACT TCA CAA GCA GC
	rbcl636F ⁹	GCG TTG GAG AGA TCG TAT CT
	rbcl724R ⁹	TGC CAT GTA CCT GCA GTA GC
<i>rps12–rpL20*</i>	5'rps12 ⁷	ATT AGA AAN RCA AGA CAG CCA AT
	rpL20 ⁷	CGY YAY CGA GCT ATA TAT CC
<i>rpoB–trnC*</i>	rpoB ⁷	CKA CAA AAY CCY TCR AAT TG
	trnC ^(GCA) R ⁷	CAC CCR GAT TYG AAC TGG GG
<i>rpS16 intron*</i>	rps16F ⁷	AAA CGA TGT GGT ARA AAG CAA C
	rps16R ⁷	AAC ATC WAT TGC AAS GAT TCG ATA
<i>rps16–trnK</i>	rps16x2F2 ⁵	AAA GTG GGT TTT TAT GAT CC
	trnK ^(UUU) x1 ⁵	TTA AAA GCC GAG TAC TCT ACC
<i>trnD–trnT</i>	trnT ^(GGU) 7	CTA CCA CTG AGT TAA AAG GG
	trnE ^(UUC) 7	AGG ACA TCT CTC TTT CAA GGA G
	trnY ^(GUA) 7	CCG AGC TGG ATT TGAACC A
	trnD ^(GUC) F ⁷	ACC AAT TG ACT ACA ATC CC
<i>trnS–trnfM*</i>	trnS ^(UGA) 7	GAG AGA GAG GGA TTC GAA CC
	trnfM ^(CAU) 7	CAT AAC CTT GAG GTC ACG GG
<i>trnS–trnL*</i>	trnS ^(GGA) 7	TTA CCG AGG GTT CGA ATC CCT C
	5'trnL ^(UAA) R(TabB) ⁷	TCT ACC GAT TTC GCC ATA TC
<i>trnV–ndhC*</i>	trnV ^(UAC) x2 ⁵	GTC TAC GGT TCG ART CCG TA
	ndhC ⁵	TAT TAT TAG AAA TGY CCA RAA AAT ATC ATA
Notes to accompany Table 2-3: Primer sources: ¹ Song & Mitchell-Olds (2007) ² Slotte <i>et al.</i> (2006) ³ Koch <i>et al.</i> (2001) ⁴ Beilstein <i>et al.</i> (2006) ⁵ Shaw <i>et al.</i> (2007) ⁶ Beilstein <i>et al.</i> (2008) ⁷ Shaw <i>et al.</i> (2005) ⁸ Dobeš <i>et al.</i> (2004) ⁹ Savolainen <i>et al.</i> (2000) ¹⁰ Taberlet <i>et al.</i> (1991) ¹¹ Primer designed in this study Base codes for degenerate primers: A/C: M; A/G: R; A/T: W; C/G: S; C/T: Y; G/T: K.		

In the pilot study, all markers except *ndhF* were amplified as single sequences using a single primer pair. For *ndhF* the amplification primers pairs were ndhF5F/ndhF989R, ndhF989F/ndh1703R and ndhF1410F/ndhF2100R. For *trnL–trnF* the amplification primers were trnF^(GAA)F and 5'trnL^(UAA)F. For *trnD–trnT* the amplification primers were trnD^(GUC)F and trnT^(GGU). All marker regions for which

multiple amplification or sequencing primer pairs were employed in either the pilot or the full study are illustrated in Figure 2-1. Primer oligonucleotides were supplied by Eurofins MWG Operon (Anzingestr. 7a, Ebersberg, Germany).

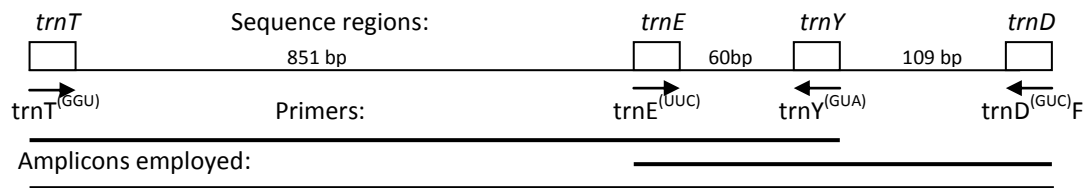
PCRs were performed in PTC 200 (MJ Research, Waltham, USA) or DNA Engine (Bio-Rad Laboratories Ltd, Herts, UK) Peltier thermocyclers, initially using the '*rpl16*' protocol of Shaw *et al.* (2007). The low annealing temperature (50 °C) led to multiple products in some cases, and here the temperature was increased (typically to 51 or 52 °C, but in some cases to as high as 60 °C) in order to obtain a single product. A typical PCR programme involved: 5 min at 80 °C; 30 cycles of: 1 min at 95 °C, 1 min at 52 °C, ramp of 0.3 °C s⁻¹ to 65 °C, 4 min at 65 °C; 5 min at 65 °C, and hold at 4 °C.

PCR products were visualized via agarose gel electrophoresis, using 1% (w/v) agarose gels stained with ethidium bromide (1 µg ml⁻¹). Gels were run for 30 minutes at 100 V (max current 0.2 A), and then photographed on a UV illuminator. PCR product DNA concentration and size were estimated by comparison with a 100 bp DNA ladder (New England Biolabs (UK) Ltd., Herts, UK).

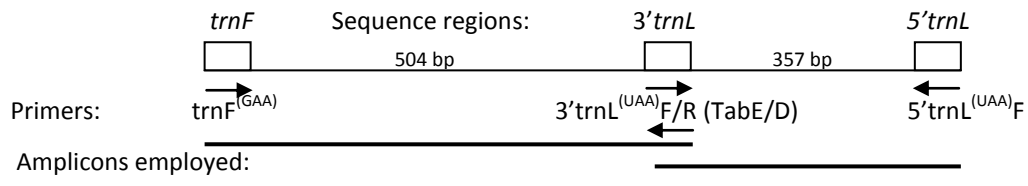
DNA Sequencing

Cycle sequencing was carried out using the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems Inc., California, USA), using reaction volumes of 10 µl in either 0.2 ml tubes or in 96-well plates. The reaction mixture included: 5.7 µl distilled water, 1.5 µl 5× sequencing buffer, 0.5 µl BigDye 3.1 solution, 0.3 µl of one sequencing primer and 2 µl template DNA solution. Template DNA was not subject to purification or dephosphorylation reactions before addition. In the pilot study, all PCR products were sequenced using the same primers used in the amplification steps. A separate sequencing reaction was carried out for each of the two sequencing primers employed. Thermal cycling was carried out using thermocyclers detailed above and following the standard PCR programme for the BigDye v.3.1 kit.

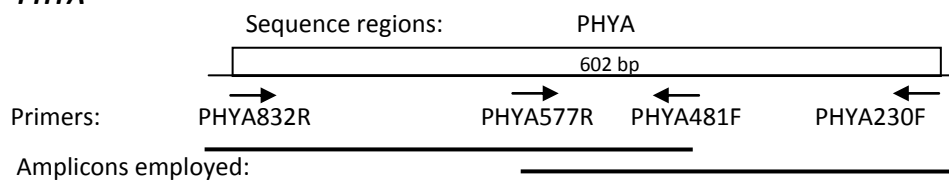
trnD-trnT



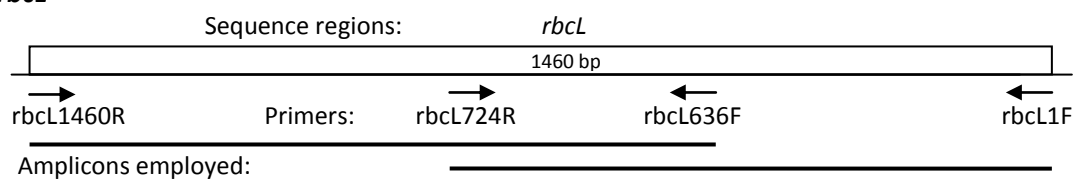
trnL-trnF



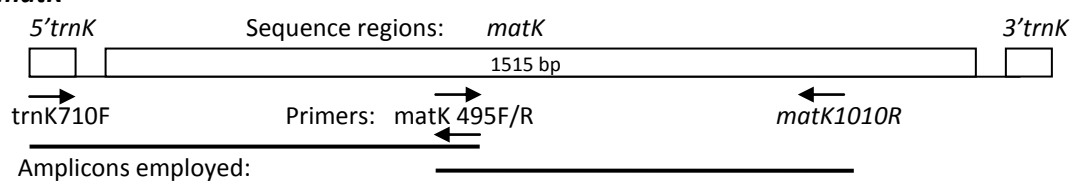
PHYA



rbcL



matK



ndhF

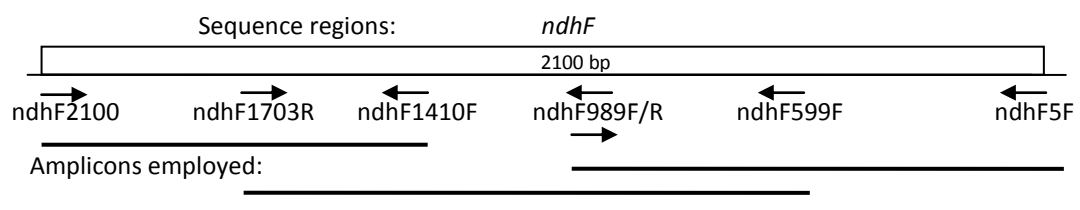


Figure 2-1. Schematic representation of marker regions for which > 2 amplification or sequencing primers were employed, indicating approximate locations of primer-binding sites, amplified regions and sequencing reads. Based on Shaw *et al.* 2005; 2007.

After cycle sequencing, sequencing products were purified and precipitated. To each 10 µl volume was added 1 µl of precipitation buffer (containing 1.5 M sodium acetate and 250 mM EDTA, adjusted to pH 8.0 using 1 M sodium hydroxide solution and glacial acetic acid), and 40 µl of 100% ethanol. After sealing and mixing, tubes were refrigerated at –20 °C for 15 min, and centrifuged at 1500 *g* for 45 min. The supernatant was then removed by inverting the tubes/plates and centrifuging at 300 *g* for 1 min. Remaining ethanol and water were removed by heating the open tubes/plates at 70 °C for 10 min on a GeneAmp PCR System 2700 Peltier cycler (Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California, USA). Sequencing products were sent to the DNA Sequencing Unit at the Department of Zoology, University of Oxford for re-suspension and visualization using an ABI 3730xl DNA Analyzer (Applied Biosystems). The output format for chromatograms was .ab1.

Sequence Assembly

Sequence files were loaded into Chromas Lite (version 2.01) (Technelesium Pty Ltd, 701/8 Cordelia St., Brisbane, Australia) and saved in FASTA format. Sequences were BLAST searched in GenBank in order to confirm their identity. The .ab1 files were loaded into Sequencher (version 4.5) (Gene Codes Corp., 775 Technology drive, Suite 100A, Ann Arbor MI 48198, USA), and the ‘Trim Ends’ function was used to trim the 3’ and 5’ ends of each sequence, removing bases until there were <2 ambiguous bases in the terminal 25 bases. For a few cases it was necessary to reduce the stringency of this criterion, to <4 ambiguous base calls in 25 in order to retain a useful length of sequence. In many cases chromatograms did not meet these criteria, and these were rejected at this stage. Sequencing was repeated from the sequencing reaction onwards for these accession/sequencing primer combinations; in most cases the second or third chromatogram obtained was satisfactory.

All suitable sequence reads from the same accession and DNA region (i.e., typically one for each sequencing primer used) were assembled to give a consensus sequence using the 'Assemble Automatically' function in Sequencher. Conflicting base calls between sequences were generally few in number, and most could be resolved by manual base calling. A small number of bases remained ambiguous and were coded using the standard ambiguous base codes (A/C: M; A/G: R; A/T: W; C/G: S; C/T: Y; G/T: K).

Sequence Alignment

Consensus sequences were saved in FASTA format, and loaded into MEGA (version 4) (Kumar *et al.*, 2008). GenBank sequences for two non-Alysseae species from the Brassicaceae (*Arabidopsis thaliana* accessions NC000932.1 and NC000423.1; *Lobularia maritima* accessions AP009375 and AP009274.1) were added to the alignments (complete chloroplast genome sequences are available for these two species). Sequences were initially aligned to the *Arabidopsis thaliana* sequence and to amplification and sequencing primer sequences using ClustalW (Larkin *et al.*, 2007) from within MEGA. The set of sequences was then aligned using MUSCLE (Edgar, 2004). All amplification primer sites were cut from the alignment along with any sequence data from outside the target sequence. The *A. thaliana* and *L. maritima* sequences were then removed to leave alignments containing only sequences of Alysseae.

Data Analysis

Aligned sequence lengths were determined in the MEGA Alignment Explorer via the 'Site # with Gaps' command. The number of variable sites was determined in MEGA by manual counting (non-variable sites are asterisked in the column headings in this computer program). Gaps were coded using the simple gap coding approach of Simmons *et al.* (2001). This was carried out using SeqState (version 1.4.1) (Muller, 2005), which appends the gap coded data matrix to the end of the initial alignment. The new data matrix was then opened in Winclada (version 1.00.08) (Nixon, 2002), and

the total number of gap characters was manually counted. The 'Mop Uninformative Characters' and 'Delete Selected Characters' commands were used to display parsimony-informative characters (PICs) only, which were then manually counted for both substitutions and gap characters. The minimum number of informative character-state changes ($\Delta_{\min}$) (Wortley & Scotland, 2006) was determined from the parsimony-informative character matrix by visual inspection.

Full Scale Study

Taxon Sampling

Alysseae

The aim of the phylogenetic work in this study was to produce a robust and well-sampled phylogeny of the Alysseae, as a basis for studying the evolution of nickel hyperaccumulation in this tribe. An up-to-date species list for the Alysseae is presented in Appendix 1, and indicates that the tribe contains a total of 255 species in the following 16 genera: *Alyssoides*, *Alyssum*, *Aurinia*, *Berteroa*, *Bornmuellera*, *Clastopus*, *Clypeola*, *Degenia*, *Fibigia*, *Galitzkya*, *Hormathophylla*, *Leptoplax*, *Phyllolepidum*, *Physocardamum*, *Physoptychis* and *Straussiella*. It was decided that taxon sampling in the tribe should aim to include as many species as practical from these genera. Multiple accessions were included for a small number of species in order to test the consistency of species placement, but the main emphasis in sampling was to include a high number of species. This was because geographic and trait data (important for later analyses) was mainly available in the literature at the species level. Also, an attempt was made to obtain sequence data for at least one species of each of the 16 genera in the tribe in order to give a high level of taxonomic representation from across the whole of the tribe Alysseae because phylogenetic relationships within the tribe are not yet fully resolved.

Plant tissue samples were obtained from herbaria and from seed-grown plants (grown at the Department of Plant Sciences, University of Oxford, under the glasshouse conditions outlined in the previous section). In a few cases (where seeds would not germinate), the supplied seeds were used themselves as tissue for genomic DNA extraction. Material that had a voucher herbarium specimen and for which the wild collection location and date were recorded was preferred to material of unknown origin, but it was not possible to obtain such material in all cases. For a small number of species it was necessary to use seed accessions without such information. Where possible, voucher herbarium specimens were made from plants grown from seed from the same accession, and were deposited at the University of Oxford Herbaria (OXF). As in the pilot study, plant leaf tissue taken from living plants was either used fresh or was dried using silica gel and thereafter stored at -20°C . Herbarium tissue from recent collections was preferred over older material in order to reduce the likelihood of DNA degradation. The oldest herbarium material for which DNA extraction was attempted here dated from 1934 (*A. gehamense*, *Balls* 1313 from Edinburgh). Extracted DNA was obtained from other researchers for a small number of species, and was supplied as an aqueous solution. Full details of source, tissue type, collection data and voucher specimen are included in Appendix 2 for all of the 206 tissue samples or extracted DNA obtained in this study. A total of 189 samples were obtained from Alysseae taxa (representing 170 species), and 16 further species were sampled from the Brassicales. The sampling breakdown across the 16 genera of the Alysseae is indicated in Table A1-2 in Appendix 1.

Additional Brassicaceae species

In order to root Alysseae trees, it was necessary to include outgroup taxa in the study. In order to facilitate sequence alignment, such taxa ideally would be closely related to, but definitely outside the Alysseae. However, given that the sister relationships between the Alysseae and other tribes within the Brassicaceae are uncertain (Beilstein *et al.*, 2006; Warwick *et al.*, 2008; Beilstein *et al.*, 2010; Couvreur *et al.*, 2010), it was considered appropriate to sample a number of species from the

Brassicaceae for potential inclusion in phylogenetic analyses. The inclusion of species from several Brassicaceae tribes was also considered likely to increase the ease and quality of sequence alignment of Alysseae taxa with sequences from species outside the Brassicaceae, which were included as part of the molecular clock-based analyses (described in the following sections). The taxonomy and authorities for all species sampled are detailed in Appendix 1.

Brassicaceae species sampled here from outside the Alysseae included *Iberis saxatilis* (tribe Iberideae) and *Chorispora purpurascens* (tribe Chorisporae) because at the time of sampling there was some evidence that these tribes may be closely related to the Alysseae (Beilstein *et al.*, 2006; Khosravi *et al.*, 2009). Four species formerly included in *Alyssum* were also included in the analysis, because at the time of sampling their phylogenetic relationships were uncertain: *Farsetia stylosa* (tribe Malcolmieae), *Ladakiella klimesii* (tribe Camelinae), *Stevenia tenuifolia* and *S. canescens* (tribe Arabideae). Tissue or extracted DNA was obtained for these species from the sources listed in Appendix 2. Three species, *Thlaspi arvense*, *Pseudocamelina glaucophylla* and *Alliaria petiolata* (all in tribe Thlaspidae), were sampled in order to provide appropriate nodes for age calibration (in this case the stem node of the genus *Thlaspi*, to which *Pseudocamelina* is putatively sister, and *Alliaria* is closely related), as described in following sections. Leaf tissue or extracted DNA was obtained for these species from the sources listed in Appendix 2. Extensive sequence data was available in GenBank for *Arabidopsis thaliana* (tribe Camelinae) and *Lobularia maritima* (tribe Malcolmieae) (the latter genus was a former member of the Alysseae), and was included in the study (described under *Data from Published Sources* below). Sampling was carried out and/or sequences were obtained from GenBank for a total of 10 Brassicaceae species from outside the Alysseae.

Additional Brassicales species

Further sampling from other families within the order Brassicales was carried out in order to allow the inclusion of further nodes of potential use for molecular clock calibration. The inclusion of *Akania bidwillii* and *Bretschneidera sinensis* (both from family Akaniaceae) and *Tropaeolum majus* (Tropaeolaceae) allowed the inclusion of the *Akania* and Akaniaceae stem nodes (because *Akania* and *Bretschneidera* are sister genera in the family Akaniaceae, and Akaniaceae and Tropaeolaceae are sister families (De Craene & Haston, 2006; Beilstein *et al.*, 2010). The inclusion of *Capparis spinosa* (Capparaceae), *Batis maritima* (Bataceae) and *Carica papaya* (Caricaceae) allowed the inclusion of nodes close to the crown node of the Brassicales, which have previously been dated using the fossil plant *Dressiantha bicarpellata* (Franzke *et al.*, 2009; Beilstein *et al.*, 2010; Couvreur *et al.*, 2010). Leaf tissue or extracted DNA was obtained for these species from the sources listed in Appendix 2. Sampling was carried out and/or sequences were obtained from GenBank for a total of six Brassicales species from outside the Brassicaceae.

Non-Brassicales species

In order to root trees that included species from non-Brassicaceae families within the Brassicales (including species from the most basally diverging lineage in that order), it was necessary to include a species closely related-to but definitely outside the Brassicales. *Gossypium hirsutum* and *G. gossypioides* (family Malvaceae, order Malvales) were chosen because this genus fulfils these criteria; there is strong evidence that Malvales is sister to Brassicales (Bremer *et al.*, 2009). Also, abundant genome sequence data are available for *Gossypium* species in GenBank.

Taxonomy, Identification and Voucher Specimens

The taxonomy of the Alyseae used in this study is discussed in detail, and a full list of the species accepted here are included in Appendix 1. The taxonomy is largely based on the Brassicaceae

species checklist of Warwick *et al.* (2006) and *The Plant List* (www.theplantlist.org). The infra-specific taxonomy of the Alyseae is not addressed in detail in this study, and all diversification analyses and trait assignments were carried out at the species level (as described in following sections).

Species identifications of sample material (e.g. from herbarium labels or determination slips and from notes accompanying seed accessions) were verified where possible using descriptions and identification keys in the *Flora Europaea* (Tutin & Ackroyd, 1993), *Flora Iberica* (Casareovioje, 1993), *Flora of Turkey* (Davis, 1965) and Dudley's (1964a) synopsis of *Alyssum*. For 24 samples (representing 20 species) obtained from herbarium specimens at the Royal Botanic Garden, Edinburgh, the actual specimens were not seen. However, all herbarium specimens sampled from this source were scanned (by staff at that institution) and digital images are available via the online herbarium catalogue (<http://elmer.rbge.org.uk/bgbase/vherb/bgbasevherb.php>) and were used for species verification. The majority of the herbarium specimens from Edinburgh have been determined by authorities on Alyseae taxonomy (I. Hedge, R.D. Reeves and T.R. Dudley) and therefore the identification of these specimens is considered likely to be reliable. Voucher specimens were taken for the majority of seed-grown plants and have been deposited in the Oxford Herbaria (OXF), although not all species reached the flowering or fruiting stage often necessary for reliable identification. Herbarium specimen accession numbers are included in Appendix 2. One seed accession labelled as *Alyssum dasycarpum* (accession PI304980, obtained from USDA North Central Regional Plant Introduction Station, Ames, Iowa, USA) was omitted from the study because the plants grown from this accession were clearly identified as *Lobularia maritima* (i.e., the 'sweet alyssum' of horticulture).

Choice of DNA Regions

Between August 2009 and October 2010, DNA sequencing was attempted for all available Alysseae species for four potentially highly variable chloroplast regions: *trnD–trnT*, *trnL–trnF*, 3′*rps16–5′trnK* (hereafter *rps16–trnK*), and *matK* (hereafter ‘the four variable regions’). Marker choice was based on the pilot study described above and in *Chapter 3: Results*. Regions *trnD–trnT* and *rps16–trnK* were chosen for their high variability and relatively straightforward sequencing. Regions *trnL–trnF* and *matK* had a lower variability but were chosen because they were particularly straightforward to sequence, and were considered likely to provide reliable sequence data from the older herbarium tissue that was sampled. In addition, these latter two regions have been widely sampled in plant species from the Brassicaceae, and at the time of sampling this was considered potentially useful in allowing the Alysseae dataset to be combined with published data from the family. Typical sequence lengths for these four chloroplast regions in seed plants are 922, 880, 786 and *ca.* 1024 base pairs (bp), respectively (Koch *et al.*, 2001; Shaw *et al.*, 2007). Between October 2010 and March 2011, in order to expand the phylogenetic analysis, DNA sequencing was also attempted for these four variable chloroplast regions for the above species from outside the Alysseae.

Three additional, potentially more conserved regions were also sequenced for the non-Alysseae species, and also from a set of 14 species from within the Alysseae (except for a minority of sequences which were available from published sources, see *Data from Published Sources* below). These regions (hereafter the ‘three conserved regions’) included: part of the chloroplast gene *rbcl* (which codes for the enzyme Rubisco (Bedbrook *et al.*, 1979)), the chloroplast gene *ndhF* (which codes for the NADH dehydrogenase subunit 5 (Wissinger *et al.*, 1988)), and part of the nuclear gene *PHYA* which codes for the phytochrome A pigment (Hershey *et al.*, 1985). These DNA regions were chosen because they have been shown to be relatively conserved (Savolainen *et al.*, 2000; Beilstein *et al.*, 2008; 2010), a feature that facilitates sequencing across distantly-related taxa, and the

alignment of sequences. Of these three, the *ndhF* region was included in the pilot study, and was found to be relatively straightforward to sequence.

Sequence paralogy could be caused by the processes of lineage sorting, gene duplication/extinction and horizontal transfer (Maddison, 1997). Such processes may cause the incongruence that is sometimes observed in trees constructed using different DNA regions (e.g. Goodson *et al.* 2011; Smedmark & Bremer, 2011; Xu *et al.* 2012), and hence may lead to a divergence between reconstructed and actual phylogenies. Because of the risk of the occurrence of these processes, the majority of recent phylogenetic analyses include multiple sequence regions. Since the process of organelle capture may lead to whole organellar genomes being transferred between lineages (Acosta & Premoli, 2010; Stegemann *et al.* 2012), separate chloroplast regions cannot be regarded as evolutionarily independent. It was therefore considered important to include some nuclear sequence data in this study, in order to allow independent tests of phylogeny. It was partly for this reason that the *PHYA* region was sequenced here; and that published *ITS* data were obtained and analyzed (as described under *Data from Published Sources* below). The use of chloroplast and two separate nuclear regions in this study provides independent corroboration of some parts of the Alysseae phylogeny, in particular the relationships between the major Alysseae clades. Also, the *ndhF* and *PHYA* regions have been used in several Brassicaceae-wide phylogenetic analyses (Beilstein *et al.* 2006; 2008; 2010), allowing the potential for combining published sequence data with sequences obtained here.

Because of their expected low variability, it was not considered appropriate to sequence the three conserved regions for all Alysseae species. The choice of 14 Alysseae species for sampling was based on the results of the initial phylogenetic analyses, and this selection included representatives of the major clades identified within the tribe (the larger clades were represented by multiple

species; Table 2-4). Sequencing of these regions was also carried out for four additional Brassicaceae species from outside the Alyseae.

The taxon sampling described was considered appropriate for a combined (i.e., supermatrix) analysis of the variable and conserved sequence data (see discussion under *Sequence Concatenation*) below.

Table 2-4. List of species and accessions for which sequencing of the three conserved regions (*ndhF*, *PHYA* and *rbcl*) was carried out.

Major Alyseae Clade	Species (& Accession) Sequenced for <i>ndhF</i> , <i>PHYA</i> and <i>rbcl</i>	Major Alyseae Clade	Species (& Accession) Sequenced for <i>ndhF</i> , <i>PHYA</i> and <i>rbcl</i>
A1a	<i>Alyssum corsicum</i> (accession 2)	A4	<i>Hormathophylla longicaulis</i>
	<i>Alyssum lesbiacum</i>	A5	<i>Bornmuellera tymphaea</i>
	<i>Alyssum malacitanum</i>		<i>Physocardamum davisii</i>
A1b	<i>Alyssum blepharocarpum</i>	A6	<i>Aurinia saxatilis</i> (accession 1)
A1c	<i>Clypeola aspera</i>	N/A	<i>Chorispora purpurascens</i>
A2	<i>Alyssum dasycarpum</i>	N/A	<i>Farsetia stylosa</i>
	<i>Alyssum macrocalyx</i>	N/A	<i>Iberis saxatilis</i>
	<i>Alyssum wulfenianum</i>	N/A	<i>Pseudocamelina glaucophylla</i>
A3	<i>Degenia velebitica</i>	Note: Clades A1, A2, A3, A4 and A5 are described in Chapter 1: Introduction. Clades A1a, A1b and A1c are described in Chppter3: Results.	
	<i>Fibigia clypeata</i>		

DNA Extraction, Amplification and Sequencing

DNA extraction, PCR amplification and DNA sequencing procedures were as for the pilot study described above. The plant tissue samples from which DNA was extracted included herbarium specimens and fresh or dried tissue from glasshouse-grown plants (grown from seed accessions). Four seed accessions (of *Alyssum cuneifolium*, *A. oxycarpum*, *Alyssoides utriculata* and *Fibigia triquetra*) failed to germinate, and DNA was extracted directly from the seed and/or fruit material. Many of the tissue samples obtained from herbarium specimens were very small (< 0.5 mg). PCR amplification of the seven target DNA regions was achieved using the DNA oligonucleotide primers listed in Table 2-3. DNA was sequenced directly from PCR products, and purification and cloning

were not carried out. A minimum of two sequence reads (in either the same, or in opposite directions) were attempted for all sections of sequence under investigation in this study, though for a small number of samples (<5%) only a single read was obtained.

Sequence Assembly

DNA sequence assembly was carried out using Sequencher. Sequence ends were cut automatically using the trim ends facility and the stringency settings described for the pilot study. Additional end sections were cut out manually as necessary in order to exclude regions of poor quality sequence.

All assembled and edited sequences were BLAST searched in order to check their broad taxonomic identity. All had close matches with Brassicaceae sequences except for one set, from *A. homalocarpum*. Sequences from this sample had a high level of similarity to published sequences from species in the Asteraceae. This DNA had been extracted from a herbarium specimen (*Collenette* 1985, obtained from the herbarium at the Royal Botanic Garden, Edinburgh) that appeared rather sun-bleached, and a low DNA concentration could have made this sample susceptible to contamination. The Church Laboratory, in the Department of Plant Sciences at Oxford in which all DNA work was carried out, had recently been used for analysis of various tissue types (including pollen) of several *Senecio* species (Asteraceae). Sequence data from this accession was excluded from further analysis.

Data from Published Sources

Variable Regions

Sequence data were obtained from *GenBank* for *Arabidopsis thaliana*, *Lobularia maritima* and *Gossypium hirsutum* for all four of the highly variable chloroplast regions. Sequence data were also obtained from this source for seven other species (Table 2-5).

Table 2-5. Sequence data (GenBank accession numbers) obtained from published sources for four highly variable chloroplast regions.

Species (& Accession)	<i>trnD-trnT</i>	<i>trnL-trnF</i>	<i>rps16-trnK</i>	<i>matK</i>
Brassicaceae				
<i>Alliaria petiolata</i>	-	-	-	AF144363.1
<i>Arabidopsis thaliana</i>	C000932.1	NC000932.1	NC000932.1	NC00932.1
<i>Lobularia maritima</i>	NC009274.1	NC009274.1	NC009274.1	NC009274.1
<i>Thlaspi arvense</i>	-	DQ821410.1	-	GQ424602.1
Other Brassicales				
<i>Batis maritima</i>	-	-	-	AY483219.1
<i>Bretschneidera sinensis</i>	-	-	-	AY483220.1
<i>Capparis spinosa</i>	-	AY122422.2	-	EU371772.1
<i>Carica papaya</i>	-	-	-	AY483221.1
<i>Tropaeolum majus</i>	-	-	-	AY483224.1
Malvales				
<i>Gossypium hirsutum</i>	DQ345959.1	HQ696725.1	NC007944.1	DQ345959.1
NB Dashes (-) indicate that sequence data were not available.				

Conserved Regions

Sequence data were obtained from GenBank for *ndhF* and *rbcL* for a number of non-Alysseae species (Table 2-6).

Table 2-6. Sequence data (GenBank accession numbers) obtained from published sources for *ndhF* and *rbcL*.

Species (& Accession)	<i>ndhF</i>	<i>rbcL</i>
Brassicaceae		
<i>Alliaria petiolata</i>	AF144363.1	FJ395597.1
<i>Arabidopsis thaliana</i>	NC00932.1	AP000423.1
<i>Lobularia maritima</i>	NC009274.1	AP0009375.1
<i>Pseudocamelina glaucophylla</i>	DQ288817.1	-
<i>Thlaspi arvense</i>	GQ424602.1	FN594829.1
Other Brassicales		
<i>Akania bidwillii</i>	-	L13568.2
<i>Batis maritima</i>	AY483221.1	M88341.1
<i>Bretschneidera sinensis</i>	AY483224.1	M95753.2
<i>Capparis spinosa</i>	EU371772.1	AY167985.1
<i>Carica papaya</i>	AY483221.1	-
<i>Tropaeolum majus</i>	AY483224.1	L14706.1
Malvales		
<i>Gossypium hirsutum</i>	U55340.1	X15886.1
NB Dashes (-) indicate that sequence data were not available.		

ITS Data

To date, all phylogenetic studies with significant species-level sampling in the Alyseae have used sequences from the nuclear *ITS* region, as discussed in full in *Chapter 1: Introduction*. *ITS* sequences of species of Alyseae sequenced in the following studies were retrieved from GenBank: Mengoni *et al.* (2003), Warwick *et al.* (2008), German *et al.* (2009) and Cecchi *et al.* (2010). Where available, sequence data for the *ITS1*, *ITS2* and the *5.8S* regions were obtained. An *ITS* sequence for *Arabidopsis thaliana* was also obtained from GenBank in order to allow this species to be employed as an out-group. The full list of GenBank accession numbers is included in Table A2-7.

Sequence data generated in the study of Khosravi *et al.* (2009) had not been published at the time of analysis, and this data was supplied by K. Mummenhoff (one of the authors of the 2009 study). These species are also included in Table A2-7. Since the quality of the *ITS* sequences generated and used by Mengoni *et al.* (2003) have been questioned (Bailey *et al.*, 2006; Warwick *et al.*, 2008), these particular 37 sequences were subject to a separate phylogenetic and pseudogene detection analysis, which is fully detailed in Appendix 5.

Table 2-7. *ITS* sequence data (GenBank accession numbers) obtained from published sources.

Publication	Taxon	Sequence Accession
Cecchi <i>et al.</i> (2010)	<i>Alyssum argenteum</i>	GQ284855.1, GQ284854.1
	<i>Alyssum baldaccii</i>	GQ284857.1, GQ284858.1, GQ284856.1
	<i>Alyssum bertolonii</i>	GQ284868.1, GQ284859.1
	<i>Alyssum bertolonii</i> ssp. <i>scuterinum</i>	GQ284867.1
	<i>Alyssum corymbosoides</i>	GQ284892.1, GQ284878.1
	<i>Alyssum densistellatum</i>	GQ284879.1, GQ284880.1
	<i>Alyssum desertorum</i>	GQ284881.1
	<i>Alyssum euboicum</i>	GQ284882.1
	<i>Alyssum fragillimum</i>	GQ284883.1
	<i>Alyssum heldreichii</i>	GQ284884.1
	<i>Alyssum markgrafii</i> (= <i>A. murale</i>)	GQ284866.1, GQ284864.1, GQ284865.1, GQ284863.1
	<i>Alyssum murale</i>	GQ284887.1, GQ284871.1, GQ284862.1, GQ284870.1, GQ284861.1, GQ284872.1, GQ284873.1, GQ284875.1, GQ284879.1, GQ284877.1, GQ284860.1, GQ284874.1,

Table 2-7. ITS sequence data (GenBank accession numbers) obtained from published sources.

Publication	Taxon	Sequence Accession
		GQ284876.1, GQ284885.1.
	<i>Alyssum orbelicum</i>	GQ284888.1
	<i>Alyssum robertianum</i>	GQ284889.1
	<i>Alyssum sibiricum</i>	GQ284890.1
	<i>Alyssum smolikanum</i>	GQ284891.1
	<i>Alyssum troodi</i>	GQ284893.1
	<i>Aurinia saxatilis</i>	GQ284894.1
	<i>Bornmuellera baldaccii</i>	GQ284895.1
	<i>Leptoplax emarginata</i>	GQ284897.1, GQ284896.1.
	<i>Physoptychis cyclocarpum</i> (= <i>Phyllolepidum cyclocarpum</i>)	GQ284851.1
	<i>Physoptychis rupestre</i> (= <i>Phyllolepidum rupestre</i>)	GQ284852.1
German <i>et al.</i> (2009)	<i>Alyssum dasycarpum</i>	FM164503.1, FM164502.1.
	<i>Alyssum lenense</i>	FM164506.1, FM164507.1.
	<i>Alyssum linifolium</i>	FM164508.1, FM164509.1.
	<i>Alyssum obovatum</i>	FM164511.1, FM164510.1.
	<i>Berteroa incana</i>	FM164646.1
	<i>Fibigia suffruticosa</i>	FM164657.1
	<i>Galitzkya potaninii</i>	FM164524.1, FM164525.1.
	<i>Galitzkya spathulata</i>	FM164534.1, FM164535.1, FM164656.1.
Khosravi <i>et al.</i> (2009)	<i>Alyssoides utriculata</i>	unpublished
	<i>Alyssum bracteatum</i>	unpublished
	<i>Alyssum dasycarpum</i>	unpublished
	<i>Alyssum desertorum</i>	unpublished
	<i>Alyssum heterotrichum</i>	unpublished
	<i>Alyssum inflatum</i> (= <i>Alyssum tortuosum</i>)	unpublished
	<i>Alyssum linifolium</i>	unpublished
	¹ <i>Alyssum marginatum</i> (= <i>A. szovitsianum</i>)	unpublished
	<i>Alyssum polycladum</i>	unpublished
	<i>Alyssum szovitsianum</i>	unpublished
	<i>Clastopus vestitus</i>	unpublished
	<i>Clypeola aspera</i>	unpublished
	<i>Clypeola dichotoma</i>	unpublished
	<i>Clypeola lappacea</i>	unpublished
	<i>Fibigia macrocarpa</i>	unpublished
	<i>Fibigia multicaulis</i>	unpublished
	<i>Fibigia suffruticosa</i>	unpublished
	<i>Fibigia umbellata</i>	unpublished
	² <i>Physoptychis gnaphalodes</i> (= <i>P. caspica</i>)	unpublished
Mengoni <i>et al.</i> (2003)	<i>Alyssum alpestre</i>	AY237957.1
	<i>Alyssum alyssoides</i>	AF401114.1
	<i>Alyssum anatolicum</i>	AY237956.1
	<i>Alyssum argenteum</i>	AY237955.1, AY237955.1.
	<i>Alyssum bertolonii</i>	AY237954.1
	<i>Alyssum bertolonii</i> ssp.	AY237930.1

Table 2-7. ITS sequence data (GenBank accession numbers) obtained from published sources.

Publication	Taxon	Sequence Accession
	<i>scuterinum</i>	
	³ <i>Alyssum biovulatum</i> (= <i>A. obovatum</i>)	AY237953.1
	<i>Alyssum caricum</i>	AY237952.1
	<i>Alyssum condensatum</i>	AY237951.1
	<i>Alyssum corsicum</i>	AY237949.1, AY237950.1.
	<i>Alyssum cypricum</i>	AY237948.1
	<i>Alyssum davisianum</i>	AY237947.1
	<i>Alyssum fallacinum</i> (= <i>A. baldaccii</i>)	AY237946.1
	<i>Alyssum floribundum</i>	AY237945.1
	<i>Alyssum huber-morathii</i>	AY237943.1
	<i>Alyssum lesbiacum</i>	AY237942.1
	<i>Alyssum malacitanum</i>	AY237941.1
	<i>Alyssum minus</i>	AY237939.1
	<i>Alyssum montanum</i>	AY237938.1
	<i>Alyssum murale</i>	AY237936.1, AY237937.1.
	<i>Alyssum nebrodense</i>	AY237935.1
	<i>Alyssum oxycarpum</i>	AY237934.1
	<i>Alyssum peltarioides</i>	AY237932.1, AY237933.1
	<i>Alyssum pinifolium</i>	AY240871.1
	<i>Alyssum pintodasilvae</i>	AY237929.1
	<i>Alyssum pterocarpum</i>	AY237931.1
	<i>Alyssum serpyllifolium</i>	AY237923.1, AY237944.1, AY237922.1
	<i>Alyssum sibiricum</i>	AY237927.1, AY237928.1.
	<i>Alyssum tenium</i>	AY237926.1
	<i>Alyssum virgatum</i>	AY237925.1
	<i>Alyssum wulfenianum</i>	AY237924.1
O'Kane <i>et al.</i> (1996)	<i>Arabidopsis thaliana</i>	U43225.1
Warwick <i>et al.</i> (2008)	<i>Alyssoides cretica</i>	EF514592.1
	<i>Alyssoides utriculata</i>	EF514593.1
	<i>Alyssum alyssoides</i>	EF514595.1, EF514594.1, EF514596.1.
	⁴ <i>Alyssum americanum</i> (= <i>A. obovatum</i>)	EF514600.1, EF514598.1, EF514597.1, EF514599.1.
	<i>Alyssum aureum</i>	EF514601.1
	<i>Alyssum baumgartnerianum</i>	EF514602.1
	<i>Alyssum borzaeanum</i>	EF514603.1
	<i>Alyssum dasycarpum</i>	EF514604.1
	<i>Alyssum desertorum</i>	EF514606.1, EF514607.1, EF514605.1.
	<i>Alyssum lenense</i>	EF514610.1
	<i>Alyssum linifolium</i>	EF514611.1
	<i>Alyssum menioides</i>	EF514612.1
	<i>Alyssum murale</i>	EF514613.1, EF514614.1, EF514615.1.
	<i>Alyssum obovatum</i>	EF514621.1, EF514617.1, EF514618.1, EF514619.1, EF514620.1, EF514616.1.
	<i>Alyssum repens</i>	EF514622.1
	<i>Alyssum serpyllifolium</i>	EF514623.1
	<i>Alyssum simplex</i>	EF514624.1
	<i>Alyssum tortuosum</i>	EF514625.1

Table 2-7. ITS sequence data (GenBank accession numbers) obtained from published sources.

Publication	Taxon	Sequence Accession
	<i>Aurinia saxatilis</i>	EF514630.1, AF401115.1.
	<i>Berteroa incana</i>	AY254544.1, EF514631.1, EF514632.1, EF514633.1
	<i>Berteroa orbiculata</i>	EF514634.1
	<i>Bornmuellera baldaccii</i>	EF514635.1, EF514636.1, EF514637.1.
	<i>Bornmuellera tymphaea</i>	EF514640.1, EF514639.1, EF514638.1.
	<i>Clastopus vestitus</i>	EF514641.1
	<i>Clypeola aspera</i>	EF514642.1
	<i>Clypeola cyclodontea</i>	EF514643.1
	<i>Clypeola jonthlaspi</i>	EF514644.1
	<i>Clypeola lappacea</i>	EF514645.1
	<i>Degenia velebitica</i>	EF514646.1, DQ249857.1.
	<i>Fibigia clypeata</i>	DQ249852.1
	<i>Fibigia macrocarpa</i>	EF514653.1
	<i>Fibigia suffruticosa</i>	EF514654.1
	<i>Galitzkya macrocarpa</i>	EF514655.1
	<i>Galitzkya potaninii</i>	EF514656.1
	<i>Hormathophylla halimifolia</i>	EF514658.1
	<i>Hormathophylla longicaulis</i>	EF514660.1, EF514659.1.
	<i>Hormathophylla reverchonii</i>	EF514661.1
	<i>Hormathophylla spinosa</i>	EF514662.1
	<i>Physoptychis caspica</i>	EF514682.1

Notes to accompany Table 2-7

¹*Alyssum marginatum* Steud. is considered a synonym of *Alyssum szovitsianum* Fisch. & C.A.Mey. by *The Plant List* with medium confidence; the latter is an accepted species in this study (see Appendix 1).

²*Physoptychis gnaphalodes* Boiss. is considered a synonym of *Physoptychis caspica* V.V.Botschantz by *The Plant List* with medium confidence; the latter is an accepted species in this study (see Appendix 1).

³*Alyssum biovulatum* N.Busch is considered a synonym of *Alyssum obovatum* (C.A.Mey.) Turcz. by *The Plant List* with medium confidence; the latter is an accepted species in this study (see Appendix 1).

⁴*Alyssum americanum* Greene is considered a synonym of *Alyssum obovatum* (C.A.Mey.) Turcz. by *The Plant List* with medium confidence; the latter is an accepted species in this study (see Appendix 1). All other synonymy indicated in the table is discussed in Appendix 1.

***ndhF* and *PHYA* Data**

The study of Beilstein *et al.* (2010) included the sequencing and phylogenetic analysis of *ndhF* and *PHYA* data from 144 Brassicaceae species from a wide range of tribes, and a further 32 Brassicales species. Aligned datasets were obtained for both of these regions from M. A. Beilstein.

The *ndhF* dataset supplied contained 173 sequences, representing 171 species. These included no species of Alysseae, 140 species of Brassicaceae, 30 further species of Brassicales and *Gossypium gossypoides*. The *PHYA* dataset supplied contained 136 sequences, representing 134 species,

including 3 species of Alyseae (*Alyssum parviflorum* (= *A. simplex*), *Clypeola aspera* and *Fibigia suffruticosa*), 118 further species of Brassicaceae, 12 further species of Brassicales, and *Gossypium gossypioides*.

Sequence Alignment

Sequence data for separate sequence regions were aligned separately. For *trnD–trnT*, *trnL–trnF*, *rps16–trnK*, *matK* and *rbcl*, all sequences were loaded into the MEGA alignment Explorer (one alignment per DNA region) and approximately aligned using ClustalW (default settings). Uniform sequence orientation was achieved at this stage using the reverse complement command on groups of sequences that were clearly highly divergent. Sequence alignments for *trnD–trnT*, *trnL–trnF*, *rps16–trnK* and *matK* were then aligned using MUSCLE (default settings). Due to the highly conserved nature of the *rbcl* sequences, alignment in MUSCLE was considered unnecessary and this step was omitted. Alignments were visually checked in MEGA and manually edited in order to correct obvious alignment errors.

Sequence data obtained here for *PHYA* and *ndhF* were added to the Brassicaceae-wide alignments obtained from M. A. Beilstein (as used in Beilstein *et al.*, 2010), and aligned using ClustalW in MEGA. Due to high levels of sequence conservation, alignment in MUSCLE was considered unnecessary for these datasets.

Alignment Quality and Editing

Alignments were annotated in MEGA by adding primer sequences and by adding *Arabidopsis thaliana* reference sequences from GenBank (see Tables 2-6 and 2-7). This annotation was carried out in order to verify the correct orientation and identity of sequences, and to allow the identification of amplification primer regions for removal. Annotated alignments for each DNA region were subsequently edited to remove these annotations, regions of poor alignment (typically at the ends of the sequence region) and external primer regions. Sections of sequence that were

cut from the alignment are described in the following paragraphs. The base position numbering used here relates to positions in the annotated alignments. Sections of sequence described here are inclusive of the end base positions, which are stated.

Coding regions (*matK*, *PHYA*, *ndhF* and *rbcL*)

These regions aligned without problems. It was not necessary to exclude any sections/sequences due to poor alignment. Sequence quality was checked for these coding regions by double-checking chromatograms for all frame-shift indels and stop codons. All frame-shift indels detected (a total of *ca.* 70 for all regions and sequences) were found to be chromatogram reading errors and were corrected. A total of 66 stop codons were found, thought to be the result of chromatogram reading errors; of these 30 were corrected manually, and 36 could not be corrected (due to ambiguous chromatograms) and were removed by sequence end-trimming of the first 5 to 16 bases from these sequences (alignment positions 1–70 bp). The intron region in the *PHYA* sequence (at 1547–1744 bp inclusive) was identified from the *Arabidopsis thaliana* reference sequence and was excluded from the alignment for all sequences. End-trimming removed the following sections: *ndhF*: 1–63 and 2132–2318 bp; *matK*: 1–58 and 1123–1166 bp; *rbcL*: 1–32 and 1376–1447 bp; *PHYA*: 1–33 and 1956–2035 bp.

ITS

The *ITS* region as usually sequenced consists of two spacer regions (*ITS1* and *ITS2*) separated by a shorter rDNA (*5.8S*) which codes for the 5.8S rRNA. The latter region is sometimes removed for phylogenetic analyses because it is highly conserved. Here the entire *ITS* region (i.e. including the *5.8S* region) was used in analyses, except for the sequences obtained from the Khosravi *et al.* (2009) study for which this section had been removed. Much of the *ITS* region was relatively conserved in the sequences analyzed here, but the two following sections (within the *ITS1* and *ITS2* spacer regions respectively) aligned poorly and were removed: 150–194 bp and 583–651 bp. End-trimming removed sections 1–41 and 1008 bp onwards. The following sequences aligned poorly in

some regions of the alignment and were removed for the following sections: *Alyssum dasycarpum* (from Khosravi *et al.*, 2009; no GenBank accession number) and *Aurinia rupestris* (= *Phyllolepidum rupestre*) (GenBank accessions GQ284851 and GQ284852) 826 bp onwards; *Aurinia saxatilis* (GQ284894) 821 bp onwards; *Bornmuellera baldaccii* (GQ284895) 911 bp onwards; *Fibigia multicaulis* and *F. umbellata* (both from Khosravi *et al.*, 2009; no GenBank accession number) 323–610 bp; and *Leptoplax emarginata* (GQ284896 and GQ284897) 911 bp onwards. The following sequences had long strings of missing data (i.e. strings of N characters) towards one end of the alignment and these sections were removed: *Alyssum corymbosoides* (GQ284892) 802 bp onwards; *Alyssum orbelicum* (GQ284888) 911 onwards; *Alyssum murale* (GQ284886) 663 bp onwards; and *Alyssum robertianum* (GQ284889) 541 bp onwards.

Since all of the the *ITS* sequences obtained in this study (all of which were from published sources) were obtained from separate individual plants (i.e. no accessions were repeat sequenced, e.g. using cloning), it was not possible to investigate the presence or absence of intra-individual variation in *ITS* in Alysseae.

trnL-trnF

The majority of this region consists of the *trnL* intron plus part of the *trnL-trnF* intergenic spacer (IGS). The *trnL* region aligned without problems for all accessions. The following three sections of the IGS region aligned poorly and were excluded from the alignment for all sequences: 776–782, 796–851 and 941–1026 bp. End-trimming removed sections 1–63 and 1347–1379 bp.

rps16-trnK

Because the majority of this region consists of highly variable IGS it contains many indels, including many repeated regions of variable length. Homology of the following sections of the alignment was uncertain and they were excluded for all sequences: 212–225, 298–316, 300–414, 791–812, 870–1270 and 1419–1457 bp. The following sequences aligned poorly towards one end of the alignment

and for the following sections were removed: *Alyssum umbellatum* and *Alyssum simplex* (accession 1): 1–1270 bp; *Aurinia saxatilis* (accessions 1 and 2) and *Aurinia corymbosa*: 1–1207 bp; *Alyssum niveum* and *Alyssum thymops*: 941 bp onwards, *Alyssum propinquum*: 982 bp onwards. Non-Brassicaceae accessions aligned poorly from 414 bp onwards and were therefore excluded from the alignment beyond this point. End-trimming removed sections 1–73 and 1636–1832 bp.

trnD–trnT

This region consists of relatively conserved tRNA coding regions (*trnT*, *trnE*, *trnY* and *trnD*) alternating with highly variable IGS regions. The following sections of the alignment aligned poorly and were excluded for all sequences: 597–669, 1632–1666, 1847–1868 and 2151–2184 bp. End-trimming removed sections 1–52 and 2295–2469 bp. The sequence for *Alyssum thymops* aligned poorly towards one end of the alignment and was removed from 1021 bp onwards.

Non-contiguous & Partial Sequences

Across all seven DNA regions sequenced here, 39 sequences were non-contiguous (i.e. were built from fragments that do not meet, and hence contain artifactual gaps). These include 1 from *matK*, 1 from *rbcl*, 4 from *ndhF* and 34 from *trnD–trnT* (and none from *PHYA*, *rps16–trnK* and *trnL–trnF*). Many more of the sequences are partial because short regions (typically 10–30 bp) at one or both ends are missing; this creates artifactual gaps on concatenation of sequences. Although these gaps represent missing data, they are not expected to bias or otherwise adversely affect phylogenetic analyses because indel characters were not employed in subsequent analyses. Indel characters were not evaluated because utilizing this data in maximum likelihood and Bayesian phylogenetic analyses is both methodologically and theoretically problematic due to the lack of robust models for the evolution of gap characters. In addition, the number of DNA sequences that were trimmed, or for which parts were excluded for some sequences (a consequence of the use of highly variable markers, for which alignment was problematic for some regions), would be likely to make gap coding problematic due to the presence of artifactual gaps in the alignment.

Alignment End-trimming

The ends of the alignment were trimmed as indicated above to exclude all external amplification primer regions, and to remove sections represented by very few (i.e. <5) taxa. The editing described in the paragraphs above resulted in a final alignment for each DNA region. Key information about the size and taxonomic coverage of these alignments is included in Table 2-8, further details are included in *Chapter 3: Results*.

Table 2-8. Alignment lengths, numbers of DNA sequences and accessions, and total dataset size for the eight DNA regions investigated.

DNA Region	Alignment length (bp)	No. of sequences (No. of accessions)					Total Dataset Size (bp)
		Alysseae	Further Brassicaceae	Further Brassicales	Ex-Brassicales	Total	
<i>ITS</i>	721	181 (88)	1 (1)	0 (0)	0 (0)	182 (89)	92 272
Variable Regions							
<i>matK</i>	1064	166 (181)	10 (10)	5 (5)	1 (1)	182 (197)	173895
<i>rps16-trnK</i>	987	160 (178)	9 (9)	6 (6)	1 (1)	176 (194)	191478
<i>trnD-trnT</i>	2054	164 (188)	8 (8)	5 (5)	1 (1)	178 (202)	414908
<i>trnL-trnF</i>	1133	164 (178)	10 (10)	5 (5)	1 (1)	180 (194)	219802
Conserved Regions							
<i>ndhF</i>	2068	13 (13)	143 (146)	30 (30)	2 (2)	188 (191)	394988
<i>PHYA</i>	1724	13 (13)	120 (122)	13 (13)	1 (1)	147 (149)	256876
<i>rbcl</i>	1343	12 (12)	7 (7)	6 (6)	1 (1)	26 (26)	34918

Sequence Concatenation

These alignments were subject to separate phylogenetic analysis and to concatenation followed by phylogenetic analysis (see *Phylogenetic Analysis* below). Prior to concatenation, alignments were padded with blank sequences as necessary to ensure that all had the same number of rows, and rows were ordered identically for all regions. Sequences were then concatenated by copying and pasting alignments one after the other into a new concatenated alignment file in the MEGA Alignment Explorer. Alignments for the above regions were concatenated as detailed in the following paragraphs in order to produce the three concatenated alignments, A–C described below. *ITS* sequences were excluded from concatenated alignments. This was done because of uncertainty about the reliability of some of the published *ITS* sequence data for *Alyssum*, as

discussed in *Chapter 1: Introduction*. Exclusion of *ITS* data gives the present study a high degree of independence from previous phylogenetic work in the Alysseae.

The concatenation of sequence alignments for different DNA regions and containing different sets of taxa carried out is consistent with the ‘supermatrix approach’ (sometimes termed the ‘total evidence approach’) frequently used in modern phylogenetic analyses (reviewed by de Quieroz & Gatesy, 2007). This method appears to be replacing the ‘supertree approach’, in which multiple phylogenetic trees were combined in order to produce a single combined tree (von Haeseler, 2012). The supermatrix approach maximizes the availability of phylogenetic information (whereas there can be considerable loss of information in supertree analyses), and is generally robust to high proportions of missing data caused by incomplete coverage of DNA regions across taxa, so long as there is a common set of DNA regions in the dataset that can generate a scaffold, and taxa are not represented by single regions that show low phylogenetic signal (which can result in ‘rogue’ taxa and loss of resolution) (McMahon and Sanderson 1996, Wiens & Morrill, 2011). The approach is particularly useful for combining sets of DNA sequences collected at different phylogenetic levels, or having different evolutionary rates – for example, to combine a well-sampled species-level dataset with a genus- or family-level dataset. This can be achieved through the use of a limited number of representative or placeholder taxa common to both datasets, whilst the majority of taxa in the two datasets may differ. Supermatrices have frequently been used to combine nuclear and organellar sequence data (e.g. Beilstein *et al.* 2006, 2010; Marquinez *et al.*, 2009; Fisher-Reid & Wiens, 2011; Perez-Gutierrez *et al.*, 2012). However, in concatenating sequences from different DNA regions, a key issue is the extent to which a single phylogenetic analysis of multiple genes may hide incongruities between the different DNA regions. Such incongruities are not uncommon between nuclear sequences (e.g. Baker *et al.*, 2011), and between nuclear and chloroplast sequences (e.g. German *et al.*, 2011; Tippery & Les, 2011; Willyard *et al.*, 2011), although incongruence between chloroplast regions is generally not expected due to the lack of

recombination in chloroplast genomes (Birky, 1995). A common precautionary approach is to carry out separate phylogenetic analyses for each of the component DNA regions in order to assess congruence. The trees produced may be assessed qualitatively, by inspection, or through various quantitative methods (e.g. the incongruence length difference (ILD) test of Farris (1995)), although the latter generally require the separate DNA regions to have a high level of overlap in taxon sampling. In addition the ILD test has been criticized for a number of reasons (e.g. its limited power for datasets exhibiting heterogeneity in site-to-site substitution rates (Darlu & Lecointre, 2001), its inability to predict the utility of concatenation of datasets (Barker and Lutzoni, 2002), and its inability to identify clear cases of incongruence (Yoder *et al.*, 2001)), and is now little used. In this study, separate trees were constructed for all of the eight DNA regions analyzed. Tree congruence was then assessed qualitatively, by inspection, before concatenation (as discussed in *Chapter 3: Results*).

Concatenated Alignment A

The alignment included all of the 189 Alysseae accessions (representing 170 Alysseae species) sequenced in this study. It also included 11 further Brassicaceae and 6 further Brassicales species sequenced here or obtained from GenBank. *Gossypium hirsutum* was also included. This gave a total of 207 sequences. The alignment included sequence data for all seven DNA regions sequenced in this study (i.e. *matK*, *ndhF*, *PHYA*, *rbcl*, *rps16-trnK*, *trnL-trnF* and *trnD-trnT*). The total alignment length was 10 342 bp. For *matK*, *rps16-trnK*, *trnD-trnT* and *trnL-trnF*, sequence data were included (where available) for all accessions in the alignment. For *ndhF*, *PHYA* and *rbcl*, sequence data were included (where available) for only the non-Alysseae species plus the 14 representatives of the major clades of the Alysseae; data were therefore not included for these three DNA regions for the majority of Alysseae species. Missing data constituted 50% of the alignment. This alignment was produced in order to allow an Alysseae-wide phylogenetic analysis, with a simultaneous primary

molecular clock calibration based on published ages of three fossils from the Brassicales. Trees produced using this alignment were rooted on the Brassicales–*Gossypium hirsutum* branch.

Concatenated Alignment B

This alignment was produced by removing all non-Alysseae species from Alignment A, with the exception of *Iberis saxatilis*. Of the Brassicaceae species sampled here, the latter species was consistently outside (yet closely-related to) the Alysseae (see *Chapter 3: Results*) and was retained in this alignment for tree-rooting. This alignment contained a total of 190 accessions (representing 170 Alysseae species and *Iberis saxatilis*). The alignment had a total length of 10 342 bp. In order to facilitate sequence partitioning, gap-only sites that arose from removal of the non-Alysseae sequences were not deleted from the alignment. Missing data constituted 52% of the alignment. This alignment was produced in order to allow an Alysseae-wide phylogenetic analysis, with a simultaneous secondary molecular clock calibration based on published age estimates for the Alysseae crown node. Trees produced using this alignment were rooted on the Alysseae–*Iberis saxatilis* branch.

Concatenated Alignment C

This alignment included all 180 accessions (representing 177 Brassicales species and *Gossypium gossypioides*) included in the Brassicaceae-wide analysis of *PHYA* and *ndhF* of Beilstein *et al.* (2010). To this were added *ndhF* and *PHYA* sequences (where available) for the 14 Alysseae species representing the major Alysseae clades, the three additional Brassicaceae species (listed in Table 2-4), and *Gossypium hirsutum*. The total alignment therefore contained 198 accessions. The total alignment length was 3 713 bp. Missing data (from all sources, including indels) constituted 19% of the alignment. This alignment was produced for the first part of a two-stage calibration procedure for the Alysseae. This involved a Brassicales-wide analysis with a primary clock calibration to estimate the ages of key nodes in the Alysseae. In the first stage, trees were rooted on the

Gossypium–Brassicales branch. The second part of this analysis employed Concatenated Alignment B and trees were rooted on the *Iberis saxatilis*–Alysseae branch.

Geographic and Character Data

Tissue Sample Collection Locations

Descriptions of sample collection locations were obtained from herbarium/seed accession labels including latitude/longitude where available. For samples without latitude/longitude information, these values were obtained from location descriptions using Google Earth (version 6.2.2.6613) (Google Inc.) mapping software and/or the UN Gazetteer (<http://dma.jrc.it/services/gazetteer/>).

Nickel Hyperaccumulator Status

Nickel hyperaccumulator status data were collected from the literature (where available) for all species of Alysseae; full lists of publications consulted and data obtained are provided in Appendix 3. Nickel hyperaccumulator status was coded at the species level using two separate regimes. Under the first character-state coding regime, all Alysseae species were coded as *nickel hyperaccumulators* (character state 1), *non-accumulators* (character state 0), or *missing data* (symbol -). The Reeves (1992) definition of a nickel hyperaccumulator was used to distinguish the two states, in which a species is deemed to be a nickel hyperaccumulator if at least one wild-collected plant has been recorded with $> 1000 \text{ mg kg}^{-1}$ nickel content by dry mass in an aerial part. Under the second character-state coding regime, all species were coded as *hyperaccumulators* (character state 1), *non-accumulators* (character state 0), *polymorphic species* (character state 2) or *missing data* (symbol -). Under this system, hyperaccumulators were those species for which all wild-collected specimens had nickel content $> 1000 \text{ mg kg}^{-1}$. Non-accumulators had $< 1000 \text{ mg kg}^{-1}$ for all wild-collected specimens, and polymorphic species had one or more specimen above and one or more below the 1000 mg kg^{-1} threshold value for nickel. Maximum recorded nickel content

was also recorded for all species. Due to variation in the form of published values (which variously included individual measurements, means, medians and ranges) it was not possible to determine mean published values for the nickel shoot content of each species.

Leaf tissue samples were available here for four species for which shoot nickel content has not been reported in the literature (*Alyssum flahaultianum*, *Chorispura purpurascens*, *Fibigia umbellata* and *Physocardamum davisii*), and for nine species reported as non-accumulators but for which few (i.e., only one or two) measurements have ever been made (*Alyssum aizoides*, *A. filiforme*, *A. fragillimum*, *A. haussknechtii*, *A. mouradicum*, *A. nebrodense*, *A. obtusifolium*, *A. polycladum* and *A. tetrastemon*). Nickel content was therefore measured for these samples here. Samples of two additional species (*A. cilicicum* and *A. dubertretii*) were included as positive controls, and samples of two further species (*A. stribryni* and *A. umbellatum*) were included as negative controls. These samples were subject to digestion in concentrated (69%, v/v) nitric acid and then to atomic absorption spectrophotometry using the apparatus and procedure described by Roosens *et al.* (2003). This analysis was carried out by J.A.C. Smith and E. Bailes at the Department of Plant Sciences, University of Oxford.

Serpentine endemic status

Serpentine endemic status data were collected where available from the literature for all Alyseae species; full lists of publications consulted and data obtained are provided in Appendix 3. Serpentine endemic status was coded using the following criteria: *serpentine endemic* (character state 1), *non-endemic* (character state 0), or *missing data* (symbol -). Serpentine endemics included all species reported only from serpentine substrates. Species recorded from non-serpentine substrates (typically limestone or gypsum), even though they may also have been reported from serpentine, were categorized as *serpentine non-endemics*. Species for which floras,

herbarium specimens or other literature make no specific mention of serpentine endemism were coded as missing data.

Life-History Strategy

Life-history strategy data were collected where available from the literature for all Alysseae species; full lists of publications consulted and data obtained are provided in Appendix 3. Life-history strategy was coded using the following criteria (based on Drummond *et al.*, 2012): *semelparous species* (including annuals and biennials; character state 1), *iteroparous species* (i.e., perennials; character state 0), or *missing data* (symbol -). There were some conflicts in the literature consulted. Species reported as both annuals and biennials were categorized as semelparous. Species reported as both biennials and perennials were categorized as iteroparous species.

Species Range Area

For Alysseae species restricted to Europe, a measure of species range area (at the 50 km × 50 km scale) was determined by counting the number of 50 km x 50 km grid squares included in the native range (represented as individual dots in the species distribution maps of *Atlas Flora Europaea* Vol. 10: Jalas & Suominen, 1994). The full dataset is provided in Appendix 3.

Linear Range Size

Since detailed estimates of species range area were only possible for species restricted to Europe (distribution maps of a similar quality do not exist for the majority of areas in which Alysseae species are present), a second, simpler measure of range size was also employed. For all Alysseae species, including those in the *Atlas Flora Europaea*, a linear range size was estimated by measuring the distance connecting the two most distant points (as measured by minimum surface distance using Google Earth) on the perimeter of their geographic range. Species distributions were

determined from the published literature (typically floras), including both distribution maps and descriptions. For each species the geographic distribution is summarized in text, and the longitude and latitude of the two measurement points was recorded. For many species endemic to Turkey, species distributions have been described using the $2^{\circ} \times 2^{\circ}$ degree grid system detailed in the *Flora of Turkey* (Davis, 1965), and the minimum resolution possible was the diagonal distance across one of these cells (typically around 280 km, but varying with latitude). In order to minimize the effect of bias resulting from such variation in the minimum level of resolution of these data, a minimum value of 290 km was employed for linear range size in all analyses except for the correlation described below. Data were not available on the limits of the geographic distribution of 63 (i.e. 25%) of the 255 Alyseae species accepted here (as indicated in Appendix 3); these species were therefore excluded from this analysis. Two additional species were excluded from the analysis: *Alyssum bertolonii*, because it has a strongly disjunct distribution (it is present on serpentine formations in north-west Italy and in the western Balkans), and *A. alyssoides* because its native range is obscured by widespread introductions. Full lists of publications consulted and data obtained are provided in Appendix 3.

Range Area and Linear Range Size Correlation

In order to determine the extent to which estimated linear range size (km) is a useful predictor of species range area (no. of 50×50 km grid squares in which present), a linear regression and Pearson correlation analysis of these two variables were carried out in R (version 2.15.0) (www.r-project.org) using $\log_{10}$ transformed data, and residual values were plotted in order to assess normality.

Phylogenetic Analysis

Evolutionary Model Selection

The suitability of 24 evolutionary models for modelling sequence evolution in the eight DNA regions was assessed using JModelTest (version 0.0.1) (Posada, 2008). Substitution schemes used included JC, HKY and GTR, each employing all possible combinations (i.e., eight) of up to three additional parameter sets: F (estimated base frequencies), I (proportion of invariant sites), and Γ (distribution of rates shape parameter). These combinations therefore include the F81, K80 and SYM models. Maximum likelihood was used to produce the base tree for likelihood calculations. Due to the large size of the alignments and the abundance of gap data (due to both indels and missing data), it was not possible to test a larger number of models within a reasonable period of time. For the same reasons, hierarchical likelihood ratio tests were not performed. Alignments for the seven separate DNA regions were tested separately.

Maximum Likelihood-based Analysis (RAxML)

Maximum likelihood trees were produced for all seven DNA regions sequenced in this study, for the *ITS* alignment, and for Concatenated Alignments A and B using RAxML (version 7.2.8 Black Box) (Stamatakis *et al.*, 2008), accessed online via the Phylobench server (at <http://phylobench.vital-it.ch/>) or the Teragrid computer grid, accessed via the CIPRES Science Gateway (<http://www.phylo.org>). Only the GTR model is available in the online version of this program. For all DNA regions for which Γ and I parameters were present in the model chosen by AIC scores (i.e. *ITS*, *matK*, *ndhF*, *trnL-trnF* and *trnD-trnT*), these two parameters were included in the RAxML model. For the remaining three regions (*PHYA*, *rbcL* and *rps16-trnK*) the I parameter was not included in the model chosen by AIC scores, and therefore the GTR + Γ model was used in the RAxML analysis. For Concatenated Alignment A, the GTR + Γ + I model was employed, and parameters were optimized separately for each of the 7 DNA regions. Branch support values were obtained *via* rapid bootstrapping within RAxML.

Parsimony-based Analysis (WinClada)

Parsimony trees were constructed for Concatenated Alignments A and B using NONA (version 2) (Goloboff, 1999) run from within WinClada (version 1) (Nixon, 1999). A 1000-replication tree search was performed, with 5 starting trees and 50 trees kept per replication. The TBR + TBR branch-swapping search strategy was employed. Branch support values were obtained via 1000-replicate bootstrap analyses, with 5 starting trees and 10 trees searched per replication, with other settings in default positions. When more than one most parsimonious tree was found, results were summarized on a strict consensus tree indicating bootstrap support values.

Bayesian Analysis (MrBayes)

Bayesian inference phylogenetic analyses were carried out for Concatenated Alignments A and B using MrBayes (version 3.1.2) (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) run on the Teragrid computer grid, accessed *via* the CIPRES Science Gateway (<http://www.phylo.org>). The GTR model was employed for all DNA regions for which this model was chosen by AIC scores (i.e. all but *PHYA*). The HKY model was employed for *PHYA*. The Γ parameter was included for all regions. The invariant sites parameter I was included for *ndhF*, *matK*, *trnD-trnT* and *trnL-trnF*. For Concatenated Alignment A, the GTR + Γ + I model was employed, and parameters were optimized separately for each of the 7 DNA regions. Two runs of eight MCMC chains were used with a heating parameter of 0.02. Chains were sampled every 100 steps. Convergence of separate MCMC chains was monitored via the plot of tree posterior probability against step number (no upward trend indicates convergence), and the average standard deviation of split frequencies was (a value <0.05 indicates convergence). Analyses were run employing 100 000, 2 million and 5 million MCMC steps. Run times were *ca.* 50h. Convergence of MCMC chains was not achieved for either Concatenated Alignment A or B after 5 million MCMC steps (final standard deviations of split frequencies were 0.135 and 0.148 respectively). It was not considered necessary to run longer analyses in MrBayes

(in order to achieve convergence of MCMC chains) because it was decided to carry out Bayesian inference of phylogenies using a BEAST (see *Molecular clock-based analyses using beast*, below).

Tree Rooting

WinClada, RAxML and MrBayes trees were rooted as follows. The *ITS* alignment contained only Alyseae species plus *Arabidopsis thaliana* (Camelineae); *ITS* trees were therefore rooted on the branch connecting *A. thaliana* to the remainder of the tree. Alignment B contained only Alyseae species plus *Iberis saxatilis* (tribe Iberidae) and was rooted on the branch connecting *I. saxatilis* to the remainder of the tree. The remaining alignments contained Brassicales species plus *Gossypium gossypioides* (Malvales) (*ITS* and *PHYA*) or *Gossypium hirsutum* (Malvales) (remaining DNA regions). These trees were therefore rooted on the *Gossypium*–Brassicales (i.e. Malvales–Brassicales) branch.

Molecular Clock-based Analysis using BEAST

Use of Molecular Clocks to Estimate Lineage Divergence Times

Molecular clock-based techniques are used to estimate divergence dates of lineages, and provide phylogenies with a time-calibrated axis. This allows phylogenetic structure to be interpreted in a historical context, and is essential for the analysis of species diversification rates. The concept of a molecular clock was based on observations that differences between macromolecular sequences are related to the amount of time since their divergence (Zukerkandl & Pauling, 1965). Whilst many studies have shown that strict (i.e., regular) molecular clocks are rare, the use of more flexible molecular clock models ('relaxed molecular clocks') is now widespread in phylogenetic analysis (reviewed by Renner, 2005), subject to the availability of suitable calibrations, which may take the form of primary calibrations (i.e., direct fossil-based calibration of nodes within tree) or secondary calibrations (i.e., use of estimates of node age or rate of sequence change obtained from other

phylogenetic studies). Relaxed molecular clocks allow clock rate variation between branches, and increasingly this variation is being modelled using probability distributions which are parameterized during the analysis. In a relaxed uncorrelated lognormal molecular clock model (Drummond *et al.*, 2006), the clock rate on any branch is independent of other branches, and is distributed as a lognormal distribution, the mean of which represents the average rate of molecular change across the tree. The computer program BEAST (Bayesian Evolutionary Analysis Sampling Trees; Drummond & Rambaut, 2007) is increasingly used for Bayesian phylogenetic analyses employing molecular clocks because of its power, flexibility and ease of use. Here, a relaxed uncorrelated lognormal molecular clock model was used in BEAST (version 1.6.1) to simultaneously infer phylogeny and divergence dates within the Alysseae.

Fossil Calibration of Molecular Clocks

The placement of fossil-based calibration dates onto phylogenetic trees has received much attention in the literature. It is widely agreed that fossils can only set minimum ages for clades, because the earliest known fossil for a group is likely to post-date the actual age of the group by an unknown but possibly substantial period of time. Whether the fossil-derived date should be applied to the crown or the stem node of a clade is more problematic. A common approach has been to apply the date to the stem node, since plant fossils are usually only fragmentary and therefore only some of the characters which define a clade can be assessed. It is possible that characters that are not available for analysis may not match the crown group character-state suite, and therefore the fossil represents a lineage in the stem group (Magallón & Sanderson, 2001; Magallón, 2004). However, as Smith *et al.* (2010) have pointed out, this approach may bias analyses towards younger dates for nodes, since in some cases the crown node may well be a more appropriate node for fossil-derived date calibration. Unfortunately, there is rarely a justifiable reason to place the fossil at the crown node, and such a placement could therefore cause the opposite bias (i.e., the minimum bound used may, in fact, be older than the real minimum age for a

clade). As a precaution, Rutschmann *et al.* (2007) proposed and used a ‘multicalibration’ approach which compared the effects of applying dates to different nodes within a phylogeny. However, fossil dates can be used with confidence as minimum ages for stem nodes (which cannot be younger than a fossil from that clade, but not for the crown nodes, which could possibly be younger). This approach, in which stem node age is modelled probabilistically above a minimum bound, has been used in a number of studies in the angiosperms (e.g. Magallón & Castillo, 2009; Simon *et al.*, 2009; Bell *et al.*, 2010). Typically, a lognormal distribution is employed for primary calibrations, because it allows the mode to be offset somewhat above the minimum bound (minimum ages of fossils are generally expected to be somewhat older than the minimum age of the taxon they represent due to the low likelihood of the fossilization process) combined with a rapidly decaying probability that has no hard upper bound. Secondary calibrations, in which an estimated age and associated 95% confidence interval are used to constrain a node in the analysis, are typically modelled using a normal distribution. However, as Ho & Phillips (2009) have pointed out, at least one calibration point in an analysis does require a hard upper bound (or a point calibration) in order to avoid divergence times taking arbitrarily high upper values.

Phylogenies in the Brassicaceae have been subject to a limited number of options for fossil calibrations. Franzke *et al.* (2009) used a secondary calibration to date their Brassicales tree. They used the published age estimate for the Moringaceae/Brassicaceae split (*ca.* 70 Mya) that was obtained by Wikström *et al.* (2001) in their angiosperm-wide tree. (The latter tree was calibrated using the fossils *Protofagacea* (Herendeen *et al.*, 1995) and *Antiquacupula* (Sims *et al.*, 1998), which show a number of affinities with the Fagales, to fix the age of the Cucurbitales/Fagales split at 84 Mya.) These authors suggested that the *Dressiantha bicarpellata* fossil (the oldest known fossil flower) (*ca.* 90 Mya: Gandolfo *et al.*, 1998) provides indirect support for this calibration, since it has been assigned to the Brassicales and shows affinities with *Moringa*. In a separate analysis, these authors also used the age of the *Rorippa* (Mai, 1995; publication not seen) fossil to constrain

the *Rorippa/Cardamine* split to 2.5–5.0 Mya, but considered that this resulted in an unreasonably high age estimate for the Brassicaceae. Couvreur *et al.* (2009) used the *Dressiantha bicarpellata* fossil as a primary calibration point to date their Brassicales tree. They constrained the Brassicales crown node to a normal distribution with a mean of 89.5 Mya (95% CI: 86–91 Mya). They argued that using this date as the mean of a normal prior distribution (rather than as a minimum age for the split) better incorporates uncertainty about fossil age. Beilstein *et al.* (2010) reviewed the utility of 21 fossils in a number of orders for the calibration of their Brassicales tree, and chose to employ the following: *Dressiantha bicarpellata*, three *Akania* fossils (47–61 Mya; Romero & Hickey, 1976; Gandolfo *et al.*, 1988; Iglesias *et al.*, 2007) and *Thlaspi primaevum* (ca. 30 Mya; Becker *et al.* 1961; Wing 1987). They noted that the fossil pollen reported from *Rorippa* is not verifiable, and did not consider its use in molecular clock calibration justified. Their use of the above fossils is discussed in more detail in the following sections.

Fossil Calibrations Employed Here

In this analysis, the lower bounds of the *Akania* sp. (Iglesias *et al.*, 2007) and *Thlaspi primaevum* (Manchester & O’Leary, 2010) fossil age estimates were used as minimum age limits for the *Akania* stem node and the *Thlaspi* stem node, respectively. The *Dressiantha* fossil was used as a minimum age for the Brassicales crown node.

***Akania* sp.**

The *Akania* sp. fossil was collected from the Danian (Paleocene) Formation of Palacio de les Loros in southern Chubut, Argentina, and its generic assignment is supported by leaf shape and venation pattern (Iglesias *et al.*, 2007). The age of this fossil is constrained to 61.7 ± 0.2 Mya (via the magnetic polarity chron 26–27 boundary: Gradstein *et al.*, 2004; Iglesias *et al.*, 2007). Here, the lower bound of this age estimate was used as a minimum age for of the *Akania* stem node. This is the node at which the monospecific genus *Akania* and its monospecific sister genus *Bretschneidera*

converge. This sister relationship is strongly supported in the molecular phylogenetic study of Gadek *et al.* (1992).

Additional fossils from *Akania* (*A. americana* (Gandolfo *et al.*, 1988) and *A. patagonica* (Romero *et al.*, 1976)) were employed in the fossil-calibrated phylogeny of Beilstein *et al.* (2010). In that study, these two latter fossils were used to provide a minimum age of 47 Mya for the *Akania* stem node, whilst the *Akania* sp. fossil was used to provide a minimum age of 61 Mya the Akaniaceae stem node (the node joining Akaniaceae with its sister family, Tropaeolaceae (a relationship supported by the angiosperm-wide analysis of Bremer *et al.* (2009))). This procedure was not used here because it is considered that the date of the *Akania* sp. fossil can be more informatively used as a minimum age for the *Akania* stem node than the Akaniaceae stem node, and therefore that the minimum age provided by the *A. americana* and *A. patagonica* fossils is redundant. The procedure used by Beilstein *et al.* (2010) may have caused an underestimation of node ages throughout their tree.

Thlaspi primaevum

The *Thlaspi primaevum* fossil was collected from the Oligocene Ruby Basin flora of southwestern Montana, USA (Becker, 1961) dated 30.8–29.2 Mya (Wing, 1987). The assignment of this fossil to *Thlaspi* (Brassicaceae, tribe Thlaspideae) by Becker (1961) was based on its angustiseptate winged fruits. The generic assignment of this fossil has been questioned by Franzke *et al.* (2011), who note the convergence of winged fruits in several unrelated angiosperm families, and within the Brassicaceae itself. However, Manchester & O’Leary (2010) and Beilstein *et al.* (2010) provided additional evidence in support of the assignment of this fossil to Brassicaceae (superior ovary, bicarpellate construction, and single style) and to the genus *Thlaspi* (multiple concentrically striate seeds in each locule, narrow pedicel, venation of the paired fruit wings). In particular, the latter

authors stressed the combination of concentrically striate seeds and winged fruit; a character combination which occurs in the Brassicaceae only in the genus *Thlaspi*.

Here, the lower bound of this age estimate was used as a minimum age for the *Thlaspi* stem node. This is the node at which the genus *Thlaspi* and its sister group diverged. However, the sister-group of *Thlaspi* is somewhat unclear in the literature, due in part to low levels of sampling in this part of the Brassicaceae, and in part to the lack of monophyly of some Brassicaceae genera. The tree of Koch *et al.* (2001) gave strong support for *Thlaspi* and *Alliaria* (tribe Thlaspidaceae) as sister taxa, but this tree had relatively low sampling (only 21 genera of Brassicaceae were represented). In contrast, the tree of Khosravi *et al.* (2009) gave strong support for *Pseudocamelina glaucophylla* as sister to *Thlaspi arvense* + *T. ceratocarpon*, in preference to species from five other genera in tribe Thlaspidaceae (*Peltaria turkmena*, *Alliaria petiolata*, *Graellsia saxifragifolia*, *Camelinopsis campylopoda* and *Didymophysa aucheri*). The *ndhF*/*PHYA* tree of Beilstein *et al.* (2010) gave *Graellsia saxifragifolia* as sister to *T. arvense*, although this analysis did not include any true representatives of *Pseudocamelina* (*P. campylopoda*, which was included, is now considered a synonym of *Camelinopsis campylopoda* (Bornm. & Gauba) A.G.Mill. (tribe Camelinae): Miller, 1978). Because of the uncertainty as to whether *Pseudocamelina* or *Graellsia* is sister to *Thlaspi*, *P. glaucophylla* was included in the sampling of non-Alysseae Brassicaceae species described above (*Graellsia saxifragifolia* is already included in the *ndhF* dataset of Beilstein *et al.* (2010) that was analyzed here). On the basis of the results obtained here (see the maximum likelihood tree in Chapter 3: Results), *Pseudocamelina* was considered the most likely sister to *Thlaspi*, and therefore the *Thlaspi* stem node was represented in the dated analysis by the node linking *T. arvense* and *P. glaucophylla*.

This fossil placement differs from that used by Beilstein *et al.* (2010). These authors used the date of the *T. primaevum* fossil to provide a minimum age for the node which joins *T. arvense* and

Alliaria petiolata. The only other genus in the Brassicaceae having concentrically striate seeds is *Alliaria*, which has latiseptate fruits. Beilstein *et al.* (2010) proposed that angustiseptate fruits evolved on the branch leading to *Thlaspi*, and that striated seeds evolved on the branch leading to *Thlaspi* and *Alliaria*. On this basis, the authors used the age of the *T. primaevum* fossil to constrain the origin of the crown group *Thlaspi* + *Alliaria*. The proposal regarding fruit evolution is supported by these authors' study of the evolution of fruit characters in the Brassicaceae (which indicates that no close relatives of *Thlaspi* or *Alliaria* have angustiseptate fruits); however, their latter proposal is unsupported (none of the genera that are more closely related to *Thlaspi* than is *Alliaria* has striated seeds). The fact that the *T. primaevum* fossil can be assigned to *Thlaspi* based on multiple other characters means that a more informative use of this fossil-derived date is as a minimum age for the *Thlaspi* stem node. This decision of Beilstein *et al.* (2010) may have resulted from the failure of the accession labelled '*Pseudocamelina campylopoda*' to be monophyletic with *Thlaspi*, combined with a failure by these authors to recognize that this *P. campylopoda* is a synonym of a species in a different genus and tribe, and therefore that *Pseudocamelina* proper was not represented on their tree. The node linking *Thlaspi* and *Alliaria* is deeper in the phylogeny than the true *Thlaspi* stem node. This may have caused an underestimation of node ages across the dated phylogeny of Beilstein *et al.* (2010).

Dressiantha bicarpellata

The affinities of the *Dressiantha bicarpellata* fossil (Gandolfo *et al.*, 1998) have also been debated in the literature. This fossil was collected from the Turonian (Upper Cretaceous) Raritan formation in New Jersey, USA (an age which spans the period 93.6–89.3 Mya). In the original description of this species, Gandolfo *et al.* (1998) placed it within the order Capparales (now family Capparaceae within the order Brassicales (Bremer *et al.*, 2009)), due it having a set of characters only found elsewhere in the Capparales (the presence of a gynophore, 2 1 2 arrangement of sepals, unequal petals, monotheical anthers, and a bicarpellate gynoecium). However, a tree-based study by de

Craene *et al.* (2006) placed *Dressiantha* as sister to *Anacardium* in the order Sapindales. A tree-based analysis by Beilstein *et al.* (2010) placed *Dressiantha* sister to *Moringa oleifera* (family Moringaceae, Brassicales) in the most-visited topology, and sister to *Carica papaya* (Caricaceae, Brassicales) or *Batis maritima* (Bataceae, Brassicales) in other frequently visited topologies. On this basis, Beilstein *et al.* (2010) used the age of the *Dressiantha* fossil to constrain several nodes towards the base of the Brassicales (including nodes joining the following families with the Brassicaceae: Bataceae, Caricaceae, Gyrostemonaceae and the Tropaeolaceae + Caricaceae clade). These authors found that the different placements had little effect on age estimates throughout their Brassicaceae tree. Here, the *Dressiantha* derived age is used conservatively as a minimum bound for the crown node of the Brassicales, in agreement with the final position chosen by Beilstein *et al.* (2010) in their Brassicales-wide molecular clock analysis. The order Malvales (represented in this study by accessions from the genus *Gossypium*) is sister to Brassicales (Bremer *et al.*, 2009), and in this study the Brassicales stem node is represented by the node at which the Brassicales and *Gossypium* lineages converge.

Because of the uncertainties surrounding fossil-calibrated molecular clock analyses, three separate fossil calibration strategies were employed, as described in the following sections.

Calibration Strategy 1: Primary Calibration

BEAST (version 1.6.1) (Rambaut & Drummond, 2007) was used to simultaneously infer phylogeny and divergence dates using a relaxed uncorrelated lognormal molecular clock model. A primary calibration was employed in BEAST by using fossil evidence to set prior age distributions for three nodes. Lower bounds of the estimated ages of fossils of *Akania* sp. (61.3 Mya), *Thlaspi primaevum* (29.2 Mya) and *Dressiantha bicarpellata* (89.3 Mya) were used to set lower bounds for lognormal prior distributions of age for the *Akania* stem node, the *Thlaspi* stem node and the Brassicales crown node, respectively ($\mu = 1$ Mya and $\sigma = 0.5$ throughout). Phylogenies were built using

Concatenated Alignment A, and were rooted on the Brassicales–*Gossypium branch*. A uniform prior age distribution was set for the root node, extending from 89.3 Mya (based on the *Dressiantha* fossil-derived minimum age for the Brassicales crown group) to 125 Mya (the oldest record of eudicot pollen (Brenner, 1996), as employed by Beilstein *et al.* (2010)). Sequence data were input and priors and other operators were set using BEAUti (Bayesian Evolutionary Analysis Utility) (version 1.6.1) (Rambaut & Drummond, 2007) to create the .xml input file for BEAST.

A starting tree was produced in BEAST by initially running an analysis as below but without a molecular clock or any date constraints, and using one MCMC chain of 200 000 steps. The output tree was edited (as necessary) in TreeEdit (version 1.0a10; Rambaut & Charleston, 2004) and Mesquite (version 2.74; Maddison & Maddison, 2011) in order to ensure the compatibility of nodes with the chosen age constraints in the later, dated runs.

Alignment A was partitioned into its seven component regions and appropriate evolutionary models were applied to each partition based on the results of Jmodeltest analyses (GTR + Γ + I for *matK*, *ndhF*, *trnD–trnT*, and *trnL–trnF*; GTR + Γ for *rbcL* and *rps16–trnK*; and HKY + Γ for *PHYA*). A relaxed uncorrelated lognormal clock rate was estimated separately for each partition. A birth–death species diversification model was employed; the birth rate was given a uniform prior between 0 and positive infinity, and a starting value of 1, and the relative death rate was given a uniform prior between 0 and 1 with a starting value of 0.5. All other prior and operator values were set at default values.

A total of 14 independent MCMC chains were run using a combination of desktop computers and the *Hal* and *Sal* clusters at the Oxford Supercomputing Centre (OSC), University of Oxford. Seven desktop analyses each ran for 30 million steps and each took between 100 and 168 h to complete. Seven supercomputer analyses ran for 59 to 75 million steps, and each took 160 to 170 h to

complete. Log statistics and trees were saved every 2000 steps for runs on desktop computers, and every 3000 steps for runs on clusters. Appropriate burn-in within chains and likely convergence between chains were determined using the tree posterior probability trace plot and ESS values in Tracer (version 1.5.0) (Rambaut & Drummond, 2007). Convergence between the separate MCMC chains was poor, and due to differences in log likelihood values, combining data from separate runs reduced their total effective sample size (ESS). Even the run yielding the highest mean log likelihood (-73761) and highest ESS for the tree posterior (169.0) after burn-in (42 million steps) had an ESS below the minimum of value of 200 recommended by Drummond *et al.* (2007). Therefore the results of this analysis are not reported here.

Calibration Strategy 2: Secondary Calibration

A secondary calibration was employed by setting the prior age distribution for the Alysseae crown node to a normal distribution ($\mu = 23.1$ Mya, $\sigma = 7.00$ Mya), based on a recent published age estimate for this node extracted from the Brassicaceae-wide phylogeny of Beilstein *et al.* (2010). This analysis employed Concatenated Alignment B, and therefore excluded all non-Alysseae species except *Iberis saxatilis*. A uniform prior age distribution was set for the root node (the node joining the Alysseae with *Iberis saxatilis*), extending from 23.1 Mya (the estimated age of the Alysseae crown node in Beilstein *et al.*, 2010) to 54.3 Mya (the estimate for the age of the Brassicaceae crown node in Beilstein *et al.*, 2010). The alignment was partitioned into its seven component regions and appropriate evolutionary models were applied to each (as for Calibration Strategy 1, see above), and parameters were estimated separately for each partition. A relaxed uncorrelated lognormal clock rate was estimated separately for each partition. The speciation model was as described in Calibration Strategy 1, and all other prior and operator values were set at default values.

Four independent MCMC chains of 30 million steps were run on desktop computers. Each chain took 100–175 h to run, depending upon the machine used. Log statistics and trees were saved every 2000 steps, giving 15 000 samples per run. Convergence within and between chains was determined using the tree posterior trace plot and ESS values in Tracer. Based on the trace plot, results from the first 5 million steps in each run (i.e., the initial 2500 log statistics or trees) were counted as burn-in and were excluded from further analysis. Log statistics and trees for the remaining 25 million steps (i.e. 12 500 log statistics and trees) in each run were combined using LogCombiner (version 1.6.1) (Rambaut & Drummond, 2007), and re-sampled at a lower frequency to give a total of 10 000 log files and trees. The final sample had an ESS for the tree posterior of 297.7. This value is above the minimum ESS value of 200 recommended by Drummond *et al.* (2007). A maximum clade credibility (MCC) tree was produced from this sample of trees using TreeAnnotator (version 1.6.1) (Rambaut & Drummond, 2007) with node heights for clades based on their mean heights.

Calibration Strategy 3: Two-stage Calibration

A two-stage calibration strategy was also employed. This involved first producing a widely sampled, dated phylogenetic tree of the Brassicales based on Concatenated Alignment C and employing fossil-dated nodes. This alignment included *ndhF* and *PHYA* sequences from Beilstein *et al.* (2010) from a wide range of Brassicales taxa; it also included 14 representatives of the major Alysseae clades, allowing estimated ages of multiple nodes within the Alysseae to be obtained. A second dated phylogenetic tree was then constructed from Concatenated Alignment B (in a similar manner to Calibration Strategy 2) using these nodes as secondary calibration points.

Stage 1

The first phylogenetic analysis employed Concatenated Alignment C containing *PHYA* and *ndhF* sequences, and was similar to the analysis carried out by Beilstein *et al.* (2010). It differed in the

inclusion of sequence data for an additional 14 Alysseae species and in the fossil age calibration procedure. Three fossil-dated nodes were employed here, using the nodes, age limits and probability distributions discussed under Calibration Strategy 1 above. Two important problems with the Beilstein *et al.* (2010) analysis were: (1) uniform priors with high upper limits were used for fossil-dated nodes, which is likely to lead to overestimates of ages throughout the tree; and (2) nodes used for calibration of the tree were sub-optimal for two of the fossils (i.e. the nodes used were more basal than the stem node of the smallest clades in which the *Thlaspi primaevum* and *Akania* sp. fossils can be placed (the genera *Thlaspi* and *Akania*, respectively)). This is likely to underestimate ages for nodes in these areas of the tree. To address these flaws, the present analysis used fossil calibration as described in Calibration Strategy 1. Unlike the analysis of Beilstein *et al.* (2010), the *Akania* sp. fossil (but not the *A. patagonica* and *A. americana* fossils) was used for calibration. The prior for the root node (i.e. the node joining *Gossypium* to the remainder of the tree) was uniform: 89.3–125 Mya, as in Calibration Strategy 1. The alignment was partitioned into its two component regions (i.e. *PHYA* and *ndhF*), and appropriate evolutionary models applied to each (as for Calibration Strategy 1); associated parameters were estimated separately for each partition. The molecular clock and species diversification models were as for Calibration Strategy 1. All other prior and operator values were set at default values.

Four independent MCMC chains of 50 million steps were run on the OSC *Hal* and *Sal* clusters and each took 100–125 h to complete. Log statistics and trees were saved every 2000 steps, giving 25000 samples per chain. Convergence within and between runs was determined using the tree posterior trace plot and ESS values in Tracer. Based on the trace plot, results from the first 10 million steps in each run were counted as burn-in and were excluded from further analysis. Log statistics and trees for the remaining 40 million steps (i.e. 20 000 log statistics and trees) in each run were combined using LogCombiner and were re-sampled at a lower frequency to give a total of 10 000 log files and trees. The final sample had an ESS for the tree posterior of 250.1. This value is

above the minimum ESS value of 200 recommended by Drummond *et al.* (2007). An MCC tree was generated from the final sample as described under Calibration Strategy 1 above.

Three nodes within the Alysseae clade in this tree were selected as calibration points for the second stage of the analysis. These were: (1) the Alysseae crown node; (2) the A1 clade crown node; and (3) the A5 clade crown node. All had posterior probabilities > 0.99, and their estimated ages were 38.5 (95% CI = 24.8–54.6), 26.7 (95% CI = 13.2–39.0) and 11.3 (95% CI = 3.1 to 19.3) Mya, respectively (see *Chapter 3: Results* for further details).

Stage 2

This tree-building procedure followed a similar process to the secondary calibration described under Calibration Strategy 2 above, except that three (rather than one) calibration points were employed. The three secondary calibration nodes were employed by setting the prior age distribution for the Alysseae crown node, A1 clade crown node and A5 crown node to normal distributions with μ = 38.5, 26.7 and 11.3 Mya, respectively, as estimated in Stage 1 of this analysis. Values for σ were chosen to ensure that the upper and lower bounds of the 95% CI for the prior matched those of the estimates obtained in Stage 1 (σ values were 8.5, 8.0 and 5.0 Mya, respectively). This analysis employed Concatenated Alignment B, and therefore excluded all non-Alysseae species except *Iberis saxatilis*. A uniform prior age distribution was set for the root node (the node joining the Alysseae with *Iberis saxatilis*) extending from 38.5 Mya (the estimated age of the Alysseae crown node from Stage 1 of this analysis,) to 54.3 Mya (the estimate for the age of the Brassicaceae crown node in Beilstein *et al.*, 2010). The alignment was partitioned into its seven component regions, and appropriate evolutionary models applied to each (as for Calibration Strategy 1); associated parameters were estimated separately for each partition. The molecular clock and species diversification models were as for Calibration Strategy 1. All other prior and operator values were set at default values.

Four independent MCMC chains of 60 million steps were run on OSC *Hal* and *Sal* clusters and each took 98–100 h to complete. Log statistics and trees were saved every 2000 steps, giving 30 000 trees and log files per run. Convergence within and between runs was determined using the tree posterior trace plot and ESS values in Tracer. Based on the trace plot, results from the first 20 million steps (for three chains) and 42 million steps (for one chain) (i.e. the initial 10 000 and 21 000 log statistics and trees) were counted as burn-in and were excluded from further analysis. Log statistics and trees for the remaining 138 million steps were combined using LogCombiner, and the resulting 69 000 trees and log files were re-sampled at a lower frequency to give a total of 10 000 trees and log files. The final sample had an ESS for the tree posterior of 281.0. This value is above the minimum ESS value of 200 recommended by Drummond *et al.* (2007). An MCC tree was generated from the final sample as described under Calibration Strategy 2 above. This MCC tree was used in further analyses (described below). Reasons for using this tree rather than those from Calibration Strategies 1 or 2 are presented in *Chapter 3: Results*.

Character Optimization

Maximum likelihood character optimization was carried out in Mesquite in order to reconstruct ancestral characters states for the following characters: (1) nickel hyperaccumulator status (with polymorphic species coded as nickel hyperaccumulators); (2) nickel hyperaccumulator status (with polymorphic species coded as third character state); (3) serpentine endemic status (coded as a binary character, with species of unknown serpentine endemic status coded as such); and (5) life-history strategy. The maximum likelihood analysis employed a 2-parameter asymmetric model (allowing separate rates for the gains and losses of characters), except for (3) above, where the >2 character states necessitated the single parameter Mk1 model in Mesquite. Character optimization was carried out using the MCC Alysseae tree obtained from *Calibration Strategy 3* (as opposed to the maximum likelihood tree obtained from RAxML, the parsimony tree from Nona/Wincalda, or a consensus tree) because it is fully resolved and indicates node ages. Parsimony reconstruction was

not carried out because the cost-weighting of character gains versus losses would have been arbitrary, and because this approach offers no obvious method for determining levels of certainty of character states of ancestral nodes, especially under phylogenetic uncertainty (see below).

Because of the critical importance of the ancestral state of the Clade A1a and A5a crown nodes in this study (these were the smallest clades containing all hyperaccumulator species in *Alyssum* and in *Bornmuellera* + *Leptoplax*, respectively), these states were further investigated using the Multistate package of BayesTraits (version 1.0) (Pagel & Meade, 2006). Ancestral state certainties for these node were determined for nickel hyperaccumulation (coded as a binary character, with polymorphic species coded as nickel hyperaccumulators) and for serpentine endemism (coded as a binary character, with species of unknown status coded as such).

Maximum likelihood and MCMC-based analyses were carried out on the MCC tree obtained using Calibration Strategy 3 described above. Initially, an unconstrained maximum likelihood analysis using 10 repetitions was carried out. Further maximum likelihood analyses were carried out with the A1a and A5a nodes each fixed as hyperaccumulator and non-accumulator, and then as serpentine-endemic and non serpentine-endemic. For nickel hyperaccumulation status and for serpentine endemic status, these analyses were repeated using an MCMC-based Bayesian approach employing MCMC chains of 2–10 million steps. A burn-in of 1–5 million steps was discarded, and samples were logged every 500–1000 steps. Rate deviation parameters of 0.1 (for the nickel hyperaccumulation dataset) and 0.2 (for the serpentine endemism dataset) were employed, chosen to allow appropriate acceptance ratios (20–40%; Pagel & Meade, n.d.), on the basis of several trial runs. A reversible-jump hyperprior (Pagel & Meade, 2006) was used to set prior distributions for the rate parameters. Priors for rate parameter means and deviations were taken from an exponential distribution between 0 and 20. Limits were chosen to include the range

of values obtained in the separate maximum likelihood runs. For each estimation, three additional MCMC chains (with different random seed numbers) were run to ensure consistency of results.

In order to incorporate phylogenetic uncertainty into the analysis, a second ML analysis was carried out on a set of 1000 trees sub-sampled from the set of 10 000 trees from which the MCC tree was obtained. However, inter-tree heterogeneity in likelihood values meant that it was not possible to carry out an MCMC-based analysis for multiple trees. During attempts to do this, acceptance ratios were consistently too low for efficient searches of parameter space (<1%, rather than the 15–40% recommended by Pagel & Meade (n.d.), and separate MCMC chains (run for up to 100 million steps) did not converge on similar likelihood values.

Character Association Analysis

Maximum likelihood and MCMC sampling procedures were used in the BayesDiscrete package of BayesTraits to test for an association between nickel hyperaccumulation and serpentine endemism. Nickel hyperaccumulation and serpentine endemism were coded as binary characters as described in the previous section. The Discrete package within BayesTraits was used to estimate rate parameters for the gain and loss of these two characters. An independent run was carried out in which one gain rate and one loss rate are employed to model the evolution of each of the two parameters on the tree (i.e., four parameters in total). A dependent run was then carried out, in which two gain and two loss rates were employed for each character. The additional parameters allowed rate gains and losses to be optimized separately for lineages with different states for the other character.

Maximum likelihood and MCMC-based analyses was carried out on the MCC tree obtained using Calibration Strategy 3 described above. The maximum likelihood analyses involved 10 repetitions per tree. Likelihood values for dependent and independent runs were compared using the

likelihood ratio test statistic, for which p values were obtained from a chi-squared distribution. Four separate MCMC chains were also run using the MCC tree, each of 10 million steps. A burn-in of 5 million steps was discarded, and samples were logged every 1000 steps. Rate deviation of 0.1 was employed, chosen to allow an appropriate acceptance ratio (20–40%), on the basis of several trial runs (of 1 million steps each). A reversible-jump hyperprior (Pagel & Meade, 2006) was used to set prior distributions for the rate parameters. Priors for rate parameter means and deviations were taken from an exponential distribution between 0 and 20. Limits were chosen to include the range of values obtained in the maximum likelihood runs.

In order to incorporate phylogenetic uncertainty into the analysis, a second ML analysis was carried out on a set of 1000 trees sub-sampled from the set of 10 000 trees from which MCC tree was obtained. As for the ancestral state analysis above, Inter-tree heterogeneity in likelihood values meant that it was not possible to carry out an MCMC-based analysis for multiple trees (see previous section).

Diversification Rate Analyses

MEDUSA

In order to determine net diversification rate and relative extinction rate for the Alysseae, and to identify nodes where there is evidence of rate shifts, the MEDUSA (Modeling Stepwise Diversification Using Stepwise AIC) procedure of Alfaro *et al.* (2009) was employed. This was implemented as an R script that employs the APE (Paradis *et al.*, 2004) and Geiger (Harmon *et al.*, 2008) packages to model lineage diversification using the species birth–death modelling approach of Rabosky *et al.* (2007). This method determined the likelihood values associated with trees that have 1 to 20 different rates of net species diversification rate and relative species extinction rate

(with breakpoints placed at their optimal nodes on the tree), and then uses AIC scores to determine the optimum number of breakpoints.

The MEDUSA analysis was based on the MCC tree obtained using Calibration Strategy 3. Exemplar species were nominated from the following major Alysseae clades, essentially allowing the analysis to be carried out on a pruned tree represented by 10 terminal clades: A1a, A1b, A1c, A2, A3, A4, A5, A6, A7 and A8. Clade sizes were determined by counting the numbers of species present in each clade (on the MCC tree) and assigning unsampled species to clades based on their taxonomy (i.e., their genus or infra-generic section) (detailed in full in Appendix 4). Two species outside clades A1–A6 in the trees obtained here were themselves treated as terminal clades: *Phyllolepidum cyclocarpum* (A7) and *Clypeola ciliata* (A8). The former clade also has one unsampled species assigned to it (*Phyllolepidum rupestre*), and the latter consisted of a single member. Table 2-9 indicates the number species assigned to each of the terminal clades. Of the 255 Alysseae species accepted in this study, 239 (i.e. 94%) were included in the MEDUSA analysis; 16 species could not be assigned to terminal clades because information about their infra-generic placement within *Alyssum* was not available. In order to verify that the results were robust to these missing data, the MEDUSA analysis was also run under the assumption that the 16 *Alyssum* species of unknown sectional membership belonged to (1) the A1b clade and (2) the A2 clade. These assignments were chosen because in addition to Clade A1a, clades A1b and A2 are the only two to contain *Alyssum* species (all other Alysseae clades contain species from other genera of Alysseae). The hyperaccumulator status of all these 16 taxa is unknown.

Table 2-9. Species richness of the major clades of Alyseae, used as input data for the MEDUSA species diversification rate analysis.

Alyseae Clade	Corresponding Taxa	Sampled species	Unsampled species	Total Species
A1a	<i>Alyssum</i> sect. <i>Odontarrhena</i> + 3 spp. from sect. <i>Alyssum</i> + 1 spp. from sect. <i>Meniocus</i>	63	15	78
A1b	<i>Alyssum</i> sect. <i>Meniocus</i> (except 1 spp. above)	4	1	5
A1c	<i>Clypeola</i>	4	4	8
A2	<i>Alyssum</i> sect. <i>Alyssum</i> (except 3 spp. above) & sect. <i>Gamosepalum</i>	59	30	89
A3	<i>Alyssoides</i> , <i>Clastopus</i> , <i>Degenia</i> , <i>Physoptychis</i> and <i>Straussiella</i>	17	4	21
A4	<i>Hormathophylla</i>	6	4	10
A5	<i>Bornmuellera</i> , <i>Leptoplax</i> and <i>Physocardamum</i>	7	2	9
A6	<i>Aurinia</i> , <i>Berteroa</i> , <i>Galitzkya</i>	8	8	16
A7	<i>Phyllolepidum</i>	1	1	2
A8	<i>Clypeola ciliata</i> only	1	0	1
Total Assigned	All above	170	68	239
Unassigned	Various	0	16	16
Total		170	84	255

Whole-Clade Diversification Rates

Based on the clade size estimates in Table 2-9, the whole-clade estimator of Magallón & Sanderson (2001) was used to estimate diversification rates for the following selected clades within the Alyseae: A1a, A2, *Alyssum s.l.* (i.e. A1a + A1b + A1c + A2), A5 and A5a (i.e. the hyperaccumulator clade within the A5 clade). The diversification rate of the Alyseae clade (i.e., 255 species) was also estimated. These clades were selected in order to compare the species diversification rates of the two hyperaccumulator clades (i.e. A1a and A5a) with several higher-order clades in which they are nested. The analysis was performed using the R package GEIGER (Harmon *et al.*, 2008) based on the equations in Magallón & Sanderson (2001) (incorporating the erratum published in Magallón & Sanderson (2006)). Diversification rate estimates were based on both crown and stem node ages and under relative extinction rates of both 0 and 0.90. Rates were determined using clade age estimates obtained from all three fossil-calibration strategies.

BiSSE Modelling

In order to test the hypothesis that nickel hyperaccumulation and serpentine endemism are associated with an increase in species diversification rate in the Alysseae, the BiSSE (binary state speciation model) (Maddison *et al.*, 2007) was employed. A maximum likelihood search was implemented using this model in R using the Diversitree (version 1) package (Fitzjohn *et al.* 2009), which allows analyses using incompletely sampled phylogenies. This method works by testing the hypothesis that lineages with different states for a binary character have different rates of absolute species diversification (λ). In this procedure, maximum likelihood values under two scenarios are compared: (1) where λ can have different values for lineages with different character states; and (2), where λ is constrained to take a single value across all lineages. AIC scores are used to compare the two models.

Where the binary character has no homoplasy, BiSSE tests a hypothesis similar to Magallón & Sanderson's (2001) whole clade estimator, i.e., that the diversification rate in a particular clade differs from that in the rest of the phylogeny (albeit that the latter estimates net diversification rate, whereas BiSSE uses within-clade branching patterns to estimate absolute diversification and extinction rates for all branches). Since there is homoplasy in the distribution of nickel hyperaccumulation in Alysseae, the BiSSE method tests a slightly different hypothesis. BiSSE is being used here to test the hypothesis that nickel hyperaccumulator lineages have a higher absolute rate of species diversification than non-accumulator lineages, wherever they occur in the Alysseae phylogeny. In this analysis, the nickel hyperaccumulation trait was coded as a binary character as described above.

Due to the extent of missing data (i.e., unsampled taxa were assigned to all but one terminal clade of the tree), a suitable starting point could not be determined using the 'starting.point' command. Therefore, an initial unconstrained analysis was employed using starting values based on Fitzjohn

(2009) (i.e., λ_0 (diversification rate of non-accumulator lineages) = 0.1 splits lineage⁻¹ Mya⁻¹; λ_1 (diversification rate of hyperaccumulator lineages) = 0.2; μ_0 (extinction rate of non-accumulator lineages) = 0.03 extinctions lineage⁻¹ Mya⁻¹; μ_1 (extinction rate of hyperaccumulator lineages) = 0.03; q_{01} (probability of [change from non-accumulator to hyperaccumulator] lineage⁻¹ Mya⁻¹) = 0.01; q_{10} (probability of [change from hyperaccumulator to non-accumulator] lineage⁻¹) = 0.01 Mya⁻¹). Resulting parameter estimates were used as starting values in subsequent unconstrained and constrained analyses. As in the MEDUSA analysis, this analysis was based on the MCC tree produced in BEAST using fossil-calibration strategy 3. This tree was pruned in R to yield single terminals representing Alyseae clades A1a, A1b, A1c, A2, A3, A4, A5 and A6. The two species lying outside these clades (*Phyllolepidum cyclocarpum* and *Clypeola ciliata*) were treated as separate terminals (A7 and A8, respectively). Table 2-10 shows the number of hyperaccumulator, non-accumulator and total species assigned to each of the above clades.

Two further Alyseae-wide BiSSE analyses were carried out in order to incorporate the 16 species to which a terminal clade could not be assigned. In one analysis these 16 species were assigned to the A1b clade, and in the other they were assigned to the A2 clade. Otherwise, these analyses were similar to that described above.

In addition to the Alyseae-wide analysis, separate BiSSE analyses were also carried out for (1) the *Alyssum s.l.* clade (which contains clades A1, A2, A6 and A8, and hence the hyperaccumulator clade within *Alyssum s.l.* (i.e. clade A1a)), and (2) the non-*Alyssum s.l.* Alyseae clade (which contains clades A3, A4, A5 and A7, and hence also the hyperaccumulator clade within A6 (i.e. clade A6a)). These separate analyses were carried out in order to determine whether speciation and extinction rates differed for the two separate origins of nickel hyperaccumulation within the Alyseae. AIC scores for constrained and unconstrained BiSSE runs using maximum likelihood were compared using ANOVA in R.

Table 2-10. Numbers of hyperaccumulators species, non-accumulator species and species for which the hyperaccumulator status is unknown, shown for the major clades of Alyseae, and used as input data for the BiSSE species diversification rate analysis.

Alyseae Clade	Hyperaccumulator	Non-accumulator	Status unknown	Total Species
A1a ¹	51	18	9	78
A1b ¹	0	5	0	5
A1c ¹	0	7	1	8
A2 ¹	0	75	14	89
A3 ²	0	16	5	21
A4 ²	0	8	2	10
A5 ²	5	3	1	9
A6 ¹	0	12	4	16
A7 ²	0	1	1	2
A8 ¹	0	0	1	1
Unassigned	0	0	16	16
Total (Alyseae)	56	145	54	255
Total (<i>Alyssum s.l.</i>) ¹	52	105	25	181
Total (non- <i>Alyssum s.l.</i>) ²	5	28	25	58
¹ Clades included in <i>Alyssum s.l.</i> -wide BiSSE analysis.				
² Clades included in non- <i>Alyssum s.l.</i> Alyseae-wide analysis.				

In order to verify that the results were robust to missing data (i.e., the 16 Alyseae species that could not be assigned to *Alyssum* sections, and hence to major Alyseae clades), the BiSSE analysis was re-run assuming that the 16 *Alyssum* species of unknown *Alyssum* section membership belong to (1) the A1b clade, and (2) the A2 clade, as described for the MEDUSA analysis above.

QuaSSE Modelling

The Quantitative State Speciation and Extinction (QuaSSE) model of Fitzjohn *et al.* (2010) was employed to test for a relationship between linear range size and species diversification rate in Alyseae. The model was implemented using the R package Diversitree.

Maximum likelihood optimization of species diversification model parameters was carried out with species diversification rate as (1) a linear function of the log linear range estimator, (2) a sigmoidal function of the log linear range estimator, and (3) a constant value with respect to the log₁₀ linear range size. Analyses were carried out both including and excluding a drift parameter (which allows

for underlying change in linear range size values over time). In QuaSSE analyses, the standard deviation (*sd*) parameter accounts for uncertainty in the character state measurements, e.g., due to within-species variation and measurement error. Since linear range size is a property of species rather than individuals, within-species variation is not relevant and uncertainty in character state measurements is limited to measurement error. To estimate this, the standard deviation of the residuals obtained from a linear regression of $\log_{10}$ linear range size on $\log_{10}$ species range area (0.3218), was employed for the *sd* parameter in these analyses.

QuaSSE analyses cannot be performed on datasets containing missing character data, or trees containing negative branch lengths. Therefore the MCC Alysseae tree obtained from fossil Calibration Strategy 3 was pruned in R to remove terminal taxa for which linear range size values were unavailable. Species for which multiple accessions were available were arbitrarily pruned to leave one terminal node per species. A total of 119 species were present in the pruned tree. One negative branch length (of -27 800 years, for the branch subtending a clade of *A. pterocarpum*, *A. masmenaeum*, *A. obtusifolium*, *A. cilicicum* and *A. callichroum*) was removed from the tree in Mesquite by changing it to a small positive value (+10 000 years), and reducing the length of the two branches immediately tipward accordingly. The extent of representation of each of the major Alysseae clades is indicated in Table 2-11. With the exception of clade A8, which contains a single species, representation of clades varied from 43.8% for clade A6 to 75.3% for clade A1a. Since QuaSSE makes the assumption that missing taxa are randomly distributed across the tree, such variation in representation could bias the analysis. For this reason, branches from over-represented clades were randomly pruned in order to equalize the representation of all clades to approximately 50%. Due to the low absolute numbers of species in some clades, the adjusted representation values (excluding A8, which was 100%) varied between 47.6% and 55.6%. The numbers of species available for the analysis, and the adjusted samples for each of the major Alysseae clades, are shown in Table 2-11.

Table 2-11. Number of species available for sampling in QuaSSE analysis, shown for each of the major Alysseae clades. Because the representation of clades varied due to missing data (and this was considered likely to bias diversification rates), species were randomly removed from over-represented clades. Adjusted numbers of species are shown.

Alysseae Clade	Species available for QuaSSE analysis	Total Species	% Representation	Adjusted no. of species for QuaSSE analysis (no. of species randomly excluded) for representation of <i>ca.</i> 50%	Adjusted % Representation
A1a	58	78	74.3	38 (i.e., -20)	48.7
A1b	4	5	66.7	3 (i.e., -1)	60.0
A1c	4	8	50.0	4 (i.e., -0)	50.0
A2	50	89	56.2	45 (i.e., -5)	50.6
A3	10	21	47.6	10 (i.e., -0)	47.6
A4	6	10	60.0	5 (i.e., -1)	50.0
A5	5	9	55.6	5 (i.e., -0)	55.6
A6	7	16	43.8	7 (i.e., -0)	50.0
A7	1	2	50.0	1 (i.e., -0)	100
A8	1	1	100	1 (i.e., -0)	100
Unassigned	0	16	00.0	0 (i.e., -0)	00.0
Total	146	255	57.3	119 (i.e. -27)	50.4

The adjusted (i.e. pruned) tree contained 119 species (i.e. 46.7% of Alysseae species). Phylogenetic uncertainty due to the incomplete sampling of Alysseae species was taken into account using the sampling frequency parameter ('sampling.f' = 119/255). AIC values from the separate QuaSSE optimizations runs were compared using an ANOVA in R.

Chapter 3: Results

Phylogenetic Utility of DNA Sequence Regions for Alysseae

Sequence Variability

Results of the pilot study to screen DNA sequence regions in terms of their phylogenetic utility in Alysseae are presented in Table 3-1. Successful amplification and high-quality sequences were obtained for 13 of the 21 DNA regions screened in the pilot study: *trnD-trnT*, *rps16-trnK*, *rpl32-trnL*, *ndhF*, *trnH-psbA*, *matK*, *trnV-ndhC*, *ndhF-rpl32*, *trnL-trnF*, *rpl16*, *rps12-rpl20*, *trnG-trnS*, and *trnQ-rps16*. Amplification and sequencing were unsuccessful for all three nuclear regions (*LUMINIDEPENDENS*, *Dpa1* and *Nuo*) screened. Sequence lengths for each region ranged from 317 bp (*trnQ-rps16*) to 1983 bp (*ndhF*).

The number of variable characters varied from 40 (*trnQ-rps16*) to 276 (*rpl32-trnL*), and as a percentage of aligned length from 7% (*trnQ-rps16* and *ndhF*) to 30% (*rpl32-trnL*). Three regions each had > 200 variable characters (*trnD-trnT*, *rps16-trnK* and *rpl32-trnL*). The number of parsimony-informative (PI) substitutions varied between 2 (*trnQ-rps16*) and 124 (*trnD-trnT*); three regions had > 100 PI characters (*trnD-trnT*, *rps16-trnK* and *rpl32-trnL*). The $\Delta_{\min}$ value, i.e., the minimum number of PI character-state, as opposed to character, changes, varied between 2 (*trnQ-rps16*) and 124 (*trnD-trnT*); three regions had > 100 PI character-state changes (*rps16-trnK*, *trnD-trnT* and *rpl32-trnL*). The number of indel characters varied between 0 (*ndhF*) and 37 (*rps16-trnK*). Three regions each had >30 indels (*rps16-trnK*, *trnV-ndhC* and *rpl32-trnL*). The number of PI indels varied between 0 (*ndhF*) and 28 (*rpl32-trnL*); two regions had 20 or more informative gap characters (*trnD-trnT* and *rps16-trnK*).

Ease of Amplification and Sequencing

In addition to levels of sequence variation, the ease with which a DNA region can be amplified and sequenced for a given taxonomic group has an important bearing on its practical phylogenetic utility. Very high levels of variability could potentially reduce the value of regions if that variability also extends to the primer regions, making amplification and/or sequencing unreliable in some taxa. For this reason, in addition to the quantitative measures of sequence variability discussed above, a qualitative assessment of the ease of use of DNA regions (with regions graded high, moderate or poor) was employed (Table 3-1). This assessment includes consideration of amplification and sequencing difficulties (e.g., due to the presence of microsatellite regions), and ease of alignment. Ease of use was graded high for four regions (*rps16–trnK*, *matK*, *rps12–rpl20* and *trnQ–rps16*), moderate in six regions (*trnD–trnT*, *ndhF*, *trnH–psbA*, *ndhF–rpl32*, *trnL–trnF* and *rpl16*), and the remaining regions were graded poor.

Selection of DNA Sequence Regions for Alysseae

The region *rps16–trnK* has high sequence variability in Alysseae and high ease of use; *matK* has moderate variability and high ease of use; *trnD–trnT* has high variability and moderate ease of use. On this basis these three regions were selected for their high phylogenetic utility in the Alysseae. One additional region, *trnL–trnF*, has high ease of use and somewhat lower sequence variability, and was considered to be of some phylogenetic utility in the Alysseae. Based on this survey to screen candidate sequence loci, the four selected loci provide a total of 338 PI substitution characters and 38 informative gap characters for the eight accessions that were sampled, and have the potential to yield a much more fully resolved and robustly supported Alysseae phylogeny than previous studies that relied solely on *ITS* (which had 212 to 310 PI characters in 38 to 78 accessions (Mengoni *et al.*, 2003; Cecchi *et al.*, 2010)). Because of the need to include species from outside the Alysseae (in order to root trees, and to allow the inclusion of nodes deeper in the Brassicales for

molecular clock calibration) in addition to the four highly variable chloroplast regions above, an additional two chloroplast DNA regions, *ndhF* and *rbcl*, and a one single-copy nuclear region, *PHYA*, were also selected. The choice of these three regions, and of taxon sampling from Alysseae and the wider Brassicales for all seven selected DNA regions is discussed in detail in Chapter 2.

Phylogenetic Analysis of Alysseae

Summary of DNA Sequence Data

In order to reconstruct the phylogeny of Alysseae, sequence data were obtained from the literature or generated in this study for the seven DNA regions described in the previous section. Sequence data was also obtained entirely from published sources for an eighth region: *ITS*. Table 3-2 lists the numbers of sequences (including partial sequences) obtained for Alysseae, other Brassicaceae, and other Brassicales species. For the four highly variable chloroplast regions *matK*, *rps16-trnK*, *trnD-trnT* and *trnL-trnF*, 194–202 accessions of 177–181 species were obtained. These included 178–188 accessions of 161–170 Alysseae species (representing 67% of species in the tribe), all of which were sequenced in this study. For the more conserved regions *ndhF*, *PHYA* and *rbcl*, 26–191 accessions of 26–187 species were obtained. These included 12–13 accessions of 12–13 Alysseae species, of which 10–13 accessions of 10–13 species were sequenced here. For the *ITS* region, 182 accessions of 89 species were obtained. These included 181 accessions of 88 Alysseae species. This dataset represents the largest quantity of Alysseae DNA sequence data assembled to date.

Table 3-1. Phylogenetic utility of 21 candidate DNA sequence loci in Alysseae.

Region (selected loci are indicated by an *)	Amplification Success	Sequencing Success	Ease of use	Sequence lengths (bp)	Alignment length	Substitutions			Indels	
						Variable sites (%)	PICs (%)	$\Delta_{\min}$	Total	PICs
<i>trnD-trnT</i> *	✓	✓	moderate	1144–1298	1552	240 (15)	124 (8)	124	23	10
<i>rps16-trnK</i> *	✓	✓	high	775–821	934	221 (24)	109 (12)	109	37	20
<i>rpl32-trnL</i>	✓	✓	poor	778–803	935	276 (30)	107(11)	108	32	28
<i>ndhF</i>	✓	✓	moderate	1983	2014	137 (7)	77 (4)	77	0	0
<i>trnH-psbA</i>	✓	✓	moderate	334–480	585	116 (20)	69 (12)	70	15	6
<i>matK</i> *	✓	✓	high	998	1057	117 (7.8)	64 (6)	64	3	1
<i>trnV-ndhC</i>	✓	✓	poor	641–765	883	99 (11)	55 (6)	55	35	17
<i>ndhF-rpl32</i>	✓	✓	moderate	728–853	953	128 (13)	52 (5)	52	20	19
<i>trnL-trnF</i> *	✓	✓	moderate	631–725	744	51 (7)	41 (6)	41	9	7
<i>rpl16</i>	✓	✓	moderate	998	807	119 (15)	38 (5)	40	19	7
<i>rps12-rpl20</i>	✓	✓	high	904-1005	697	82 (12)	35 (5)	35	13	9
<i>trnG-trnS</i>	✓	✓	poor	480–518	573	45 (8)	18 (3)	18	21	11
<i>trnQ-rps16</i>	✓	✓	high	317–541	573	40 (7)	2 (0)	2	11	2
<i>rpoB-trnC</i>	✗	✗	poor	-	-	-	-	-	-	-
<i>trnS-trnL</i>	✗	✗	poor	-	-	-	-	-	-	-
<i>rpl32-trnF</i>	✗	✗	poor	-	-	-	-	-	-	-
<i>trnS-trnF</i>	✗	✗	poor	-	-	-	-	-	-	-
<i>rps16</i> intron	✗	✗	poor	-	-	-	-	-	-	-
<i>LUMINIDEPENDE</i>	✗	✗	poor	-	-	-	-	-	-	-
<i>Dpa1</i>	✗	✗	poor	-	-	-	-	-	-	-
<i>Nuo</i>	✗	✗	poor	-	-	-	-	-	-	-

Alignment Statistics

The aligned lengths, missing data and sequence variability statistics for the eight single-region and three concatenated DNA alignments are indicated in Table 3-3. For the eight separate DNA regions, alignment length in Alysseae ranged from 721 bp (for *ITS*) to 2022 bp (for *ndhF*). Numbers of variable sites for total were highest for *ndhF* and *PHYA* (1115 and 1167, respectively), reflecting the fact that these alignments contained sequences from a wide range of species from across the Brassicales (whereas in the alignments of the highly variable chloroplast regions, the majority of sequences were from species of Alysseae). (Due to such differences in taxon sampling, and in the absolute number of sequences in each alignment, the sequence variability statistics in Table 3-3 are not directly comparable between alignments.) Numbers of variable sites for Alysseae-only alignments were highest in *trnD-trnT* (836). Of the four highly variable chloroplast regions, numbers of parsimony informative characters (PICs) within Alysseae ranged from 164 (for *trnL-trnF*) to 485 (for *trnD-trnT*). For the more conserved regions, the number of PICs within Alysseae ranged from 27 (in *rbcl*) to 153 (in *ndhF*); the *ITS* region had 291.

The quantity of missing data in the alignments for the separate DNA regions ranged from 4.1% (for *PHYA*) to 11% (for *matK*). The quantity missing data in the Concatenated alignments was much higher (50%, 52% and 19% in Concatenated Alignments A, B and C, respectively). This is because one or more of the component DNA regions were entirely missing for many of the accessions in these alignments. For example, in *Concatenated Alignment A*, sequence data for *ndhF*, *PHYA* and *rbcl* were only included for 28, 24 and 25 accessions, respectively, and therefore the majority of accessions in the alignment have missing data for these regions. The supermatrix approach to data concatenation used in this study is generally robust to significant levels of missing data caused by incomplete coverage of DNA regions across taxa (as discussed under *Sequence Concatenation*, Chapter 2).

Table 3-2. Summary of sequence coverage for each DNA sequence locus.

Region	Full or partial sequences obtained: No. species (No. accessions)										
	Alysseae		Other Brassicaceae		Other Brassicales		Ex-Brassicales		Total sequenced here	Total	
	Sequenced here	Published data	Sequenced here	Published data	Sequenced here	Published data	Sequenced here	Published data		Alysseae	Total
<i>ITS</i>	0 (0)	88 (181)	0 (0)	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	88 (181)	89 (182)
<i>matK</i>	166 (181)	0 (0)	6 (6)	4 (4)	0 (0)	5 (5)	0 (0)	1 (1)	172 (187)	166 (181)	182 (197)
<i>rps16-trnK</i>	160 (178)	0 (0)	7 (7)	2 (2)	6 (6)	0 (0)	0 (0)	1 (1)	173 (191)	160 (178)	176 (194)
<i>trnD-trnT</i>	164 (188)	0 (0)	6 (6)	2 (2)	5 (5)	0 (0)	0 (0)	1 (1)	175 (199)	164 (188)	178 (202)
<i>trnL-trnF</i>	164 (178)	0 (0)	7 (7)	3 (3)	4 (4)	1 (1)	0 (0)	1 (1)	175 (189)	164 (178)	180 (194)
<i>ndhF</i>	13 (13)	0 (0)	3 (3)	140 (143)	0 (0)	30 (30)	0 (0)	1 (2)	16 (16)	13 (13)	187 (191)
<i>PHYA</i>	10 (10)	3 (3)	2 (2)	118 (120)	1 (1)	12 (12)	0 (0)	1 (1)	10 (10)	13 (13)	147 (149)
<i>rbcL</i>	12 (12)	0 (0)	3 (3)	4 (4)	0 (0)	6 (6)	0 (0)	1 (1)	15 (15)	12 (12)	26 (26)

Table 3-3. Summary statistics for sequence alignments.

Alignment	Sequence lengths (bp)	Alignment size		Whole alignment		Alysseae species only		% Gap characters	% Missing	No. species (No. accessions)	
		No. Sequences	Length (bp) (Alysseae only)	Variable sites (%)	PICs (%)	Variable sites (%)	PICs (%)			Alysseae	Total
<i>ITS</i>	552–610	182	721 (720)	446 (62)	292 (40)	441 (61)	291 (40)	22	8.0	88 (181)	89 (182)
<i>matK</i>	992–998	197	1064 (1031)	614 (58)	412 (39)	437 (42)	291 (28)	6.3	11	165 (181)	181
<i>rps16–trnK</i>	540–821	194	987 (944)	567 (57)	417 (42)	516 (55)	326 (35)	38	6.8	161 (178)	177
<i>trnD–trnT</i>	926–1358	203	2054 (1879)	1207 (59)	773 (38)	836 (44)	485 (26)	46	8.0	170 (188)	181
<i>trnL–trnF</i>	492–791	194	1133 (768)	557 (49)	555 (49)	307 (40)	164 (21)	46	5.8	165 (178)	181
<i>ndhF</i>	1992–2025	191	2068 (2022)	1115 (54)	806 (39)	296 (15)	153 (8)	2.5	9.4	13 (13)	187
<i>PHYA</i>	1692–1712	149	1724 (1706)	1167 (68)	879 (51)	290 (17)	132(8)	1.1	4.1	13 (13)	147
<i>rbcL</i>	1342–1343	26	1343 (1342)	262 (20)	126 (9.4)	71 (5.3)	27 (2.0)	0.10	5.2	12 (12)	26 (26)
Concatenated A*	N/A	207	10342 (9711)	4581 (44)	2761 (27)	2722 (28)	1570 (16)	18	50	170 (189)	181
Concatenated B*	N/A	190	10342 (9711)	2806 (27)	1589 (15)	2722 (28)	1570 (16)	18	52	170 (189)	171
Concatenated C*	N/A	198	3791 (3728)	2282 (60)	1646 (43)	586 (16)	285 (7.6)	2.2	19	16 (17)	195

*Concatenated alignments contain the following regions: A and B: *matK*, *ndhF*, *PHYA*, *rbcL*, *rps16trnK*, *trnD–trnT* and *trnL–trnF*; C: *PHYA* and *ndhF*.

Selecting Appropriate Models of Sequence Evolution

Table 3-4 shows the evolutionary (i.e., DNA substitution rate) model with the lowest and second-lowest Akaike information criterion (AIC) scores for each of the eight DNA regions. Models with the lowest AIC score were used in all subsequent model-based (i.e., maximum likelihood and Bayesian) phylogenetic analyses. The results of AIC- and Bayesian information criterion (BIC) based model selection were in agreement for five of the eight DNA regions: for *matK*, *ndhF* and *trnL–trnF*, the GTR + I + Γ model had the lowest AIC and BIC scores; for *rps16–trnK*, the GTR + Γ model had the lowest AIC and BIC scores; and for *PHYA*, the HKY + Γ model had the lowest AIC and BIC scores. For two of the eight regions, the AIC and BIC methods selected different models: these were GTR + Γ and HKY + Γ (respectively) for *rbcl*, and GTR + I + Γ and GTR + Γ (respectively) for *trnD–trnT*. The HKY + Γ model differs from the GTR + Γ + I model in only allowing substitution rates to differ between transitions and transversions, and not between all possible pairs of bases (Lanave *et al.*, 1984; Hasegawa *et al.*, 1985). The GTR + Γ model differs from the GTR + Γ + I model only in the lack of a parameter for the proportion of invariant sites (no sites are invariant under the former)(Gojobori *et al.*, 1981).

Table 3-4. Optimal models of sequence evolution based on testing 24 models using *JModeltest*.

DNA Region	Lowest AIC Model (Second-lowest AIC Model)	AIC Score (Second-lowest AIC score)
<i>ITS</i>	HKY + I + Γ (SYM + Γ)	18351.9 (18483.6)
<i>matK</i>	GTR + I + Γ (GTR + Γ)	22791.2 (22864.3)
<i>ndhF</i>	GTR + I + Γ (GTR + Γ)	57848.6 (57879.2)
<i>PHYA</i>	HKY + Γ (SYM + I + Γ)	78105.5 (78290.1)
<i>rbcl</i>	GTR + Γ (GTR + I + Γ)	8636.1 (8636.74)
<i>rps16–trnK</i>	GTR + Γ (GTR + I + Γ)	21115.7 (21117.8)
<i>trnD–trnT</i>	GTR + I + Γ (GTR + Γ)	37963.0 (37966.0)
<i>trnL–trnF</i>	GTR + I + Γ (GTR + Γ)	18069.0 (18097.5)

Congruence between DNA Regions

In order to investigate the congruence between the eight separate DNA regions that were selected, separate maximum likelihood trees were constructed (using RAxML) for each DNA region and are presented in Figures A6-1 to A6-8 in Appendix 6. Due to the large numbers of sequences in most of these trees it is not possible to discuss congruence between species relationships in detail, and due to the differences in taxon sampling between the eight trees, it was not possible to use formal tests of congruence. Given the importance of relationships between the major clades of Alysseae (Clades A1–A6 of Warwick *et al.* (2008) and Clades A1a, A1b, A1c and A5a defined in this study (see *Multi-locus phylogeny of Alysseae* below)), congruence was assessed here in terms of the membership of and relationships between these major clades. In order to focus only on robustly-supported incongruence, branches receiving low support (<70% bootstrap support (bs)) were not considered in this assessment.

Alysseae Clades A1–A6 were robustly supported in the majority of trees, with the exception of that based on *trnL–trnF* which had very little resolution. With the exception of *Phyllolepidum*, relationships between the major Alysseae clades were congruent, although generally not well resolved. *Phyllolepidum* was sister to Clade A3 in three trees (based on *matK* (83% bs), *rps16–trnK* (72% bs) and *trnD–trnT* (72% bs)) but sister to Clade A5 in one tree (based on *ITS* (78% bs)).

The species compositions of clades were consistent between trees based on different DNA regions, except for the following five species. In the tree based on *matK*, *Alyssum cassium* was in Clade A4, but in trees based on *rps16–trnK* and *trnD–trnT* it was present in Clade A1 (with 100% bs in both). In the *matK* alignment, this species was represented by an unusually short partial sequence (314 bp) (with no obvious alignment errors), and it is possible that this could account for the incongruence. In trees based on *rps16–trnK* and *trnD–trnT*, *Berteroa incana* and *Bornmuellera kiyakii* were in the A1 clade (with 100% bs in both cases), but in trees based on *ITS*

and *matK* they were in Clade A6 (99% and 100% bs, respectively). In trees based on *matK*, *A. pinifolium* was sister to Clade A2 (77% bs), but in trees based on *ITS*, *rps16–trnK* and *trnD–trnT* it was present in Clade A1 (with 88%, 100% and 100% bs, respectively). These results are unexpected, and possible causes are discussed below.

In the tree based on *ndhF*, an accession of *A. simplex* (sampled under the name *A. parviflorum* by Beilstein *et al.* (2010)) is sister to Clade A1a. However, in trees based on *ITS*, *matK*, *rps16–trnK* and *trnD–trnT* (in which *A. simplex* was represented by three different accessions, two of which were sequenced here) it was in Clade A2 (with 100%, 97%, 100%, 100% bs, respectively). This difference may have resulted from a misidentification of the species in Beilstein *et al.* (2010).

PHYA was successfully sequenced for 10 species. Amplification and sequencing could not be achieved for *Alyssum blepharocarpum*, *Batis maritima*, *Carica papaya*, *Farsetia stylosa*, *Iberis saxatilis*, and *Pseudocamelina glaucophylla*. Apart from the *Alyssum simplex* accession of Beilstein being sister to clade A1 (with 72% bs) (as described for *ndhF* above), the *PHYA* tree, was congruent with the plastid-based Alysseae trees (see Figure A6-4 in Appendix 6). There was therefore no indication of paralogy, hybridization or polyploidy in Alysseae.

Apart from the relationships of *Phyllolepidum* and the five Alysseae species mentioned, there is a high level of congruence between Alysseae trees generated from different DNA regions, although the resolution of relationships between the major clades of the tribe were poorly resolved in some trees (particularly those based on *ITS* and *trnL–trnF*). On this basis, and in order to increase resolution and branch support, it was decided to concatenate sequence data from *matK*, *ndhF*, *PHYA*, *rbcl*, *rps16–trnK*, *trnD–trnT* and *trnL–trnF* as described under *Sequence Concatenation* in Chapter 2. The *ITS* region was not included in the concatenated dataset because it was considered useful to produce a phylogeny of Alysseae that was completely

independent of previous analyses of the tribe, and also because of the potential problems of paralogy associated with the *ITS* region (discussed under *Reliance on ITS sequence data* in Chapter 1 and investigated in Alysseae in Appendix 5).

The lack of congruence between plastid regions in the relationships of accessions of *Phyllolepidum*, *A. cassium*, *A. pinifolium*, *A. simplex*, *Berteroa incana* and *Bornmuellera kiyakii* is unexpected given that the chloroplast is considered to be a non-recombining locus. For *A. cassium* this result is considered likely to result from a short partial sequence for *matK*, and is therefore not considered significant here. For *Phyllolepidum*, the branch support was not strong, and this lack of congruence is therefore also not considered significant here. For the other four accessions, in which incongruence had high branch support, causes such as sample contamination, or (in the case of *A. simplex*) species misidentification, cannot be fully ruled out without additional laboratory work. However, these sequences were not identical to those of any other accession in the study, suggesting that simple mislabelling of samples was not a factor. There is evidence that *matK* may be a pseudogene in some non-photosynthetic orchid taxa (e.g., the presence of frame-shifts in this region in Corallorhizinae (Freudensteinia & Senyo, 2008) and the presence of somewhat *matK*-like non-functional regions in *Neottia* Guett. (Logacheva *et al.*, 2011)). However, this regions does appear to be functional in a wide range of plants (Barthet & Hilu, 2008), and for paralogy to be possible explanation of the incongruence seen in the *MatK* tree would require multiple copies to be (or to have been) present, something for which there is no evidence for the Brassicaceae. Although unexpected, incongruence between plastid loci is sometimes reported. For example G. Reeves *et al.* (2001) found incongruence between *rbcl* and a combined dataset also containing *rps4* and *trnL-trnF* in a parsimony-based analysis. This incongruence had high bootstrap support, despite the two possible placements of taxa in the combined tree differing by only three steps.

As a precautionary measure, in order to investigate the effect that these four accessions (*A. cassium*, *A. simplex*, *Berteroa incana* and *Bornmuellera kiyakii*) have on trees constructed from concatenated datasets, Alysseae trees were constructed from datasets both including and excluding these accessions, as described in the next section.

Multi-locus Phylogeny of Alysseae

In order to maximise the resolution and support for species relationships within the Alysseae, multi-locus phylogenies of the tribe were constructed using maximum likelihood, parsimony and Bayesian inference. These analyses employed Concatenated alignment B, containing sequence data from all seven DNA regions sequenced in this study (i.e., *matK*, *ndhF*, *PHYA*, *rbcL*, *rps16-trnK*, *trnL-trnF* and *trnD-trnT*). The maximum likelihood tree is shown in Figure 3-1. This tree had a log likelihood value of -49704.56. Clades A1, A2, A3, A4, A5 and A6 are labelled as in Warwick *et al.* (2008). Within Clade A1 three further clades are recognised here: A1a (which comprises almost exclusively *Alyssum* section *Odontarrhena*), A1b (which comprises *Alyssum* section *Meniocus*) and A1c (which comprises species of *Clypeola*). *Alyssum s.l.* is defined as the clade comprising clades A1, A2, A6 and *Clypeola ciliata*, and is the smallest clade to contain all *Alyssum* species. Within Clade A5, one further clade is recognised here: A5a, which includes all nickel hyperaccumulator species in A5.

Clades A1, A1b, A1c, A2, A3, A4, A5 and A6 all had strong support (with 98% or higher bs) in the maximum likelihood tree. Clades A1a and A5a had moderate support (with 81% and 87% bs respectively). Relationships between Alysseae clades were generally well supported. Clade A1 consisted of clades A1a, A1b, A1c and *Clypeola ciliata*. Clades A1a and A1b, and *Clypeola ciliata* formed a clade with strong support (92% bs). Clade A1c was sister to A1a + A1b + *C. ciliata* with strong support (100% bs). Clade A6 was sister to A1 with moderate support (85% bs). Clade A2 was sister to A1 + A6 with strong support (97% bs). Clade A3 was sister to *Phyllolepidium cyclocarpum* with moderate support (89% bs). Clade A4 was sister to A3 + *P. cyclocarpum* with

strong support (96% bs). Clade A5 was sister to A3 + *P. cyclocarpum* + A4 with strong support. Alyseae was split basally into the clades A1 + A6 + A2 and A3 + *P. cyclocarpum* + A4 + A5.

Resolution within clade A1a was very poor, with almost all nodes receiving bootstrap support value of < 50% (see Figure 3-1b). No species for which multiple accessions were sampled in this clade (i.e., *Alyssum corsicum* (2 accessions), *A. gehamense* (2), *A. giosnanum* (2), *A. murale* (6) and *A. pintodasilvae* (2)) were monophyletic, although support for non-monophyly in these species was low (all had <60% bs). Branch lengths were particularly short in the A1a clade, indicating relatively little sequence divergence between species in this clade. No clade within Clade A1a had strong (>90% bs) in this tree. Relationships within Clade A1b were fully resolved and all nodes had bs of 99% or higher. Within Clade A1c, two nodes had bs >98% and the third had 73%.

Clade A2 was quite well resolved (the majority of nodes had bs values >60%, and many were >80%) (see Figure 3-1c). This clade was split into two large sub-clades, each with strong support (both had 100% bs). *A. montanum* (represented by four accessions) was non-monophyletic with high bootstrap support. Of the four accessions sampled, accessions 2 and 3 were sister with low support (67% bs); accessions 1 and 4 were each outside of a clade having 81% bs that contained accessions 3 and 4. The only other species in Clade A2 for which multiple accessions were sampled (*A. simplex*) was non-monophyletic with moderate support (88% bs). Relationships within Clade A3 were generally well supported (the majority of clades had bs >70%, and many had bs >90%). The two accessions of *Alyssoides utriculata* (the only species in this clade represented by multiple accessions) were sister with strong support (100% bs). *Straussiella purpurea* was nested within Clade A3. This monotypic genus has not previously been included in any phylogenetic study. Clade A4 was relatively poorly resolved (all nodes had 61% or lower bs). Clade A5 was relatively well resolved (three of the four nodes had 87% or greater bs). Clade A6 was well resolved in all trees (all nodes had 88% or higher bs). Two species were represented by multiple accessions (*Aurinia uechtritziana* (2 accessions) and *Aurinia saxatilis* (2)); both were

non-monophyletic with high (92 and 98% bs respectively). Possible causes of the lack of species cohesion observed in this study are discussed in Chapter 4.

In order to investigate the effect of including or excluding the four Alysseae accessions that showed incongruence between plastid regions (see previous section), particularly on the relationships between the major Alysseae clades, a maximum likelihood tree was also constructed with these four accessions excluded (tree not shown). This tree had an identical species composition of clades (except for a lack of the excluded species) and identical relationships between the major Alysseae clades. Branch support values for these were very similar (i.e., within 3%). These results suggest that the observed incongruence has not caused any significant problems in the concatenated analysis, and these taxa were therefore not excluded from the further analyses of concatenated datasets.

Congruence with other Tree Search Methods

In order to investigate congruence between trees produced using other tree search methods, trees were also produced for Concatenated Alignments A and B using parsimony (implemented in Nona/Winclada) (Figures A6-11 and A6-12 in Appendix 6) and for Concatenated Alignment B using Bayesian inference (implemented in BEAST and incorporating a molecular clock, as described more fully under *Divergence Time Estimation* below) (Figure 3-3 and Figure A7-1). The strict consensus maximum parsimony tree for Concatenated Alignment A had a total tree length of 9067 steps, CI: 0.48 and RI: 0.82. The strict consensus maximum parsimony tree for Concatenated Alignment B had a total tree length of 4872 steps, CI: 0.45 and RI: 0.87. For ease of comparison, branch support values obtained using the three tree-search methods are shown on the maximum likelihood tree in Figure 3-1.

Clades A1, A1b, A1c, A2, A3, A4, A5 and A6 all received strong branch support in all three methods (>98% bs in all cases under maximum likelihood, >96% bs under parsimony, and 1.0 posterior probability (pp) under Bayesian inference). Clade A1a is present in all three trees; it

received moderate support (81% bs) under maximum likelihood, strong support (1.0 pp) under Bayesian tree, but only low support in the parsimony tree (48% bs).

The relationships between the major Alysseae clades A1, A2, A3, A4, A5 and A6 are completely congruent under all three methods. Within Clade A1, the sister relationship of A1a and A1b receives a rather variable level of branch support. Under parsimony and Bayesian inference the clade A1a + A1b it receives moderate support (73% bs and 0.89 pp, respectively), but under maximum likelihood it receives low support (33% bs). However, the clade which includes A1a, A1b and the somewhat labile *C. ciliata* does receive strong support (92% bs) under maximum likelihood, and therefore, apart from the position of *C. ciliata*, the relationships between clades A1a, A1b and A1c can be considered robust.

The low level of support that was present for the majority of branches within clade A1a under maximum likelihood is also the case under parsimony and Bayesian inference. No clades received moderate or strong support (i.e., >79% bs or >0.89% pp) under all three tree search methods, and only three clades received moderate or strong support in two of the trees: *A. floribundum* + *A. murale* (5); *A. pterocarpum* + *A. murale* (2); and *A. bracteatum* + *A. turgidum*. As under maximum likelihood, there was a relatively high level of well-supported resolution in Clade A2 under parsimony and Bayesian inference. In Clade A2, the non-monophyly of *A. montanum* received strong support (1.0 pp) under Bayesian inference.

There was no significant incongruence between multi-locus trees constructed using the three tree search methods. More detailed discussion about the correspondence between the Alysseae tree obtained here and those of previous studies, and about the taxonomic implications of these results are included in Chapter 4.

Brassicales-wide Phylogeny

Concatenated Alignment A contained all of the sequence data in Concatenated Alignment B plus additional accessions from non-Alysseae tribes in the Brassicaceae and from other families in the Brassicales. These were included in order to provide the nodes necessary under date Calibration Strategy 1. Concatenated Alignment C contained a large number of taxa from across the Brassicales. This alignment was employed in order to obtain estimates of the age of key nodes in the Alysseae clade (including the Alysseae crown node), under stage 1 of Calibration Strategy 3.

In order to investigate the congruence between Concatenated Alignments A, B and C, and to investigate the relationships between the non-Alysseae taxa, maximum likelihood trees were also produced from the latter two alignments (Figures A6-9 and A6-10 in Appendix 6). Within Alysseae there is complete congruence in the membership and relationships of the major Alysseae clades between trees based on Concatenated Alignments A and B. In the latter tree, the Alysseae is monophyletic with strong support (100% bs); the Alysseae clade forms a polytomy (with moderate support; 84% bs) with *Chorispora purpurascens* (tribe Chorisporaeae) and with a weakly supported clade (66% bs) containing *Arabidopsis thaliana* (tribe Cameliniidae) and *Ladakiella klimesii* (tribe Arabideae). Relationships between the remaining Brassicales species that were sampled are generally well resolved and robustly supported in both trees (eight of the ten nodes receive 99% bs or higher). In all cases where there is moderate or strong branch support (i.e., 70% bs or higher), these relationships are congruent with the Brassicales tree of Beilstein *et al.* (2010). This was also the case for trees constructed from Concatenated Alignment A using parsimony (Fig. A6-12 in Appendix 6). In the analysis of Concatenated alignment A using Bayesian inference and Calibration Strategy 1, separate MCMC chains failed to converge in a reasonable time period. This is likely to be due to the large size of this dataset, and the large amount of missing data due to the presence of large numbers of taxa for which sequences were only present for two of the eight loci). The Bayesian tree for

Concatenated Alignment A/Calibration Strategy 1 is therefore likely to be unreliable and is not considered here.

The relationships within Alysseae in the tree based on Concatenated Alignment C are generally robustly supported (most receive >80% bs). All are consistent with Concatenated Alignments A and B apart from the relationship of an accession of *A. simplex* sampled by Beilstein *et al.* (2010). As discussed for the separate *ndhF* tree above, this accession was sister to Clade A1a in the analysis based on Concatenated Alignment C, whereas it was within Clade A2 in other analyses (in this case those based on Alignments A and B). This is unsurprising given that the *ndhF* locus made up > 50% of the data in Concatenated Alignment C. Relationships across other parts of the Brassicales had variable branch support; where there was moderate or strong support (i.e., >70% bs) they were congruent with the tree of Beilstein *et al.*, (2010).

In general there is very strong congruence between the three concatenated alignments. Importantly, the two internal Brassicales nodes required for date-calibration under Calibration Strategy 2 (the *Akania-Bretschneidera* split within the Akaniaceae, and the *Thlaspi-Pseudocamelina* split within the Thlaspidae) both received strong support in all trees in which they were tested. (Testing the third node that was used, the crown node of the Brassicales is beyond the scope of this study).

Divergence Time Estimation

In order to estimate divergence times within Alysseae, time-calibrated phylogenetic trees were produced using a relaxed uncorrelated lognormal molecular clock implemented in BEAST. Three clock calibration strategies were employed. Calibration Strategy 1 was a primary calibration in which three fossil-dated nodes were used to calibrate a single tree containing Alysseae sequences and selected additional sequences from other parts of the Brassicales. Under this strategy, separate MCMC chains did not converge on the same posterior distribution within a

reasonable time period. This result may have been caused by the presence of small numbers of distantly related Brassicales species present in the same tree as large numbers of closely-related Alyseae species. Because of this lack of MCMC convergence, the results obtained under Calibration Strategy 1 are not presented or considered further here.

Calibration Strategy 2 was a secondary calibration in which an estimate for the age of the Alyseae crown node (obtained from Beilstein *et al.*, 2010) was used to calibrate an Alyseae tree. MCMC chains from separate analyses converged on the same posterior distributions (and samples could be pooled for both stages, yielding a high effective sample size (ESS) of 297.7. Problems with the choice of fossil-calibrated nodes in Beilstein *et al.* (2010) (discussed in full under *Molecular Clock-based Analysis using BEAST* in Chapter 2) are likely to make this date calibration unreliable, and therefore the results obtained under Calibration Strategy 2 are not discussed in detail here. The maximum clade credibility tree obtained under Calibration Strategy 2 is presented in Appendix 7.

Calibration Strategy 3 was a two-stage calibration strategy employing a Brassicales tree (largely based on the sequence data of Beilstein *et al.* (2010)) (Fig. 3-2a) that was calibrated using three fossil-dated nodes. The estimated ages of three nodes from this tree were then used to calibrate an Alyseae tree (Figure 3-2b). MCMC chains from separate analyses converged on the same posterior distributions (and samples could be pooled for both stages, yielding high ESS values; Stage 1: 250.1; Stage 2: 281.0). Since Calibration Strategy 3 did not have the MCMC convergence problems of Calibration Strategy 1, or the overly deep allocation of fossil-dated nodes of Calibration Strategy 2, the tree obtained under Calibration Strategy 3 is considered to be the most reliable of the three time calibrated trees generated in this study. The maximum clade credibility (MCC) tree produced using Calibration Strategy 3 is shown in Figs 3-2 and 3-3, and is discussed further below. This tree was used in subsequent character optimization and species diversification analyses.

Average posterior log likelihood values for the sets of trees from which MCC trees were produced were: -49136 (Calibration Strategy 2); -67748 Calibration Strategy 3 (Stage 1); and -49178 Calibration Strategy 3 (Stage 2). Only likelihood values from Calibration Strategy 2 and stage 2 of Calibration Strategy 3 are directly comparable. These two trees were based on the same alignment (Alignment B) and had very similar log likelihood values, despite different age calibration methods.

Brassicales Tree Topology and Resolution

The tree from Stage 1 of Calibration Strategy 3 had a topology and resolution consistent with Beilstein *et al.* (2010). The Alysseae (represented by 17 accessions) was monophyletic. Except for the sister relationship of *Alyssum simplex*, all nodes receiving posterior probabilities > 0.8 were consistent with the non time-calibrated maximum likelihood tree presented in Figure 3-1. *Alyssum simplex* (for which *ndhF* sequence data had been obtained from M.A. Beilstein, under the name *A. parviflorum*) was placed within *Clade A1*, rather than *Clade A2*. In other trees, all other accessions of *A. simplex* were placed in *Clade A2*.

Alysseae Tree Topology and Resolution

In the tree from Stage 2 of Calibration Strategy 3 Alysseae clades A1, A1a, A1b, A1c, A2, A3, A4, A5, A5a and A6 received strong support (pp of 1.0 in all cases). Two species were outside these clades: *Phyllolepidum cyclocarpum* was sister to *Clade A4* with low support (pp of 0.87) and *Clypeola ciliata* was sister to *Clades A1c* without support (pp of <0.8). *Clades A1b, A1c, A2, A3, A4, A5* and *A6* were well resolved; the majority of nodes received strong support. Resolution within *Clade A1a* was poor, with the majority of nodes unsupported (pp of <0.8). Six clades within *A1a* had strong support (i.e., > 0.94 pp): *A. pterocarpum* + *A. murale* (2); *A. turgidum* + *A. bracteatum*; *A. pintodasilvae* (2) + *A. malacitanum*; *A. floribundum* + *A. murale* (5); *A. argenteum* + *A. euboicum* (0.96 pp); and *A. murale* (4) + *A. lesbiacum* + *A. pintodasilvae* (1).

Apart from the relationships of *Phyllolepidum cyclocarpum* and *Clypeola ciliata*, the relationships between Alysseae clades are congruent with the multi-locus non time-calibrated maximum likelihood tree presented in the previous section.

Node Ages for Alysseae Clades

Estimated ages of selected nodes obtained under calibration strategies 2 and 3 are listed in Table 3-5, along with their 95% confidence intervals (CIs). These indicate that divergence time estimates are sensitive to the calibration strategies used and these differences are consistent across nodes. Divergence time estimates under Calibration Strategy 2 were younger than those obtained under Calibration Strategy 3. The magnitude of the differences between calibration strategies is greater for the older clades, with a much more limited range for younger clades. For example, Clade A1a (of particular interest here as it contains all nickel hyperaccumulators in *Alyssum*, see *Evolution of Nickel Hyperaccumulation* below) had a crown node age estimate of 2.71 (95% CI: 1.78–3.73) Mya under Calibration Strategy 2 and 3.27 (95% CI: 2.06–4.72) Mya under Calibration Strategy 3. The young ages of estimates under Strategy 2 are consistent with the selection of nodes for fossil-based dating by Beilstein *et al.* (2010) that are too deep in the phylogeny, as discussed in Chapter 2, and the observed differences in age estimates obtained under the two strategies are unsurprising. The estimates obtained under Calibration Strategy 3 are the preferred results and these were used in subsequent species diversification analyses.

Table 3-5. Divergence time estimates for selected nodes, produced using data calibration strategies 2 and 3, as described in the text.

Clade	Divergence time estimates with their 95% confidence intervals (Mya)					
	Strategy 2 (Secondary calibration)		Strategy 3 (Two-stage calibration)			
			Stage 1 (Brassicales tree)		Stage 2 (Alysseae tree)	
	Crown node	Stem node	Crown node	Stem node	Crown node	Stem node
A1a	2.71 (1.78–3.73)	6.61 (4.55–8.97)	N/A	N/A	3.27 (2.06–4.72)	8.31 (5.16–11.88)
A2	11.6 (8.34–15.24)	21.3 (16.3–26.8)	N/A	N/A	14.1 (9.71–19.5)	25.3 (18.2–33.5)
A5	7.28 (5.01–9.76)	17.6 (13.2–22.6)	N/A	N/A	8.78 (5.62–12.31)	20.7 (14.7–27.8)
A5a	5.16 (3.9–7.36)	7.28 (5.01–9.76)	N/A	N/A	6.31 (3.80–9.31)	8.78 (5.62–12.3)
<i>Alyssum s.l.</i>	21.3 (16.3–26.8)	23.3 (18.5–29.6)	N/A	N/A	25.28 (18.2–33.5)	27.5 (20.2–36.5)
Alysseae	23.3 (18.5–29.6)	N/A	38.4 (24.9–54.8)	58.32 (47.0–71.8)	27.47 (20.2–36.5)	N/A
Brassicales	N/A	N/A	101 (90.3–116)	N/A	N/A	N/A

Plant Traits

In order to investigate their evolution in Alyseae, data were collected on four plant traits for species in this tribe: maximum shoot nickel concentrations, serpentine endemism status, life history strategy, and species range size. The full dataset is included in Appendix 3.

Shoot Nickel Concentration Measurements

In order to ensure as complete and reliable a dataset as possible for shoot nickel concentration, measurements were taken using atomic absorption spectrophotometry for 17 species for which tissue was available. These include four species not previously tested, nine tested only once or twice previously, and four control species for which published data is available. Results are shown in Table 3-6. Of the total of 17 species tested, only the two positive control samples gave nickel concentrations above the hyperaccumulation threshold of 1 000 mg kg⁻¹ by dry mass. These results therefore provide evidence that another fifteen species are non-accumulators. However, leaf samples of *A. aizoides*, *Alyssum obtusifolium*, *A. flahaultianum*, and *A. nebrodense* were obtained from plants grown in standard commercial soil-based compost without any additional nickel added, and therefore may not be comparable with plants from serpentine soils. Of these, *A. flahaultianum* has not been tested previously, and given that only one specimen of this species has therefore now been tested, the result for this species should be regarded as provisional.

Nickel Hyperaccumulation in Alyseae

Nickel hyperaccumulation status data were assembled from the literature for 201 Alyseae species (i.e., 78.8 %), showing that there are thirty-nine confirmed nickel hyperaccumulator species (34 in *Alyssum s.l.*, four in *Bornmuellera*, and one in *Leptoplax*) out of a total of 255 species; and that an additional 17 of the *Alyssum s.l.* species were polymorphic (Table 3-7) for this trait. As previously demonstrated, nickel concentrations show a bimodal distribution both for all 201 species for which data are available (Fig. 3-4a), and for the subset of 164 species sampled in the current phylogeny (Fig. 3-4b).

Table 3-6. Nickel concentration measurements of shoot dry biomass.

Species	Tissue or seed source	Botanical voucher collector name and number / seed accession number	Ni content (mg kg ⁻¹ dry mass)	Hyperaccumulator status (Ni > 1000 mg kg ⁻¹)
Positive Controls:				
<i>Alyssum cilicicum</i>	E*	Reeves 2185	11570	Hyperaccumulator
<i>Alyssum dubertretii</i>	E*	Reeves 2171	16210	Hyperaccumulator
Negative Controls:				
<i>Alyssum stribryni</i>	AGS ¹	1255	1	Non-accumulator
<i>Alyssum umbellatum</i>	E	Meikle 2709	890	Non-accumulator
Test Species:				
<i>Alyssum aizoides</i>	P ¹	Jurasek 2005: 341	3	Non-accumulator
<i>Alyssum obtusifolium</i>	UPM ¹	1340-68	4	Non-accumulator
<i>Alyssum filiforme</i>	E*	Reeves 2223	299	Non-accumulator
<i>Alyssum flahaultianum</i>	UPM ¹	2184-74	57	Non-accumulator
<i>Alyssum fragillimum</i>	E	Davis 18127A	27	Non-accumulator
<i>Alyssum haussknechtii</i>	E	Coode & Jones 1349	247	Non-accumulator
<i>Alyssum mouradicum</i>	E*	Reeves 1983	6	Non-accumulator
<i>Alyssum nebrodense</i>	UPM ¹	6950-85	9	Non-accumulator
<i>Alyssum polycladum</i>	E	Sharif	37	Non-accumulator
<i>Alyssum tetrastemon</i>	P	Pavelka 2008: 57	1	Non-accumulator
<i>Chorispora purpurascens</i>	E	Jallad 207	30	Non-accumulator
<i>Fibigia umbellata</i>	E	Aryavand <i>et al.</i> 1421	20	Non-accumulator
<i>Physocardamum davisii</i>	E	Tatli 5806	165	Non-accumulator
Sources: Plant material obtained from: AGS: Alpine Garden Society; P: Private seed collection; UPM: Crucifer Seedbank at the Polytechnic University of Madrid; E: Herbarium material, Royal Botanic Gardens, Edinburgh; E* Dried leaf material provided by R.D. Reeves taken from herbarium specimens prior to deposition at E. Full addresses are provided in Appendix 2. ¹ Plants grown from seed in commercial soil-based compost (i.e., without added nickel).				

Table 3-7. Nickel hyperaccumulation data for genera of Alysseae.

Genus	Nickel hyperaccumulator status				Total
	Hyperaccumulator	Polymorphic	Non-accumulator	No data	
<i>Alyssoides</i>	0	0	2	0	2
<i>Alyssum</i>	34	17	98	39	188
<i>Aurinia</i>	0	0	6	2	8
<i>Berteroa</i>	0	0	5	0	5
<i>Bornmuellera</i>	4	0	2	1	7
<i>Clastopus</i>	0	0	2	0	2
<i>Clypeola</i>	0	0	7	2	9
<i>Degenia</i>	0	0	1	0	1
<i>Fibigia</i>	0	0	7	6	13
<i>Galitzkya</i>	0	0	1	2	3
<i>Hormathophylla</i>	0	0	8	2	10
<i>Leptoplax</i>	1	0	0	0	1
<i>Phyllolepidum</i>	0	0	2	0	2
<i>Physocardamum</i>	0	0	1	0	1
<i>Physoptychis</i>	0	0	2	0	2
<i>Straussiella</i>	0	0	1	0	1
Total	39	17	145	54	255

Serpentine Endemism in Alyseae

Data on serpentine endemism status were assembled from the literature for 156 (i.e. 61.2 %) of the 255 Alyseae species (Table 3-8), and show that the species endemic to areas of serpentine substrate are in the same genera and include almost the same numbers of species as nickel hyperaccumulators, although the proportion of species of unknown status is higher (40 % cf. 20 %) for serpentine occurrence. Of the 40 serpentine-endemic species, 35 (i.e., 88%) were nickel hyperaccumulators, two were polymorphic for nickel hyperaccumulation (*Alyssum euboicum* and *A. floribundum*), and one species was a non-accumulator (*A. densistellatum*).

Table 3-8. Serpentine endemic status data for genera of Alyseae.

Genus	No. species			Total
	Serpentine-endemic	Not serpentine-endemic	No data	
<i>Alyssoides</i>	0	2	0	2
<i>Alyssum</i>	35	84	69	188
<i>Aurinia</i>	0	6	2	8
<i>Berteroa</i>	0	4	1	5
<i>Bornmuellera</i>	4	2	1	7
<i>Clastopus</i>	0	0	2	2
<i>Clypeola</i>	0	6	3	9
<i>Degenia</i>	0	0	1	1
<i>Fibigia</i>	0	2	11	13
<i>Galitzkya</i>	0	0	3	3
<i>Hormathophylla</i>	0	8	2	10
<i>Leptoplax</i>	1	0	0	1
<i>Phyllolepidum</i>	0	1	1	2
<i>Physocardamum</i>	0	0	1	1
<i>Physoptychis</i>	0	0	2	2
<i>Straussiella</i>	0	0	1	1
Total	40	116	99	255

Life-History Strategy in Alyseae

Life-history strategy data were assembled from the literature covering 222 (i.e., 87.1 %) of the 255 Alyseae species (Table 3-9), and indicate that the majority of species in the tribe (i.e., at least 174; 68%) are iteroparous.

Table 3-9. Life-history strategy data for genera of Alysseae.

Genus	Life-History Strategy			Total
	Semelparous	Iteroparous	No Data	
<i>Alyssoides</i>	0	2	0	2
<i>Alyssum</i>	38	122	28	188
<i>Aurinia</i>	0	8	0	8
<i>Berteroa</i>	2	3	0	5
<i>Bornmuellera</i>	0	7	0	7
<i>Clastopus</i>	0	2	0	2
<i>Clypeola</i>	9	0	0	9
<i>Degenia</i>	0	1	0	1
<i>Fibigia</i>	0	9	4	13
<i>Galitzkya</i>	0	3	0	3
<i>Hormathophylla</i>	0	10	0	10
<i>Leptoplax</i>	0	1	0	1
<i>Phyllolepidum</i>	0	2	0	2
<i>Physocardamum</i>	0	1	0	1
<i>Physoptychis</i>	0	2	0	2
<i>Straussiella</i>	0	1	0	1
Total	49	174	32	255

Species Range Size in Alysseae

Species range area was estimated as the number of 50 × 50 km grid squares in native species ranges. These data were assembled from *Atlas Flora Europaeae* (Jalas & Suominen, 1994) for 63 Alysseae species, including 11 nickel hyperaccumulators (of which two were polymorphic), 43 non-accumulators and nine species of unknown nickel hyperaccumulation status (full dataset in Table 3-2 in Appendix 3). Range areas extended from one 50 × 50 km grid square, the minimum resolution of this dataset (for 12 species: two hyperaccumulators, eight non-accumulators and two species of unknown status) to 203 grid squares (for the non-accumulator *Aurinia saxatilis*). The frequencies of species range areas for nickel hyperaccumulator (including polymorphic species) and non-accumulator species suggest that hyperaccumulators tend to occupy smaller ranges than non-accumulators (Fig. 3-5a). A two-tailed *t*-test on the log-transformed dataset (sample variances constrained to be equal) indicated no significant difference between means of nickel hyperaccumulator and non-accumulator species, both when the former included polymorphic species ($p = 0.12$, $n = 11$ and 43, respectively) and excluded them ($p = 0.13$, $n = 9$

and 43 respectively). Because of the presence of negative skew in the frequency distributions (Fig. 3-5a), Wilcoxon rank sum tests were also carried out, and indicated no significant difference in range size between nickel hyperaccumulator and non-accumulator species when polymorphic species were included (as hyperaccumulators) ($p = 9.8 \times 10^{-2}$), or excluded ($p = 9.4 \times 10^{-2}$).

A second measure of species range size was employed: linear range size. This permitted inclusion of a larger sample of species: 172 *Alyseae* species, including 36 nickel hyperaccumulators, 17 polymorphic species, 119 non-accumulators and 18 species of unknown nickel hyperaccumulation status. The frequency distribution of this measure (further details of which are included in Chapter 2) (Fig. 3-5b) suggests a difference in range size between hyperaccumulator and non-accumulator species. In this analysis the Ni hyperaccumulator *Alyssum troodi* had the smallest linear range (8 km, although this was converted to the minimum value of 290 km as described in Chapter 2 for all analyses except the linear correlation described below). The non-accumulator *A. simplex* had the largest linear range (9 241 km). Two-tailed *t*-tests on the log-transformed data (sample variances constrained to be equal) indicated that nickel hyperaccumulator species occupied smaller mean linear ranges than non-accumulator species, both when the former included polymorphic species ($p = 3.5 \times 10^{-3}$, $n = 53$ and 119, respectively) and when it excluded them ($p = 3.2 \times 10^{-8}$, $n = 36$ and 119 respectively). Because of the presence of negative skew in the frequency distributions (Fig. 3-5a), Wilcoxon rank sum tests were also carried out, and also indicated a significant difference in range size between nickel hyperaccumulator and non-accumulator species ($p = 4.0 \times 10^{-4}$ and 2.0×10^{-8} , including and excluding polymorphic species respectively). Using the linear measure of species range size (which allowed a much larger dataset, and hence analyses with greater statistical power than the extent of occurrence measure), there is strong evidence that nickel hyperaccumulator species have smaller range sizes than non-accumulator species.

In order to ensure that the linear measure of species range size was correlated with the species range area values obtained from *Atlas Flora Europaeae*, a correlation analysis was carried out. A significant correlation between linear range size and extent of occurrence for the 63 species for which both measures were available was found (Pearson correlation coefficient was: $r^2 = 0.67$ ($p = 3.09 \times 10^{-16}$) (Fig. 3-6a), justifying the use of the former measure (which is readily estimated from available range data) as an alternative to the latter measure (which is not readily estimated and requires a level of collection data which is lacking for many Alysseae species). The least squares linear regression line had an x coefficient = 0.882 ($p = 3.09 \times 10^{-16}$) and y intercept = -1.35 ($p = 4.75 \times 10^{-9}$). Residual values from the correlation analysis are shown in Figure 3-6b, and indicate (by inspection) an approximately normal distribution (and hence that the assumption of normality made under a linear regression analysis is justified). Two additional non-parametric correlation analyses also both strongly rejected the null hypothesis of no correlation (Spearman's rank correlation coefficient was 0.840 ($p = 2.2 \times 10^{-16}$), and Kendall's tau coefficient was 0.690 ($p = 8.66 \times 10^{-15}$)).

Plant Trait Optimization

In order to investigate the evolutionary history of nickel hyperaccumulation, serpentine endemism and life history strategy in the Alysseae, these traits were optimized onto the maximum clade credibility tree obtained from the two-stage molecular clock calibration (Calibration Strategy 3) using maximum likelihood and Bayesian methods implemented in Mesquite and BayesTraits (for further details see Chapter 2). Results of these analyses are discussed in the following sections, and illustrated in Fig. 3-7, 3-8 and 3-9, and Fig. A8-1 in Appendix 8.

Evolution of Nickel Hyperaccumulation

Maximum likelihood optimisation of nickel hyperaccumulation, with polymorphic species coded as hyperaccumulators, shows independent origins of nickel hyperaccumulation on the branches leading to Clade A5a in *Bornmuellera s.l.* and to Clade A1a in *Alyssum s.l.* (Fig. 3-7). Nickel hyperaccumulation is postulated to have evolved within *Bornmuellera s.l.* on the branch subtending the clade comprising *Bornmuellera kiyakii*, *B. tymphaea*, *B. baldaccii* and *Leptoplax emarginata*, here referred to as the A5a clade, with no hypothesized reversals to non-accumulation. Nickel hyperaccumulation apparently also evolved independently within *Alyssum s.l.*, on the branch leading to *Clade A1a*.

Maximum likelihood analysis (using BayesTraits and based on the MCC tree), yielded a certainty of 0.99951 for the crown node of Clade A5a being a nickel hyperaccumulator, and a certainty of 0.99998 for the crown node of Clade A1a being a nickel hyperaccumulator. Estimates for the rate of character change were 4.47×10^{-3} changes $\text{Mya}^{-1} \text{branch}^{-1}$ for changes from non-accumulator to nickel hyperaccumulator, and 1.33×10^{-1} changes $\text{Mya}^{-1} \text{branch}^{-1}$ for changes from nickel hyperaccumulator to non-accumulator. Constraining the crown node of Clade A5a to be a nickel hyperaccumulator and then a non-accumulator gave log likelihood values of -52.69 and -56.27, respectively (a difference of 3.58 log likelihood units). Constraining the crown node of Clade A1a to be a nickel hyperaccumulator and then a non-accumulator gave log likelihood values of -52.68 and -59.48, respectively (a difference of 3.34 log likelihood units). These differences are both >2 log likelihood units, suggesting significance (Pagel, 1999), and providing evidence that the crown node of Clade A1a represents a nickel hyperaccumulator species.

MCMC-based Bayesian analysis yielded a mean certainty of 0.99756 (95% CI: 0.99090–0.99997) for the crown node of Clade A5a being a nickel hyperaccumulator, and mean certainty of 0.99991 (95% CI: 0.99963–0.99999) for the crown node of Clade A1a being a nickel

hyperaccumulator. Estimates for the rate of character change had means 8.11×10^{-3} (95% CI: 1.31×10^{-3} – 1.91×10^{-2}) changes $\text{Mya}^{-1} \text{branch}^{-1}$ for changes from non-accumulator to nickel hyperaccumulator, and 1.41×10^{-1} (95% CI: 9.01×10^{-2} – 2.04×10^{-1}) changes $\text{Mya}^{-1} \text{branch}^{-1}$ for changes from nickel hyperaccumulator to non-accumulator. Constraining the crown node of Clade A5a to be a nickel hyperaccumulator and then a non-accumulator gave harmonic mean log likelihood values of -55.03 and -59.27, respectively. The Bayes factor statistic (8.48) of >5 provides evidence (Pagel & Meade n.d.) that the A5a crown node has a higher probability of having been a nickel hyperaccumulator than a non-accumulator. Constraining the crown node of Clade A1a to be a nickel hyperaccumulator and then a non-accumulator gave harmonic mean log likelihood values of -54.94 and -62.77, respectively. The Bayes factor statistic (15.66) of >10 provides strong evidence (Pagel & Meade n.d.) that the A1a crown node has a higher probability of having been a nickel hyperaccumulator than a non-accumulator.

In the MCC tree employed in the above analyses, the A1a clade contains 59 accessions of 49 nickel hyperaccumulator species (including polymorphic species) and 16 accessions of 15 non-accumulator species. However, since the majority of branches within this clade receive only weak support (<0.8 pp) the species relationships within the clade remain largely unknown (Fig. 3-3c). Depending on the resolution of these relationships it could be necessary to invoke up to 16 separate losses or multiple gains of the nickel hyperaccumulation trait in this clade. In order to explore the impact of this phylogenetic uncertainty upon the nickel hyperaccumulation status of the A1a crown node, maximum likelihood analyses were also carried out on a sample of 1000 posterior trees. For these trees, the certainty of the crown node of Clade A1a being a nickel hyperaccumulator had a mean of 0.99949 and a range of 0.97323–1.0. Constraining the crown node of Clade A1a to be a nickel hyperaccumulator gave log likelihood values higher than when it was constrained to be a non-accumulator in all but one (i.e., 99.9%) of the trees (in the single exception it was 0.32 log units lower). Values under enforced hyperaccumulator status of at the A1a crown node were significantly higher (i.e. >2 log likelihood units; Pagel 1999; note

that formal log likelihood tests are not possible for non-nested models) than under enforced non-accumulator status in 949 (i.e., 94.9%) of the trees. Unfortunately there is no statistically meaningful way to combine results across trees under maximum likelihood (Pagel and Meade, n.d.), and MCMC-based analysis over multiple trees was not possible for this dataset (possibly due to inter-tree heterogeneity in likelihood values, see Chapter 2 for further details).

In order to explore the impacts of the occurrence of polymorphism in nickel hyperaccumulation in some species in Clade A1a, a second optimisation was carried out using maximum likelihood, this time coding species polymorphic for nickel hyperaccumulation as third character state. This analysis also suggested origins of nickel hyperaccumulation on the branches leading to Clade A5a in *Bornmuellera* and to Clade A1a in *Alyssum s.l.* The results are shown in Fig. A8-1 in Appendix 8. Since all members of Clade A5a are hyperaccumulators, and none are polymorphic, for *Bornmuellera* this analysis yields results identical to those above for this clade. The A1a clade includes 34 accessions of 32 nickel hyperaccumulator species, 25 accessions of 17 polymorphic species, and 16 accessions of 15 non-accumulator species). Depending on resolution of relationships within Clade A1a it could be necessary to invoke multiple independent losses (up to 12 separate losses) of the nickel hyperaccumulation or polymorphic traits, and multiple shifts from hyperaccumulator to polymorphic states in this clade under this three character state coding regime.

Constraining the crown node of Clade A1a to be a nickel hyperaccumulator, polymorphic species and then a non-accumulator gave log likelihood values of -94.20, -94.10 and -100.26 respectively (a difference of 6.06 and 6.16 log likelihood units between the first and last and between the second and last of these, respectively; these differences are both >2 log likelihood units, suggesting significance (Pagel, 1999)). This result suggests that including a separate character state for polymorphic species does not alter the conclusion that the crown node of Clade A1a was a nickel hyperaccumulator or a polymorphic species. The similarity in likelihood

values under enforced nickel hyperaccumulator status and polymorphic status mean that it not clear precisely which of these two character states was likely to have been present at the A1a crown node.

None of the above analyses differ in their key conclusions: two clear origins of nickel hyperaccumulation within Alyseae, although a more detailed exploration of the evolutionary history of nickel hyperaccumulation within Clade A1a is beyond the scope of this study due to the lack of resolution in this clade.

Evolution of Serpentine Endemism

Maximum likelihood optimisation of serpentine endemism shows this trait to be present in three Alyseae clades (Fig. 3-8). There is one origin of serpentine endemism in *Bornmuellera s.l.* on the branch subtending Clade A5a. There is one origin within Clade A2, on the branch leading to *Alyssum densistellatum* (the only serpentine endemic species in Clade A2). All other serpentine endemics within Alyseae are placed within Clade A1a, and the crown node of this clade is inferred to be serpentine endemic, indicating that this trait evolved on the branch subtending this node.

Maximum likelihood analysis (using BayesTraits and based on the MCC tree), yielded a certainty of 0.96804 for the crown node of Clade A5a being serpentine endemic, and a certainty of 0.99980 for the crown node of Clade A1a being serpentine-endemic. Estimates for the rate of character change were 2.43×10^{-2} changes $\text{Mya}^{-1} \text{branch}^{-1}$ for changes from non-serpentine endemic to serpentine-endemic, and 3.35×10^{-1} changes $\text{Mya}^{-1} \text{branch}^{-1}$ for changes from serpentine endemic to non-serpentine endemic. Constraining the crown node of Clade A5a to be serpentine-endemic and then non serpentine-endemic gave log likelihood values of -65.94 and -66.56, respectively (a difference of 0.62 log likelihood units). Constraining the crown node of Clade A1a to be serpentine endemic and then a non serpentine-endemic gave log likelihood

values of -65.60 and -71.01, respectively (a difference of 5.41 log likelihood units). Of these differences, only the latter is >2 log likelihood units, suggesting significance (Pagel, 1999). The small number of species present in the A5a clade is likely responsible for the lack of significance of the former difference.

MCMC-based Bayesian analysis yielded a mean certainty of 0.88873 (95% CI: 0.67314–0.99163) for the crown node of Clade A5a being serpentine-endemic, and mean certainty of 0.99880 (95% CI: 0.99561–0.99995) for the crown node of Clade A1a being serpentine-endemic. Estimates for the rate of character change had means 4.07×10^{-2} (95% CI: 1.25×10^{-2} – 8.57×10^{-2}) changes $\text{Mya}^{-1} \text{branch}^{-1}$ for changes from non serpentine-endemic to serpentine-endemic, and 3.79×10^{-1} (95% CI: 2.45×10^{-1} – 5.38×10^{-1}) changes $\text{Mya}^{-1} \text{branch}^{-1}$ for changes from serpentine endemic to non serpentine-endemic. Constraining the crown node of Clade A5a to be serpentine-endemic and then non serpentine-endemic gave log likelihood values of -69.27 and -69.96, respectively (a difference of 0.69 log likelihood units). Constraining the crown node of Clade A1a to be serpentine-endemic and then a non serpentine-endemic gave log likelihood values of -68.21 and -71.14, respectively (a difference of 2.93 log likelihood units). Of these difference, only the latter is >2 log likelihood units, suggesting significance (Pagel, 1999). The small number of species present in the A5a clade is likely to be responsible for the lack of significance of the former difference.

In the MCC tree employed in the above analyses, Clade A1a contains 32 accessions of 30 serpentine endemic species. Depending on the resolution of species relationships within Clade A1a, it could be necessary to invoke losses or gains of serpentine endemism within this clade. In order to explore the impact of the phylogenetic uncertainty in Clade A1a on the serpentine endemic status of the A1a crown node, maximum likelihood analyses were also carried out on a sample of 1000 posterior trees. For these trees, the certainty of the crown node of Clade A1a being serpentine endemic had a mean of 0.99586 and a range of 0.84739–0.99999.

Constraining the crown node of Clade A1a to be serpentine endemic gave log likelihood values higher than constraining it to be a non serpentine-endemic in all but four (i.e., 99.6%) of the trees, and significantly higher (i.e. >2 log likelihood units difference, Pagel 1999) in 893 (i.e., 89.3%) of the trees. Unfortunately there is no statistically meaningful way to combine results across trees under maximum likelihood (Pagel and Meade, n.d.), and MCMC-based analysis over multiple trees was not possible for this dataset (possibly due to inter-tree heterogeneity in likelihood values, see Chapter 2 for further details).

Evolution of Life-History Strategy

Maximum likelihood optimisation of life history strategy (coded as semelparous and iteroparous), using the same MCC tree as in the above character analyses, shows that the maximum likelihood ancestral state for Alysseae as a whole is iteroparous (Fig. 3-9), and no members of clades A3, A4 and A5 are semelparous. There are multiple transitions to the derived semelparous condition across the tree (within clades A2, A1 and A6, as well as within clade A1a) (Fig. 3-9). The ancestral state of clade A2 is unclear since the ancestral state of subclades A2a and A2b are iteroparous and semelparous respectively. Clade A1a contains three semelparous species, with all others being iteroparous; and trait optimisation implies an ancestral iteroparous state for this clade. However, the general lack of resolution in clade A1a, precludes any precise estimate of the number of independent origins of serpentine endemism within this clade.

Character Association

In order to investigate a possible correlation between nickel hyperaccumulation and serpentine endemism, maximum likelihood optimisations were carried out in BayesTraits. Both traits were coded as binary characters, with species polymorphic for nickel hyperaccumulation coded as nickel hyperaccumulators. This analysis yielded log likelihood values of -99.80 and -124.3 for

character-dependent and character-independent maximum likelihood optimisations, respectively. The log likelihood ratio (49.07) indicates that the character-dependent model provides a significantly better fit than the independent model ($p < 0.005$, d.f. = 4).

MCMC-based Bayesian analysis gave harmonic means of log likelihoods of -130.9 and -104.27 under character dependent and character independent optimizations, respectively. The Bayes factor statistic (53.26) provides strong evidence that the character-dependent model provides a better fit than the independent model. Maximum likelihood optimisation also yielded a significantly better fit ($p < 0.05$) for the dependent model than for the independent model across all 1000 posterior trees (sampled from the set of posterior *Alysseae* trees obtained under Calibration Strategy 3). Dependent model likelihood values had the range -109.26 to -93.54 and independent model likelihood values had the range -139.42 to -116.59 for these trees, indicating that the association between traits is robust to phylogenetic uncertainty within *Alysseae*. Together, the above results provide good evidence for a correlation between nickel hyperaccumulation and serpentine endemism.

Species Diversification Rate Analyses

Whole-Clade Diversification Rates

In order to investigate patterns of species diversification within Alysseae, the whole-clade diversification methods of Magallón & Sanderson (2001; 2006) were used to estimate species diversification rates for selected clades within the tribe, and to indicate any clades which are larger or smaller than expected, given the background diversification rate of the Alysseae. Results are presented in Table 3-10, and on the diversity tree in Fig. 3-10. These indicate that Clade A1a has the highest whole-clade average net species diversification rate under both calibration strategies 2 and 3 (ranging from 0.52 to 1.35 splits lineage⁻¹ million years⁻¹ under zero extinction to 0.33 to 0.78 splits lineage⁻¹ million years⁻¹ under high extinction (a relative extinction rate of 0.9)). For the reasons discussed under *Divergence Time Estimation*, above, estimates of species diversification rates based on ages obtained from Calibration Strategy 3 are the preferred to those based on Calibration Strategy 2.

Under both calibration strategies 2 and 3, Clade A1a (which contains all of the nickel hyperaccumulators in *Alyssum*) has a net species diversification rate approximately four times as high as Clade A2 (which contains the majority of the non-accumulator species in *Alyssum*). Only the A1a clade had a size outside the 95% confidence interval for the expected value (based upon the whole-clade species diversification rate for the Alysseae). Clade A1a was larger than the upper limit, implying that species diversification has proceeded with a higher average rate in Clade A1a than the average (background) rate in the tribe Alysseae.

Table 3-10. Whole-clade diversification rates for selected clades in Alysseae.

Clade	Net species diversification rate				Clade Size (Species)	95% CI for Expected Clade Size* $\epsilon = 0$ ($\epsilon = 0.9$)
	Calibration Strategy 2 Secondary calibration		Calibration Strategy 3 Two-stage calibration			
	Crown node	Stem node	Crown node	Stem node		Crown node
A1a	1.35 (0.78)	0.66 (0.33)	1.12 (0.65)	0.52 (0.26)	78	2.1–8.4 (1.2–36)
A2	0.33 (0.19)	0.21 (0.11)	0.27 (0.16)	0.18 (0.090)	89	4.5–69 (4.1–456)
<i>Alyssum s.l.</i>	0.21 (0.14)	0.22 (0.13)	0.18 (0.11)	0.19 (0.11)	181	24.3–526 (26–3633)
A5	0.29 (0.12)	0.16 (0.05)	0.24 (0.098)	0.13 (0.044)	16	2.7–25.7 (2.1–154)
A5a	0.13 (0.039)	0.19 (0.036)	0.14 (0.032)	0.067 (0.013)	4	2.3–15.9 (1.6–86.6)
Alysseae	0.21 (0.14)	N/A	0.18 (0.12)	N/A	255	N/A
N.B. Values assume an extinction rate (ϵ) of 0 and in parentheses an extinction rate of 0.9. Diversification rates are in units of lineage splits lineage ⁻¹ million years ⁻¹ . All values were calculated in the GEIGER package of R (Harmon <i>et al.</i> , 2008).						
*The 95% confidence interval for the expected species diversification rate was determined from the Alysseae clade crown node rate obtained under Calibration Strategy 3, using the method of Magallón and Sanderson (2001, 2007) implemented in the R package GEIGER.						

Detecting Species Diversification Rate Shifts

In order to detect shifts in species diversification and extinction rates within Alysseae, the stepwise maximum likelihood modelling procedure MEDUSA (Alfaro *et al.*, 2009) was employed. In order to maximise the representation of Alysseae taxa, species that were not sampled in the current phylogeny, but for which hyperaccumulation data were available were assigned to terminal clades based on the correspondence between the major Alysseae clades and the generic and sectional taxonomy of the tribe. All but 16 of the 255 Alysseae species (i.e., 94%) could be assigned a terminal clade (see *Diversification Rate Analyses* section in Chapter 2 and Table A5-1 in Appendix 5 for full details).

The analysis in which the 16 unassigned species were excluded gave an optimal model (based on AIC scores corrected for finite dample size (AICc)) with two diversification rates (i.e., one rate shift) (Table 3-11). The optimal location of the breakpoint between these two rates was on the branch leading to Clade A1a. For Clade A1a, the estimated species diversification rate (r) was 0.40 splits lineage⁻¹ million years⁻¹ and the estimated relative extinction rate (ϵ) was 0.65 extinctions lineage⁻¹ million years⁻¹. For the remainder of the tree (i.e., excluding Clade A1a), r was 0.16 and ϵ was 1.23×10^{-7} .

Table 3-11. Log likelihood and AICc scores for the six models with the lowest AICc scores obtained from the diversification analysis excluding the 16 unassigned *Alyssum* species.

Model No.	No. of diversification rates	No. parameters	Log likelihood	AICc score*
1	1	2	-64.29	133.3
2	2	5	-55.76	126.1
3	3	8	-54.04	138.5
4	4	11	-52.67	165.1
5	5	14	-52.05	237.1
6	6	17	-51.70	749.4
Results for the optimal model (lowest AICc score) are shown in bold.				
* AICc score is AIC score with a correction for finite sample sizes.				

A likelihood ratio test using χ^2 gave $p = 6.8 \times 10^{-4}$, indicating a significant difference between the likelihood values of the one-rate and two-rate models. These results provide evidence for a 2.5-fold increase in species diversification rate and a decrease in relative extinction rate on the branch leading to Clade A1a.

Assigning all 16 unassigned species to Clade A1b, still resulted in an optimal model with two diversification rates (i.e., one rate shift) (Table 3-12). However, in this case the rate shift was located on the branch leading to the A1a + A1b clade. For the A1a + A1b clade, r was 0.25 and ϵ was 0.85. For the remainder of the tree (i.e., excluding the A1a clade), r was 0.15 and ϵ was 3.4×10^{-7} . A likelihood ratio test using χ^2 gave $p = 7.1 \times 10^{-4}$, indicating a significant difference between the likelihood values of the one-rate and two-rate models. This assignment represents an extreme case and was carried out in order to test the maximum possible impact upon the MEDUSA analysis of the 16 unassigned taxa. Due to the small size of Clade A1b (five species assigned in the previous analysis), it is considered extremely unlikely that all of the 16 unassigned species would be members of this clade.

Table 3-12. Log likelihood and AICc scores for the six models with the lowest AICc scores obtained from the diversification analysis in which the 16 unassigned *Alyssum* species were placed in clade A1b.

Model No.	No. of Diversification Rates	No. Parameters	Ln Likelihood	AICc score*
1	1	2	-65.59	135.9
2	2	5	-57.09	128.8
3	3	8	-55.41	141.2
4	4	11	-54.10	167.9
5	5	14	-53.53	240.1
6	6	17	-53.15	752.3
Results for the optimal model (lowest AICc score) are shown in bold.				
* AICc is AIC with a correction for finite sample sizes.				

Given the large size of Clade A2 (i.e., the largest of Alysseae clades A1–A6, with 89 species assigned in the previous analysis), it is considered likely that the majority of the unassigned

species will be members of that clade. A third scenario, in which all 16 unassigned species were placed in Clade A2, gave similar results to the analysis excluding these 16 species. The optimal model had two diversification rates (i.e., one rate shift) (Table 3-13), and the rate was shift located on the branch leading to the A1a clade. For the A1a clade, r was 0.40 and ϵ was 0.65 (i.e., identical to the results of the first MEDUSA analysis). For the remainder of the tree (i.e., excluding Clade A1a), r was 0.159 and ϵ was 1.09×10^{-6} . A likelihood ratio test using χ^2 gave $p = 7.6 \times 10^{-4}$, indicating a significant difference between the likelihood values of the one-rate and two-rate models.

Table 3-13. Log likelihood and AICc scores for the six models with the lowest AICc scores obtained from the diversification analysis in which the 16 unassigned *Alyssum* species were placed in clade A2.

Model No.	No. of Diversification Rates	No. Parameters	Ln Likelihood	AICc score*
1	1	2	-64.48	133.0
2	2	5	-56.06	122.1
3	3	8	-54.31	124.6
4	4	11	-52.90	127.8
5	5	14	-52.24	132.5
6	6	17	-51.86	137.7
Results for the optimal model (lowest AICc score) are shown in bold.				
* AICc is AIC with a correction for finite sample sizes.				

Under all three MEDUSA analyses, including two which attempted to account for species that could not be assigned to terminal clades, there was a significant increase in species diversification rate within Clade A1. These results are in agreement with the whole-clade diversification rates described in the previous section for Clade A1 (which was larger than the range of expected values for a clade of that age).

Because MEDUSA employs stem ages in the calculation of net diversification rates in unresolved terminal clades (Alfaro *et al.*, 2009), it is likely to underestimate rates in clades having long stem

lineages (as is the case for Clade A1a; Fig. 3-3). The higher diversification rate obtained for the A1a clade using the crown node-based whole-clade approach (in the previous section) is consistent with this (0.65–1.12 as opposed to 0.40 splits lineage⁻¹ million years⁻¹) and should be used in preference to the MEDUSA rate in comparisons with other taxa.

Testing Associations between Diversification Rate and Trait Evolution using BiSSE

In order to investigate the possible correlation of nickel hyperaccumulation with species diversification and extinction rates in the Alyseae, a maximum likelihood based BiSSE model (Maddison *et al.*, 2007; Fitzjohn *et al.*, 2009) was employed. Species birth (λ) and death (μ) rates estimated across the Alyseae, *Alyssum s.l.* and non-*Alyssum s.l.* Alyseae clades (Table 3-14) show that a two- λ -rate model yields higher species diversification rates for hyperaccumulator versus non-accumulator lineages in both Alyseae and *Alyssum s.l.* clade-wide analyses. Likelihood ratio tests using χ^2 gave $p = 2.10 \times 10^{-11}$ and $p = 4.01 \times 10^{-8}$ (for Alyseae and *Alyssum s.l.*, respectively); the change in AIC values (i.e., ΔAIC) from the one-rate models to the two-rate models were both negative (–42.88 and –28.15, for Alyseae and *Alyssum s.l.* respectively), i.e., the lower AIC scores were for the two-rate models in both cases, indicating that the two-rate models gave a better fit to the data. This suggests a significant association between the derived evolution of nickel hyperaccumulation and an elevated species diversification rate within *Alyssum s.l.*

In contrast, there was no significant difference between species diversification rates for hyperaccumulator and non-accumulator species in the non-*Alyssum s.l.* Alyseae clade-wide analysis (the likelihood ratio test using χ^2 gave $p = 0.787$, and $\Delta AICc$ from the one-rate to the two-rate model was +1.93, i.e., the one-rate model had a lower score, and hence a better fit to the data.

The hypothesis that species diversification rates are equal for nickel hyperaccumulator and non-accumulator lineages can therefore be rejected for the *Alyssum s.l.* clade, where the species diversification rate is higher for hyperaccumulator than for non-accumulator lineages. The null hypothesis of equal rates cannot be rejected for the non-*Alyssum s.l.* Alyseae clade, suggesting that the evolution of nickel hyperaccumulation is not associated with an increase in species diversification rate in the A5a (i.e., *Bornmuellera* + *Leptoplax* + *Physocardamum*) hyperaccumulator clade.

Table 3-14. Birth-death parameter estimates obtained in BiSSE analysis excluding the 16 unassigned *Alyssum* species.

Analysis	Model	Diversification Rate (bifurcations lineage ⁻¹ million years ⁻¹)		Extinction Rates (extinctions lineage ⁻¹ million years ⁻¹)	
		λ ₀	λ ₁	μ ₀	μ ₁
Alysseae-wide	Two-λ-rate	0.160	2.18	1.30 × 10 ⁻⁶	1.79
	Constrained λ	0.277		1.08 × 10 ⁻¹	1.31
Alyssum s.l.-wide	Two-λ-rate	0.217	2.83	5.63 × 10 ⁻²	2.34
	Constrained λ	0.225		5.26 × 10 ⁻²	1.76
Non-Alyssum s.l. Alysseae wide	Two-λ-rate	0.140	0.214	6.72 × 10 ⁻⁹	0.152
	Constrained λ	0.142		3.56 × 10 ⁻⁶	4.21 × 10 ⁻²
Model parameters: λ ₀ = species diversification rate in non-accumulator lineages; λ ₁ = species diversification rate in hyperaccumulator lineages; μ ₀ = relative species extinction rate in non-accumulator lineages; μ ₁ = relative species extinction rate in hyperaccumulator lineages.					

The Alyseae clade-wide BiSSE analysis in which the 16 species that could not be assigned to a terminal clade were assigned to clade A1b (Table 3-15) still supported the two- λ -rate model whereby higher species diversification rates are significantly associated with hyperaccumulator versus non-accumulator lineages. The likelihood ratio test using χ^2 gave $p = 6.40^{-12}$; Δ AIC from the one-rate to the two-rate model was -54.24, i.e., the lower score was for the two-rate model (Table 3-15), indicating that it gave a better fit to the data.

Table 3-15. Birth-death parameter estimates obtained in BiSSE analysis, with the 16 unassigned *Alyssum* species assigned to clade A1b.

Analysis	Model	Diversification rate (bifurcations lineage ⁻¹ million years ⁻¹)		Extinction rates (extinctions lineage ⁻¹ million years ⁻¹)	
		λ_0	λ_1	μ_0	μ_1
Alysseae-wide	Two- λ -rate	0.287	3.31	0.148	3.04
	Constrained λ	0.318		0.0838	2.91
Model parameters: λ_0 = species diversification rate in non-accumulator lineages; λ_1 = species diversification rate in hyperaccumulator lineages; μ_0 = relative species extinction rate in non-accumulator lineages; μ_1 = relative species extinction rate in hyperaccumulator lineages.					

Similarly, when the 16 unassigned species were assigned to clade A2 the two- λ -rate model whereby higher species diversification rates are associated with hyperaccumulation is supported (Table 3-16). The likelihood ratio test using χ^2 gave $p = 1.26^{-11}$; Δ AIC from the one-rate to the two-rate model was -43.87, i.e., the lower score was for the two-rate model, indicating that it gave a better fit to the data.

Table 3-16. Birth-death parameter estimates obtained in BiSSE analysis, with 16 unassigned *Alyssum* species assigned to clade A2.

Analysis	Model	Diversification rate (bifurcations lineage ⁻¹ million years ⁻¹)		Extinction rates (extinctions lineage ⁻¹ million years ⁻¹)	
		λ_0	λ_1	μ_0	μ_1
Alysseae-wide	Two- λ -rate	0.163	2.33	1.10×10^{-6}	1.95
	Constrained λ	0.248		6.79×10^{-2}	1.80
Model parameters: λ_0 = species diversification rate in non-accumulator lineages;; λ_1 = species diversification rate in hyperaccumulator lineages; μ_0 = relative species extinction rate in non-accumulator lineages; μ_1 = relative species extinction rate in hyperaccumulator lineages.					

Testing Associations between Diversification Rate and Species Range Size using QuaSSE

Quantitative state speciation and extinction (QuaSSE) modelling (Fitzjohn, 2010) was used to investigate the association of species diversification rate and linear range size of species in Alysseae by fitting models in which diversification rate a) was independent of linear range size, and b) was related to linear range size via linear and sigmoidal functions. Linear and sigmoidal models which included a drift parameter (allowing an underlying change in speciation rate over

time) were also included in the analysis. The two models in which species diversification rate was a function of linear range size both had a significantly better fit to the data than the constant diversification rate model (Table 3-17).

Table 3-17. ANOVA and AIC results for QuaSSE analyses.

Alysseae species sampling	Model	d.f.	Log likelihood	AIC	<i>p</i> value P ($AIC_{\text{model}} = AIC_{\text{constant}}$) (based on χ^2)	ΔAIC
119/255	Constant	3	-257.37	520.73	N/A	N/A
	Linear	4	-247.11	502.23	5.94×10^{-6}	-18.5
	Linear (including drift parameter)	5	-246.47	502.94	1.85×10^{-5}	-17.8
	Sigmoidal	6	-255.10	522.19	2.09×10^{-1}	-1.46
	Sigmoidal (including drift parameter)	7	-250.33	514.65	7.05×10^{-3}	-7.04

The ΔAIC from the constant to linear and sigmoidal models were -18.5 and -1.46, respectively (-17.8 and -7.04 when a drift parameter was included), i.e., scores for all variable-rate models were lower than for the constant-rate model; the likelihood ratio test using χ^2 gave $p = 5.94 \times 10^{-6}$ and $p = 2.09 \times 10^{-1}$ for linear and sigmoidal models, respectively (1.85×10^{-5} and 7.05×10^{-3} , respectively, when a drift parameter was included). Of the five models tested, the linear model (without a drift parameter) gave the best fit to the data.

In the linear models, the negative relationship between species diversification rate and linear range size is indicated by the negative values of the gradient parameter ($l.m$) in the two linear models (Table 3-18). In the sigmoidal models, this negative relationship is indicated by the high and low values, respectively, of diversification rate asymptote parameters at the lower and upper limits of species range ($l.y_0$ and $l.y_1$). The inflection point ($l.x.mid$) for the sigmoidal model (without drift) was at $3.28 \log_{10}$ km, equating to a linear range of 1905 km. This is the species

range size at which a change in range size leads to the greatest change in species diversification rate under the sigmoidal model.

Table 3-18. Parameter estimates obtained from QuaSSE analyses.

QuaSSE species diversification rate model	Parameter estimates
Constant	$\lambda = 0.484$, $\mu = 0.293$, diffusion = 2.48×10^{-3}
Linear	<i>l.c</i> = 2.83, <i>l.m</i> = -0.829, <i>m.c</i> = 9.90×10^{-3} , diffusion = 3.14×10^{-3}
Linear (including drift parameter)	<i>l.c</i> = 3.30, <i>l.m</i> = -0.980, <i>m.c</i> = 1.95×10^{-5} , diffusion = 2.19×10^{-3} , drift = -3.25×10^{-3}
Sigmoidal	<i>l.y0</i> = 0.858, <i>l.y1</i> = -2.18×10^{-2} , <i>l.x.mid</i> = 3.28, <i>l.r</i> = 1.00, <i>m.c</i> = 0.281, diffusion = 3.30×10^{-3}
Sigmoidal (including drift parameter)	<i>l.y0</i> = 0.984, <i>l.y1</i> = -1.45×10^{-2} , <i>l.x.mid</i> = 2.92, <i>l.r</i> = 1.05, <i>m.c</i> = 3.56×10^{-2} , diffusion = 2.89×10^{-3} , drift = -9.49×10^{-2}
Model parameters diffusion = underlying rate of character (i.e., species range size) change over time; drift = underlying direction of character (i.e., species range size) over time. Constant rate models: λ = species diversification rate ('birth rate'); μ = relative species extinction rate ('death rate'). Linear models: <i>l.c</i> = constant; <i>l.m</i> = x coefficient (i.e. gradient). Sigmoidal models: <i>l.y0</i> = asymptotic species diversification rate for low range sizes; <i>l.y1</i> = asymptotic species diversification rate for high range sizes; <i>l.x.mid</i> = inflection point (in terms of species range size); <i>l.r</i> = steepness parameter; <i>m.c</i> = relative species extinction rate.	

Geographic Distribution of Tissue Sample Collection Locations

The collection locations of plant tissue samples employed in this study are shown in Figure 3-11a. Figures 3-11b and 3-11c show collection locations of hyperaccumulator and non-accumulator species in *Alyssum s.l.* and Clade A5, respectively. Descriptions of the geographic distribution of Alyseae species are included in Table A3-2 in Appendix 3. Nickel hyperaccumulator species in *Alyssum s.l.* are widely distributed from Iberia in the western Mediterranean, through the eastern Mediterranean (especially Turkey) to SW and central Asia. This distribution spans much of the region in which non-accumulator species in *Alyssum s.l.* (and indeed the genus as a whole) are present (Fig 3-11b). (One species of non-accumulator, *Alyssum obovatum* in Clade A2, does reach Alaska and northern Canada, though in this study, this species was sampled from a specimen that was collected in central Asia). In contrast, hyperaccumulators and non-accumulators in Clade A5 are restricted to a relatively small region which encompasses the Balkans and Turkey (Fig. 3-11c).

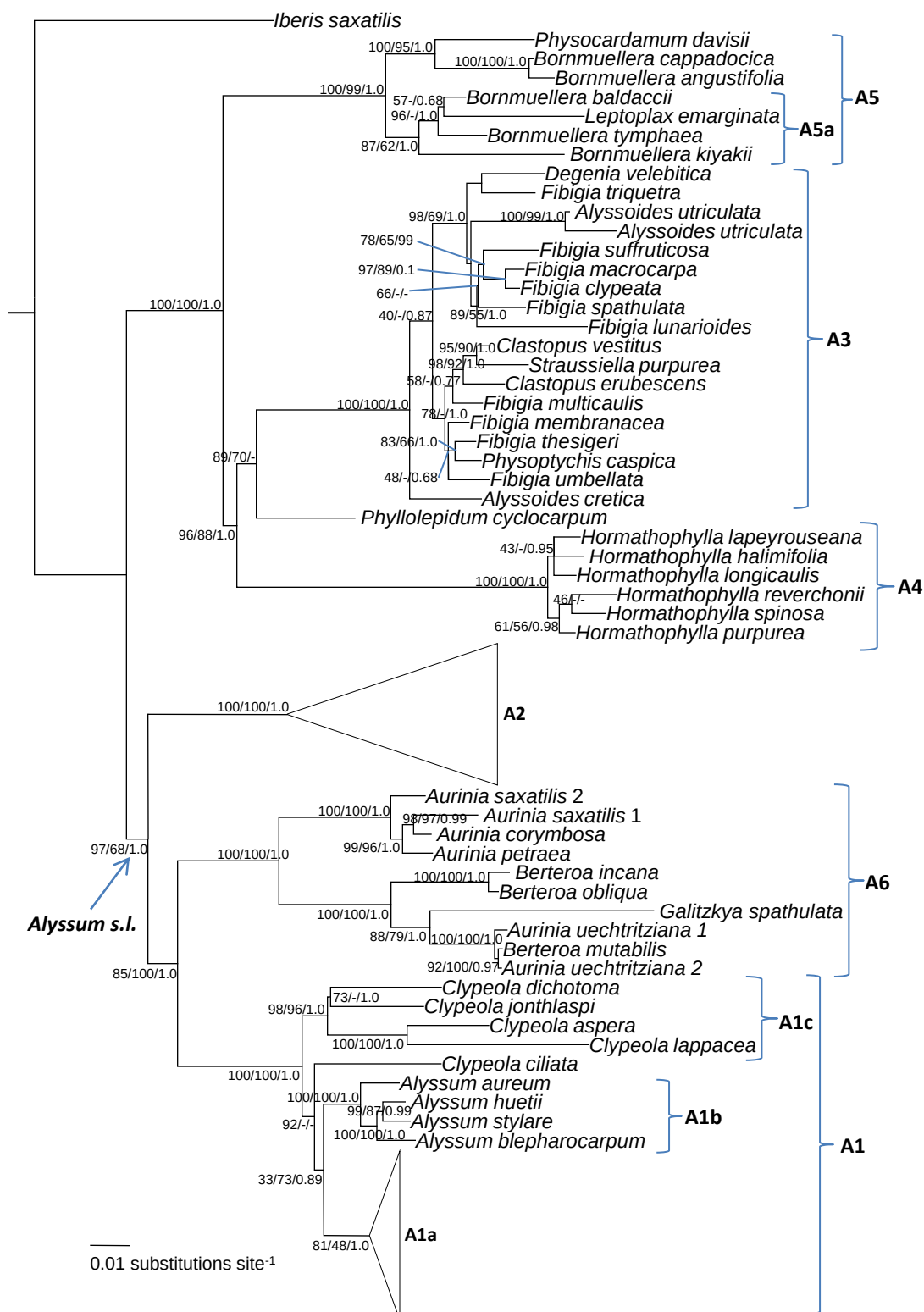


Figure 3-1a. Maximum likelihood tree of Alysseae produced using RAxML, based on Concatenated Alignment B. Figures indicate branch support values (in the following order: bootstrap support (bs) under maximum likelihood; bs under parsimony; posterior probability (pp) under Bayesian inference; dashes indicate clade absence). Values at branches receiving <50% bs or <0.5 pp are not shown. For parsimony and Bayesian inference trees see Appendix 6 and Figure 3-3 respectively. Rooted on *Iberis saxatilis*. Alysseae clades A1 to A6 are indicated, as described in the text. Detail of clades A1a and A2 is included in Figures 3-1b and 3-1c. Scale bar (for branch lengths) indicates substitutions site⁻¹.

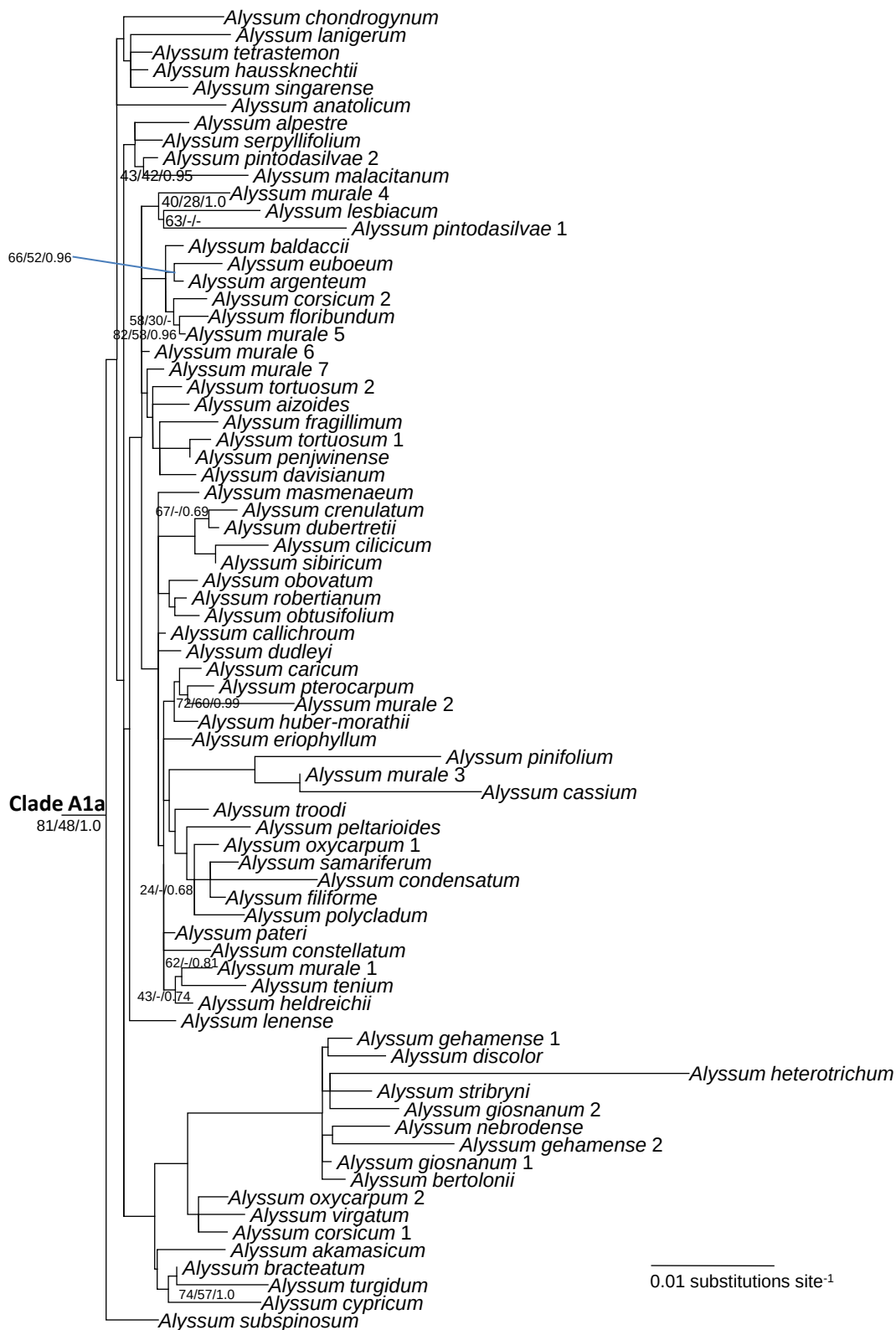


Figure 3-1b. Detail of clade A1a from Figure 3-1a. For further details see legend at Figure 3-1a.

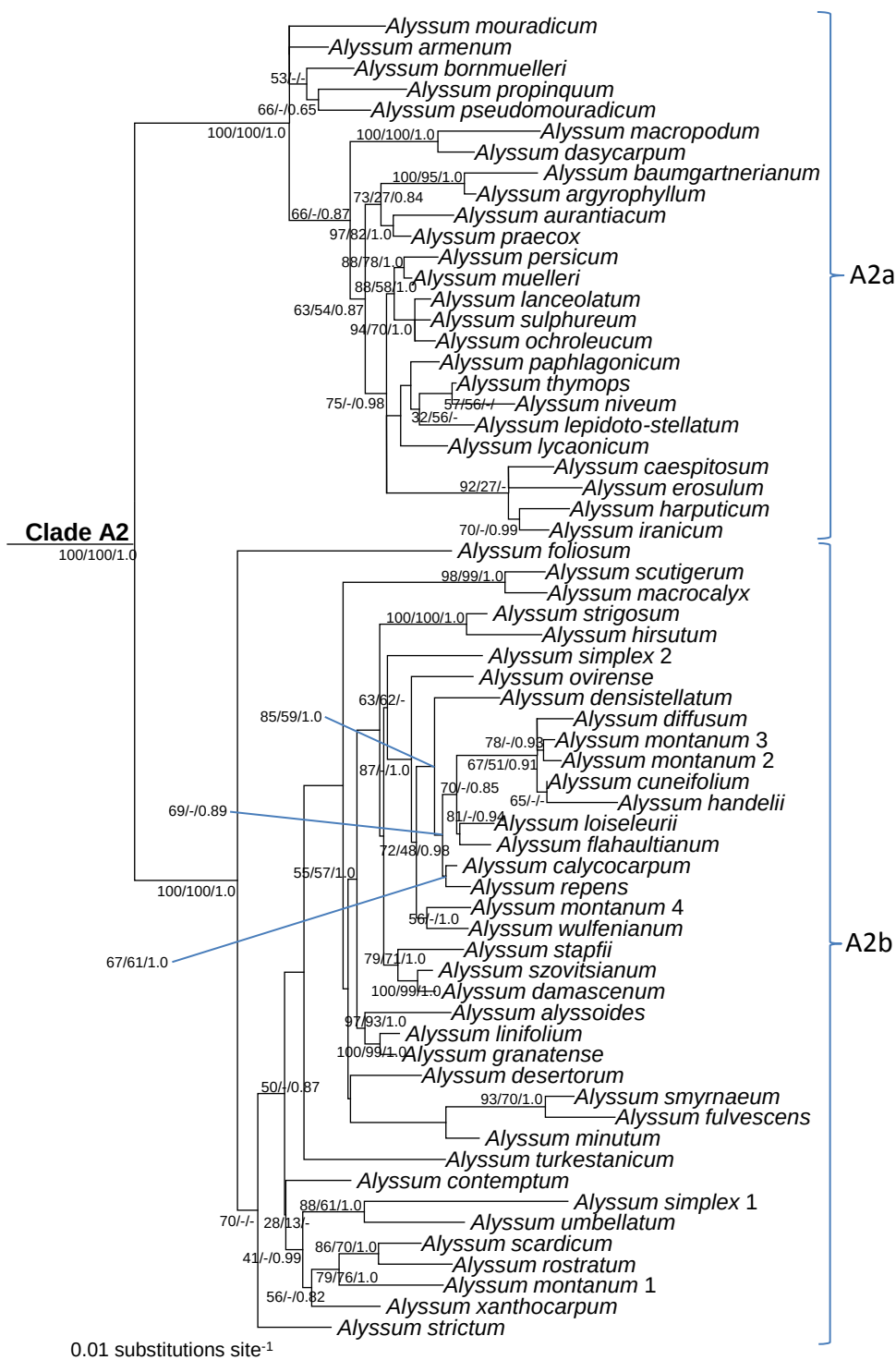


Figure 3-1c. Detail of clade A2 from Figure 3-1a. For further details see legend at Figure 3-1a.

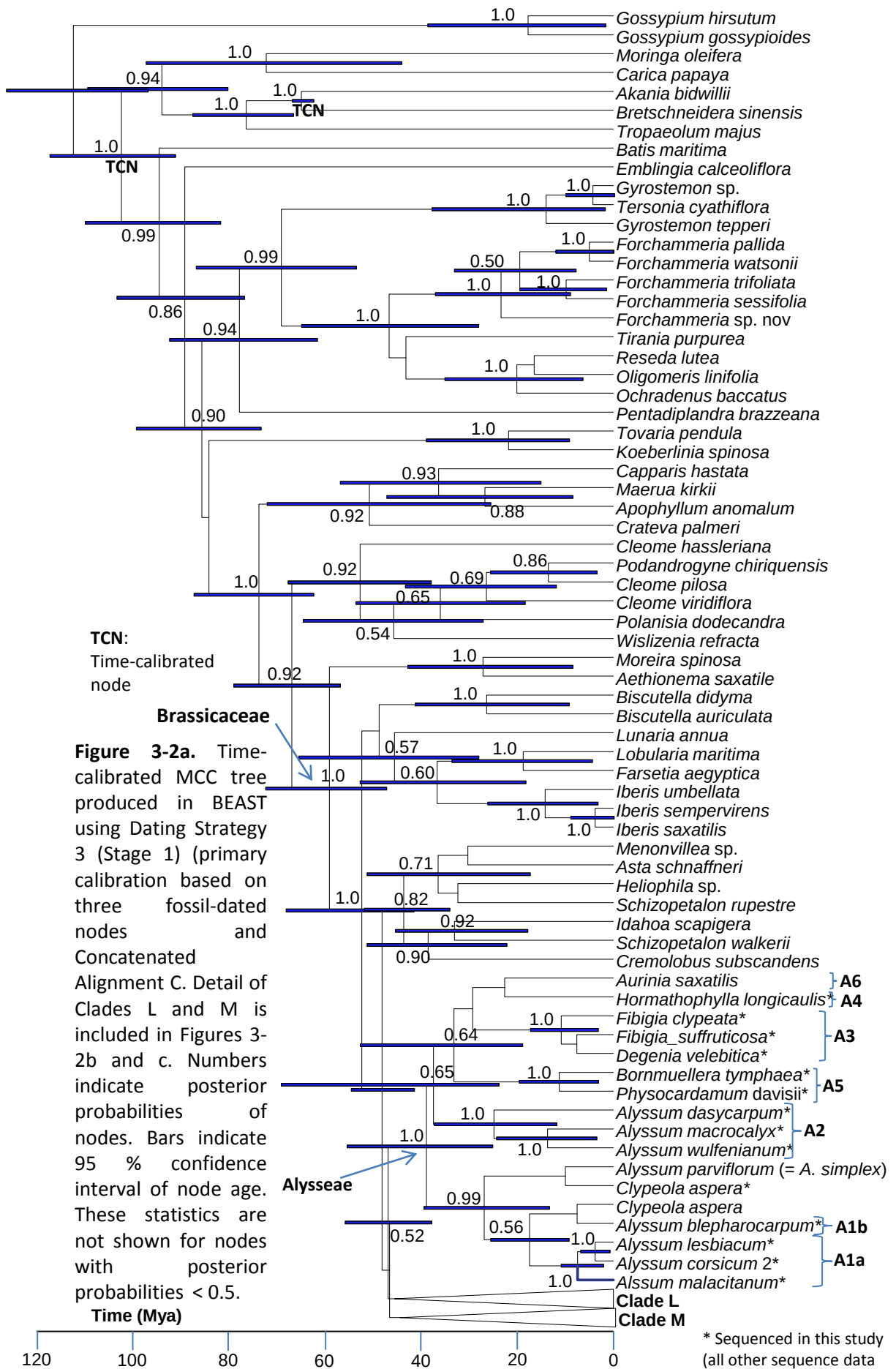
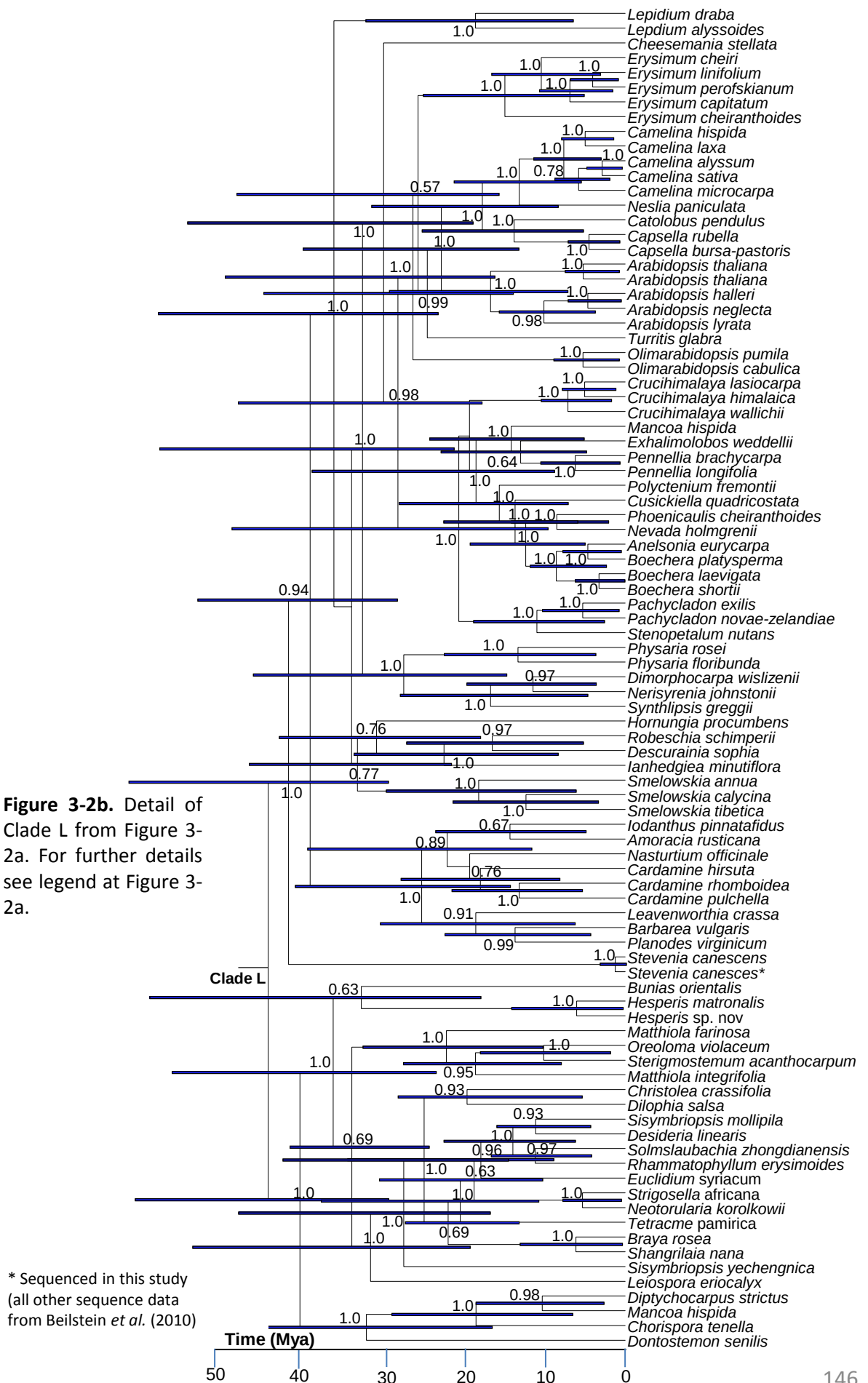
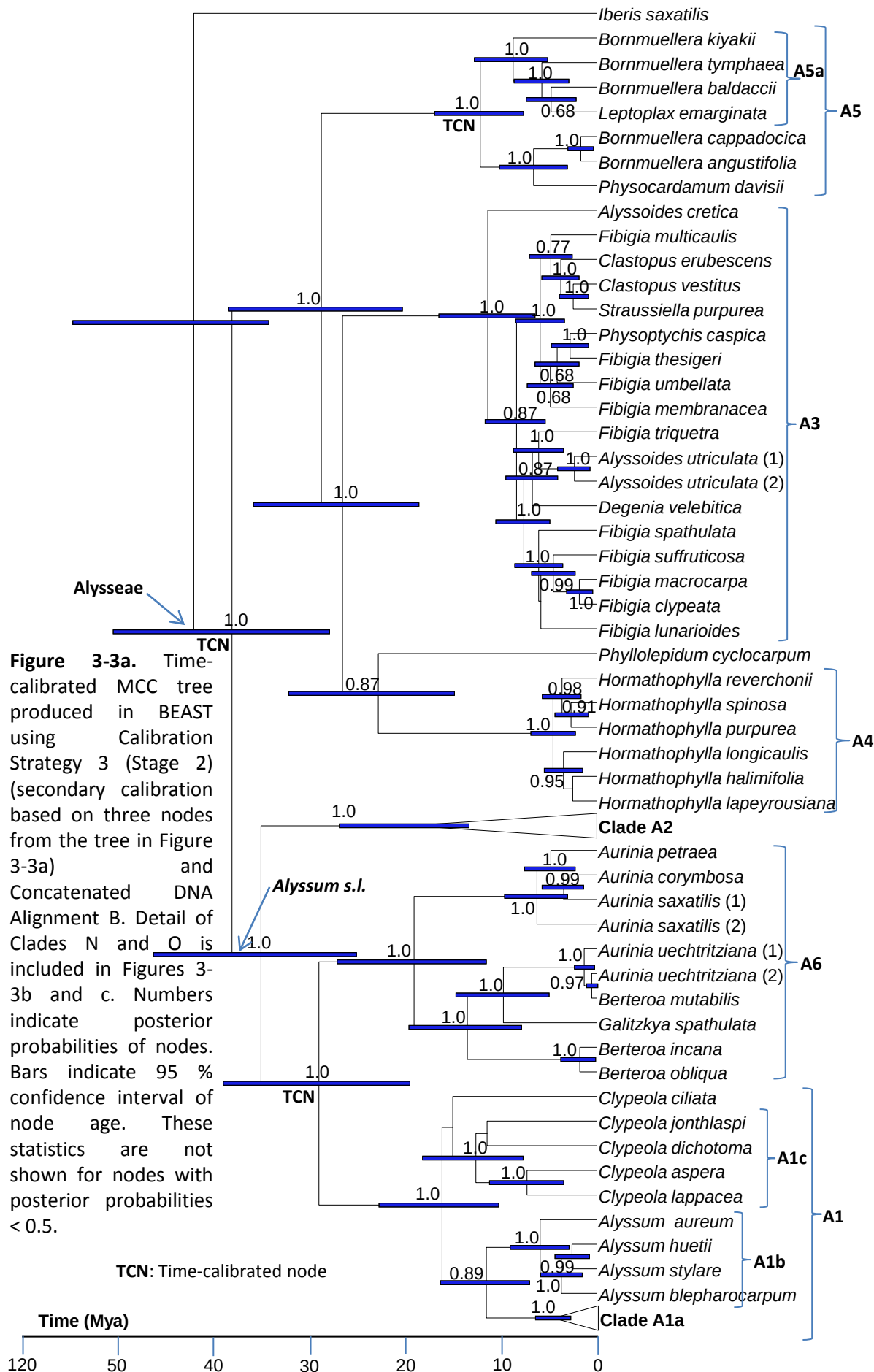


Figure 3-2b. Detail of Clade L from Figure 3-2a. For further details see legend at Figure 3-2a.





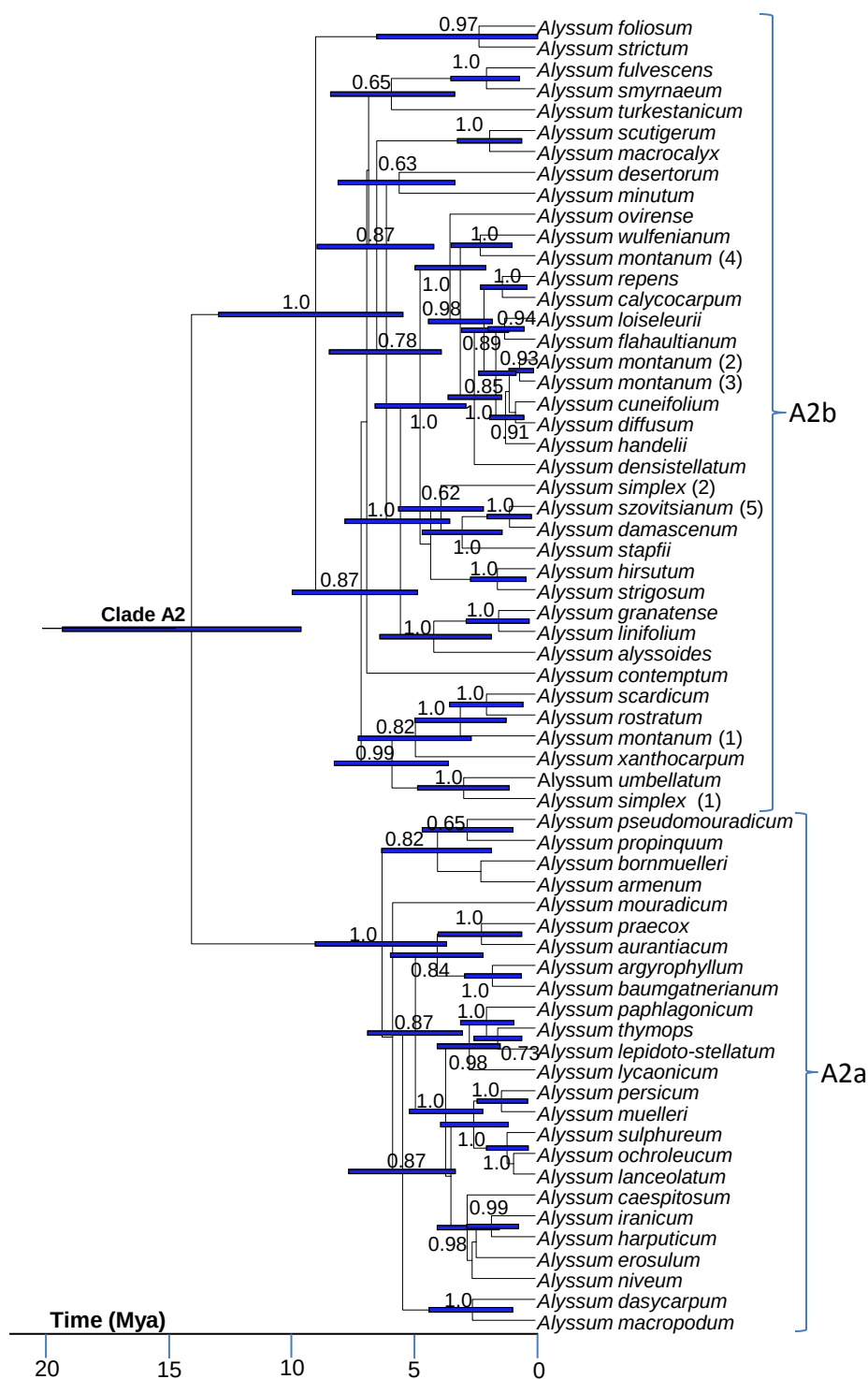


Figure 3-3b. Detail of Clade A2 from Figure 3-3a. For further details see legend at Figure 3-3a.

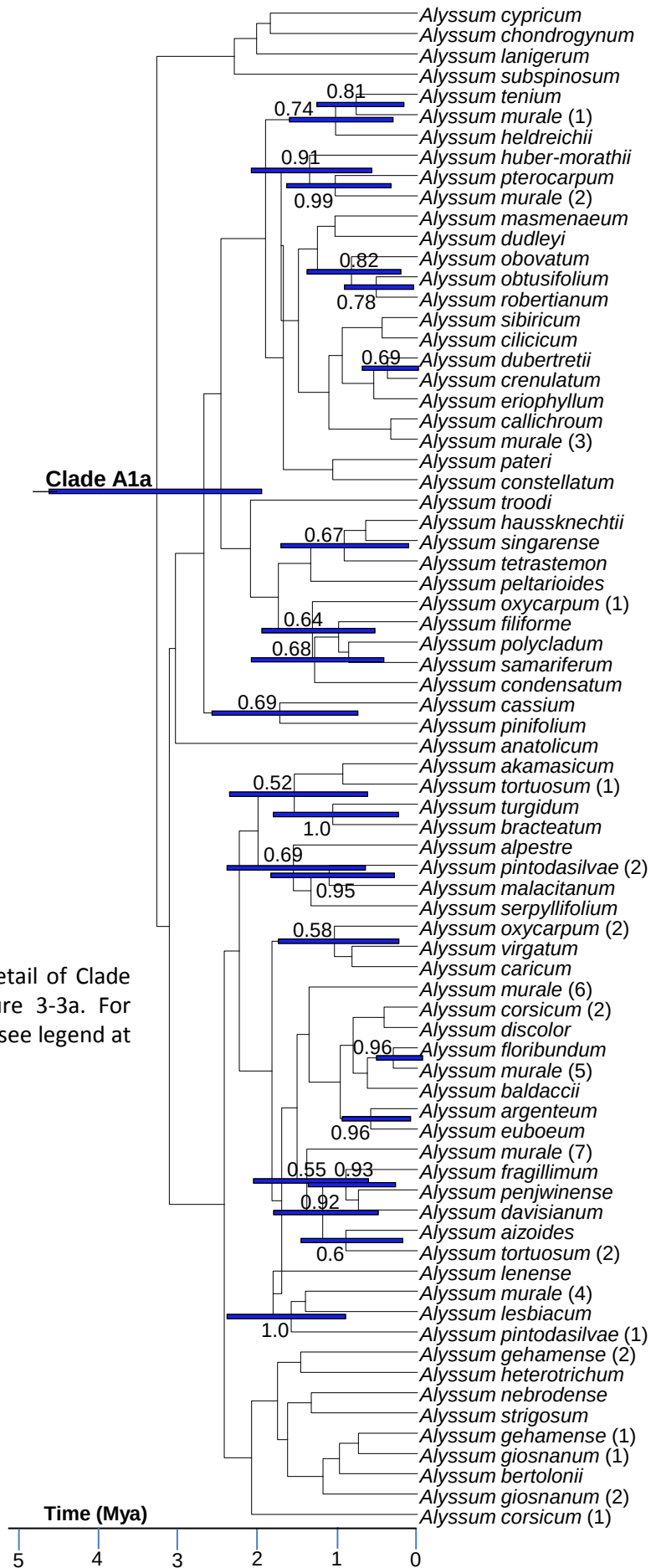


Figure 3-3c. Detail of Clade A1a from Figure 3-3a. For further details see legend at Figure 3-3a.

Maximum Ni concentrations in Alysseae species

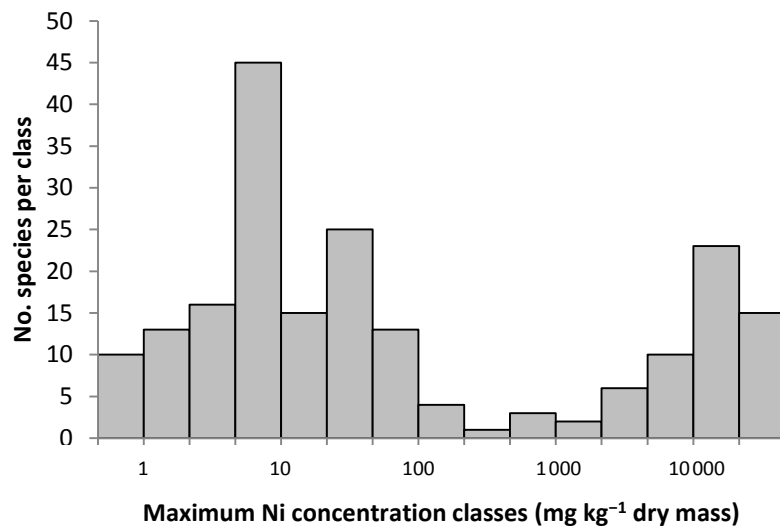


Figure 3-4a. Distribution of maximum shoot nickel concentrations reported for 201 Alysseae species. Data for 3 species were obtained in this study. Data for the remaining 198 species were obtained from the literature. Full dataset, including sources is included in Table A3-1 in Appendix 3.

Maximum Ni concentrations in Alysseae species sampled in this study

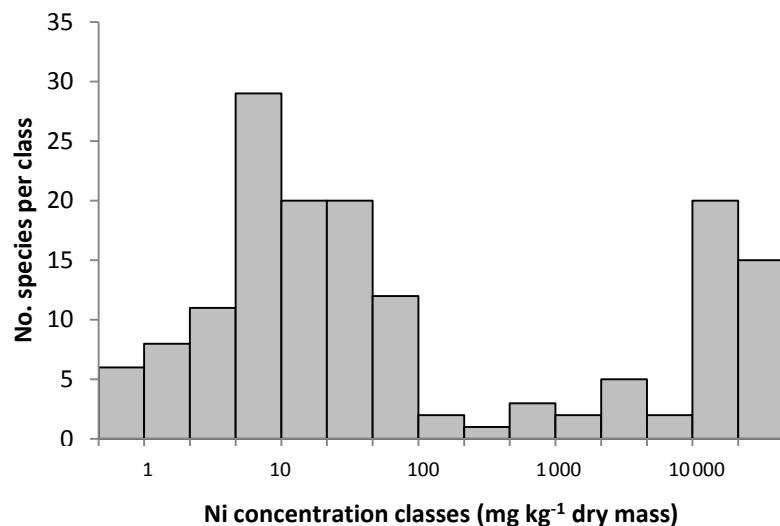


Figure 3-4b. Distribution of maximum shoot nickel concentrations reported for 164 Alysseae species included in the phylogenetic analyses in this study. Data for 3 species were obtained in this study. Data for the remaining 161 species were obtained from the literature. Full dataset, including sources is included in Table A3-1 in Appendix 3.

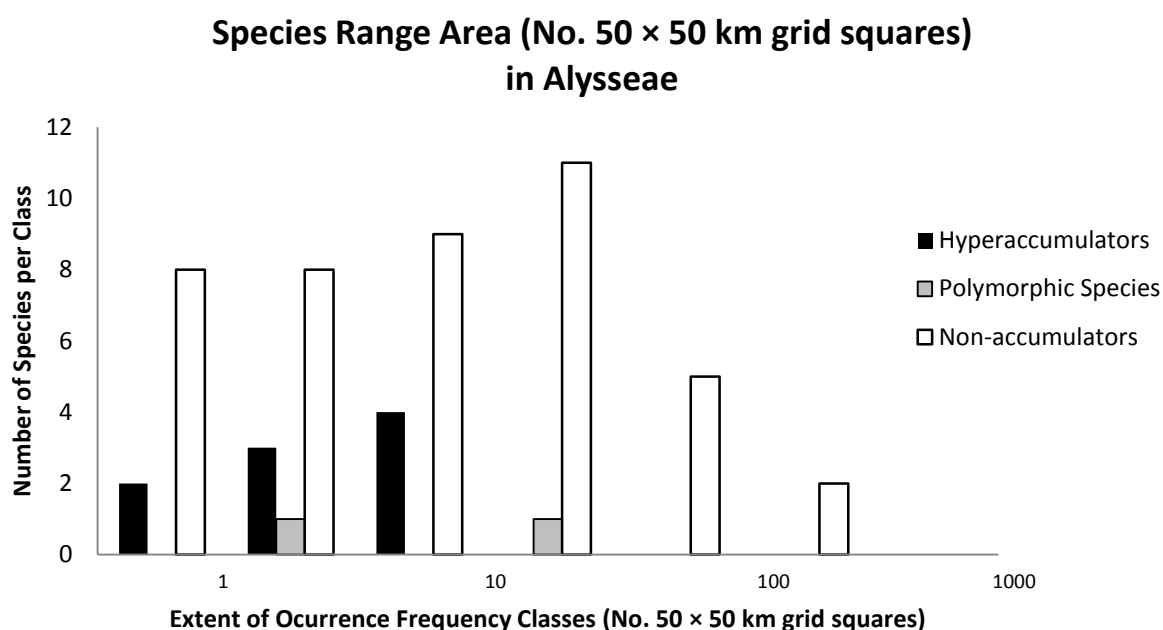


Figure 3-5a. Frequency distribution of extent of species range area (no. of 50 × 50 km grid squares in which present) for nine Ni hyperaccumulator, 43 non-accumulator and 2 polymorphic species of Alysseae that have native ranges restricted to the area covered in the *Atlas Flora Europaeae* (Jalas & Suominen, 1994).

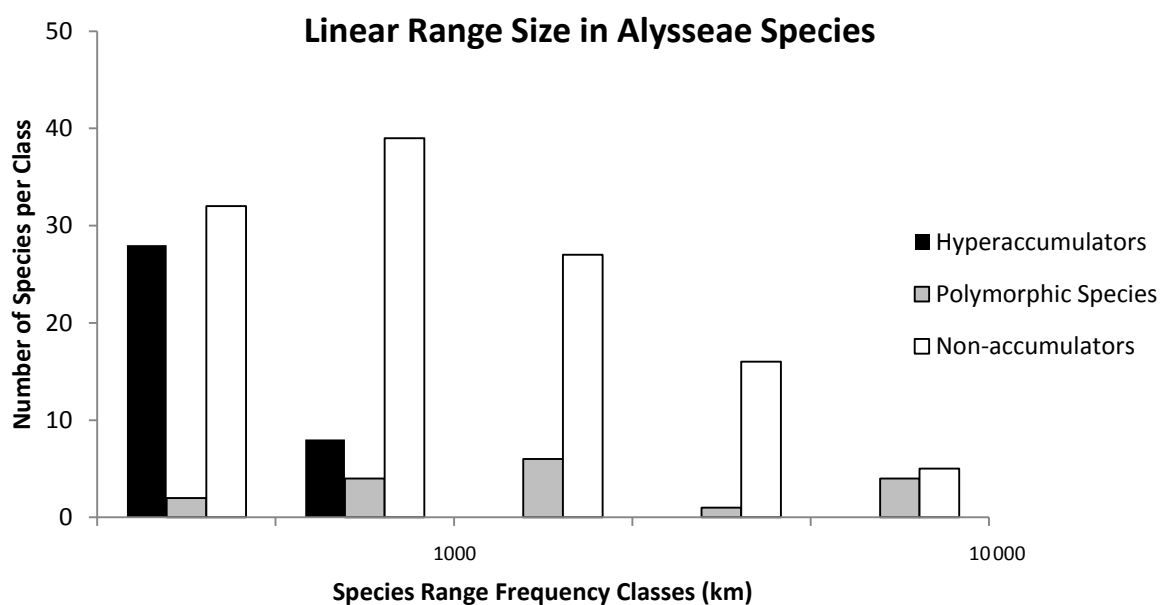


Figure 3-5b. Frequency distribution of estimated linear range size (km) for 36 nickel hyperaccumulator, 119 non-accumulator and 17 polymorphic species of Alysseae. Note that a minimum value of 290 km was employed in this analysis, in order to avoid bias from variation in the accuracy of estimates at small values (see *Chapter 2 Materials and Methods* for further details).

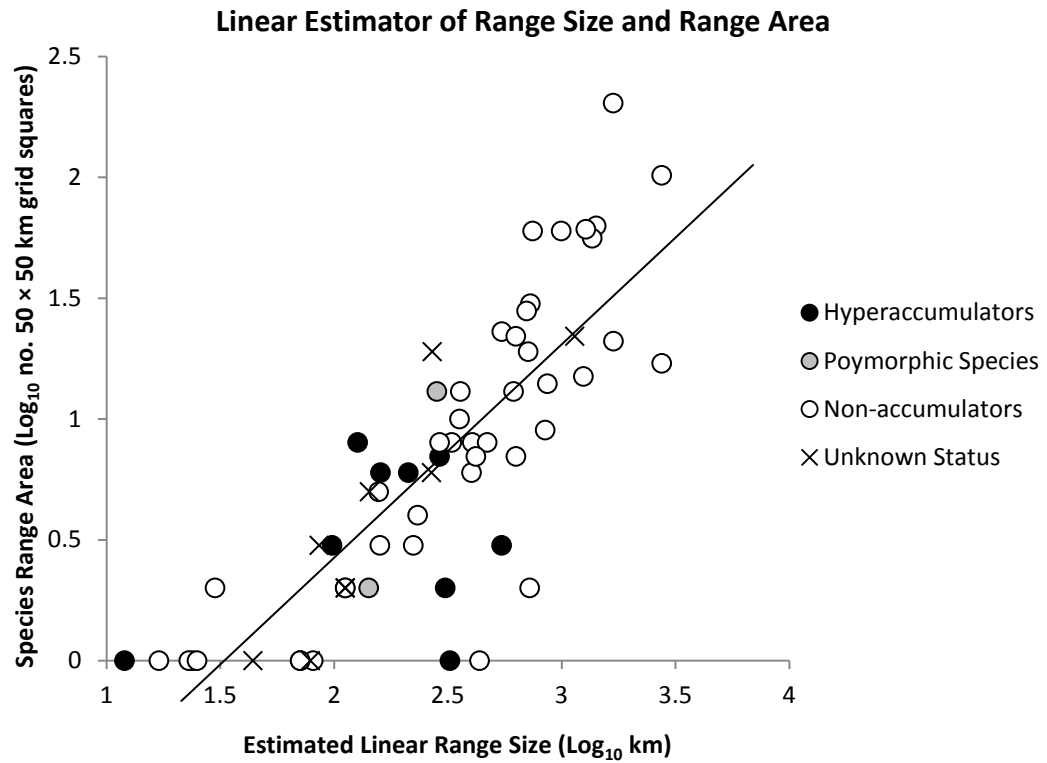


Figure 3-6a. Species range area (Log_{10} no. 50×50 km grid squares) plotted against estimated linear range size (Log_{10} km) for 63 *Alysseae* species that are restricted to Europe. The line indicates the linear model obtained from linear regression (see main text for further details).

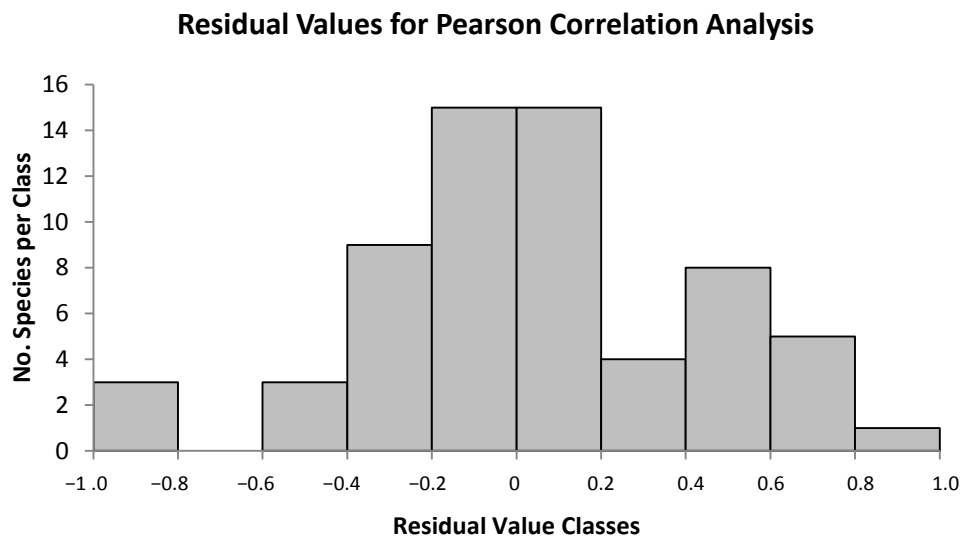


Figure 3-6b Histogram showing the frequency distribution of residual values obtained in the linear regression analysis illustrated in Figure 3-6a and described in the text. This indicates that the distribution is approximately normal, and that a linear regression analysis is justified.

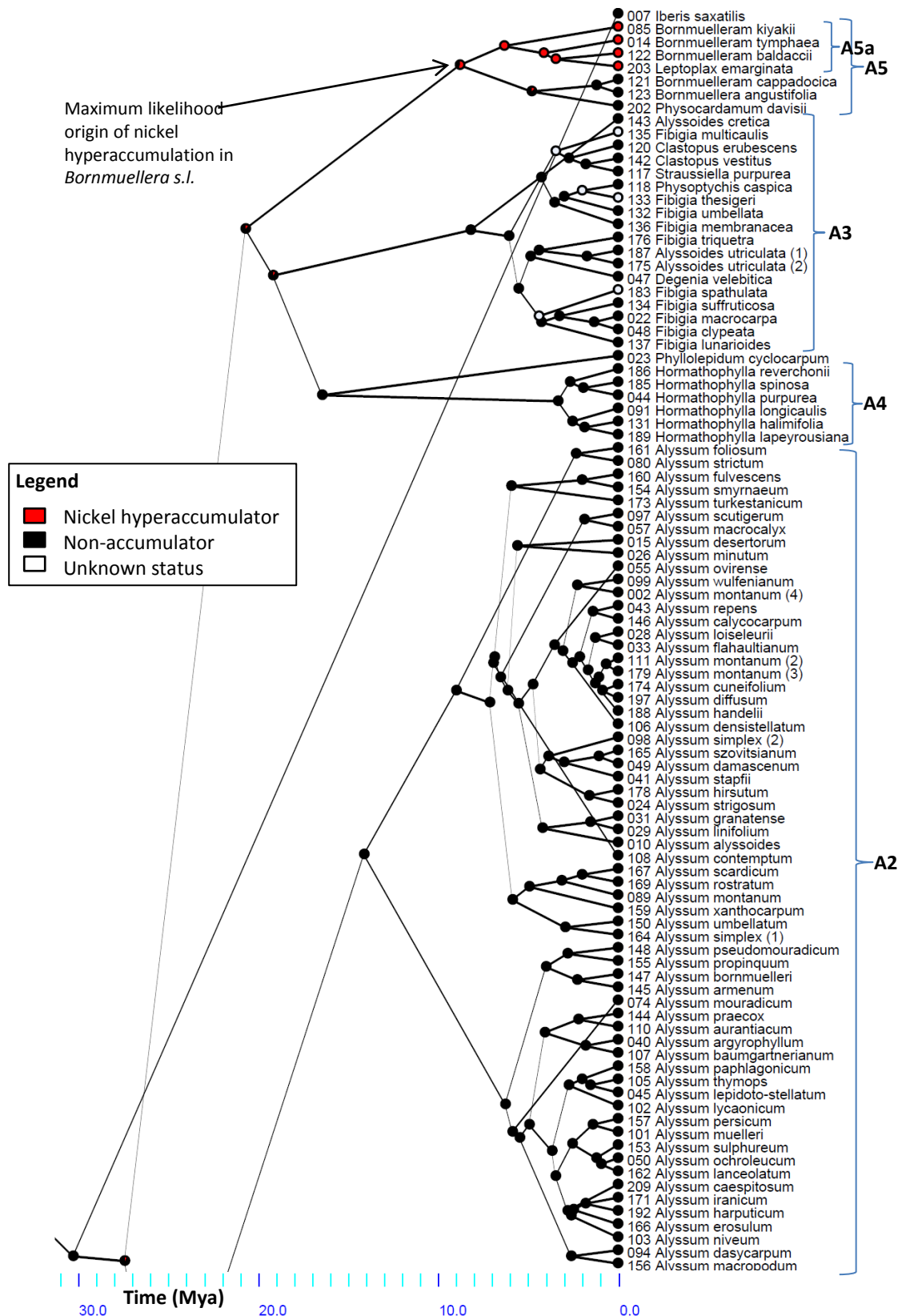


Figure 3-7a. Nickel hyperaccumulation status optimised using maximum likelihood (two-rate asymmetric model) and two character states onto the tree of Alyseae obtained using Concatenated Alignment B and Calibration Strategy 3.

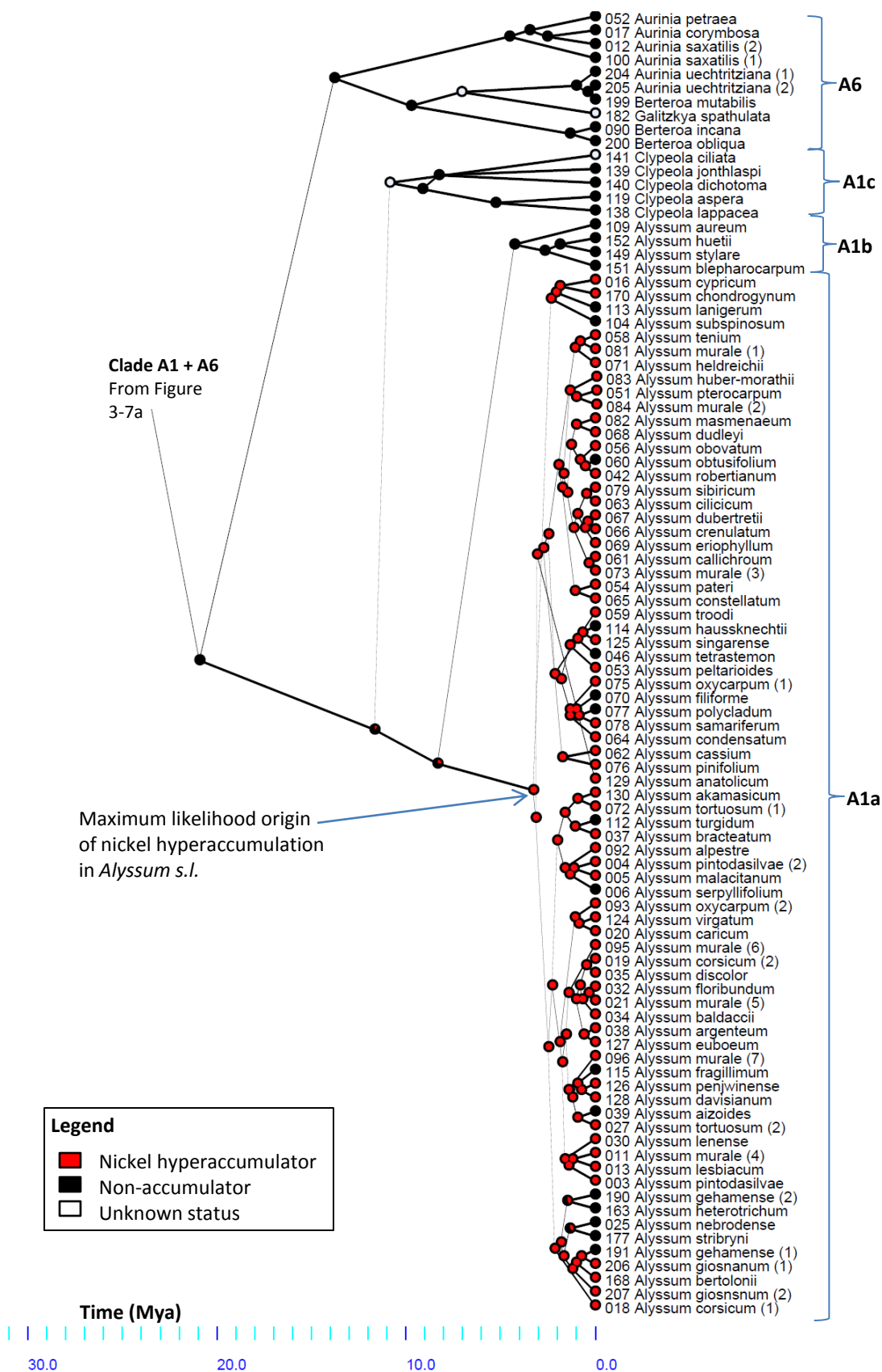


Figure 3-7b. Detail of Clade A1 + A6 from Figure 3-7a. Nickel hyperaccumulation status optimised using maximum likelihood and two character states onto the tree of Alyseae obtained using Concatenated Alignment B and Calibration Strategy 3.

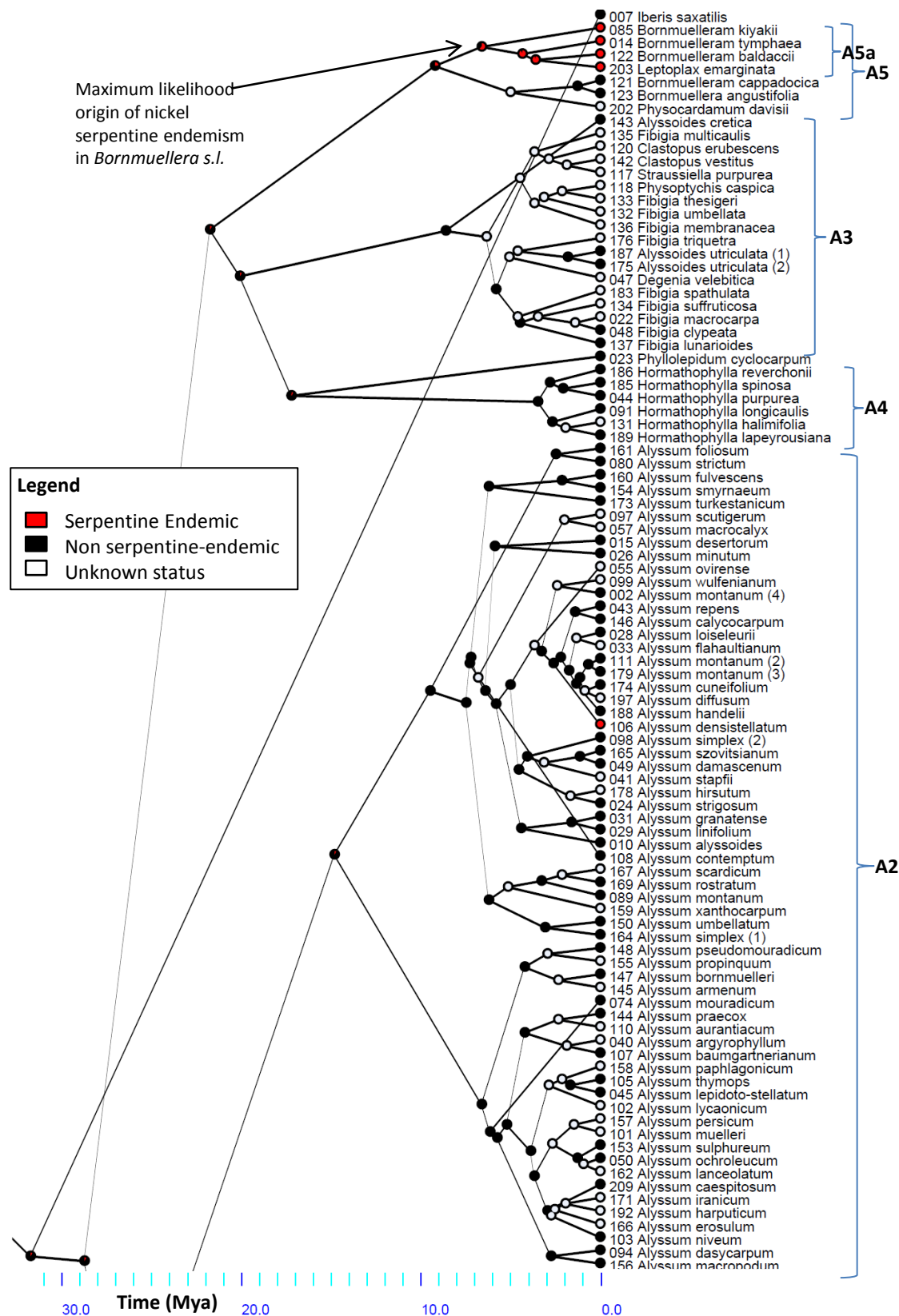


Figure 3-8a. Serpentine endemism status optimised using maximum likelihood (two-rate asymmetric model) and two character states onto the tree of Alyseae obtained using Concatenated Alignment B and Calibration Strategy 3.

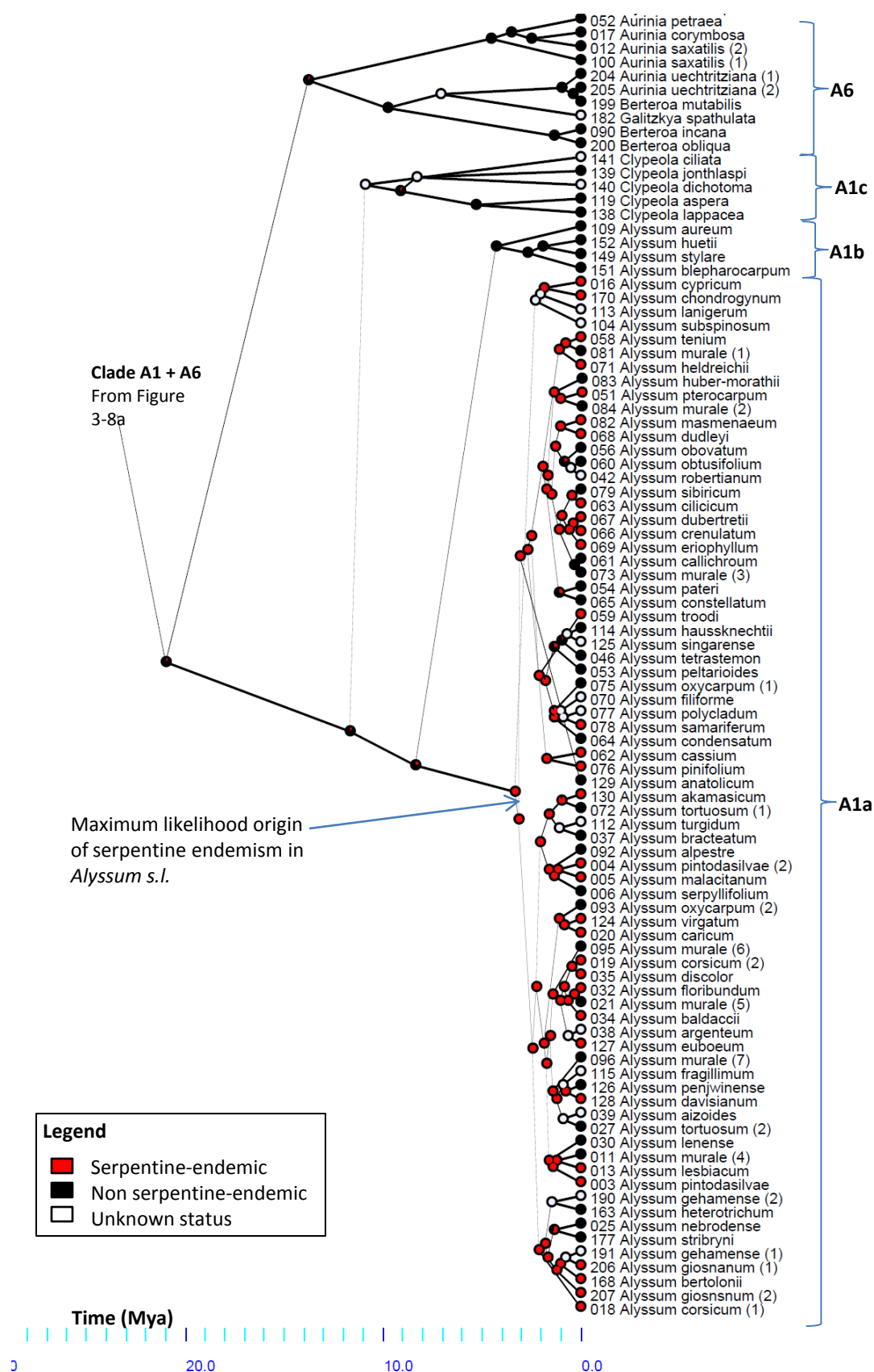


Figure 3-8b. Detail of Clade A1 + A6 from Figure 3-8a. Serpentine endemism status optimised using maximum likelihood and two character states onto the tree of Alyseae obtained using Concatenated Alignment B and Calibration Strategy 3.

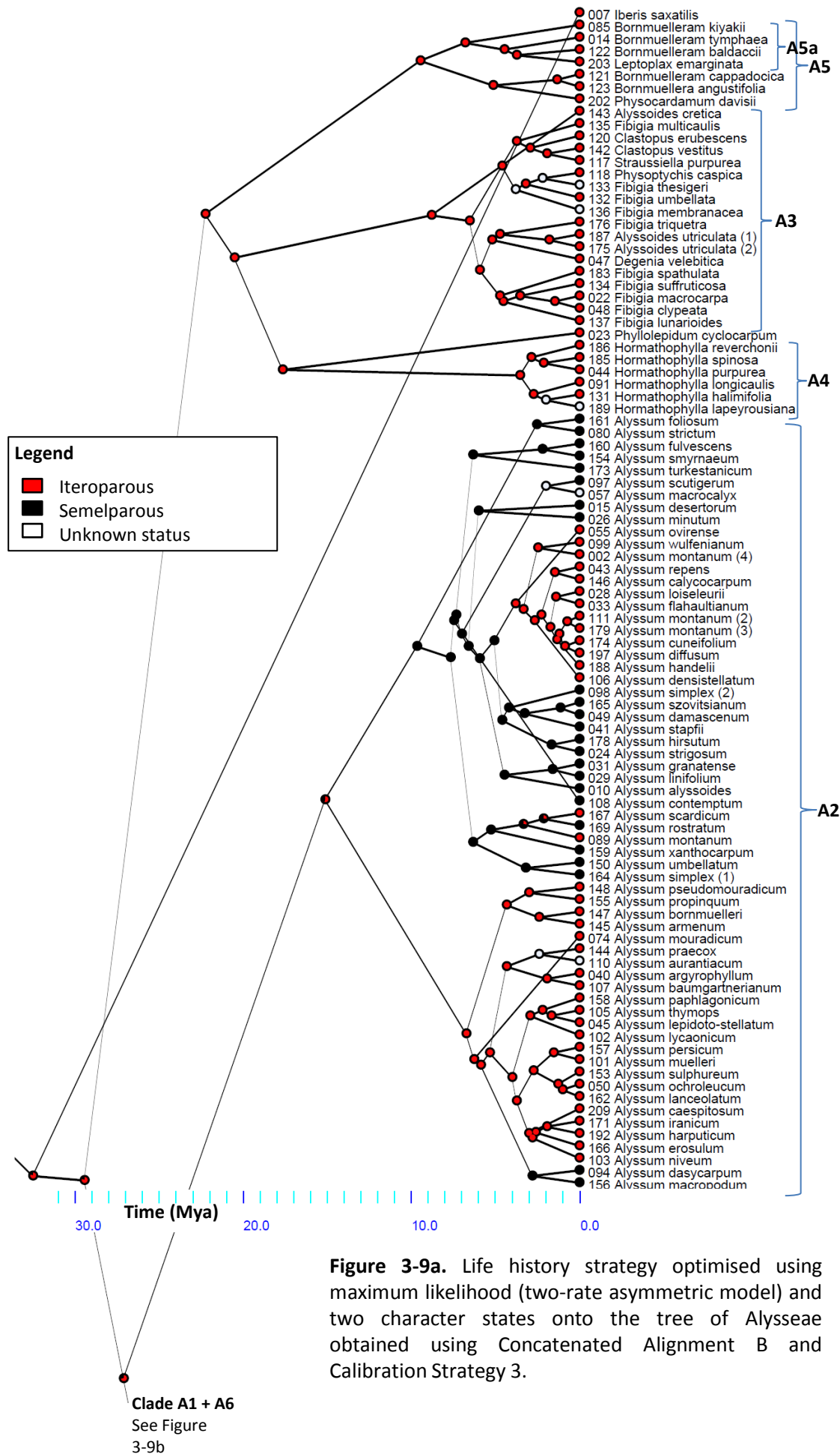


Figure 3-9a. Life history strategy optimised using maximum likelihood (two-rate asymmetric model) and two character states onto the tree of Alyseae obtained using Concatenated Alignment B and Calibration Strategy 3.

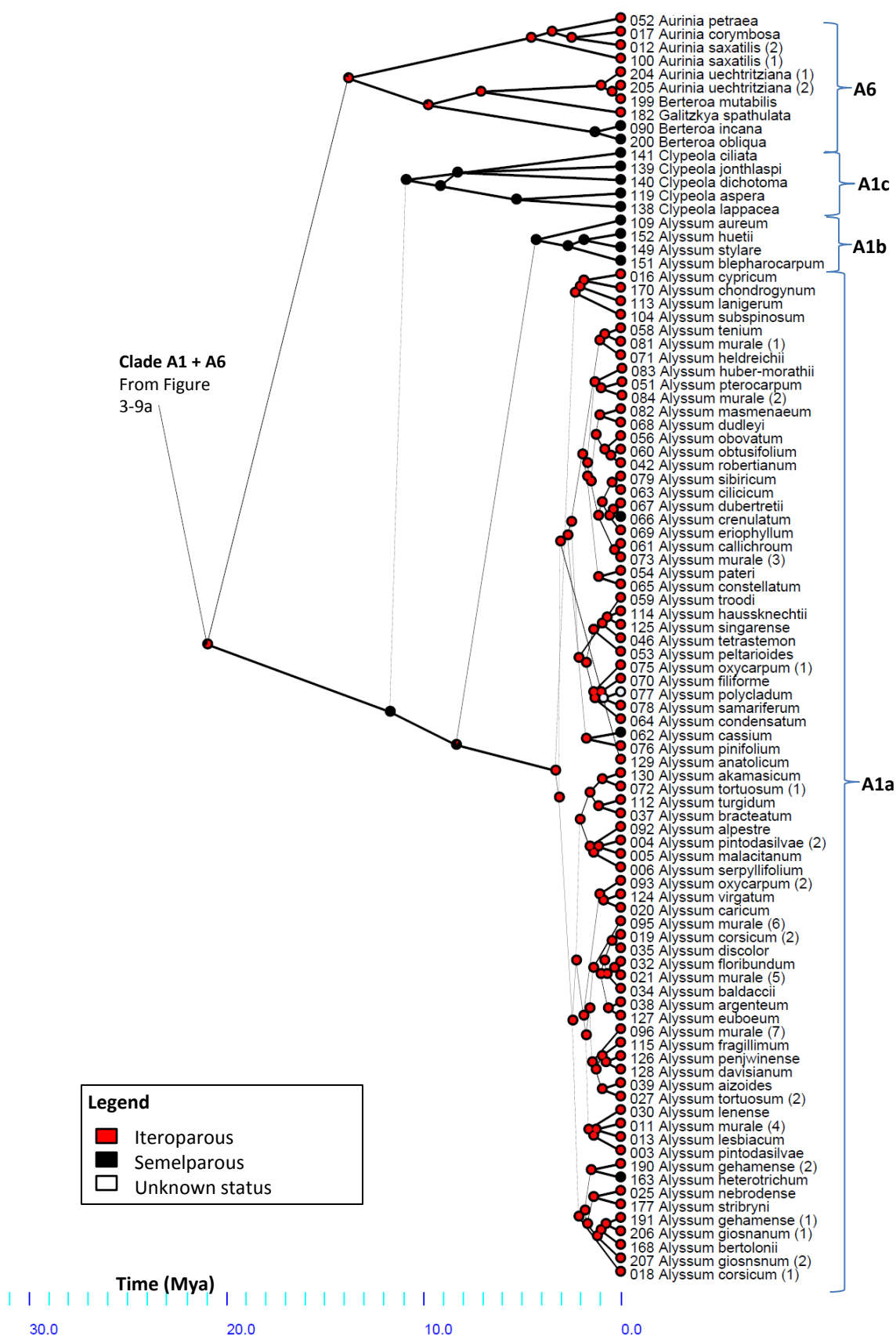


Figure 3-9b. Detail of Clade A1 + A6 from Figure 3-7a. Life history strategy optimised using maximum likelihood and two character states onto the tree of Alyseae obtained using Concatenated Alignment B and Calibration Strategy 3.

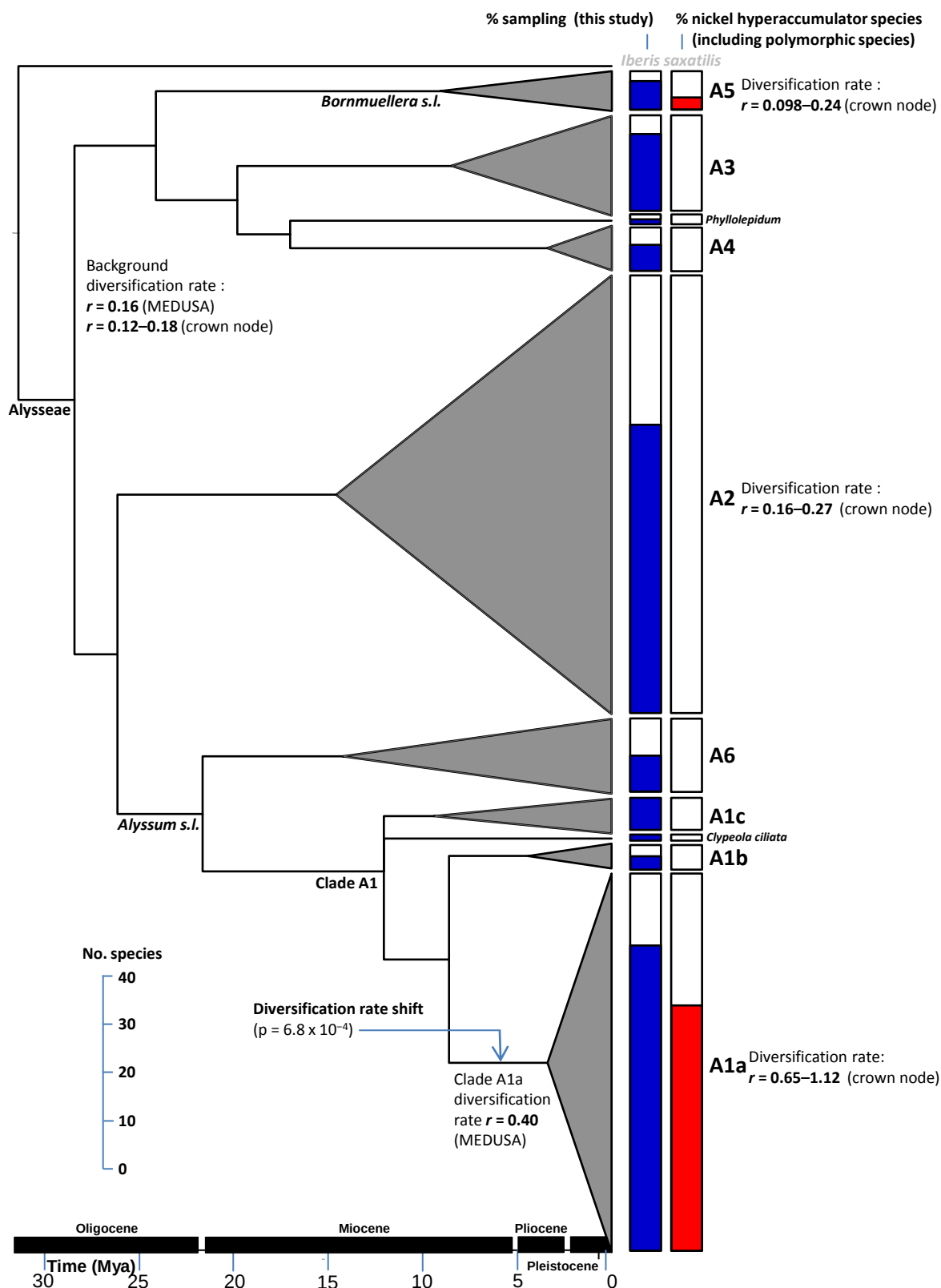


Figure 3-10. Diversity tree based on time-calibrated MCC tree produced in BEAST using Concatenated Alignment B and Calibration Strategy 3 (Stage 2) (see Fig. 3-3). Nodes receiving <0.8 pp have been collapsed. Terminal nodes have been collapsed into the major Alysseae Clades A1-A6 of Warwick *et al.* (2008) and Clades A1a, A1b, A1c described in Chapter 3. Diversification rates are indicated for selected clades, in units of lineage splits lineage⁻¹ million years⁻¹; rates based on stem nodes were calculated in MEDUSA (Alfaro *et al.*, 2009); rates based on crown nodes were calculated using the whole-clade method of Magallón and Sanderson (2001; 2006) implemented in the R package GEIGER (Harmon *et al.*, 2008). Bars to the right indicate % sampling (based on the allocation of unsampled species to clades detailed in Appendix 4) and % nickel hyperaccumulator species (including polymorphic species) for the major Alysseae clades.

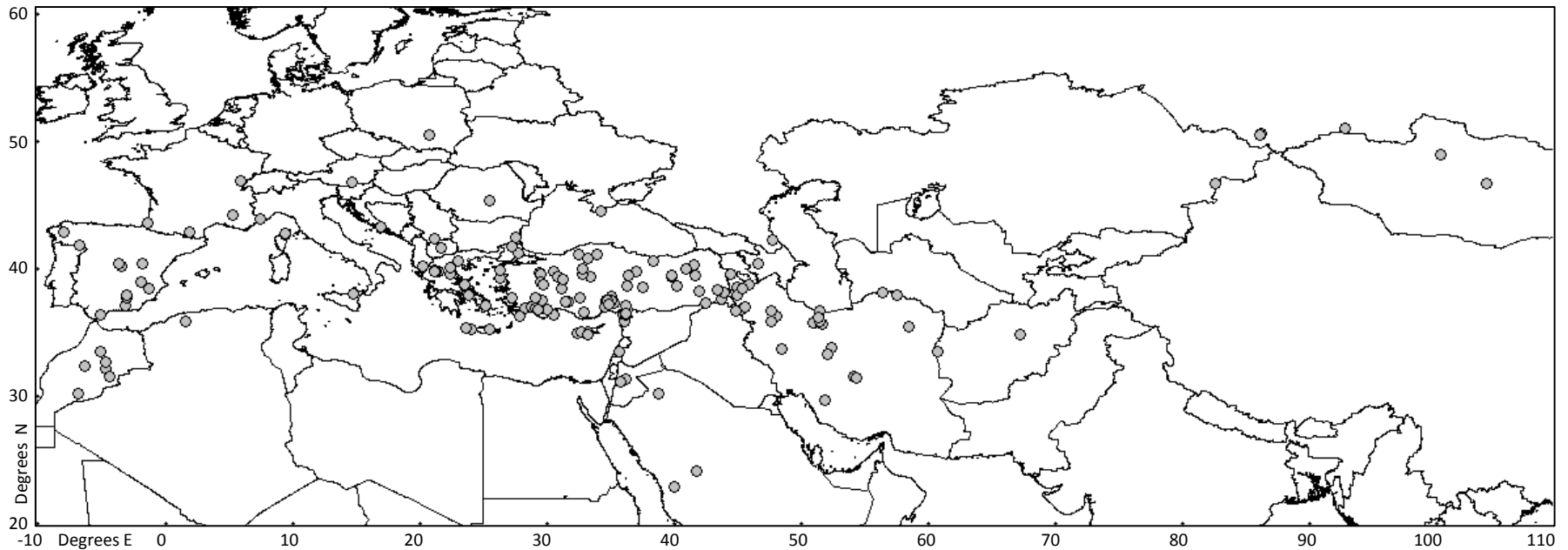


Figure 3-11a. Collection locations for the 175 Alysseae tissue, seed or DNA samples used in this study (out of a total of 189) for which such geographic data were available (collection location is therefore not shown for 14 samples). Map projection: equirectangular (i.e., equal map distances between lines of longitude and latitude). Map source and copyright: diva-gis (2011) global country boundaries (<http://www.diva-gis.org/Data>).

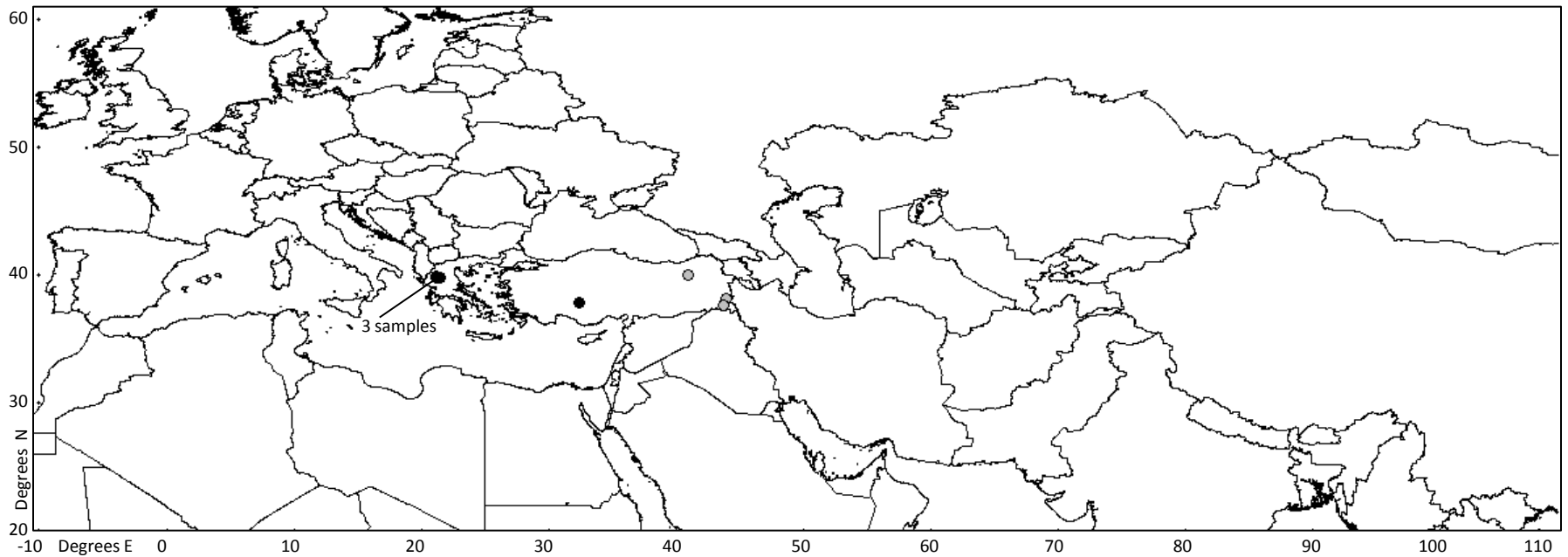


Figure 3-11b. Collection locations for the seven Clade A5 (i.e., *Bornmuellera*, *Leptoplax* and *Physocardamum*) tissue and seed samples used in this study. ● Nickel hyperaccumulator species (four samples). ○ Non-accumulator species (three samples). Map projection: equirectangular (i.e., equal map distances between lines of longitude and latitude). Map source and copyright: diva-gis (2011) global country boundaries (<http://www.diva-gis.org/Data>).

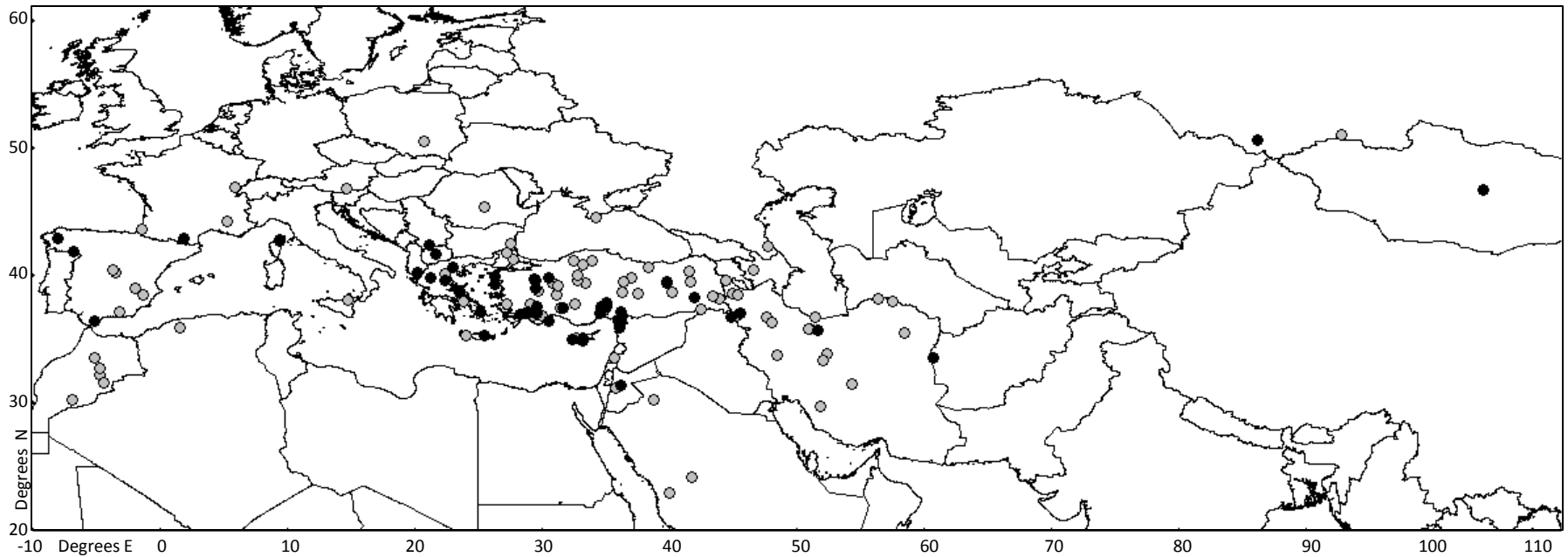


Figure 3-11c. Collection locations for the 146 *Alyssum s.l.* tissue, seed or DNA samples used in this study (out of a total of 162) for which such geographic data were available (collection location is therefore not shown for 16 samples). ● Nickel hyperaccumulator species (55 samples). ○ Non-accumulator species (91 samples). Map projection: equirectangular (i.e., equal map distances between lines of longitude and latitude). Map source: diva-gis (2011) global country boundaries (<http://www.diva-gis.org/Data>).

Chapter 4: Discussion

The evolution of complex physiological plant traits and their impacts on the evolutionary dynamics and biogeography of angiosperm diversification are central questions in evolutionary biology. Phylogenetic approaches are providing new insights into these questions for an increasing set of key adaptive plant traits, such as succulence (Arakaki *et al.*, 2011), C4 photosynthesis (Kadereit *et al.*, 2012), crassulacean acid metabolism (Crayn *et al.*, 2004) amongst others. However, lack of robust phylogenies for many plant lineages means that the evolution of many other important traits remains poorly understood. This is the case for the evolution of metal hyperaccumulation.

The evolution of nickel hyperaccumulation in Alysseae – and especially *Alyssum*, which contains more nickel hyperaccumulator species than any other plant genus – remains poorly understood. In large part this lack of understanding has been due to the absence of a well-resolved, robustly supported and densely sampled phylogeny of Alysseae, and especially of the genus *Alyssum*. Previous phylogenetic studies have remained sparsely sampled and mainly based on single DNA sequence regions (especially *ITS*), such that lack of resolution has been a persistent problem in this group. In this study, I present the most densely sampled and well-resolved phylogeny of Alysseae and *Alyssum* to date, with 67% taxon sampling of both Alysseae and *Alyssum*, obtained using a larger character-set based on multiple phylogenetically informative DNA sequence regions. The previous lack of evidence about when, where and especially how many times the ability to hyperaccumulate nickel evolved within Alysseae means that it has been impossible to assess the impacts of this trait on species diversification, and especially on the geotemporal trajectory of diversification in a group that occupies a widespread but fragmented geographic distribution across the Mediterranean and south-west Asian regions.

The new phylogeny of Alyseae presented here represents the most comprehensive exploration of generic relationships across the tribe and species relationships within *Alyssum* realized to date. This new phylogeny provides a powerful framework for investigating when and how many times nickel hyperaccumulation evolved in this clade and its impacts on patterns, rates and processes of lineage and species diversification, and specifically addresses the question of why there are so many nickel hyperaccumulator species in the single genus *Alyssum*.

Phylogeny of Alyseae

As for the majority of angiosperm families, tribal and generic relationships, and especially generic delimitation in Brassicaceae, remain in a state of flux, and the non-monophyly of genera remains common. The new phylogeny of Alyseae presented here provides the most rigorous test of generic monophyly in this tribe to date.

Monophyly of Alyseae

The Alyseae phylogeny presented here includes denser taxon sampling of both *Alyssum* (67%) and *Alyseae* species (67%) than previous studies (10–15% and 11–21% respectively (Mengoni *et al.*, 2003; Warwick *et al.*, 2008; Cecchi *et al.*, 2010)). Where they included the appropriate non-Alyseae species, all trees generated here support the monophyly of Alyseae *s.str.* (i.e., *Alyssoides*, *Alyssum*, *Aurinia*, *Berteroa*, *Bornmuellera*, *Clastopus*, *Clypeola*, *Degenia*, *Fibigia*, *Galitzkya*, *Hormathophylla*, *Leptoplax* and *Physoptychis*, as discussed in Chapter 1), including *Phyllolepidum* (in agreement with Cecchi (2011)), *Physocardamum* (tentatively placed in tribe Lepideae by Al-Shehbaz *et al.*, (1986), but weakly supported within the Alyseae in the phylogeny of Warwick *et al.* (2010)) and *Straussiella* (tentatively placed in tribe Alyseae (Al-Shehbaz *et al.*, 2006) but not included in any previous phylogenetic studies). The trees generated using Concatenated Alignment C (Figs 3-2 and A6-11), which included representatives of 109

Brassicaceae genera outside Alysseae provides particularly strong support for a monophyletic Alysseae, consisting of the 16 genera listed above.

Sister Relationship of Alysseae

Although the three major Brassicaceae lineages I, II and III of Beilstein *et al.* (2006), have been supported in other recent studies, the branching order of these and other clades in the family remains uncertain, poorly resolved and weakly supported, due to short branch lengths in these parts of the tree (Koch *et al.*, 2007; Franzke *et al.*, 2007; German *et al.*, 2009; Lysak *et al.*, 2009). This has also been the case for Alysseae, which is placed outside these three major lineages (Beilstein *et al.*, 2010), and whose sister group remains unclear. In the tree of Khosravi *et al.* (2009), which sampled 19 Brassicaceae tribes, Alysseae was sister to Chorispora + Hesperidae with moderate support. In the tree of Beilstein *et al.* (2010), which sampled more widely across the Brassicaceae (number of tribes not stated), Alysseae was sister to the large Brassicaceae Lineage I + *Stevenia* (whereas the representative of Chorispora was in lineage III), but branch support was not stated.

In the trees based on Concatenated Alignments A (Figs 3-2 and A6-11), and C (Figs A6-9,12), which included various non-Alysseae genera from the Brassicales, all three of the major Brassicaceae lineages were resolved as monophyletic groups with strong support, although as found by Beilstein *et al.* (2010) many deeper nodes in the family were unsupported, and the relationships of Alysseae were unresolved, shedding no further light on the likely sister group of the tribe. In agreement with previous *ITS* studies (Warwick *et al.*, 2008; Khosravi *et al.*, 2009), the genera *Farsetia*, *Stevenia* (Figs 3-2, A6-9,11,12) and *Ladakiella* (Figs A6-9,12) were placed outside Alysseae *s.str.* in this study.

Major Alysseae Clades

In the following discussion, the results of Mengoni *et al.* (2003) are disregarded due to the potential unreliability of the data in that study (see *Phylogeny of Alysseae* in Chapter 1 and Appendix 5 for details). All of the major Alysseae clades A1-A6 of Warwick *et al.* (2008) form robustly supported monophyletic clades (bootstrap support (bs) of 100%, Fig 3-1; posterior probabilities (pp) of 1.0, Fig. 3-3) in this study. All of these clades have received high support in at least one previously published (ITS-based) tree (see discussion under *Phylogeny of Alysseae* in Chapter 1). Warwick *et al.* (2008) noted the congruence between clades A1–A6 and the genera of Alysseae and sections of *Alyssum*, although due to limited sampling of *Alyssum* species in previous studies, such congruence has not yet been thoroughly explored. The greater species sampling in this study provides further evidence for a strong, but not complete, congruence between these major Alysseae clades and the genera of Alysseae and sections within *Alyssum*. All species of *Hormathophylla* sampled to date are placed in Clade A4. All species of *Bornmuellera* sampled to date, along with the monospecific genera *Leptoplax* and *Physocardamum*, and are placed in Clade A5. All species of *Aurinia*, *Berteroa* and *Galitzkya* sampled to date are placed in Clade A6. All species of *Alyssoides*, *Clastopus*, *Fibigia*, and *Physoptychis* and the monospecific genera *Degenia* and *Straussiella* are present in Clade A3 (*Straussiella* had not been sampled in any previous phylogenetic study). All species of *Clypeola* sampled to date except for *C. ciliata* are present in Clade A1c. Species of *Alyssum* are placed in either Clade A1 or Clade A2. All 59 species of *Alyssum* sect. *Odontarrhena* sampled here are placed in Clade A1. Of the 46 *Alyssum* sect. *Alyssum* species sampled here, 43 were present in Clade A2, while *A. aizoides*, *A. lenense*, *A. stribryni* were all placed in Clade A1. Of the nine *Alyssum* sect. *Gamosepalum* species sampled, eight were in Clade A2 (the exception, *A. tetrastemon*, was in Clade A1). Of the six *Alyssum* sect. *Meniocus* species sampled, four were in Clade A1b (*A. heterotrichum* was in Clade A1a, and *A. linifolium* was in Clade A2). All four *Alyssum* sect. *Psilonema* species sampled in this study were in Clade A2.

Relationships between Major Alysseae Clades

Compared to previous studies, the phylogeny of Alysseae presented here provides important new evidence about relationships of the major clades within Alysseae. *Phyllolepidum* was placed as sister to Clade A3 with moderate support (89% bs; Fig. 3-1) in the maximum likelihood tree, but as sister to clade A4 with low support in the BEAST tree (0.87 pp; Fig. 3-3). Furthermore, the two alternative placement of *Phyllolepidum* were both inconsistent with Cecchi *et al.* (2010) and Cecchi (2011), where *Phyllolepidum* was sister to Clade A5, albeit with low/moderate support. Thus, while there is no doubt about the placement of *Phyllolepidum* in the combined A3+A4 clade, its relationships to either A3 or A4 remain uncertain. The relationships between all other major Alysseae clades received strong support in the both maximum likelihood (bs of 100%; Fig. 3-1) and BEAST (pp of 1.0; Fig. 3-3) trees. This contrasts with previous analyses, in which relationships between the major Alysseae clades received low or no support (Warwick *et al.*, 2008, Kosravi *et al.*, 2008, Cecchi *et al.*, 2010, Cecchi, 2011). The novel but robustly supported placement of Clade A6 as sister to Clade A1, and hence nested within *Alyssum* (Fig. 3-1), has important ramifications in terms of the non-monophyly of *Alyssum*, as discussed below.

Species Relationships in Alysseae

This study provides a high degree of resolution and support for species relationships within some of these Alysseae clades, and notably within clades A3, A4, A5 and A6. In Clade A3, the three genera *Fibigia*, *Clastopus*, and *Alyssoides* are all non-monophyletic (*Degenia* and *Straussiella* are monospecific, and only one *Physoptychis* accession was included) (Fig. 3-1). In Clade A5 the two monospecific genera *Leptoplax* and *Physocardamum* are nested within *Bornmuellera* with robust support, rendering the latter genus non-monophyletic as currently defined (Figs 3-1, 3-3). *Leptoplax* was sister to *B. baldaccii*, with weak support (57% bs, Fig. 3-1; 0.68 pp, Fig. 3-3). In contrast, Cecchi *et al.* (2010) found *Leptoplax* to be sister to *B. baldaccii* + *B. tymphaea*. Within

Clade A6 neither of the genera *Berteroa* or *Aurinia* are monophyletic (only one accession of *Galitzkya* was sampled).

Clade A2 comprises two robustly supported large subclades (clades A2a and A2b; Fig 3-1), and within each subclade many additional clades are also resolved with robust support (Figs 3-1). Three of the four species in *Alyssum* sect. *Gamosepalum* series *Connata* (*A. lepidoto-stellatum*, *A. paphlagonicum* and *A. thymops*) form a clade which receives strong support in the BEAST tree (Fig. 3-3) (although they do not in the maximum likelihood tree, in which branch support is generally lower within Clade A2; Fig. 3-1). The fourth species in this series, *A. tetrastemon*, is placed in Clade A1a (Figs 3-1, 3-3). All five species of sect. *Gamosepalum* series *Libera* that were sampled (*A. baumgartnerianum*, *A. harputicum*, *A. lycaonicum*, *A. niveum* and *A. sulphureum*) were placed within Clade A2a (Figs 3-1, 3-3), however these five were not monophyletic. No such subsections or series have been delimited in sect. *Alyssum*, and a detailed analysis of morphological data, in the context of the molecular results, would be useful in order to investigate whether clades A2a and A2b (and their various subclades) can be distinguished morphologically. Species from Clade A2a are restricted geographically to Turkey and neighbouring countries (indeed, the majority of species in this clade are endemic to Turkey), while species from Clade A2b come from Turkey and wider geographic area, and many of these species themselves have wide geographic ranges.

The *Alyssum montanum* complex is known to be highly variable in morphology, ploidy level and environmental requirements (Španiel *et al.*, 2011a,b). However, population-genetic analyses (Španiel *et al.*, 2011a,b; 2012) have only looked at central European populations, and previous phylogenies sampled only a single accession. The four accessions sampled here are all placed in Clade A2b, but the species itself is not monophyletic on the tree, with different accessions scattered across the Clade A2b; accessions 2 and 3, from a seed sample from Spain and a herbarium specimen from Morocco, respectively, are sister to each other with moderate support.

Accession 1 was from a herbarium specimen that had been clearly identified as *A. montanum*. Accession 2 was from a USDA seed accession from which a voucher specimen had not been taken, and the species identification is therefore questionable. Together, the results obtained here for *A. montanum* suggest that the alliance may be more appropriately treated as several distinct species, in line with its variable morphology and ploidy levels and wide geographic distribution. However, misidentification or hybridization/chloroplast capture cannot be ruled out as causes of this non-monophyly, and a much more detailed analysis including multiple accessions from multiple populations across the range of the complex would be needed to address this question properly and to arrive at a more satisfactory delimitation of species.

In contrast to previous studies (Warwick *et al.*, 2008; Khosravi *et al.*, 2009; Cecchi *et al.*, 2010), where relationships within clade A1 remained unclear due to sparse taxon sampling and lack of resolution and support, here a much more complete hypothesis of relationships is revealed for the first time. Clade A1 comprises three subclades and *Clypeola ciliata* (Figs 3-1, 3-3). Given the strong support for these subclades (except for Clade A1a under parsimony) and their congruence to genera or generic sections within *Alyssum*, the three subclades have been informally labelled A1a, A1b and A1c here. A strongly supported clade containing *Alyssum* sect. *Odontarrhena* species (i.e., Clade A1a) was present in Cecchi *et al.*, 2010. The presence of Clade A1c (i.e., *Clypeola*) nested within Clade A1 in the present study agrees with the *ITS* phylogenies of Warwick *et al.* (2008), Khosravi *et al.* (2009), Cecchi *et al.* (2010) and Cecchi (2011).

In addition to five species from other sections of *Alyssum* (*A. aizoides*, *A. lenense* and *A. stribryni* from sect. *Alyssum*, *A. tetrastemon* from sect. *Gamosepalum* and *A. heterotrichum* from sect. *Meniocus*), Clade A1a contains exclusively species from *Alyssum* sect. *Odontarrhena*. Furthermore, species from this section are not present in any other part of the tree. Section *Odontarrhena* is diagnosed by its uniovulate locules (species in other sections all have two or more seeds per locule

of the silicle) (Dudley, 1964a), and all members are biennial or perennial. However, for Clade A1a, these characters are not diagnostic, and may be homoplastic, since *A. aizoides*, *A. lenense*, *A. stribryni* and *A. tetrastemon* have locules that are 2-ovulate, and *A. heterotrichum* has locules that are 4-6 ovulate (Dudley, 1964a; Warwick *et al.*, 2008) and is an annual (see Appendix 3). Clade A1b contains four of the six species sampled from sect. *Meniocus*, and no others. Clade A1c contains four of the five species sampled from *Clypeola*; the fifth species, *C. ciliata* is sister to A1c without support in the BEAST tree (<0.5 pp; Fig 3-3) but sister to A1a + A1b with strong support in the maximum likelihood tree (92% bs Fig. 3-1). Clades A1a and A1b are sister to each other with low support in both the maximum likelihood and BEAST trees (33% bs, Fig 3-1; 0.89 pp, Fig 3-3).

In line with previous studies, there is a striking lack of well-supported resolution in Clade A1a (Figs 3-1, 3-3), due to lack of phylogenetically informative variation for the A1a clade species for the DNA sequence regions utilized. No clades received strong or moderate support under all three tree search methods and therefore the lack of cohesion in the *Odontarrhena* species for which multiple accessions were sampled could result from the generally poor resolution and short branch lengths in this clade.

There are a number of other possible explanations for the lack of cohesion of species within Clade A1a. Species delimitations that do not reflect phylogenetic history, for example due to the presence of cryptic species, although more widespread in animals, are known in the plant kingdom (e.g., Liao *et al.*, 2011; Liu *et al.*, 2011; Dong *et al.* 2012; Heinrichs *et al.*, 2012), including in serpentine endemic species (Yost *et al.*, 2012). This is especially likely for geographically widespread polymorphic alliances such as *A. murale*. Alternatively, lineage sorting, whereby shared ancestral alleles span multiple species (Avise *et al.*, 1983) is likely to be particularly prevalent in recent radiations such as that of the A1a clade (Maddison & Knowles, 2006). Hybridization, introgression and chloroplast capture (Wolfe & Elisens, 1995) are further possible explanations.

Lastly, errors in species identification could be responsible for the apparent lack of cohesion of species in phylogenies. For example, a voucher specimen was not taken for accession 4 of *A. montanum* (obtained from a USDA seed source) or for *A. pintodasilvae* accession 1, and therefore the identity of these accessions cannot be confirmed. The lack of a monograph for *Alyssum*, and subsequent reliance of regional floras for identification, increases this risk, especially given that the geographic distribution of many species is poorly known.

For the above reasons, especially the lack of resolution in Clade A1a, a much more detailed phylogenetic analysis, particularly one employing additional highly variable DNA regions (e.g. additional non-coding chloroplast regions, and nuclear single nucleotide polymorphisms), and dense sampling of populations across their entire geographic ranges is likely to be necessary to increase the resolution of phylogenetic relationships in Clade A1a. Whilst the detail of species relationships in this clade is clearly interesting and relevant in the investigation of the detailed evolutionary history of nickel hyperaccumulation in *Alyssum*, they do not affect the broader conclusions of this study, since all *Alyssum* section *Odontarrhena* species, and all nickel hyperaccumulator species from this genus were strongly supported within the A1a clade.

Together, the results obtained here provide strong support for the five major Alyseae clades (A1–A5) of Warwick *et al.* (2008), and the newly recognized sub-clades A1a, A1b, A1c, A2a and A2b. They demonstrate the strong congruence between these clades and genera of the tribe and the generic sections within *Alyssum*. They also provide strong evidence for the sister relationships between these clades, although the exact placement of *Phyllolepidum* and *Clypeola ciliata* remain uncertain. Despite the broad congruence in terms of placement of genera and these major clades, there is also ample evidence to suggest that seven of the 16 genera of Alyseae are non-monophyletic, suggesting that generic delimitation across the tribe remains in a state of considerable flux, and the need for a new generic classification of Alyseae. The resolution of

species-level relationships within these clades is variable between clades, and is particularly poor in Clade A1a, which contains almost exclusively species from *Alyssum* sect. *Odontarrhena*.

Taxonomic Implications

Alysseae

This study supports the findings from previous *ITS*-based studies that (based on the taxonomy in *Appendix 1*), Alysseae *s.str.* contains all sampled species of the following 15 genera: *Alyssoides*, *Alyssum*, *Aurinia*, *Berteroa*, *Bornmuellera*, *Clastopus*, *Clypeola*, *Degenia*, *Fibigia*, *Hormathophylla*, *Galitzkya*, *Leptoplax*, *Phyllolepidum*, *Physoptychis*, and *Physocardamum*. The genus *Straussiella*, which is included in an explicit phylogenetic analysis here for the first time, is also placed within the Alysseae *s.str.* There is now ample evidence to suggest that the genera *Asperuginoides*, *Didymophysa*, *Farsetia*, *Lobularia* and *Ptilotrichum* (two former species of the last of these are now placed in the genus *Stevenia*) are not closely related to Alysseae and lie outside the tribe. Current evidence therefore suggests that the Alysseae should be re-circumscribed to include these 16 genera plus *Lepidotrichum* (see below). However, finding a morphological character or character set which defines this tribe is likely to require further herbarium work, since none of the 19 morphological characters investigated in Alysseae by Warwick *et al.* (2008) is unique to the above genera within the Brassicaceae. For clarity it is recommended that the term 'Alysseae' should be used rather than Alysseae *s.str.*, for this taxon, and that the term 'Alysseae (*sensu* Al-Shehbaz *et al.* (2006))' should be used for the earlier delimitation in that study, in preference to Alysseae *s.l.*

Alysseae Genera

The denser sampling and greater resolution obtained here within Alysseae than in previous studies, provides a good foundation for reassessment of generic delimitation across the tribe. This study provides further evidence for the monophyly of *Hormathophylla*. The species *H. purpurea*,

previously placed in the segregate genus *Nevadensia*, is nested within *Hormathophylla* with strong support, suggesting that resurrection of the genus *Nevadensia* for this species, as proposed by Rivas-Martínez *et al.* (2002) based on morphological and karyological characters, and also supported by Cecchi (2011) (where this species was sister to the other four species sampled, with low support), is unnecessary.

As previously noted by Cecchi (2011), additional evidence presented here suggests that *Phyllolepidum* (formerly two species in *Aurinia*) is not closely related to *Aurinia*, and that resurrection of *Phyllolepidum* is indeed justified. Both *Berteroa* and *Aurinia* are non-monophyletic in the analyses presented here (Fig. 3-3), with additional evidence that *Aurinia uechtritzi* is not closely related to other *Aurinia* species as found by Cecchi (2011), who resurrected the genus *Lepidotrichum* to accommodate this species and account for the non-monophyly of *Aurinia*. However, in the tree presented here the two accessions sampled (only one was sampled by Cecchi, 2011) form a paraphyletic grade with *Berteroa mutabilis*, suggesting the need for more complete sampling and phylogenetic work on these two genera before formal taxonomic changes are made. Indeed this will be needed to ascertain the status of the genera in clade A6.

The placements of *Leptoplax* and *Physocardamum*, nested within *Bornmuellera* in clade A5, render *Bornmuellera* non-monophyletic. New morphological work targeted on this clade is needed to explore what diagnostic synapomorphies can be found upon which to base a new delimitation of genera in this clade (either sinking *Leptoplax* and *Physocardamum* into *Bornmuellera*, or potentially establishing two genera corresponding to the Clade A5a and its sister clade. Pending this further work, *Bornmuellera s.l.* is used hereafter to refer to the clade comprising *Bornmuellera*, *Leptoplax* and *Physocardamum*, as an interim solution that satisfies the needs of the subsequent analyses presented in this thesis.

A similar lack of monophyly characterizes the genera *Alyssoides*, *Fibigia* and *Clastopus* in clade A3 (Fig. 3-3a). Five species of *Fibigia* were separated based on morphology into a new segregate genus *Irania* by Hadač & Chrtek (1973). This segregate was not accepted by Warwick *et al.* (2006), but was supported by the phylogeny of Khosravi *et al.* (2009). However, this latter study sampled only two of these five species (*F. multicaulis* and *F. umbellata*) plus two other *Fibigia* species, and all of the branches separating the two clades received low support. In the present study, three of these five species (*F. membranacea*, *F. multicaulis* and *F. umbellata*) and seven other *Fibigia* species were sampled, and were placed with high support in three distinct subclades within clade A3. However, none of these is congruent with the concept of Hadač & Chrtek (1973) for the new genus *Irania*, which is not supported in this analysis. It seems likely that the calyx, filament and seed characteristics used by Hadač & Chrtek (1973) to define *Irania* are in fact homoplastic.

The non-monophyly of *Fibigia*, *Alyssoides* and *Clastopus* in clade A3, and of *Aurinia* and *Berteroa* in Clade A6, suggests the need for a new generic classification of Alyseae. However, denser taxon sampling and a detailed cladistic analysis of morphology would be desirable before a revised set of genera are delimited and new nomenclatural combinations proposed.

The non-monophyly of *Alyssum*, with clade A1c (i.e. *Clypeola*) nested within Clade A1, as well as Clade A6 (i.e. *Aurinia*, *Berteroa* and *Galitzkya*) sister to A1 (and hence nested within *Alyssum s.l.*), suggests a need for taxonomic changes to all these genera. Two possible options include: (1) referring all of the genera in clades A1, A2 and A6 to *Alyssum*; or (2) recognizing clades A1a, A1b, A1c, A2 and A6 at the generic level, for example, as *Odontarrhena* (a taxon first described by Meyer (1831)), *Meniocus*, *Clypeola*, *Alyssum* and *Aurinia*, respectively. Given the considerable nomenclatural changes implied by either of these options, detailed cladistic analysis of morphological data in conjunction with the new molecular phylogeny presented here is needed to establish diagnosability of any such taxa before a new classification is proposed. Pending this

further work, it is recommended that *Alyssum s.l.* is used to refer to the clade comprising clades A1, A2 and A6, as an interim solution that satisfies the needs of the subsequent analyses presented in this thesis.

Lack of resolution in the Clade A1a precludes any assessment of the monophyly of the formal subsections and series of sect. *Odontarrhena*, or of the taxonomy of the *Alyssum murale* and *A. serpyllifolium* species complexes. In Clade A2 there is a higher level of resolution, and some congruence to the infrageneric taxonomy. The lack of monophyly of the *A. montanum* accessions suggests that recognition of more than one species to account for that alliance may be appropriate. However, a more detailed and densely sampled study involving more than four accessions, spanning the full geographic range is needed to resolve this species complex.

Age Estimates in Alyseae

Alyseae Crown Node

A number of published studies have estimated divergence times and the ages of genome duplication events within the Brassicaceae using molecular clock-based techniques (Yang *et al.*, 1999; Koch *et al.*, 2000, 2001; Eremolaeva *et al.*, 2003; Henry *et al.*, 2006; Schranz and Mitchell-Olds, 2006; Couvreur *et al.*, 2009; Fawcett *et al.*, 2009; Beilstein *et al.*, 2010; Franzke *et al.*, 2010). Relatively few, however have produced dated phylogenies in which the Alyseae is represented by multiple species, and hence can be used to date any of the nodes within this tribe.

As detailed in Table 3-5 and described in Chapter 3, age estimates obtained here for the crown node of Alyseae (based on Calibration Strategy 3) are 38.4 Mya (95% CI: 24.9–54.8 Mya) (in the Stage 1, Brassicales-wide tree (Fig. 3-2)) and 27.5 Mya (95% CI: 20.2–36.5 Mya) (in the Stage 2, Alyseae tree Fig. 3-3). These are older than previous estimates (19.3 Mya (95% CI: 12.3–26.7 Mya)) in Couvreur *et al.* (2009) and 23.1 Mya (95% CI: ca.10–ca.35 Mya) in Beilstein *et al.* (2010)).

The fact that older estimates were obtained here than in the Beilstein *et al.* (2010) study is likely to be due to the differences in the placement of fossil calibration points (rather than say, the small amount of additional sampling of Alyseae species carried out here in Stage 1 (Fig. 3-2)). Here, the published *Akania* and *Thlaspi* fossil ages were used as minimum age constraints for more recent nodes than those selected by Beilstein *et al.* (2010) (i.e., for *Akania*: the stem node of the genus *Akania* (here) versus the stem node of the Akaniaceae (in Beilstein *et al.*, 2010); for *Thlaspi*: the stem node of the genus *Thlaspi* (here) versus the *Thlaspi-Alliaria* split (in Beilstein *et al.*, 2010)). Beilstein *et al.* (2010) placed these two fossil-derived age estimates at excessively deep nodes, whereas here they were placed at the stem nodes of their genera. It is therefore considered that the placement of fossil calibrations employed here is more appropriate than that of Beilstein *et al.* (2010) (discussed fully under *Fossil Calibrations Employed Here* in Chapter 2). A key result of Beilstein *et al.* (2010) was the unexpectedly old estimates for the ages of nodes within the Brassicales. However, the estimates obtained here, based on a better-justified date calibration procedure suggest even older dates, which is a significant result. Furthermore, the precision of the Stage 2 estimate obtained here is higher than that of Beilstein *et al.* (2010) (95% confidence intervals of 20.2 to 36.5 Mya (in stage 2 of Calibration Strategy 3), as opposed to *ca.*10 to *ca.*35 Mya (in Beilstein *et al.* 2010)). However, given the relative paucity of reliable fossils in the Brassicaceae, divergence time estimates within this family are always going to be surrounded by greater uncertainty than estimates for families with abundant fossil records.

Due to the uncertainty surrounding the sister relationship of Alyseae (and hence the stem node for the clade cannot be identified with certainty), estimating the age of the stem node is problematic, and was not possible in this study.

Major Alysseae Clades

Within the Alysseae, an age estimate has been previously published for only one node. Beilstein *et al.* (2010) estimate the age of the *Clypeola aspera*/*Alyssum simplex* split (with the latter species referred to its synonym, *A. parviflorum*) to be 13.2 Mya (95% CI: ca.9–ca.29 Mya). Given that *A. simplex* is a member of section *Alyssum* and would therefore be expected to be placed in clade A2, this split would be expected to represent the *Alyssum s.l.* crown node (see Fig. 3-1). However, in the analysis undertaken here, this same *A. simplex* accession was placed in clade A1, whereas two other accessions of this species that were sequenced here were in Clade A2 (Figs 3-1, 3-3), as expected for a member of *Alyssum* sect. *Alyssum*. This suggests that the *A. simplex* accession used by Beilstein *et al.* (2010) may have been misidentified and for this reason this age estimate is considered to be unreliable.

The current study provides age estimates for the crown nodes of all major Alysseae clades and the three subclades within Clade A1 (Table 3-5). The oldest of these is Clade A2 (crown node 14.1 Mya (95% CI: 9.71–19.5 Mya)), the youngest is A1a (crown node 3.27 Mya (95% CI: 2.06–4.72 Mya)). These results suggest that the establishment of the major Alysseae lineages occurred in the Neogene, especially the mid-Miocene, and that the large majority of extant species diversity in the tribe was established only in the late Miocene or more recently. Given its more recent age, the large size (65 species) of the A1a clade is a striking feature of the time-calibrated Alysseae tree, and suggests that a striking radiation of species occurred in *Alyssum* in the Pliocene or even the Pleistocene. The absolute rate of species diversification occurring in this radiation is discussed and placed in context under *Species Diversification in Alysseae* below.

Origins of Hyperaccumulation and Serpentine Endemism in Alyseae

Phylogenetic Position

The results obtained here show that there were likely to have been two independent evolutionary origins of nickel hyperaccumulation in Alyseae: one in the Clade A5, i.e. within *Bornmuellera s.l.*, and one in Clade A1, i.e., within *Alyssum s.l.* The finding of a separate origin of nickel hyperaccumulation in *Bornmuellera* is consistent with the tentative results in Cecchi (2011), although that study suffered from very low levels of taxon sampling (as discussed under *Phylogeny of Alyseae* in Chapter 1) and included only one nickel hyperaccumulator species within *Bornmuellera*. The lineage in which hyperaccumulation developed in *Bornmuellera s.l.* subsequently diversified into the four hyperaccumulator species in that clade that were sampled here (three in *Bornmuellera* and one in *Leptoplax*).

The finding that nickel hyperaccumulation originated once in *Alyssum s.l.*, on the branch leading to Clade A1a, contrasts with previous ideas about multiple origins suggested by Mengoni *et al.* (2003) and Cecchi *et al.* (2010). However the tree topology of the Mengoni *et al.* (2003) study is dismissed as unreliable (published topologies could not be re-constructed, see *Chapter 1* and *Appendix 5*) and was relatively poorly resolved (as have been all other *ITS*-based studies in Alyseae, due to relatively low levels of phylogenetically informative sequence variation in that DNA region). The Cecchi *et al.* (2010) study suffered from severe under-sampling, low branch support in *Alyssum* and lack of formal analysis of character evolution. However, these previous studies did attempt to assign hyperaccumulation status to individual terminals on their trees (i.e., the hyperaccumulation status of the particular accessions employed in the study were determined), rather than the assignment of hyperaccumulation at the species level as adopted here. Nevertheless, polymorphism for hyperaccumulation status within species was incorporated into the character evolution analyses carried out here by using a third character state, which still supported the crown

node of Clade A1a being a nickel hyperaccumulator. The lack of robustly supported resolution within the A1a clade reduces the power of character optimizations and certainly prevents firm conclusions about the number of gains or losses of hyperaccumulation in that clade. For example, if all non-accumulator species formed a clade within Clade A1a, it would only be necessary to postulate a single loss of hyperaccumulation. However, the presence of species that are polymorphic for nickel hyperaccumulation strongly suggests the scenario of multiple independent losses.

What can be said, however, is that the results obtained here suggest that homoplasy in nickel hyperaccumulation in the A1a clade results mainly from evolutionary loss rather than from multiple independent gains of this trait, which represents a significant finding, in terms of the implications for likely drivers of diversification and geographic dispersal of clade A1a. This result shifts the likely evolutionary scenario for nickel hyperaccumulation from one of remarking upon its lability and repeated origins (Mengoni *et al.*, 2003, Cecchi *et al.*, 2010) to one of attempting to understand the reasons why a single lineage within *Alyssum s.l.* evolved nickel accumulation, why the nickel accumulation lineage appears to have subsequently undergone considerable species diversification and why nickel accumulation appears to have been retained in many species and lost in others. It is perhaps notable that this scenario more closely matches the original taxonomy-based inferences of Brooks *et al.* (1979), with all but one hyperaccumulator species in *Alyssum* belonging to sect. *Odontarrhena*, and the majority of species in *Odontarrhena* being hyperaccumulators. The single nickel hyperaccumulator in *Alyssum* outside sect. *Odontarrhena* is *A. lenense* (sampled and analyzed by Brooks & Radford (1978) under the name *A. fisherianum*), which was also placed in the A1a clade here, and hence it is not the 'exception' suggested by its taxonomy.

There is some evidence that species that have apparently lost the ability to hyperaccumulate nickel, nevertheless have retained a residual element of the trait, for example in the form of shoot

tissue nickel concentrations well above those of other (presumably ancestrally) non-accumulator species and higher tolerance of that metal (e.g., *A. serpyllifolium* (Ingle *et al.*, 2005)). The difference between species that have lost the ability to hyperaccumulate nickel and species that never had that ability may be important in comparative studies which aim to elucidate the genetic or physiological basis of hyperaccumulation, and the results obtained here will help to inform the choice of Alyseae species in such studies.

Serpentine endemism is strongly associated with nickel hyperaccumulation (as evidenced by the results of the BayesTraits analysis). Within *Bornmuellera s.l.* there is complete correlation (all four hyperaccumulators are serpentine endemics), and these species formed a clade (A5a) suggesting a single origin of this trait (Fig. 3-8). However, in *Alyssum s.l.* these two traits are not completely correlated: one serpentine endemic species, *A. densistellatum*, is placed in the A2 clade and although all serpentine endemic species in Clade A1a are nickel hyperaccumulators, the reverse is not true (i.e., many of the nickel hyperaccumulator species occur both on and off serpentine substrates and are therefore not serpentine endemic). The crown node of Clade A1a appears to have been serpentine endemic. Once again, however, the lack of well-supported resolution in Clade A1a reduces the level of confidence that can be attached to specific evolutionary scenarios. Previous phylogenetic studies have demonstrated both multiple (e.g. *Calochortus*: Givnish and Patterson, 2004; *Onosma*: Cecchi *et al.*, 2011) and single origins (e.g., *Halacsya* and *Paramoltkia*: Cecchi and Selvi, 2009) of serpentine endemism at the generic level. However, no previous phylogenetic study has encompassed the number of closely-related serpentine endemics found in *Alyssum*.

Biogeography

The geographic locations of the origins of nickel hyperaccumulation in Alyseae, and especially within *Alyssum* are difficult to determine because the lack of phylogenetic resolution, and the lack

of detailed geographic distribution data for many of these species. No formal ancestral area reconstructions (Ronquist, 1997) were carried out here. However, the fact that all *Bornmuellera* species have ranges within the Balkans or Turkey suggests that nickel hyperaccumulation within this clade evolved within this geographic region. In contrast, members of Clade A1a are distributed across a wide geographic area spanning Iberia to central Asia, although its sister clade, A1b, is restricted to Turkey and Syria. Given the young age of the A1a clade, this suggests there has been a high rate of geographic dispersal in this clade. However, lack of resolution within clade A1a precludes any more detailed analysis or definite conclusions about the biogeographic history of this clade.

Although no formal ancestral area reconstructions were carried out, it is clear that, at a broad spatial scale, the geographic distribution of nickel hyperaccumulator species in the A1a clade covers a very similar overall area to that of non-accumulators across the remainder of the Alyseae (Fig. 3-11c). These observations show that hyperaccumulator lineages in *Alyssum* have spread over a wide geographic area, and given the young age of the A1a clade, have done so in a relatively short period of time. This is somewhat surprising since, although a minority of species in sect. *Odontarrhena* have mucilaginous or winged seeds, or samaroid silicles (Dudley, 1964a), no adaptations for seed dispersal have been reported for the majority of species. A low or intermediate dispersal ability, might however, be expected to promote speciation through allopatric mechanisms (see *A Geographic Species Radiation?* below). In contrast, the much smaller clade of hyperaccumulator species in *Bornmuellera* (including *Leptoplax*) are restricted to a relatively small area of the Eastern Mediterranean in Greece and Turkey.

Time of Origin and Historical Context

The branches on which nickel hyperaccumulation evolved in *Alyssum s.l.* and *Bornmuellera* have estimated ages of 3.27–8.31 Mya (incorporating 95% CIs for each value: 2.06–11.88 Mya) and 6.31–

8.78 Mya (incorporating 95% CIs for each value: 3.80–12.3 Mya), respectively (Fig. 3-3)) (the upper and lower values are for the nodes representing the ends of these branches), i.e. in the late Miocene (11.6–5.33 Mya) and early Pliocene (2.59–5.33 Mya). There is considerable overlap in these age estimates. The evolution of metal hyperaccumulation has only been studied in a few plant taxa other than *Alyssum* (e.g., *Arabidopsis halleri* (Roux *et al.*, 2011; Pauwels *et al.*, 2012); *Noccaea caerulescens* (Lochlainn *et al.*, 2011)) although the timescales involved in these other species are much shorter, since their metal tolerance and hyperaccumulation is thought to have evolved in response to anthropogenic pollution. Cecchi *et al.* (2011) concluded that serpentine endemism in *Onosma* (Boraginaceae), which includes seven serpentine endemic species, evolved in the Pleistocene, and suggested that serpentine sites could have provided thermal refugia due to their open and free-draining nature. However, more precise estimates of the ages of serpentine species in *Onosma* are not possible as much of the Cecchi *et al.* (2011) tree was poorly resolved.

The coincidence of diverse global tectonic events on land and geological and climatic events in the ocean-atmosphere in the Neogene was instrumental in establishing the modern world and much of the world's landscape as we know it today (Potter & Szatmari, 2009). Foremost amongst these changes was the late Miocene global cooling and drying and consequent greater seasonality and expansion of dry and open habitats across the globe. In addition to global scale change in the late Miocene a number of important geological events and climate changes occurred in the Mediterranean region during this period and particularly during the Messinian (7.24–5.33 Mya).

- (1) The climate in the Mediterranean underwent considerable change causing a shift from a subtropical mesic climate with summer monsoons, to the typical Mediterranean summer-dry conditions, as we know it today (Mai, 1989, 1991). This caused dramatic changes in the vegetation from mesic subtropical or warm temperate forest (the so-called Tertiary forests or Palaeotropical geoflora) towards the typical drought- and fire-adapted sclerophyllous forest and scrub (Suc, 1984).
- (2) the collision of the African and Eurasian plates and resultant orogeny in the western

Mediterranean, combined with increasing aridification, had dramatic consequences for the physical geography of the Mediterranean region, and most significantly the drying out of the Mediterranean basin, known as the Messinian salinity crisis, 5.96–5.33 Mya (Hsü *et al.* 1973). There is much debate about the extent of drying, but a drop in sea level of c. 1.5 km is believed to have occurred at least once (Clauzon *et al.*, 1996; Krijgsman *et al.*, 1999). (3) The Messinian stage ended with the Zanclean flood (5.33 Mya), whereby the opening of the straits of Gibraltar allowed the refilling of the Mediterranean from the Atlantic, possibly in a matter of months to years (Blanc *et al.*, 2002; Garcia-Castellanos *et al.*, 2009). These changes would undoubtedly have had significant impacts on the evolutionary dynamics and fates of plant lineages, causing extinction and retreat of more mesic lineages into relictual corners that remained suitable for these lineages lacking drought adaptation and at the same time opportunities for rapid diversification of lineages adapted to long summer drought and fire. A number of plant groups characteristic of the Mediterranean are thought to have diversified, some of them very rapidly during the late Miocene, Pliocene, and Pleistocene, including tribe Cochlearieae: Miocene (Koch, 2012); tribe Asterae: late Miocene (Fiz-Palacios, 2011); *Cistus*: 0.79–1.29 Mya (Guzmán *et al.* 2009); *Dianthus*: 2.1–0.9 Mya (Valente *et al.* 2010); and *Nigella*: 1.17–0.08 Mya (Bittkau & Comes 2009)). The spectacular radiations documented during the Pleistocene for *Cistus* and *Dianthus*, two genera with very high endemism in the Mediterranean (and *Cistus* essentially endemic to that region), are especially notable in this regard. These changes could also have created the potential for plant dispersal during Mediterranean drying when many areas would have been connected, and subsequent vicariant isolation following flooding, as hypothesized to explain the high diversity of *Nigella s.l.* species in the Aegean region (Bittkau & Comes, 2009).

The geological history of the numerous serpentine outcrops across the Mediterranean and south-west Asian regions is complex. Serpentes are formed through the uplifting of ultramafic oceanic crustal regions and their emplacement in continental crust at plate boundaries during periods of

tectonic activity. These rocks are abundant along the margins of the African, Asian and Arabian plates in the Mediterranean and south-west Asian regions, and their emplacement and exposure is likely to be associated with periods of orogeny and subduction at these boundaries (Moore, 2011). Robertson (2002) notes that the well-dated ophiolites of the Eastern Mediterranean are restricted to three time intervals: Triassic, Mid-Late Jurassic, and Late Cretaceous. In Turkey, for example, which contains the greatest number of hyperaccumulator species of *Alyssum*, the ophiolites are all of Late Cretaceous origin, with dates between 102 and 74 Mya (Robertson, 2002; Dilek & Furnes, 2011). The youngest outcropping in the Mediterranean region is the Early Miocene Ronda peridotite at the western end of the Betic Cordillera in southern Spain – notable as the locality for the hyperaccumulator *A. malacitanum* – which was finally emplaced by 18–16 Mya (Mazzoli and Martín Algarra, 2011). In contrast, the ophiolites of the northwestern part of the Iberian peninsula, which are the locality of the closely related hyperaccumulator *A. pintodasilvae* – namely those of Careón, Spain (395 Mya) and Morais, Portugal (392–384 Mya) – are the oldest in the entire Mediterranean region (Vaughan and Scarrow, 2003). It is clear from these studies that serpentine habitats were present across the Mediterranean region for a long time prior the origin of nickel hyperaccumulation or serpentine endemism in the Alyseae.

Species Diversification in Alyseae

Alyseae-wide background rates of net species diversification are estimated here at 0.12–0.18 splits lineage⁻¹ million years⁻¹. This range is only slightly above the background rate for the angiosperms as a whole (0.078 to 0.091; Magallón & Castillo, 2009). In contrast, the absolute rates of net species diversification estimated for Clade A1a within *Alyssum* (0.26–1.12), are high compared to the small number of rate estimates available for plant lineages: there are very few with rates of over 1.0 (Valente *et al.*, 2010). However, they do not match the highest reported estimates for plants: up to 7.55 (based on a fossil-calibration) and up to 16.45 (based on a

secondary calibration) in Mediterranean *Dianthus* (Valente *et al.*, 2010), and 1.56–5.21 for Andean *Lupinus* (Drummond *et al.*, 2012).

A key finding of this study is the discovery of a significant increase in species diversification rate on the branch leading to the A1a clade within Alyseae (Table 3-11; Fig. 3-10), the branch on which nickel hyperaccumulation is hypothesized to have originated in *Alyssum s.l.*, whilst no increase in species diversification rate was observed on the branch leading to the A5a clade, the branch on which nickel hyperaccumulation is hypothesized to have originated in *Bornmuellera s.l.* Formal testing of this association using BiSSE showed that within Alyseae, the diversification rate of hyperaccumulator lineages is higher than in non-accumulator lineages (Table 3-14). BayesTraits analyses also showed that nickel hyperaccumulation and serpentine endemism are strongly correlated. Furthermore it is apparent that species range sizes are smaller in hyperaccumulator than in non-accumulator species (Fig 3-5), and that species diversification rate is negatively related to species range size in the Alyseae (Tables 3-17, 3-18). None of these associations has previously been tested in a phylogenetic context for any group of metal hyperaccumulator plants, although informal observations about these relationships in Alyseae were made by Brooks *et al.* (1979).

This study therefore provides the first convincing evidence of correlations between nickel hyperaccumulation, species diversification rate, serpentine endemism and species range size. It is also clear that nickel hyperaccumulation in *Bornmuellera s.l.* and *Alyssum s.l.*, and subsequent rapid species diversification in the *Alyssum* A1a clade coincide with a time of great environmental change within the Mediterranean region. Whilst these results are not necessarily conclusive about definite causes and effects, or about the speciation processes underlying rapid species diversification within the A1a clade, they do provide a set of robust hypotheses for discussion and further research. It is difficult to rule out unknown co-occurring traits or processes present within the *Alyssum* hyperaccumulator clade as significant drivers of diversification in this group. For example, although

hybridization between extant species is rare (see *Taxonomy of Alyssum and Alyseae* in Chapter 1), this process may have been important in the group historically. Furthermore, the considerable variation in chromosome numbers within Alyseae (see *Taxonomy of Alyssum and Alyseae*) suggests that polyploidy may also have played a significant role in species diversification in the group. In the following sections, all this evidence is drawn together to establish a conceptual model for the evolution of nickel hyperaccumulation and rapid species diversification in the Alyseae.

Nickel Hyperaccumulation: Is it Easier to Move or to Evolve?

The single origin of nickel hyperaccumulation in *Alyssum s.l.* and the rapid diversification of species in clade A1a, which includes all the *Alyssum* hyperaccumulator species, across a broad geographic area from Iberia to southwest Asia, suggest that nickel hyperaccumulation in *Alyssum* has apparently not evolved *de-novo* from local non-accumulator species pools on the various serpentine formations in this region, but instead appears to be phylogenetically conserved. The concept of phylogenetic niche conservatism *sensu* Harvey and Pagel (1991) implies a scenario in which niches tend to be filled by species already adapted to them, rather than through *de novo* adaptations in non-adapted lineages. Donoghue (2008) has suggested that the extent of niche conservatism across the plant kingdom is higher than might be expected, and that this factor is likely to be important in understanding global patterns of biodiversity. For example, Ackerly (2004) has shown that in addition to some *in-situ* adaptation of local lineages to the summer dry conditions, many lineages of woody plants of the California chaparral appear to have been filtered from lineages pre-adapted to drought, including some from disjunct geographic areas, that predate the post-Miocene Mediterranean climate of that region. And in many plant communities, component species are more closely related than would be expected by chance (e.g., Campbell-Webb, 2000; Prinzing *et al.* 2001; Willis *et al.*, 2010; Merwin *et al.*, 2012). Much thinking about broad scale phylogenetic niche or biome conservatism (*sensu* Donoghue, 2008 and Crisp *et al.*, 2009) stems from the idea of tropical niche conservatism, whereby across larger spatial and

temporal scales, less than 50% of plant families appear to have evolved cold tolerance (which appears to require a complex set of physiological changes: Körner, 2003), and hence to have colonized the (in many cases geographically adjacent) temperate regions. Donoghue (2008) suggested that phylogenetic conservatism may result from the fact that some shifts between ecological niches present significant adaptive barriers entailing substantial and in many cases complex physiological and biochemical changes that cannot be readily evolved.

The idea of phylogenetic niche conservatism led Donoghue (2008), to a key general question as to whether it is easier for lineages to adapt to changing environments (i.e., to evolve) or to disperse between disjunct areas with similar ecologies to which they are already adapted (i.e., to move). The results of the current study apparently provide a startling example of this in *Alyssum*, where nickel hyperaccumulator species have apparently found it easier to move between serpentine sites than the geographically adjacent non-hyperaccumulators have found it to independently evolve the trait. For a given trait and a given spatial arrangement and temporal dynamic of habitat patches, lineages presumably could lie anywhere on a hypothetical scale between extreme evolvers and extreme movers. It is the tradeoffs between adaptive barriers, trait evolution and dispersal that can shape the historical assembly of species and global patterns of biodiversity (Donoghue, 2008; Crisp *et al.* 2009). Where corridors are available for dispersal, pre-adapted lineages from distant regions will presumably be likely to move in to new habitats. Where there are barriers to dispersal, resident species, or those from adjacent habitats will presumably be more likely to evolve adaptations to the new conditions and hence be able to successfully colonize. Although there are numerous examples of lineages, given sufficient time, overcoming apparently significant barriers to dispersal, such as in the trans-oceanic dispersal of land plants (e.g., various lineages in African Rapateaceae and Bromeliaceae (Givnish *et al.*, 2004), Fabaceae (Lavin *et al.* 2004), Cucurbitaceae (Schaefer *et al.*, 2010), the palms (Baker & Couvreur, 2013), and Old World to New World post-Gondwanan dispersal events in various plant families (Hughes *et al.*, 2013)).

It is clear that the junction between serpentine and non-serpentine soils presents an apparently significant adaptive barrier because of the extreme contrast in their chemical and physical conditions (Brady *et al.*, 2005). The extreme conditions of serpentine soils have led to a complex set of physiological and biochemical adaptations in many species that have successfully colonised these formations, including nickel hyperaccumulators in *Alyssum*. Such specialization mean that serpentine specialists are unlikely to be competitive on non-serpentine soils, but that serpentine-adapted species will have an advantage in colonising other serpentine sites, providing that they are able to reach them via dispersal. A serpentine specialist lineage with a very high dispersal ability would therefore be likely to pre-empt (Silvertown *et al.*, 2005)) the colonization of empty serpentine sites by other lineages, thus preventing the adaptation of local non-serpentine lineages into serpentine specialist forms. Under this scenario we would expect to see a single serpentine specialist species with a very broad geographic distribution (i.e., distributed across all or most of the serpentine sites in a region). In contrast, in a very dispersal-limited taxon, we would perhaps expect to see multiple local origins of serpentine specialists, because pre-adapted lineages would be unable to reach empty serpentine formations before local non-specialist lineages had adapted into serpentine specialists themselves. Under this second scenario we might expect to see serpentine specialists endemic to individual serpentine formations, perhaps even drawn from a wide range of plant families.

Over evolutionary timescales, the nickel hyperaccumulator lineage in *Alyssum* is clearly able to disperse, since its overall distribution covers a wide area. However, many individual species have a relatively small range size. This apparent contradiction can perhaps be explained by a combination of intermediate dispersal and allopatric speciation, as will be discussed more fully under *A Geographic Species Radiation?* Below. Indeed, Wiens (2004) proposed that phylogenetic niche conservatism may promote speciation, because it may be associated with isolated populations restricted to suitable habitat, this isolation allowing them to undergo allopatric speciation. Kozak

and Wiens (2006) found some evidence for this process in eastern North America, in which allopatric speciation in cool-climate *Plethodon* and *Desmognathus* salamanders appears to have resulted from the contraction of a formerly-widespread cool climate zone to multiple isolated, somewhat island-like, montane areas, and the inability of these taxa to adapt to the new warmer zones between them.

The question of why nickel hyperaccumulation evolved in *Alyssum s.l.* and in *Bornmuellera s.l.* and not in other parts of Alyseae, or in more distantly-related species, is a difficult but a profound one. Explanations include the possibility of pre-adaptations in some lineages to particular evolutionary trajectories. For example, if the ancestors of *Alyssum s.l.* and *Bornmuellera s.l.* were already adapted to low-nutrient and free-draining soils, this might enable them to exploit serpentine habitats more readily than related species, and hence to evolve other adaptations to serpentine habitats such as nickel hyperaccumulation. Alternatively, the underlying genetic or physiological architecture in certain lineages may facilitate the evolution of certain traits. This explicitly genetic or physiological form of pre-adaptation is referred to as deep homology in evolutionary developmental biology, and various cryptic precursor traits that are essential prerequisites for the evolution and phenotypic expression of certain complex traits, have been documented in various taxa (see the review of Shubin *et al.*, 2009; Scotland, 2010; Marazzi *et al.* 2012). Hyperaccumulation is relatively rare within the angiosperms and its occurrence is highly homoplastic (Reeves and Baker, 2000; Krämer, 2010), but the presence of at least seven independent origins of nickel hyperaccumulation within the Brassicaceae, and at least three for zinc and/or cadmium hyperaccumulation (Krämer, 2010), i.e. exactly the sort of clustered homoplasy indicative of possible precursor traits (Marazzi *et al.*, 2012), may support the presence of some sort of pre-adaptation to metal hyperaccumulation in this family. A broad study of the origins of nickel hyperaccumulation and associated ecological adaptations across the plant kingdom and application of a cryptic precursor trait model (Marazzi *et al.*, 2012) could provide a powerful test of this idea of

some sort of genetic pre-disposition to evolve the ability to hyperaccumulate. A more detailed understanding of the genetic basis of nickel hyperaccumulation will be necessary to investigate the possibility of underlying genetic or physiological architecture in the Brassicaceae or other clades.

Nickel Hyperaccumulation: A Key Innovation in *Alyssum*?

Where increases (or decreases) in species diversification rate are discovered in a lineage, an explanation is demanded in terms of likely causes or ‘drivers’ of diversification, or more precisely causes of either the increased rate of speciation or decreased rates of extinction. Such causes may be extrinsic, i.e. environmental (and typically abiotic) factors such as changes in climate or geography (e.g., Linder & Hardy, 2004; Hughes & Eastwood, 2006; Valente *et al.*, 2010). Alternatively, rate shifts may be attributed to intrinsic evolutionary innovations which have allowed a lineage to take advantage of new or newly available niche space; such adaptations are sometimes referred to as ‘key evolutionary innovations’. Examples include floral nectar spurs in *Aquilegia* (Hodges, 1997a); fleshy fruits in tropical understory plants (Smith, 2001); bilateral floral symmetry in the angiosperms (Sargent, 2004); antifreeze glycoproteins in notothenioid fishes (Matschiner *et al.*, 2011); cushion-like growth form in *Androsace* (Boucher *et al.*, 2012); iteroparity in *Lupinus* (Drummond *et al.*, 2012); and defence mechanisms in *Coprinellus* mushrooms (Nagy *et al.*, 2012). In studies which encompass parallel or convergent origins of a trait, it is possible to statistically test the extent of association between possible traits driving diversification and shifts in diversification rate (e.g. Slowinski & Guyer, 1993; Paradis, 2005, 2012; Fitzjohn, 2009, 2010; Weber & Agrawal, 2012). However, most reports of accelerated diversification are singular observations, individual historical events in a single lineage (e.g., Hughes and Eastwood, 2006; Marazzi and Sanderson, 2010; Valente *et al.*, 2010).

Marazzi and Sanderson (2010) suggested four criteria for determining whether the evolution of a trait should be considered a key evolutionary innovation, and used these criteria to test extra-floral nectaries as a possible key innovation driving diversification in the species-rich genus *Senna* (Fabaceae): (1) the trait must be a synapomorphy for the clade, i.e., it should have evolved once, and must have persisted during the period of increased diversification; (2) it must confer an identifiable functional advantage; (3) it must be correlated with a change or expansion in adaptive zone; and (4) it must confer a higher diversification rate on lineages (or within clades) in which it is present. However, Marazzi & Sanderson (2010) also acknowledged that for single origins of a trait, it is difficult to prove the key innovation hypothesis. Meeting these four criteria would mean that a trait is a good candidate for a key innovation, but would not constitute absolute proof. For example, other important intrinsic or extrinsic factors may have been overlooked, or may have left little evidence of their impact.

The finding that a significant 2.5-fold increase in species diversification rate in *Alyssum s.l.* is located on the same branch on which nickel hyperaccumulation is estimated to have originated, suggests the possibility of a causal link between nickel hyperaccumulation and species diversification. The following paragraphs assess the significance of hyperaccumulation as a potential key evolutionary innovation using the four criteria of Marazzi and Sanderson (2010).

The most parsimonious hypothesis of a single origin of nickel hyperaccumulation on the branch subtending the A1a clade in *Alyssum s.l.* means that hyperaccumulation provides a synapomorphy for that clade even though it has subsequently been lost in some members of the clade. The lack of resolution in the A1a clade makes it impossible to determine the number of losses, nor whether non-accumulator lineages within clade A1a revert to lower rates of species diversification. However, given the relatively young age of the clade and the retention of nickel hyperaccumulation in the majority of its component species, it appears that nickel hyperaccumulation is closely

associated with the high diversification rate. The first and fourth criteria of Marazzi and Sanderson (2010) therefore appear to be met.

As discussed in *Chapter 1*, there is good reason to believe that nickel hyperaccumulation provides a fitness advantage, for example through elemental defence, and more generally, this trait can be considered an adaptation to serpentine soils. For example, there is a close association between nickel hyperaccumulation and serpentine soils (Brooks *et al.*, 1979; Boyd & Martens, 1998; Reeves and Baker, 2000). The second criterion of Marazzi and Sanderson (2010) therefore appears to be met. And although the expected competitive disadvantage of hyperaccumulators on non-metaliferous soils has not yet been proven (Meerts *et al.*, 2003) and has, in fact, been little-investigated, there is evidence for a competitive disadvantage of serpentine endemic species growing on non-serpentine soils (Gulmon, 1992; Hooper and Vitousek, 1997; Elmendorf and Moore, 2007; Anacker *et al.*, 2011).

The fact that nickel hyperaccumulators in *Alyssum* occupy many of the areas of serpentine soil that occur across the Mediterranean and south-west Asia, whereas non-accumulators are generally found on non-metaliferous soils (Brooks *et al.*, 1979; Reeves and Baker, 2000; Mengoni *et al.*, 2003; Cecchi *et al.*, 2010), means that hyperaccumulation appears to have conferred the ability to occupy a distinct adaptive zone, i.e., serpentine habitats. The third criterion for key innovation therefore appears to be met.

All this evidence taken together does indeed suggest that the evolution of nickel hyperaccumulation in *Alyssum s.l.* provided a key innovation prompting rapid species diversification in this clade and expansion of the adaptive zone occupied by the genus into the extreme environments posed by serpentine substrates. However, for studies of this kind it is difficult to rule out the presence of co-occurring traits. For example, it could be that serpentine

tolerance, which may involve tolerance to various environmental conditions associated within serpentine, including high levels of nickel, magnesium, cobalt and iron combined with low availability of calcium, drought, and low nitrogen availability (Brady *et al.*, 2005; Kazakou *et al.*, 2008), co-occur within nickel hyperaccumulation, and that it is serpentine tolerance rather than nickel hyperaccumulation which is the key innovation in *Alyssum s.l.* For example, several authors have suggested that drought tolerance may be an important pre-adaptation for serpentine tolerance (Hughes *et al.*, 2001; Pepper and Norwood, 2001; Nyberg Berglund *et al.*, 2004; Cecchi *et al.*, 2010). However, it is also apparent that many, and perhaps the majority of Mediterranean plant clades show considerable drought tolerance. Similarly, Cecchi *et al.* (2010) and Stefanovic *et al.* (2003) have suggested that adaptation to basic soils may be another adaptation facilitating serpentine occurrence, and many Alyseae species not found on serpentine are calcicoles, (e.g. Davis 1965–1985; Strid and Tan, 2002; Cecchi *et al.*, 2011). However, once again, there are many Mediterranean plant clades that span basic and calcareous substrates.

The origin of nickel hyperaccumulation in *Bornmuellera s.l.* appears to meet the first, second and third criteria of Marazzi and Sanderson (2010), but not the fourth, i.e., there was no associated increase in species diversification rate. The reasons why nickel hyperaccumulation may have been a key innovation in *Alyssum s.l.*, but not in *Bornmuellera s.l.* could be due to either intrinsic or extrinsic factors. For example, the nickel hyperaccumulation trait could involve significant differences between the two lineages in terms of its genetic or physiological basis and fitness benefits between the two lineages. As discussed above, a separate, as yet unidentified trait that is absent in *Bornmuellera* could co-occur with the diversification rate shift in *Alyssum s.l.* and be the true key innovation. Alternatively, and more subtly, the *Alyssum s.l.* lineage could have been pre-adapted to take advantage of nickel hyperaccumulation in ways that the *Bornmuellera s.l.* lineage was not. There is also the possibility that extrinsic factors, differences in the environmental context of these two origins of nickel hyperaccumulation, may have been important. Although the results

of this study indicate that the time and location of origin of nickel hyperaccumulation in *Alyssum s.l.* and in *Bornmuellera s.l.* were approximately similar, time differences of only a few tens of thousands of years, or geographic differences of only a few hundred kilometers, could have provided profoundly different ecological opportunities for diversification. Once again the synchrony of trait evolution and extrinsic opportunity in time and space may be what dictates the great among-lineage variation in species diversification rates. There is also the possibility that hyperaccumulation evolved as an adaptation to serpentine soils in *Alyssum s.l.* somewhat before it did in *Bornmuellera*, allowing the pre-emptive (Silvertown *et al.*, 2005) occupation of serpentine niches, and thus preventing a significant radiation or wide geographic spread of *Bornmuellera s.l.* or other nickel hyperaccumulator taxa, although serpentine vegetation is generally open and not apparently saturated. Finally, if *Alyssum s.l.* simply has a greater propensity for dispersal than *Bornmuellera s.l.*, this could explain the wider geographic spread of both hyperaccumulator and non-accumulator species in the former. This last hypothesis would be relatively simple to test experimentally.

The intriguing question of why nickel hyperaccumulation may have contributed to high diversification rates in *Alyssum s.l.* and yet had no such effect in *Bornmuellera s.l.* serves to caution against the idea that species radiations are predictable. Such differences may result from as yet unappreciated and perhaps subtle differences in intrinsic or extrinsic factors and/or synchrony or lack of it between the two such that ‘contingency’ played an important role in evolutionary history (Gould, 1989; Losos and Ricklefs, 2009)). In other words, ‘key innovations are likely to be context-specific’ (Hodges, 1997b), and radiations associated with key innovations dependent on availability of extrinsic opportunities (Wagner *et al.*, 2012). A much more detailed knowledge of the molecular basis of hyperaccumulation across angiosperms will be required before intrinsic factors can be ruled out, and high levels of uncertainty will likely always be associated with the interpretation of extrinsic factors. However, the homoplasious occurrence of nickel hyperaccumulation and

contrasting evolutionary trajectories of *Alyssum s.l.* and *Bornmuellera s.l.*, with their shared phylogenetic histories, life-history strategies, and likely times and locations of origin of nickel hyperaccumulation, provide a valuable study system for exploring and testing evolutionary hypotheses.

A Geographic Species Radiation?

Several studies have documented substantial species clades from the late Miocene or early Pliocene in the Mediterranean region, e.g. in *Campanula* (Roquet *et al.*, 2009) and *Centaurea* (Font *et al.*, 2009). Results for other plant taxa are varied. Several genera appear to have had fairly constant rates of species diversification over the late Miocene and Pliocene (e.g., *Anthemis* (Lo Presti and Oberprieler, 2009); *Cistus* (Guzman *et al.*, 2009); *Cyclamen* (Yesson *et al.*, 2009)), whilst others have diversified much more recently, in the Pleistocene (e.g., *Dianthus* (Valente *et al.*, 2010); *Nigella* (Bittkau and Comes, 2009)) *Ranunculus* (Paun *et al.*, 2005) and the white-flowered *Cistus* clade (Guzman *et al.*, 2009)). Some of the above authors have linked the reported diversifications to contemporaneous climatic or geographic conditions or changes (e.g. Bittkau and Combes, 2009; Roquet, 2009). Despite the variation between taxa, there does, in general, appear to be good evidence for extensive diversification in the late Miocene and Pliocene in the Mediterranean region, with some much more recent (i.e., Pleistocene) diversification (the latter with very high rates in some cases (Valente *et al.*, 2010)).

From the timing of the origins of nickel hyperaccumulation in *Alyssum s.l.* and *Bornmuellera*, and rapid species diversification of the A1a clade, it is possible that there might be some link with the late Miocene aridification or the early Pliocene flooding of the Mediterranean region. For example, the presence of land bridges across parts of the Mediterranean region during the Messinian Salinity Crisis may have facilitated dispersal, and the subsequent rise in sea levels may have led to the isolation of some populations and subsequent divergence of island endemics such as *Alyssum*

cypricum and *A. troodi* on Cyprus, *A. lesbiacum* on Lesbos, and *A. tenium* on Tinos (Strid and Tan, 2002). The climatic and geographic conditions of the late Miocene and early Pliocene could also have had other, indirect, effects on geotemporal trajectories of diversification in *Alyssum s.l.* and *Bornmuellera s.l.* The increasing extent and intensity of summer drought and the likely widespread presence of saline deposits (Clauzon *et al.*, 1996) over this period is likely to have favoured drought-adapted and basophilic species, and may help to explain how the A1a clade was able to rapidly expand and diversify. This scenario contrasts with the model of evolution of serpentine tolerance in California (Anacker and Harrison, 2012a), in which the evolution of serpentine endemism in 23 Californian plant taxa was correlated within benign periods of climate, *i.e.*, high rainfall and moderate temperature, which may have mitigated the drought-prone nature of serpentine soils. However, despite these broad scale opportunities for dispersal and diversification across the Mediterranean as a whole, many *Alyssum* species are endemic to small regions of serpentine in the mountains of Turkey, an area largely unaffected by the Messinian salinity crisis and its associated land bridges, or by the subsequent isolation by flooding. In this region it seems likely that the island-like distribution of serpentine soils is a more likely cause of geographic isolation and subsequent allopatric species divergence.

Cecchi (2011) suggested that serpentine habitats in southern Europe may have provided important thermal refugia during the Pleistocene, due to their free-draining, open rocky terrain which could have provided warmer microclimates than surrounding, more closed woodland or forest vegetation. Whilst this is a possibility, there appears to be little geographic structuring within the phylogeny of the Alyseae (Cecchi *et al.*, 2010) (although formal analyses are required to be confident of this). Other authors have suggested that serpentine habitats provided refuges from competition (Cecchi *et al.*, 2010) and from pathogens (Springer, 2009), but there has been little research on these questions so far in *Alyssum*.

The majority of species radiations have been considered to be adaptive radiations, i.e., the rapid diversification of a single lineage into multiple species, with clear diversification in life history, habitat preferences, or other traits, usually presumed to reflect diversification across different ecological niches, and thought to occur in response to ecological opportunity (Sanderson, 1998; Losos and Miles, 2002). Island adaptive radiations are classic examples, whereby the colonization of a newly formed island by a limited number of lineages, and the consequent plethora of ecological opportunity, in the form of empty niches and lack of competition, allows those lineages that do become successfully established to radiate into a wide diversity of niches. Examples of such adaptive island radiations include the Hawaiian silverswords (Baldwin and Sanderson, 1998), numerous plant genera in Macaronesia (Silvertown *et al.*, 2005), *Anolis* lizards in the Caribbean (Algar and Losos, 2011) and, perhaps the most famous of all, the cichlid fishes in African lakes (reviewed in Seehausen, 2006; Wagner *et al.*, 2012). Despite these numerous examples, (Yoder *et al.*, 2010) pointed out that there are few empirical examples that demonstrate how ecological opportunity and trait innovation promote diversification across adaptive zones, even though the way in which ecological opportunity relates to diversification is a central question in evolutionary biology.

The term radiation has also been used more generally to describe rapid episodes of in species diversification that show no obvious adaptive significance (e.g. Stanley 1979; Sanderson, 1998; Schulter 2000), although only a few authors have specifically addressed non-adaptive radiations (Gittenberger, 1991; Parent *et al.*, 2008; Bacon *et al.*, 2012). For example, Kozak *et al.* (2006) found that *Plethodon* salamanders had diversified into a large number of species through allopatric speciation across spatially and temporally fluctuating environments, at rates comparable to the fastest adaptive radiations. The radiation of *Nigella* species and subspecies on Aegean islands following Pleistocene sea level rises (Bittkau and Comes, 2009) may have resulted from similar purely allopatric speciation across identical habitats, as for the radiation of *Theba* land snails

(Greve, 2012) in Macaronesia, which the authors termed a 'cryptic' radiation due to the lack of obvious morphological or ecological diversification. Island archipelagos with appropriate levels of isolation between their component islands seem likely to promote such non-adaptive, allopatric (or geographic) radiations via simple geographic isolation.

Although spatially explicit island biogeographical models have been used to understand community assembly and dynamics since MacArthur & Wilson (1967), these approaches have only recently begun to incorporate phylogenetic factors and evolutionary processes (Chen & He, 2009; Kisel and Barraclough, 2010; Pigot *et al.*, 2010; Algar & Losos, 2011; Rosindell and Phillimore 2011). Dynamic geography – for example, the possibility of land bridges during periods of low global sea levels and isolation during periods of higher sea levels – may enhance possibilities for colonization followed by isolation. However, under appropriate combinations of geospatial scales and dispersal rates, rates of colonization and gene flow are likely to be high and low enough (respectively) to allow speciation through allopatric mechanisms in more static archipelagos (see the review of Givnish, 2010). For example, Desamore *et al.* (2012) showed that genetic diversity in *Erica scoparia* was higher in the Azores archipelago than in its wide continental range, suggesting that the opportunities for population isolation afforded by archipelagos can indeed promote genetic differentiation.

Geographic radiations of this sort can also occur across archipelagos of habitat patches as well as true islands in the strict geographic sense. Habitat islands are likely to be a useful concept where patches of suitable habitat are set within a matrix of habitats so unsuitable for the species in question that survival or reproduction in these intervening areas is very low. Lake and mountain top ('sky islands') systems provide the most obvious examples of island-like habitat formations. Plants that are extreme soil specialists, such as serpentine endemics, may fit this model, and several authors have considered the global or regional distribution of serpentine soils as

archipelagos of edaphic islands (Harrison and Inouye, 2002; Rajakaruna, 2004; Cecchi *et al.*, 2010; Jestrow *et al.*, 2012). In reviewing the role of edaphic factors in plant speciation, Rajakaruna (2004) suggested that extreme soil environments such as serpentine islands may play a central role in plant evolution and speciation through the opportunities that they offer for isolation and specialization.

As shown above, an increase in species diversification rate is strongly associated with the origin of nickel hyperaccumulation in *Alyssum s.l.*, and serpentine endemism and small geographic range sizes are also significantly correlated with species diversification rates in the Alyseae. One interpretation of these findings is that the adaptive advantage in serpentine habitats conferred by the ability to hyperaccumulate nickel, allowed nickel hyperaccumulators to colonize edaphic islands of serpentine soil across the Mediterranean and south-west Asian region. The small and isolated nature of these habitat islands would have provided a high degree of separation between populations, with the result that (possibly through genetic drift, or especially given the small size of some of these habitat islands, through founder effects (reviewed in Santos *et al.*, 2012)) the lineage underwent a rapid and region-wide process of allopatric speciation over a relatively short period of time. The large increase in extinction rate observed in the A1a clade appears consistent with the small range (and presumably population) sizes in that clade. Such a scenario would represent a non-adaptive radiation, where species diversification is accompanied by negligible adaptive disparity (Kozak *et al.*, 2006). Despite the large number of species, which do show various minor morphological differences, there appears to be a striking lack of major morphological or ecological disparity in species within the A1a clade (e.g., in life-form, pollination syndrome, dispersal mechanism, etc.); thus, within serpentine habitats, the various *Alyssum* species all appear to be occupying a very similar niches. However, there has been no quantitative comparative analysis of this aspect of *Alyssum*, and a detailed study, for example employing multivariate methods to assess such variation, would be valuable.

The intermediate dispersal model (Mayr, 1963; Price and Wagner, 2004) suggests that under a given spatial arrangement, high and low dispersal rates are likely to result in low species diversification rates, through low rates of colonization and low degrees of isolation, respectively. In contrast, an intermediate level of dispersal is likely to result in a high rate of diversification. Although numerous studies have investigated a related ecological intermediate dispersal hypothesis (where intermediate levels of dispersal maximize community level diversity; see the meta-analysis of Cadotte *et al.*, 2006), few studies have addressed or critically tested the evolutionary intermediate dispersal hypothesis (Phillimore *et al.*, 2006; Van Bocxlaer *et al.*, 2010, Claramunt *et al.*, 2012.). *Alyssum* may provide a useful model system with which to address this question. It may be that the combination of the particular spatial scale of the archipelago of serpentine soil islands and the dispersal rate of *Alyssum* species, in combination, has allowed extensive allopatric speciation to occur through the genetic isolation of populations on the many disjunct serpentine islands.

In this study, species range size and serpentine endemic status were the geographic traits chosen for ancestral trait reconstruction. However, measuring the extent of allopatry between species and analyzing its phylogenetic distribution would have provided a stronger test of the edaphic island model of geographic or allopatric radiation. Allopatry – the extent of non-overlap in species distributions – is quantifiable and can be determined (typically using GIS) for any particular pair of taxa (as: $1 - \text{the proportion of overlap in the area distributions of two species}$). Attempts have been made to carry out ancestral reconstruction of allopatry (Barracough and Vogler, 2000; Fitzpatrick and Turelli, 2006). Alternatively, the extent of isolation between species (e.g., as the minimum linear distances between their edges) could also be quantified for any two pairs of species, and might be expected to show interesting correlations with species diversification rates under ancestral reconstruction-based analyses. However, such approaches would require assembly and geo-referencing of as many herbarium specimen records as possible from herbaria to obtain

enough data with sufficient geographic resolution, something that was beyond the scope of the present study.

Conclusions

This study has generated the most comprehensive phylogeny of the Alyseae to date, which has revealed the existence of a number of well-supported clades within the tribe, corresponding to some extent with traditionally recognized genera and infrageneric taxa, as well as the non-monophyly of the genus *Alyssum*. Two independent origins of nickel hyperaccumulation were located, one in the *Alyssum s.l.* clade, and one in the A5 or *Bornmuellera s.l.* clade, both dating from the late Miocene or early Pliocene.

All nickel hyperaccumulators within *Alyssum s.l.* are placed in the poorly resolved A1a clade, which has an estimated crown age of only 3.27 Mya (95% CI: 2.06–4.72 Mya), yet its constituent species are geographically widespread across the Mediterranean and south-west Asia. Within the Alyseae, there is evidence of a significant 2.5-fold increase in species diversification rate on the branch leading to the A1a clade; there is a clear association between nickel hyperaccumulation and serpentine endemism; and nickel hyperaccumulators have smaller species range sizes than non-accumulators. There is also a clear negative relationship between species diversification rate and species range size.

The combined phylogenetic and other evidence assembled here suggests that the rapid and recent species diversification that has occurred in the A1a clade is an example of a non-adaptive, allopatric or geographic radiation, driven by the evolution of nickel hyperaccumulation, as an adaptation to serpentine habitats, which facilitated colonisation of an archipelago of serpentine habitat ‘islands’ distributed across the Mediterranean basin and south-west Asia. It further suggests that the combination of the spatial scale of this archipelago and the dispersal rate of *Alyssum s.l.* species

promoted extensive allopatric speciation through genetic isolation of populations on separate serpentine habitat islands. Under this scenario, nickel hyperaccumulation could be considered a key innovation in *Alyssum s.l.* The question of why an apparently contemporaneous, yet phylogenetically independent origin of nickel hyperaccumulation in the relatively species-poor *Bornmuellera s.l.* clade was clearly not a key innovation in quite the same way is an important one that cannot yet be answered.

The Alyseae has great potential for further research work. Translating the phylogenetic consensus for this tribe into a new and workable generic classification, based on detailed morphological analysis and nomenclatural work is clearly needed, given the substantial non-monophyly of the traditionally recognized genera discovered in this study. Genome-scale sequencing may be useful to shed light on species relationships within the A1a clade, which because of the recent ancestry of hyperaccumulator and non-accumulator species is likely to be of great use in comparative genetic and physiological studies into nickel hyperaccumulation. Furthermore, the genus *Alyssum* provides an excellent model system with which to investigate processes of allopatric speciation, and to explore evolutionary hypotheses for the divergent histories of nickel hyperaccumulators in *Alyssum* and *Bornmuellera*. The new phylogeny and associated hypotheses of the evolutionary dynamics of diversification and the associated extensive taxonomic and character data appendices provide a powerful comparative framework to address these exciting research questions.

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Appendix 1:

Taxonomy of tribe Alysseae (Brassicaceae)

Circumscription of Tribe Alysseae

A systematic review of the Brassicaceae by Al-Shehbaz *et al.* (2006) suggested that the tribe Alysseae includes 17 genera: *Alyssoides* Mill., *Alyssum* L., *Asperuginoides* Rauschert, *Aurinia* Desv., *Berteroa* DC., *Bornmuellera* Hausskn., *Clastopus* Bunge ex Boiss., *Clypeola* L., *Degenia* Hayek, *Didymophysa* Boiss., *Farsetia* Turra, *Fibigia* Medik., *Galitzkya* V.V.Botschantz., *Hormathophylla* Cullen & T.R.Dudley, *Lobularia* Desv., *Physoptychis* Boiss. and *Straussiella* Hausskn.

Molecular phylogenetic studies (Warwick *et al.*, 2008; Khosravi *et al.*, 2009; German *et al.*, 2009) have indicated that the tribe Alysseae (*sensu* Al-Shehbaz *et al.*, 2006) is polyphyletic, with four genera placed within other tribes of the Brassicaceae: *Farsetia* (Malcolmieae; Warwick *et al.*, 2008), *Lobularia* (Malcolmieae; Warwick *et al.*, 2008), *Asperuginoides* (Cochlearieae; Khosravi *et al.*, 2009) and *Didymophysa* (Thlaspidaceae; Khosravi *et al.*, 2009). Excluding these genera, there is strong support for a monophyletic Alysseae *s.str.* (*sensu* Warwick *et al.*, 2008) comprising the 12 genera *Alyssoides*, *Alyssum*, *Aurinia*, *Berteroa*, *Bornmuellera*, *Clastopus*, *Clypeola*, *Degenia*, *Fibigia*, *Galitzkya*, *Hormathophylla* and *Physoptychis*. The monospecific *Straussiella* was not sampled and its placement remains uncertain. More recently, studies suggest that the Alysseae *s.str.* clade also includes *Leptoplax* O.E.Schulz (Cecchi *et al.*, 2010a) and *Physocardamum* Hedge (Warwick *et al.*, 2010) (both monospecific). Taxonomic work by Cecchi *et al.* (2011) suggests that the name *Phyllolepidum* (Ten.) Trinajstić should be applied to some Alysseae species, as discussed below. Together, this gives a total of 16 genera in the tribe Alysseae.

Species Names in Tribe Alyseae

The *International Plant Names Index* (IPNI) database (www.ipni.org) contains 721 species names for the genus *Alyssum* alone (accessed 28 January 2012), and indicates a large amount of synonymy in this and in other genera of the Alyseae. A species checklist for the Brassicaceae was produced by Warwick *et al.* (2006). However, since 2006, numerous taxonomic changes have been suggested or made in various genera of the Alyseae, some of which are reflected in the accepted names in *The Plant List* database (www.theplantlist.org).

In order to guide taxon sampling, and to allow diversification analysis to be carried out, an up-to-date species checklist was compiled for Alyseae *s.str.* using the following procedure. A basic species list for Alyseae was produced by merging the Warwick *et al.* (2006) list with the accepted species names from *The Plant List* (as at 28 January 2012) for all 16 accepted genera of the Alyseae. Species names in the genera *Irania* Hadač & Chrtek. and *Pterygostemon* V.V.Botschantz. were also reviewed but, as discussed below, are here considered synonyms.

The taxonomy of the majority of *Alyssum* species in the Warwick *et al.* (2006) checklist were reviewed in Dudley's (1964a,b) taxonomic synopses of the genus, and are maintained as such in the checklist of Warwick *et al.* (2006). Dudley was unable to review material for 12 species (*A. antiatlanticum* Emb. & Maire, *A. brughieri* Colla, *A. djurdjurae* Chodat, *A. emarginatum* Zahl ex Vis., *A. embergeri* Quezél, *Alyssum filifolium* Wahlenb., *A. flahaultianum* Emb., *A. globosum* (Desv.) Kuntze, *A. hakaszii* Nyár, *A. heideri* Kroch., *A. odoratum* Voss, and *A. subbaicalicum* Nyár. (Dudley, 1964a)). However, Warwick *et al.* (2006) accepted many of these names.

The following paragraphs provide a summary of the agreement between Warwick *et al.* (2006) and *The Plant List* for Alyseae species names, together with a summary of any additional taxonomic

information that was available. The number of species names accepted here for each of the Alysseae genera is also indicated, and the full list is provided in Table A1-1.

Given the emphasis on the rank of species in this study, resulting from the incorporation of multiple analyses addressing species diversification rates, the following taxonomic review focuses on species concepts in Alysseae. Infra-specific taxa are not considered here in detail, although a number have been described from the tribe. A number of taxa described in *Alyssum* at the species level by some authors have also been considered to constitute lower ranking taxa (particularly subspecies and varieties) by other authors (specifically: *A. atlanticum* Desf., *A. clausonis* Pomel, *A. decoloratum* Pomel, *A. haradjianii* Rech.f., *A. numidicum* Pomel, *A. patulum* Pomel, *A. speciosum* Pomel, *A. caliacrae* Nyár., *A. fastigiatum* Heywood, *A. gynopodium* P.A.Smith, *A. trichostachyum* Rupr.). The approach taken here for such taxa is to follow the most recent review of each of these names, as detailed in the list on the following pages.

Alyssoides

There is agreement between Warwick *et al.* (2006) and *The Plant List* about both of the two species present in the genus *Alyssoides*. **Two *Alyssoides* species are therefore accepted here.**

Alyssum

Critical taxonomic treatments are available for the following *Alyssum* species:

- *Alyssum atlanticum* Desf. is treated as a sub-species of *A. montanum* L. by Bòlos & Vigo (1974) (as ssp. *atlanticum* (Desf.) O.Bolòs & Vigo), and is treated as *A. montanum* L. in this study.
- *Alyssum baldaccii* Vierh. ex Nyár., which was described from Crete is considered a synonym of *A. fallacinum* Hausskn. in *The Plant List* (with medium confidence) and by Warwick *et al.* (2006). However, a plant with some similarities to *A. fallacinum* Hausskn. from mainland

Greece is considered a hybrid by Strid & Tan (2002) (see *A. fallacinum* Hausskn., below), and distinct from *A. baldaccii* present in Crete. Following Strid & Tan (2002) therefore, *A. baldaccii* is considered here to be a distinct species, endemic to Crete.

- *Alyssum borzaeanum* Nyár. is considered a synonym of *A. obtusifolium* Steven ex DC. by *The Plant List*, and is not accepted as a separate species here.
- *Alyssum canescens* DC. is accepted by Warwick *et al.* (2006), but is considered a synonym of *Ptilotrichum canescens* (DC.) C.A.Mey (with medium confidence) in *The Plant List*. Here, this name is considered a synonym of *Stevenia canescens* (DC.) D.A.German (see discussion under *Non-Alysseae species* below).
- *Alyssum clausonis* Pomel is treated as a variety of *A. montanum* L. by Debeaux (1894) (as var. *clausonis* (Pomel) Debeaux) and is treated as *A. montanum* L. in this study.
- *Alyssum costei* Sennen & Pau is considered an unresolved name in *The Plant List*, and is accepted by Warwick *et al.* (2006). However, it is considered a synonym of *Hormathophylla lapeyrouseana* (Jord.) P.Küpfer in the *Flora Iberica* (Küpfer, 1993), and is regarded as such here.
- *Alyssum dagestanicum* Rupr. is considered a synonym of *A. armenum* Boiss. By Davis *et al.* (1965), and is considered as such here.
- *Alyssum decoloratum* Pomel is treated as a variety of *A. montanum* L. by Debeaux (1894) (as var. *decoloratum* (Pomel)) Debeaux) and is treated as *A. montanum* L. in this study.
- *Alyssum diffusum* Ten. is accepted as a distinct species by Warwick *et al.* (2006) and in *The Plant List*. An AFLP-based study by Španiel *et al.* (2011a) found strong evidence that this species is distinct from *A. montanum*, and *A. diffusum* is therefore recognised as a distinct species here.
- *Alyssum emarginatum* Zahl ex Vis. is accepted by Warwick *et al.* (2006); it is considered a synonym of *Peltaria emarginata* (Boiss.) Hausskn. in *The Plant List* (with medium

confidence). Here, this name is considered a synonym of *Leptoplax emarginata* (see discussion under *Leptoplax* below).

- *Alyssum fallacinum* Hausskn. is accepted by *The Plant List* (with medium confidence) and by Warwick *et al.* (2006); however, it is considered to be a hybrid between *A. chlorocarpum* Hausskn. and *A. heldreichii* Hausskn. (Strid & Tan, 2002), and is therefore rejected here.
- *Alyssum haradjianii* Rech.f. is treated as a variety of *Alyssum murale* Waldst. & Kit. by Dudley (1964a), (as var. *haradjianii* (Rech.) Dudley), and is treated as *A. murale* Waldst. & Kit. in this study.
- *Alyssum kaynakiae* Yilmaz was described recently (Yilmaz, 2012) and therefore post-dates the Warwick *et al.* (2006) checklist; it is not yet included in *The Plant List*. This species is accepted here.
- *Alyssum klimesii* Al-Shehbaz is accepted by *The Plant List* (with medium confidence), and by Warwick *et al.* (2006). Here, this name is considered a synonym of *Ladakiella klimesii* (Al-Shehbaz) D. A. German & Al-Shehbaz (see discussion under *Non-Alysseae species* below).
- *Alyssum malacitanum* (Rivas Goday) T.R.Dudley is considered a synonym of *A. serpyllifolium* Desf. by Warwick *et al.* (2006) and in *The Plant List*. Here, however, both *A. malacitanum* and *A. pintodasilvae* (see below) are treated as distinct species. This is based on field observations, which confirm that the two serpentine endemic species (*A. pintodasilvae* in N.W. Iberia, and *A. malacitanum* close to Malaga in S.E. Spain) show consistent morphological differences from the more widespread non-serpentine Iberian plant, as described by Dudley (1966, 1986a, 1986b), and as recognised by Mengoni *et al.* (2003).
- *Alyssum markgrafii* O.E.Schulz was considered a synonym of *A. murale* Waldst. & Kit. by Cecchi *et al.* (2010), and is not recognised as a separate species here.
- *Alyssum montanum* L. Morphological and AFLP data suggest that plants usually referred to as *A. montanum* include a widespread group and a distinct and much more restricted

group of plants found in Switzerland and southwest Germany (close to the type locality for *A. montanum*) (Španiel *et al.*, 2011a). Španiel *et al.* (2012) recognised these groups at the sub-specific level, using the names *A. montanum* ssp. *montanum* for the more restricted group (which includes the type specimen of the species) and *A. montanum* ssp. *gmelinii* (Jord. & Fourr.) Em.Schmid for the more widespread group. All members of the complex are, in addition to various subspecies that have been described from other regions of Europe and North Africa (see *A. atlanticum*, *A. clausonis*, *A. decoloratum*, *A. numidicum*, *A. patulum* and *A. speciosum*) treated under the name *A. montanum* in the present study.

- *Alyssum misirdalianum* Orcan & Binzet was described in 2009 (Orcan & Binzet, 2009) and therefore post-dates the Warwick *et al.* (2006) checklist; it is described as an unresolved name in *The Plant List*. This species name is accepted here.
- *Alyssum mughlaei* Orcan was described recently (Orcan, 2005); it is accepted in *The Plant List* with medium confidence. This species name is accepted here.
- *Alyssum numidicum* Pomel was treated as a variety of *A. montanum* Waldst. & Kit. by Maire & Weiller (1967) (as *montanum* var. *numidicum* (Pomel)), and is treated as *A. montanum* Waldst. & Kit. in this study.
- *Alyssum orophilum* Jord. & Fourr. is considered a synonym of *A. montanum* in *The Plant List*, but was not discussed by Warwick *et al.* (2006). Španiel *et al.* (2011a) found some evidence that a population of plants from the southwestern French Alps (previously generally referred to *A. montanum*) should be recognised as a separate species, for which they suggested resurrecting the name *A. orophilum*. However, these authors caution that further sampling is necessary before the taxonomic change is made formally, and therefore this name is not accepted here.
- *Alyssum parviflorum* Fisch. ex M. Bieb. is considered a synonym of *A. simplex* Rudolph by *The Plant List* (with medium confidence) and by Warwick *et al.* (2006). It is therefore rejected here.

- *Alyssum patulum* Pomel is treated as a variety of *Alyssum montanum* L. by Maire & Weiller (1967) (as var. *patulum* (Pomel) Maire & Weiller), and is treated as *A. montanum* L. in this study.
- *Alyssum pintodasilvae* T.R.Dudley is considered a synonym of *A. serpyllifolium* by Warwick *et al.* (2006) and is considered an unresolved name in *The Plant List*. Here however, *A. pintodasilvae* is treated as a distinct species, as in Dudley (1966) (as discussed under *Alyssum malacitanum* above). In addition to recognizing *A. pintodasilvae* as a distinct species, Mengoni *et al.* (2003) used the name *Alyssum gutianii* [sic] for *Alyssum* tissue collected from serpentine habitats near Melide, Galicia in N.W. Spain. *Alyssum gutianae* is the name used by Rodríguez-Oubiña & Ortiz (1991) for the form of *Alyssum* present in this area, but this name has not been validly published. After fieldwork in both this area and in N.W. Portugal, and after discussions with local botanists in both areas, the author is of the opinion that the *Alyssum* of the serpentine of Melide should be regarded as *A. pintodasilvae*, from which these plants are not morphologically distinct.
- *Alyssum pirinicum* (Stoj. & Acht.) Ančev was described in 2006 (Ančev and Goranova, 2006) and therefore post-dates the Warwick *et al.* (2006) checklist; it is accepted with medium confidence in *The Plant List*. This name is accepted here.
- *Alyssum repens* Baumg. has been considered to form a species complex with *A. montanum* by some taxonomists (Marhold, 2011). However, the morphological and AFLP data of Španiel *et al.* (2011a) suggest that the two species are distinct, and they are therefore treated as distinct species here.
- *Alyssum speciosum* Pomel was treated as a sub-species of *Alyssum montanum* L. by O.Bolòs & Vigo (1974) (as subsp. *atlanticum* (Desf.) O.Bolòs & Vigo) and is treated as *A. montanum* L. in this study.
- *Alyssum tavolarae* Briq. is considered a synonym of *Alyssum robertianum* Bernard ex Gren. & Godr. by Cecchi *et al.* (2010), and is therefore rejected as a separate species here.

- *Alyssum tenuifolium* Stephan ex Willd. is accepted by *The Plant List* (with medium confidence), and by Warwick *et al.* (2006). Here, this name is considered a synonym of *Stevenia tenuifolia* (Stephan ex Willd.) D.A.German (see discussion under *Non-Alysseae species* below).

For the remaining *Alyssum* species names, where Warwick *et al.* (2006) and *The Plant List* both accept a name, it has been accepted here. This was the case for 164 species names. Cases where these two sources differed in the acceptance of names are discussed in the following paragraphs.

The following eight species names were accepted by Warwick *et al.* (2006) but are unresolved names in *The Plant List*. These names are accepted here.

- *Alyssum austrodalmaticum* Trinajstić
- *Alyssum bargalense* Micevski
- *Alyssum brughieri* Brein ex Colla
- *Alyssum heideri* Krock.
- *Alyssum magicum* C.H.An
- *Alyssum microphylliforme* Nyár.
- *Alyssum microphyllum* (C.A.Mey.) Steud.
- *Alyssum subbaicalicum* Nyár.

The following 10 *Alyssum* species names are accepted in *The Plant List* with medium confidence, they were not discussed by Warwick *et al.* (2006); these names are tentatively accepted here:

- *Alyssum arabicum* (Boiss.) T.Durand & Schinz
- *Alyssum debareense* Micevski

- *Alyssum gevgelicense* Micevski
- *Alyssum iljinskae* V.I.Dorof.
- *Alyssum kavadarcense* Micevski
- *Alyssum musilii* Velen.
- *Alyssum serpentinum* Micevski
- *Alyssum simulans* Runemark ex Hartvig
- *Alyssum skopjense* Micevski

The following 14 *Alyssum* species names are accepted by *The Plant List* with medium or low confidence, but were considered synonyms by Warwick *et al.* (2006). Given that names are more likely to be falsely accepted (typically because synonymy information is not located or is unavailable) than falsely rejected, these names are tentatively rejected here.

- *Alyssum algeriense* Pomel is considered a synonym of *A. granatense* Boiss. & Reut.
- *Alyssum anamense* Velen. is considered a synonym of *A. singarense* Boiss. & Hausskn.
- *Alyssum caliacrae* Nyár. is treated as a subspecies of *A. tortuosum* Waldst. & Kit ex Willd. (as subsp. *caliacrae* Stojan. & Stef.), and is treated as *A. tortuosum* Waldst. & Kit ex Willd. in this study.
- *Alyssum cochleatum* Coss. & Durieu is considered a synonym of *Hormathophylla baetica* P.Küpf.
- *Alyssum curetum* Gand. is considered a synonym of *A. idaeum* Boiss. & Heldr.
- *Alyssum dimorphosepalum* Eig is considered a synonym of *A. iranicum* Hausskn. ex Baumg.
- *Alyssum fastigiatum* Heywood is treated as a subspecies of *A. montanum* L. (as subsp. *montanum* L.), and is treated as *A. montanum* L. in this study.
- *Alyssum gymnopodum* P.A.Smirn. is treated as a synonym of *A. tortuosum* subsp. *cretaceum* L., and is treated as *A. tortuosum* Waldst. & Kit ex Willd. in this study.

- *Alyssum leiocarpum* Pomel is considered a synonym of *A. minutum* Schldl. ex DC.
- *Alyssum longistylum* Grossh. is considered a synonym of *A. tortuosum* subsp. *tortuosum* Waldst. & Kit. ex Willd, and is treated as *A. tortuosum* Waldst. & Kit ex Willd. in this study.
- *Alyssum nanum* Pomel is considered a synonym of *A. simplex* Rudolph.
- *Alyssum orbiculare* Nyár. is considered a synonym of *A. simplex* Rudolph.
- *Alyssum sergievskajae* Krasnob. is considered a synonym of *Stevenia sergievskajae* (Krasnob.) Kamelin & Gubanov.
- *Alyssum trichostachyum* Rupr. is considered a subspecies of *A. repens* Baumg. (as subsp. *trichostachyum* (Rupr.) Hayek.) and is treated as *A. repens* Baumg. in this study).

The following seven species names were accepted by Warwick *et al.* (2006) but are considered synonyms in *The Plant List* with medium confidence. Given that names are more likely to be falsely accepted (typically because synonymy information is not located or is unavailable) than falsely rejected, these names are tentatively rejected here.

- *Alyssum baicalicum* Nyár. is considered a synonym of *A. obovatum* (C.A.Mey.) Turcz.
- *Alyssum dahuricum* (Peschkova) Al-Shehbaz is considered a synonym of *Ptilotrichum dahuricum* Peschkova.
- *Alyssum filifolium* Wahlenb. is considered a synonym of *A. menioides* Boiss.
- *Alyssum hakaszii* Nyár. is considered a synonym of *A. microphyllum* (C.A.Mey.) Steud.
- *Alyssum inflatum* Nyár. is considered a synonym of *A. tortuosum* Willd.
- *Alyssum nevadense* Wilmott ex P.W.Ball & T.R.Dudley is considered a synonym of *A. cuneifolium* Ten. subsp. *corymbosum* (Pau) Rivas Mart., Molero Mesa & Pérez Raya, and is treated as *A. cuneifolium* Ten. Here.
- *Alyssum odoratum* Voss is considered a synonym of *Lobularia maritima* (L.) Desv.

In total, **189 *Alyssum* species** are accepted here.

Infra-generic Sections in *Alyssum*

In the most recent infra-generic classification of *Alyssum*, Dudley (1964a) recognises the following six sections: *Alyssum* (Griseb) T.R.Dudley, *Gamosepalum* (Hauskn.) T.R.Dudley, *Meniocus* (Desv.) Hook., *Odontarrhena* (Meyer) Koch, *Psilonema* (Meyer) Hook. and *Tetradenia* (Spach) T.R.Dudley. The last of these contained only species which were later referred to the genus *Hormathophylla* Cullen & T.R.Dudley, and is therefore not recognised here. A further section, *Magicum* An, was first described at the same time as its single member *A. magicum* C.H.An (An, 1981). Where possible, sectional membership was obtained from the literature (i.e., from protologues and subsequent taxonomic treatments) for the *Alyssum* species accepted here, and is indicated in Table A1-1.

For the following seven species, protologues were consulted but contained no statement of sectional membership, and no other literature stating section membership was found:

- *Alyssum arabicum* (Boiss.) T.Durand & Schinz
- **Alyssum brughieri* Brein ex Colla
- **Alyssum embergeri* Quézel
- *Alyssum flahaultianum* Emb. ex Greuter
- **Alyssum gadoreense* P.Küpfer
- *Alyssum globosum* (Desv.) Kuntze
- **Alyssum pirinicum* (Stoj. & Acht.) Ančev

For the following 14 species, the protologues were not obtained:

- *Alyssum austrodalmaticum* Trinajstić
- *Alyssum bargalense* Micevski
- *Alyssum debareense* Micevski
- *Alyssum gevgelicense* Micevski

- *Alyssum hajastanum* Avet.
- *Alyssum heideri* Krock.
- *Alyssum iljinskae* V.I.Dorof.
- *Alyssum kavadarcense* Micevski
- *Alyssum kurdicum* (Boiss.) Nyár.
- *Alyssum microphylliforme* Nyár.
- *Alyssum musilii* Velen.
- *Alyssum serpentinum* Micevski
- *Alyssum skopjense* Micevski
- *Alyssum subbaicalicum* Nyár.

However, from morphological information or stated affinities detailed in the literature, it has been possible to tentatively assign four of the above 21 species to infrageneric sections. These species are asterisked in the list above and full details of the sectional allocation are included in Appendix 4.

Aurinia

There is agreement between Warwick *et al.* (2006) and *The Plant List* about nine species in the genus *Aurinia*. There is disagreement about *Aurinia cyclocarpa* (Boiss.) Czerep., which is accepted by *The Plant List* (with medium confidence), but is considered a synonym of *A. rupestris* ssp. *cyclocarpa* (Boiss.) Cullen & T.R.Dudley by Warwick *et al.* (2006) (following the suggestion of Dudley, 1965). The studies of Hartvig (2002) and Cecchi *et al.* (2011a) suggest that this taxon should be regarded as a distinct species. Following the taxonomic changes suggested by Cecchi *et al.* (2011a), *Aurinia cyclocarpa* is here considered a synonym of *Phyllolepidum cyclocarpum* (Boiss.) Cecchi, and *Aurinia rupestris* is here considered a synonym of *P. rupestris* (Sweet) Cullen & T.R.Dudley (see *Phyllolepidum* below). Because of the distinctive morphology of *A. uechtritziana*

(Bornm.) Cullen & T.R.Dudley, Cecchi *et al.* (2011a) have suggested resurrecting the genus *Lepidotrichum* Velen & Bornm. for this taxon. However, this latter nomenclatural change has not formally been made, and is not accepted here. **Eight species of *Aurinia* are accepted here.**

Berteroa

There is agreement between Warwick *et al.* (2006) and *The Plant List* about the five species present in the genus *Berteroa* DC. **Five *Berteroa* species are accepted here.**

Bornmuellera

There is agreement between Warwick *et al.* (2006) and *The Plant List* about the seven species present in the genus *Bornmuellera* Hausskn. The hybrid *B. × petri* Greuter., Charpin & Dittrich (a hybrid of *B. baldaccii* ssp. *rechingeri* Greuter and *B. tymphaea* (Hausskn.) Hausskn.; Greuter, 1975), is not recognised as a distinct species here. **Seven *Bornmuellera* species are accepted here.**

Clastopus

There is agreement between Warwick *et al.* (2006) and *The Plant List* about the two species present in the genus *Clastopus*. **Two *Clastopus* species are accepted here.**

Clypeola

There is agreement between Warwick *et al.* (2006) and *The Plant List* about the nine species present in the genus *Clypeola*. **Nine *Clypeola* species are accepted here.**

Degenia

There is agreement between Warwick *et al.* (2006) and *The Plant List* about the single species present in the genus *Degenia* Hayek. **One *Degenia* species is accepted here.**

Fibigia

There is agreement between Warwick *et al.* (2006) and *The Plant List* about 12 species in the genus *Fibigia*. There is disagreement about *Fibigia eriocarpa* (DC.) Boiss., which is accepted by *The Plant List* (with medium confidence), but is considered a synonym of *Fibigia clypeata* (DC.) Boiss. by Warwick *et al.* (2006). This name is rejected here. Also, *Fibigia spathulata* (Kar. & Kir.) B.Fedtsch. is accepted by Warwick *et al.* (2006), but is an unresolved name in *The Plant List*; this name is accepted here. **13 *Fibigia* species are accepted here.**

Galitzkya

There is agreement between Warwick *et al.* (2006) and *The Plant List* about three species present in the genus *Galitzkya*. **Three *Galitzkya* species are accepted here.**

Hormathophylla

There is agreement between Warwick *et al.* (2006) and *The Plant List* about three species present in the genus *Hormathophylla*. Seven further species are accepted by Warwick *et al.* (2006) but are considered synonyms by *The Plant List* (all with medium confidence): *H. baetica* P.Küpfer is considered a synonym of *Ptilotrichum baeticum* (P.Küpfer) ined.; *H. cadevalliana* (Pau) T.R.Dudley is considered a synonym of *P. cadevallianum* (Pau) Heywood; *H. longicaulis* (Boiss.) Cullen & T.R.Dudley is considered a synonym of *P. longicaule* (Boiss.) Boiss.; *H. macrocarpa* (DC.) P.Küpfer is considered a synonym of *P. macrocarpum* (DC.) Boiss.; *Hormathophylla purpurea* (Lag. & Rodr.) P.Küpfer is considered a synonym of *P. purpureum* (Lag. & Rodr.) Boiss.; *H. pyrenaica* (Lapeyr.) Cullen & T.R.Dudley is considered a synonym of *P. pyrenaicum* (Lapeyr.) Boiss., and *H. reverchonii* (Degen & Hervier) Cullen & T.R.Dudley is considered a synonym of *Ptilotrichum reverchonii* Degen & Hervier.

Of the two of these seven species that were included in the molecular phylogenetic study of Warwick *et al.* (2008), both formed a monophyletic group with two other *Hormathophylla* species. Of the three of these seven species that were included in the molecular phylogenetic study of Cecchi *et al.* (2011a), all formed a monophyletic group with two other *Hormathophylla* species. Following Warwick *et al.* (2006), all of the above *Hormathophylla* species are accepted in this study.

Rivas-Martínez *et al.* (2002) have suggested that *H. purpurea* should be segregated into a new monotypic genus as *Nevadensia purpurea* (Lag. & Rodr.) Rivas Mart. In the phylogeny of Cecchi *et al.* (2010) this species was sister to a clade containing all of the four other *Hormathophylla* species sampled. However, the bootstrap support for the latter was only moderate (76 %), and no phylogeny has yet included all of the above 10 species. In the phylogeny obtained here, this species is nested within a clade containing five other *Hormathophylla* species, with strong support (0.91 posterior probability). For these reasons, the use of the name *Nevadensia purpurea* is considered premature, and this species is called *Hormathophylla purpurea* here. **Ten *Hormathophylla* species are accepted here.**

Irania

Neither Warwick *et al.* (2006) nor *The Plant List* accept any species of the genus *Irania*. All of the five species names for this genus are considered synonyms of *Fibigia* species in these two sources (all with medium confidence in *The Plant List*). Results of their phylogenetic study led Khosravi *et al.* (2009) to claim that this genus should be recognised, but this study included only two of the five species and only two other species of *Fibigia*. Here, the following *Irania* species names are therefore considered synonyms: *Irania compacta* (Rech.f.) Hadač & Chrtek (a synonym of *Fibigia compacta* Rech.f.), *Irania membranacea* (Rech.f.) Hadač & Chrtek (a synonym of *Fibigia membranacea* Rech.f.), *Irania multicaulis* (Boiss. & Hohen.) Hadač & Chrtek (a synonym of *Fibigia multicaulis* (Boiss. & Hohen.) Boiss.), *Irania pendula* (Boiss.) Hadač & Chrtek (a synonym of *Fibigia*

pendula (Boiss.) Boiss.), and *Irania umbellata* (Boiss.) Hadač & Chrtek (a synonym of *Fibigia umbellata* (Boiss.) Boiss.). **No *Irania* species are accepted here.**

Leptoplax

Both Warwick *et al.* (2006) and *The Plant List* accept the species *Peltaria emarginata* (Boiss.) Hausskn. However, the molecular phylogeny of Cecchi *et al.* (2010) suggests that this species has no affinity with other species of *Peltaria* (which are in tribe Thlaspideae), and is sister to the two *Bornmuellera* species that were included in that study. These authors therefore accept that this species should be segregated into its former monotypic genus *Leptoplax* O.E.Schulz., as *Leptoplax emarginata* (Boiss.) O.E.Schulz and this name is accepted here. **One *Leptoplax* species is accepted here.**

Phyllolepidum

Following the recommendation of Cecchi *et al.* (2011b), the genus *Phyllolepidum* is recognized here, and two species are accepted: *P. cyclocarpum* (Boiss.) Cecchi (of which *Aurinia cyclocarpa* (Boiss.) Czerep., *Aurinia rupestris* ssp. *cyclocarpa* (Boiss.) Cullen & T.R.Dudley, and *Ptilotrichum cyclocarpum* Boiss. are considered synonyms), and *P. rupestre* (Ten.) Trinajstić (of which *Ptilotrichum rupestre* (Sweet) Boiss. and *Aurinia rupestris* (Sweet) Cullen & T.R.Dudley are considered synonyms). **Two *Phyllolepidum* species are accepted here.**

Physocardamum

The single species of the genus *Physocardamum* Hedge, *P. davisii* Hedge, is accepted by Warwick *et al.* (2006), and is an unresolved name in *The Plant List*. This species name is accepted here. **One *Physocardamum* species is accepted here.**

Physoptychis

There is agreement between Warwick *et al.* (2006) and *The Plant List* about the two species present in the genus *Physoptychis*. **Two *Physoptychis* species are accepted here.**

Pterygostemon

Botschantzeva (1977) transferred *Fibigia spathulata* (Kar. & Kir.) B.Fedtsch. to a new monotypic genus *Pterygostemon* as *Pterygostemon spathulatus* V.V.Botschantz. Warwick *et al.* (2006) do not accept this name, but these authors do accept *F. spathulata* (Kar. & Kir.) B.Fedtsch. (see *Fibigia* above). The name is listed as an unresolved name in *The Plant List*. **No *Pterygostemon* species are accepted here.**

Straussiella

Both Warwick *et al.* (2006) and *The Plant List* accept one species in the genus *Straussiella* and this species is accepted here. **One *Straussiella* species is accepted here.**

The list of 255 Alysseae species names accepted here is considered to represent an up-to-date species checklist for the tribe Alysseae, and is presented in full in Table A1-1. It should be stressed this list is based on published taxonomic literature, and not upon new herbarium work. Authorities follow *The Plant List* (where available, *IPNI* where not); abbreviation styles have been standardised here for consistency. The infrageneric section to which each *Alyssum* species has been allocated in the literature is also indicated (where available) in Table A1-1, as is the extent to which each species was successfully sequenced (for at least one region of DNA sequence) in this study. Sectional allocations are based on the publications listed at the foot of the table. A summary of the list of accepted Alysseae species names (by genus) and of the extent of sampling in this study are provided in Table A1-2.

Non-Alysseae Species

A total of 19 species outside the tribe Alysseae were sampled in this study (or sequence data were obtained from GenBank). These species are listed in Table A1-3. These include 11 Brassicaceae species, six species from the Brassicales, and two species outside the Brassicales. Nomenclature and authorities for these species follow *The Plant List*, except as discussed in the following paragraphs. The choice of non-Alysseae species sampled in this study is fully discussed in the main text of the thesis (see Chapter 2 Materials and Methods).

***Ladakiella klimesii* (Al-Shehbaz) D.A.German & Al-Shehbaz**

The name *Alyssum klimesii* Al-Shehbaz is accepted by both Warwick *et al.* (2006) and *The Plant List* (with medium confidence). However, phylogenetic work by Warwick *et al.* (2008) and German *et al.* (2009) both place this species outside the Alysseae. German *et al.* (2010) have transferred this taxon to a new monotypic genus *Ladakiella* D.A.German & Al-Shehbaz, as *Ladakiella klimesii* (Al-Shehbaz) D.A.German & Al-Shehbaz. This latter name is accepted here.

***Stevenia canescens* (DC.) D.A.German**

The name *Alyssum canescens* DC. is accepted by Warwick *et al.* (2006), but is considered a synonym of *Ptilotrichum canescens* (DC.) C.A.Mey. (with medium confidence) in *The Plant List*. The phylogenetic work of Beilstein *et al.* (2008, 2010) and Warwick *et al.* (2008) place this species outside the Alysseae in the tribe Arabideae. Krasnoborov and German (2007), German (2008) and German & Al-Shehbaz (2010) have transferred all of the Asian species of *Ptilotrichum* C.A.Mey. to *Stevenia* Fisch., including *P. canescens* (Krasnoborov and German, 2007), and therefore the name *S. canescens* is accepted here.

***Stevenia tenuifolia* (Stephan ex Willd.) D.A.German**

The name *Alyssum tenuifolium* Stephan ex Willd. is accepted by *The Plant List* (with medium confidence), and by Warwick *et al.* (2006). However, this species was transferred to the genus *Stevenia* Adams & Fisch. by German (2008) as *Stevenia tenuifolia* (Stephan ex Willd.) D.German. This latter name is accepted here.

Table A1-1. Checklist of Alyseae species showing names used and species sampled in this study.

Species name & authority	<i>Alyssum</i> section	Sequenced here
<i>Alyssoides cretica</i> (L.) Medik.	N/A	Y
<i>Alyssoides utriculata</i> (L.) Medik.	N/A	Y
<i>Alyssum aizoides</i> Boiss.	<i>Alyssum</i> ¹	Y
<i>Alyssum akamasicum</i> Burt	<i>Odontarrhena</i> ¹	Y
<i>Alyssum alpestre</i> L.	<i>Odontarrhena</i> ¹	Y
<i>Alyssum alyssoides</i> (L.) L.	<i>Psilonema</i> ¹	Y
<i>Alyssum anatolicum</i> Hausskn. ex Nyár.	<i>Odontarrhena</i> ¹	Y
<i>Alyssum andinum</i> Rupr.	<i>Alyssum</i> ¹¹	N
<i>Alyssum antiatlanticum</i> Emb. & Maire	<i>Psilonema</i> ¹	N
<i>Alyssum arabicum</i> (Boiss.) T.Durand & Schinz	Unknown	N
<i>Alyssum argenteum</i> All.	<i>Odontarrhena</i> ¹	Y
<i>Alyssum argyrophyllum</i> Schott & Kotschy	<i>Alyssum</i> ¹	Y
<i>Alyssum armenum</i> Boiss.	<i>Alyssum</i> ¹	Y
<i>Alyssum artvinense</i> Busch	<i>Alyssum</i> ¹	N
<i>Alyssum aurantiacum</i> Boiss.	<i>Alyssum</i> ¹	Y
<i>Alyssum aureum</i> (Fenzl) Boiss.	<i>Meniocus</i> ¹	Y
<i>Alyssum austrodalmaticum</i> Trinajstić	Unknown	N
<i>Alyssum baldaccii</i> Vierh. ex Nyár.	<i>Odontarrhena</i> ¹	Y
<i>Alyssum bargalense</i> Micevski	Unknown	N
<i>Alyssum baumgatnerianum</i> Bornm. ex Baumg.	<i>Gamosepalum</i> ¹	Y
<i>Alyssum bertolonii</i> Desv.	<i>Odontarrhena</i> ¹	Y
<i>Alyssum blanchianum</i> Gomb.	<i>Odontarrhena</i> ²	N
<i>Alyssum blepharocarpum</i> T.R.Dudley & Hub.-Mor.	<i>Meniocus</i> ¹	Y
<i>Alyssum bornmuelleri</i> Hausskn. & Degen	<i>Alyssum</i> ¹	Y
<i>Alyssum bracteatum</i> Boiss. & Bushe	<i>Odontarrhena</i> ¹	Y
<i>Alyssum brughieri</i> Brein ex Colla	Unknown*	N
<i>Alyssum bulbotrichum</i> Hausskn. & Bornm.	<i>Alyssum</i> ¹	N
<i>Alyssum caespitosum</i> Baumg.	<i>Alyssum</i> ¹	Y
<i>Alyssum callichroum</i> Boiss.	<i>Odontarrhena</i> ¹	Y
<i>Alyssum calycocarpum</i> Rupr.	<i>Alyssum</i> ¹	Y
<i>Alyssum caricum</i> T.R.Dudley & Hub.-Mor.	<i>Odontarrhena</i> ¹	Y
<i>Alyssum cassium</i> Boiss. & Heldr.	<i>Odontarrhena</i> ¹	Y
<i>Alyssum cephalotes</i> Boiss.	<i>Alyssum</i> ¹	N
<i>Alyssum chondrogynum</i> B.L.Burt	<i>Odontarrhena</i> ¹	Y
<i>Alyssum cilicicum</i> Boiss. & Balansa	<i>Odontarrhena</i> ¹	Y
<i>Alyssum condensatum</i> Boiss. & Hausskn.	<i>Odontarrhena</i> ¹	Y
<i>Alyssum constellatum</i> Boiss.	<i>Odontarrhena</i> ¹	Y
<i>Alyssum contemptum</i> Schott & Kotschy	<i>Alyssum</i> ¹	Y

Table A1-1. Checklist of Alyseae species showing names used and species sampled in this study.

Species name & authority	<i>Alyssum</i> section	Sequenced here
<i>Alyssum corningii</i> T.R.Dudley	<i>Gamosepalum</i> ¹	N
<i>Alyssum corsicum</i> Duby	<i>Odontarrhena</i> ¹	Y
<i>Alyssum corymbosoides</i> Formánek	<i>Odontarrhena</i> ¹	N
<i>Alyssum crenulatum</i> Boiss. & Heldr.	<i>Odontarrhena</i> ¹	Y
<i>Alyssum cuneifolium</i> Ten.	<i>Alyssum</i> ¹	Y
<i>Alyssum cypricum</i> Nyár.	<i>Odontarrhena</i> ¹	Y
<i>Alyssum damascenum</i> Boiss. & Gaill.	<i>Psilonema</i> ₁	Y
<i>Alyssum dasycarpum</i> Stephan ex Willd.	<i>Psilonema</i> ¹	Y
<i>Alyssum davisianum</i> T.R.Dudley	<i>Odontarrhena</i> ¹	Y
<i>Alyssum debarense</i> Micevski	Unknown	N
<i>Alyssum densistellatum</i> T.R.Dudley	<i>Alyssum</i> ¹	Y
<i>Alyssum desertorum</i> Stapf	<i>Alyssum</i> ¹	Y
<i>Alyssum diffusum</i> Ten.	<i>Alyssum</i> ⁴	Y
<i>Alyssum discolor</i> T.R.Dudley & Hub.-Mor.	<i>Odontarrhena</i> ¹	Y
<i>Alyssum djurdjurae</i> Chodat	<i>Alyssum</i> ¹	N
<i>Alyssum doerfleri</i> Degen	<i>Alyssum</i> ¹	N
<i>Alyssum dubertretii</i> Gomb.	<i>Odontarrhena</i> ¹	Y
<i>Alyssum dudleyi</i> N.Adigüzel & R.D.Reeves	<i>Odontarrhena</i> ⁵	Y
<i>Alyssum embergeri</i> Quézel	Unknown*	N
<i>Alyssum eriophyllum</i> Boiss. & Hausskn.	<i>Odontarrhena</i> ¹	Y
<i>Alyssum erosulum</i> Gennari & Pestal. ex Clem.	<i>Alyssum</i> ¹	Y
<i>Alyssum euboeum</i> Halácsy	<i>Odontarrhena</i> ¹	Y
<i>Alyssum fedtschenkoanum</i> Busch	<i>Odontarrhena</i> ¹	N
<i>Alyssum filiforme</i> Nyár.	<i>Odontarrhena</i> ¹	Y
<i>Alyssum flahaultianum</i> Emb. ex Greuter	Unknown*	Y
<i>Alyssum floribundum</i> Boiss.	<i>Odontarrhena</i> ¹	Y
<i>Alyssum foliosum</i> Bory & Chaub.	<i>Alyssum</i> ¹	Y
<i>Alyssum fragillimum</i> (Bald.) Rech.f.	<i>Odontarrhena</i> ¹	Y
<i>Alyssum fulvescens</i> Sm.	<i>Alyssum</i> ¹	Y
<i>Alyssum gadorense</i> P.Küpfer	Unknown*	N
<i>Alyssum gehamense</i> Fed.	<i>Odontarrhena</i> ¹	Y
<i>Alyssum gevgelicense</i> Micevski	Unknown	N
<i>Alyssum giosnanum</i> Nyár.	<i>Odontarrhena</i> ¹	Y
<i>Alyssum globosum</i> (Desv.) Kuntze	Unknown	N
<i>Alyssum granatense</i> Boiss. & Reut.	<i>Psilonema</i> ¹	Y
<i>Alyssum gustavssonii</i> Hartvig	<i>Alyssum</i> ⁶	N
<i>Alyssum hajastanum</i> Avet.	Unknown	N
<i>Alyssum handelii</i> Hayek	<i>Alyssum</i> ¹	Y

Table A1-1. Checklist of Alyseae species showing names used and species sampled in this study.

Species name & authority	<i>Alyssum</i> section	Sequenced here
<i>Alyssum harputicum</i> T.R.Dudley	<i>Gamosepalum</i> ¹	Y
<i>Alyssum haussknechtii</i> Boiss.	<i>Odontarrhena</i> ¹	Y
<i>Alyssum heideri</i> Krock.	Unknown	N
<i>Alyssum heldreichii</i> Hausskn.	<i>Odontarrhena</i> ¹	Y
<i>Alyssum heterotrichum</i> Boiss.	<i>Meniocus</i> ¹	Y
<i>Alyssum hirsutum</i> M.Bieb.	<i>Alyssum</i> ¹	Y
<i>Alyssum homalocarpum</i> Boiss.	<i>Psilonema</i> ¹	N
<i>Alyssum huber-morathii</i> T.R.Dudley	<i>Odontarrhena</i> ¹	Y
<i>Alyssum huetii</i> Boiss.	<i>Meniocus</i> ¹	Y
<i>Alyssum idaeum</i> Boiss. & Heldr.	<i>Alyssum</i> ¹	N
<i>Alyssum iljinskae</i> V.I.Dorof.	Unknown	N
<i>Alyssum iranikum</i> Hausskn. ex Baumg.	<i>Alyssum</i> ¹	Y
<i>Alyssum kavadarcese</i> Micevski	Unknown	N
<i>Alyssum kaynakiae</i> Yilmaz	<i>Gamosepalum</i> ³	N
<i>Alyssum kurdicum</i> (Boiss.) Nyár.	Unknown	N
<i>Alyssum lanceolatum</i> Baumg.	<i>Alyssum</i> ¹	Y
<i>Alyssum lanigerum</i> DC.	<i>Odontarrhena</i> ¹	Y
<i>Alyssum lassiticum</i> Halácsy	<i>Alyssum</i> ¹	N
<i>Alyssum lenense</i> Adams	<i>Alyssum</i> ¹	Y
<i>Alyssum lepidoto-stellatum</i> (Hausskn. & Bornm.) T.R.Dudley	<i>Gamosepalum</i> ¹	Y
<i>Alyssum lepidotum</i> Boiss.	<i>Alyssum</i> ¹	N
<i>Alyssum lesbiacum</i> (Candargy) Rech.f.	<i>Odontarrhena</i> ¹	Y
<i>Alyssum libanoticum</i> Nyár.	<i>Odontarrhena</i> ¹	N
<i>Alyssum linifolium</i> Stephan ex. Willd.	<i>Meniocus</i> ¹	Y
<i>Alyssum loiseleurii</i> P.Fourn.	<i>Alyssum</i> ¹	Y
<i>Alyssum luteolum</i> Pomel	<i>Alyssum</i> ²	N
<i>Alyssum lycaonicum</i> (O.E.Schulz) T.R.Dudley	<i>Gamosepalum</i> ¹	Y
<i>Alyssum macrocalyx</i> Coss. & Durieu	<i>Alyssum</i> ¹	Y
<i>Alyssum macropodum</i> Boiss. & Balansa	<i>Alyssum</i> ¹	Y
<i>Alyssum magicum</i> C.H.An	<i>Magicum</i> ¹⁴	N
<i>Alyssum malacitanum</i> (Rivas Goday) T.R.Dudley	<i>Odontarrhena</i> ¹⁵	Y
<i>Alyssum masmenaeum</i> Boiss.	<i>Odontarrhena</i> ¹	Y
<i>Alyssum menioides</i> Boiss.	<i>Meniocus</i> ¹	N
<i>Alyssum microphylliforme</i> Nyár.	Unknown	N
<i>Alyssum microphyllum</i> Steud.	<i>Alyssum</i> ¹	N
<i>Alyssum minutum</i> Schlecht. ex DC.	<i>Alyssum</i> ¹	Y
<i>Alyssum misirdalianum</i> Orcan & Binzet	<i>Alyssum</i> ⁸	N
<i>Alyssum moellendorffianum</i> Asch. ex Beck	<i>Alyssum</i> ¹	N

Table A1-1. Checklist of Alyseae species showing names used and species sampled in this study.

Species name & authority	<i>Alyssum</i> section	Sequenced here
<i>Alyssum montanum</i> L.	<i>Alyssum</i> ¹	Y
<i>Alyssum mouradicum</i> Boiss. & Balansa	<i>Alyssum</i> ¹	Y
<i>Alyssum mozaffarianii</i> Kavousi	<i>Odontarrhena</i> ⁷	N
<i>Alyssum muelleri</i> Boiss. & Buhse	<i>Alyssum</i> ¹	Y
<i>Alyssum mughlaei</i> Orcan	<i>Odontarrhena</i> ¹²	N
<i>Alyssum murale</i> Waldst. & Kit.	<i>Odontarrhena</i> ¹	Y
<i>Alyssum musilii</i> Velen.	Unknown	N
<i>Alyssum nebrodense</i> Tineo	<i>Odontarrhena</i> ¹	Y
<i>Alyssum nezaketiae</i> Aytaç & H. Duman	<i>Gamosepalum</i> ¹³	N
<i>Alyssum niveum</i> T.R.Dudley	<i>Gamosepalum</i> ¹	Y
<i>Alyssum obovatum</i> (C.A.Mey.) Turcz.	<i>Odontarrhena</i> ¹	Y
<i>Alyssum obtusifolium</i> Steven ex DC.	<i>Odontarrhena</i> ¹	Y
<i>Alyssum ochroleucum</i> Boiss. & A.Huet	<i>Alyssum</i> ¹	Y
<i>Alyssum orbelicum</i> Ančev & Uzunov	<i>Odontarrhena</i> ⁹	N
<i>Alyssum ovirense</i> A.Kern.	<i>Alyssum</i> ¹	Y
<i>Alyssum oxycarpum</i> Boiss. & Balansa	<i>Odontarrhena</i> ¹	Y
<i>Alyssum paphlagonicum</i> (Hauskn.) T.R.Dudley	<i>Gamosepalum</i> ¹	Y
<i>Alyssum pateri</i> Nyár.	<i>Odontarrhena</i> ¹	Y
<i>Alyssum peltarioides</i> Boiss.	<i>Odontarrhena</i> ¹	Y
<i>Alyssum penjwinense</i> T.R.Dudley	<i>Odontarrhena</i> ¹	Y
<i>Alyssum persicum</i> Boiss.	<i>Alyssum</i> ¹	Y
<i>Alyssum pinifolium</i> (Nyár.) T.R.Dudley	<i>Odontarrhena</i> ¹	Y
<i>Alyssum pintodasilvae</i> T.R.Dudley	<i>Odontarrhena</i> ¹	Y
<i>Alyssum pirinicum</i> (Stoj. & Acht.) Ančev	Unknown*	N
<i>Alyssum pogonocarpum</i> Carlström	<i>Alyssum</i> ¹⁰	N
<i>Alyssum polycladum</i> Rech.f.	<i>Odontarrhena</i> ¹	Y
<i>Alyssum praecox</i> Boiss.	<i>Alyssum</i> ¹	Y
<i>Alyssum propinquum</i> Baumg.	<i>Alyssum</i> ¹	Y
<i>Alyssum pseudomouradicum</i> Hauskn. & Bornm. ex Baumg.	<i>Alyssum</i> ¹	Y
<i>Alyssum pterocarpum</i> T.R.Dudley	<i>Odontarrhena</i> ¹	Y
<i>Alyssum pulvinare</i> Velen.	<i>Alyssum</i> ¹	N
<i>Alyssum repens</i> Baumg.	<i>Alyssum</i> ¹	Y
<i>Alyssum robertianum</i> Bernard ex Gren. & Godr.	<i>Odontarrhena</i> ¹	Y
<i>Alyssum rostratum</i> Steven	<i>Alyssum</i> ¹	Y
<i>Alyssum samariferum</i> Boiss. & Hauskn.	<i>Odontarrhena</i> ¹	Y
<i>Alyssum samium</i> T.R.Dudley & Christod.	<i>Odontarrhena</i> ⁹	N
<i>Alyssum scardicum</i> Wettst.	<i>Alyssum</i> ¹	Y
<i>Alyssum scutigerum</i> Durieu	<i>Alyssum</i> ¹	Y

Table A1-1. Checklist of Alyseae species showing names used and species sampled in this study.

Species name & authority	<i>Alyssum</i> section	Sequenced here
<i>Alyssum serpentinum</i> Micevski	Unknown	N
<i>Alyssum serpyllifolium</i> Desf.	<i>Odontarrhena</i> ¹	Y
<i>Alyssum sibiricum</i> Willd.	<i>Odontarrhena</i> ¹	Y
<i>Alyssum siculum</i> Jord.	<i>Alyssum</i> ⁶	N
<i>Alyssum simplex</i> Rudolph	<i>Alyssum</i> ¹	Y
<i>Alyssum simulans</i> Runemark ex Hartvig	<i>Alyssum</i> ¹⁰	N
<i>Alyssum singarense</i> Boiss. & Hausskn.	<i>Odontarrhena</i> ¹	Y
<i>Alyssum skopjense</i> Micevski	Unknown	N
<i>Alyssum smolikanum</i> Nyár.	<i>Odontarrhena</i> ¹	N
<i>Alyssum smyrnaeum</i> C.A.Mey.	<i>Alyssum</i> ¹	Y
<i>Alyssum sphacioticum</i> Boiss. & Heldr.	<i>Alyssum</i> ¹	N
<i>Alyssum stapfii</i> Vierh.	<i>Alyssum</i> ¹	Y
<i>Alyssum stipitatum</i> Kavousi & T.R.Dudley	<i>Odontarrhena</i> ⁷	N
<i>Alyssum sribryni</i> Velen.	<i>Alyssum</i> ¹	Y
<i>Alyssum strictum</i> Willd.	<i>Alyssum</i> ¹	Y
<i>Alyssum strigosum</i> Banks & Sol.	<i>Alyssum</i> ¹	Y
<i>Alyssum stylare</i> (Boiss. & Balansa) Boiss.	<i>Meniocus</i> ¹	Y
<i>Alyssum subbaicalicum</i> Nyár.	Unknown	N
<i>Alyssum subspinosum</i> T.R.Dudley	<i>Odontarrhena</i> ¹	Y
<i>Alyssum sulphureum</i> T.R.Dudley & Hub.-Mor.	<i>Gamosepalum</i> ¹	Y
<i>Alyssum syriacum</i> Nyár.	<i>Odontarrhena</i> ¹	N
<i>Alyssum szarabiacum</i> Nyár.	<i>Odontarrhena</i> ¹	N
<i>Alyssum szovitsianum</i> Fisch. & C.A.Mey.	<i>Alyssum</i> ¹	Y
<i>Alyssum taygeteum</i> Heldr.	<i>Alyssum</i> ¹	N
<i>Alyssum tenium</i> Halácsy	<i>Odontarrhena</i> ¹	Y
<i>Alyssum tetrastemon</i> Boiss.	<i>Gamosepalum</i> ¹	Y
<i>Alyssum thymops</i> (Hub.-Mor. & Reese) T.R.Dudley	<i>Gamosepalum</i> ¹	Y
<i>Alyssum tortuosum</i> Waldst. & Kit ex Willd.	<i>Odontarrhena</i> ¹	Y
<i>Alyssum trapeziforme</i> Bornm. ex Nyár.	<i>Odontarrhena</i> ¹	N
<i>Alyssum trichocarpum</i> T.R.Dudley & Hub.-Mor.	<i>Alyssum</i> ¹	N
<i>Alyssum troodi</i> Boiss.	<i>Odontarrhena</i> ¹	Y
<i>Alyssum turgidum</i> T.R.Dudley	<i>Odontarrhena</i> ¹	Y
<i>Alyssum turkestanicum</i> Regel & Schmalh.	<i>Alyssum</i> ¹	Y
<i>Alyssum umbellatum</i> Desv.	<i>Alyssum</i> ¹	Y
<i>Alyssum virgatum</i> Nyár.	<i>Odontarrhena</i> ¹	Y
<i>Alyssum wierzbickii</i> Heuff.	<i>Alyssum</i> ¹	N
<i>Alyssum wulfenianum</i> Benth. ex Willd.	<i>Alyssum</i> ¹	Y
<i>Alyssum xanthocarpum</i> Boiss.	<i>Alyssum</i> ₁	Y

Table A1-1. Checklist of Alyseae species showing names used and species sampled in this study.

Species name & authority	<i>Alyssum</i> section	Sequenced here
<i>Aurinia corymbosa</i> Griseb.	N/A	Y
<i>Aurinia gionae</i> (Quézel & Contandr.) Greuter & Burdet	N/A	N
<i>Aurinia leucadea</i> (Guss.) K.Koch	N/A	N
<i>Aurinia moreana</i> Tzanoud. & Iatroú	N/A	N
<i>Aurinia petraea</i> (Ard.) Schur	N/A	Y
<i>Aurinia saxatilis</i> (L.) Desv.	N/A	Y
<i>Aurinia sinuata</i> (L.) Griseb.	N/A	N
<i>Aurinia uechtritzi</i> (Bornm.) Cullen & T.R.Dudley	N/A	Y
<i>Berteroa gintl</i> Rohlena	N/A	N
<i>Berteroa incana</i> (L.) DC.	N/A	Y
<i>Berteroa mutabilis</i> (Vent.) DC.	N/A	Y
<i>Berteroa obliqua</i> (Sm.) DC.	N/A	Y
<i>Berteroa orbiculata</i> DC.	N/A	N
<i>Bornmuellera angustifolia</i> (Hauskn. ex Bornm.) Cullen & T.R.Dudley	N/A	Y
<i>Bornmuellera baldaccii</i> (Degen) Heywood	N/A	Y
<i>Bornmuellera cappadocica</i> (Willd.) Cullen & T.R.Dudley	N/A	Y
<i>Bornmuellera dieckii</i> Degen	N/A	N
<i>Bornmuellera glabrescens</i> (Boiss. & Balansa) Cullen & T.R.Dudley	N/A	N
<i>Bornmuellera kiyakii</i> Aytaç & A.Aksoy	N/A	Y
<i>Bornmuellera tymphaea</i> (Hauskn.) Hauskn.	N/A	Y
<i>Clastopus erubescens</i> Hauskn.	N/A	Y
<i>Clastopus vestitus</i> (Desv.) Boiss.	N/A	Y
<i>Clypeola aspera</i> (Grauer) Turrill	N/A	Y
<i>Clypeola ciliata</i> Boiss.	N/A	Y
<i>Clypeola cyclodonte</i> Delile	N/A	N
<i>Clypeola dichotoma</i> Boiss.	N/A	Y
<i>Clypeola elegans</i> Boiss. & A.Huet	N/A	N
<i>Clypeola eriocarpa</i> Cav.	N/A	N
<i>Clypeola jonthlaspi</i> L.	N/A	Y
<i>Clypeola lappacea</i> Boiss.	N/A	Y
<i>Clypeola raddeana</i> Albov	N/A	N
<i>Degenia velebitica</i> (Degen) Hayek	N/A	Y
<i>Fibigia clypeata</i> (L.) Medik.	N/A	Y
<i>Fibigia compacta</i> Rech.f.	N/A	N
<i>Fibigia heterophylla</i> Rech.f.	N/A	N
<i>Fibigia lunarioides</i> (Willd.) Sweet	N/A	Y
<i>Fibigia macrocarpa</i> (Boiss.) Boiss.	N/A	Y
<i>Fibigia membranacea</i> Rech.f.	N/A	Y

Table A1-1. Checklist of Alyseae species showing names used and species sampled in this study.

Species name & authority	<i>Alyssum</i> section	Sequenced here
<i>Fibigia multicaulis</i> (Boiss. & Hohen.) Boiss.	N/A	Y
<i>Fibigia pendula</i> (Boiss.) Boiss.	N/A	N
<i>Fibigia spathulata</i> (Kar. & Kir.) B.Fedtsch.	N/A	Y
<i>Fibigia suffruticosa</i> (Vent.) Sweet	N/A	Y
<i>Fibigia thesigeri</i> Rech.f.	N/A	Y
<i>Fibigia triquetra</i> (DC.) Boiss. ex Prantl	N/A	Y
<i>Fibigia umbellata</i> (Boiss.) Boiss.	N/A	Y
<i>Galitzkya macrocarpa</i> (Ikonn.-Gal.) V.V.Botschantz.	N/A	N
<i>Galitzkya potaninii</i> (Maxim.) V.V.Botschantz.	N/A	N
<i>Galitzkya spathulata</i> (Stephan ex Willd.) V.V.Botschantz.	N/A	Y
<i>Hormathophylla baetica</i> P.Küpfer	N/A	N
<i>Hormathophylla cadevalliana</i> (Pau) T.R.Dudley	N/A	N
<i>Hormathophylla halimifolia</i> (Boiss.) P.Küpfer	N/A	Y
<i>Hormathophylla lapeyrouseana</i> (Jord.) P.Küpfer	N/A	Y
<i>Hormathophylla longicaulis</i> (Boiss.) Cullen & T.R.Dudley	N/A	Y
<i>Hormathophylla macrocarpa</i> (DC.) P.Küpfer	N/A	N
<i>Hormathophylla purpurea</i> (Lag. & Rodr.) P.Küpfer	N/A	Y
<i>Hormathophylla pyrenaica</i> (Lapeyr.) Cullen & T.R.Dudley	N/A	N
<i>Hormathophylla reverchonii</i> (Degen & Hervier) Cullen & T.R.Dudley	N/A	Y
<i>Hormathophylla spinosa</i> (L.) P.Küpfer	N/A	Y
<i>Leptoplax emarginata</i> (Boiss.) O.E.Schulz	N/A	Y
<i>Phyllolepidum cyclocarpum</i> (Boiss.) Cecchi	N/A	Y
<i>Phyllolepidum rupestre</i> (Ten.) Trinajstić	N/A	N
<i>Physocardamum davisii</i> Hedge	N/A	Y
<i>Physoptychis caspica</i> (Hablitz) V.P.Botsch.	N/A	Y
<i>Physoptychis haussknechtii</i> Bornm.	N/A	N
<i>Straussiella purpurea</i> (Bunge ex Boiss.) Hausskn.	N/A	Y
<i>Alyssum</i> section references: ¹ Dudley, 1964a ⁷ Kavousi, 2001 ¹³ Aytac and Duman, 2000 ² Pomel, 1874 ⁸ Orcan and Binzet, 2009 ¹⁴ An, 1981 ³ Yilmaz, 2012 ⁹ Ancev and Uzunov, 2002 ¹⁵ Dudley, 1986a ⁴ Brooks <i>et al.</i> , 1979 ¹⁰ Strid and Tan, 2002 ¹⁶ Dudley, 1986b ⁵ Adigüzel and Reeves, 2002 ¹¹ Komarov <i>et al.</i> , 1939 ⁶ Ball and Dudley, 1993 ¹² Orcan, 2005		
*Asterisks indicate species for which infra-generic sections have been tentatively assigned, based on morphological information or stated affinities that are detailed in the literature. Full details of these allocations are included in Appendix 4.		

Table A1-2. Summary of Alysseae species names accepted in this study (by genus).

Genus	No. species accepted in this study	Species sampled in this study	% Coverage in this study
<i>Alyssoides</i>	2	2	100
<i>Alyssum</i>	188	126	67
<i>Aurinia</i>	8	4	50
<i>Berteroa</i>	5	3	60
<i>Bornmuellera</i>	7	5	71
<i>Clastopus</i>	2	2	100
<i>Clypeola</i>	9	5	56
<i>Degenia</i>	1	1	100
<i>Fibigia</i>	13	10	77
<i>Galitzkya</i>	3	1	33
<i>Hormathophylla</i>	10	6	60
<i>Leptoplax</i>	1	1	100
<i>Phyllolepidum</i>	2	1	50
<i>Physocardamum</i>	1	1	100
<i>Physoptychis</i>	2	1	50
<i>Straussiella</i>	1	1	100
Total (16 Alysseae Genera)	255	170	67

Table A1-3. Non-Alysseae species sampled (or sequences obtained from GenBank) in this study

Species name & authority	Sequenced in this study (N = sequences obtained from GenBank only)
Brassicaceae	
<i>Alliaria petiolata</i> (M.Bieb.) Cavara & Grande (tribe Thlaspidaceae)	Y
<i>Arabidopsis thaliana</i> (L.) Heynh. (tribe Camelinae)	N
<i>Chorisporea purpurascens</i> (Banks & Sol.) Eig (tribe Chorisporeae)	Y
<i>Farsetia stylosa</i> R.Br. (Malcolmieae*)	Y
<i>Iberis saxatilis</i> L. (tribe Iberideae)	Y
<i>Ladakiella klimesii</i> (Al-Shehbaz) D.A.German & Al-Shehbaz (tribe Camelinae*)	Y
<i>Lobularia maritima</i> (L.) Desv. (Malcolmieae*)	Y
<i>Pseudocamelina glaucophylla</i> N.Busch (tribe Thlaspidaceae)	Y
<i>Stevenia canescens</i> (DC.) D.A.German (tribe Arabideae)	Y
<i>Stevenia tenuifolia</i> (Stephan ex Willd.) D.A.German (tribe Arabideae)	Y
<i>Thlaspi arvense</i> L. (tribe Thlaspidaceae)	Y
Non-Brassicaceae Brassicales	
<i>Akania bidwillii</i> (Hend. ex Hogg) Mabb. (family Akaniaceae)	Y
<i>Batis maritima</i> L. (family Bataceae)	Y
<i>Bretschneidera sinensis</i> Hemsl. (family Akaniaceae)	Y
<i>Capparis spinosa</i> (family Capparaceae)	Y
<i>Carica papaya</i> L. (family Caricaceae)	Y
<i>Tropaeolum majus</i> L. (family Tropaeolaceae)	Y
Non-Brassicales	
<i>Gossypium hirsutum</i> L. (family Malvaceae)	N
<i>Gossypium gossypoides</i> (Ulbr.) Standl. (family Malvaceae)	N
Notes to Accompany Table A1-3	
Tribal assignments for Brassicaceae species are based on the taxonomy of Al-Shehbaz <i>et al.</i> (2006), except for asterisked (*) species which are based on the results of Warwick <i>et al.</i> (2006).	

Appendix 2:

Sampled Plant Material

Herbarium Specimens

Table A2-1 lists the 130 herbarium specimens from which DNA was obtained in this study, and includes the following details for each specimen: species name (authorities for these names are indicated in Appendix 1), collector, specimen number, source herbarium, and collection location and date. For three specimens (of *Ladakiella klimesii*, *Fibigia spathulata*, and *Galitzkya potaninii*), DNA was supplied by D. A. German (of Altai State University, Russia). For all remaining specimens, DNA was extracted by the author.

Seed-grown Plants

Table A2-2 lists the 76 seed sources used in this study, and includes the following details for each source: species name (authorities for these names are indicated in Appendix 1), a description of the tissue used for DNA extraction, details of seed source, seed accession, voucher specimen, seed collection location and date (where known). For 54 seed sources, DNA was extracted from silica-dried leaf tissue from seed-grown plants. For 16 seed sources, DNA was extracted from fresh leaf tissue from seed-grown plants. For the remaining six seed sources, for which seeds failed to germinate, DNA was extracted from seeds themselves. Voucher specimens were taken for 55 seed sources.

Herbarium Specimens

Table A2-1 Herbarium specimens from which DNA was obtained.

Species (& Accession No.)	Collector & Specimen No.	Herbarium ¹	Collection Location & Date
<i>Akania bidwillii</i>	Flynn 70	OXF	Cultivated: Go Green Rainforest Nursery, Glenview, Queensland, Australia, 2010
<i>Alliaria petiolata</i>	Flynn 62	OXF	Oxford, UK, 2010
<i>Alyssoides cretica</i>	Burbidge 270	E	Crete, Greece, 1973
<i>Alyssoides utriculata</i> (1)	Gardner & Gardner 2503	E	Makarska, Croatia, 1984
<i>Alyssum akamasicum</i>	Emonson & McClintock 2755	E	Vlambouros, Cyprus, 1979
<i>Alyssum alpestre</i>	Warburg s.n.	OXF	Laulanet, France, 1957
<i>Alyssum anatolicum</i>	Davis 43143	E	Bitlis, Turkey, 1966
<i>Alyssum armenum</i>	Cheese 1489	E	Van, Turkey, 1966
<i>Alyssum aurantiacum</i>	Hartvig et al. 23430	E	Muğla, Turkey, 1984
<i>Alyssum aureum</i>	Helbaek 2400	E	Konya, Turkey, 1962
<i>Alyssum baumgartnerianum</i>	Peat et al. 199-10-76	E	Içel, Turkey, 1976
<i>Alyssum bertolonii</i>	Gardner 4318	E	Doganovic, Kosovo, 1988
<i>Alyssum blepharocarpum</i>	Coode & Jones 90	E	Ankara, Turkey, 1965
<i>Alyssum bornmuelleri</i>	Stainton & Henderson 5832	E	Giresun, Turkey, 1960
<i>Alyssum caespitosum</i>	Davis 44623	E	Van, Turkey, 1966
<i>Alyssum callichroum</i>	Reeves 1976	E*	Seyhan, Turkey, 1998
<i>Alyssum calycocarpum</i>	Davis 33295	E	Nikita, Ukraine, 1959
<i>Alyssum cassium</i>	Reeves 2152	E*	Hatay, Turkey, 2001

Table A2-1 Herbarium specimens from which DNA was obtained.

Species (& Accession No.)	Collector & Specimen No.	Herbarium¹	Collection Location & Date
<i>Alyssum chondrogynum</i>	Edmonson & McClintock 2991	E	Ephtagonia, Cyprus, 1979
<i>Alyssum cilicicum</i>	Reeves 2185	E*	Hatay, Turkey, 2001
<i>Alyssum condensatum</i>	Reeves 2010	E*	Niğde, Turkey, 1998
<i>Alyssum constellatum</i>	Reeves 1949	E*	Içel, Turkey, 1998
<i>Alyssum contemptum</i>	Davis 43065	E	Mardin, Turkey, 1966
<i>Alyssum crenulatum</i>	Reeves 2153	E*	Hatay, Turkey, 2001
<i>Alyssum davisianum</i>	Coode 2504	E	Kütahya, Turkey, 1965
<i>Alyssum densistellatum</i>	Reeves 2397	E	Evvlá, Greece, 2002
<i>Alyssum diffusum</i>	C4107	E	Cultivated: Royal Botanic Garden Edinburgh, UK, 1962
<i>Alyssum dubertretii</i>	Reeves 2171	E*	Hatay, Turkey, 2001
<i>Alyssum dudleyi</i>	Reeves 2042	E*	Kütahya, Turkey, 1998
<i>Alyssum eriophyllum</i>	Reeves 2170	E*	Hatay, Turkey, 2001
<i>Alyssum erosulum</i>	Polunin 14025	E	Mugla, Turkey, 1976
<i>Alyssum euboicum</i>	Reeves 2395	E	Evvlá, Greece, 2002
<i>Alyssum filiforme</i>	Reeves 2223	E*	Tunceli, Turkey, 2001
<i>Alyssum foliosum</i>	Davis 41741	E	Izmir, Turkey, 1965
<i>Alyssum fragillimum</i>	Davis 18127A	E	Crete, Greece, 1950
<i>Alyssum fulvescens</i>	Davis 41752	E	Izmir, Turkey, 1965
<i>Alyssum gehamense</i> (1)	Mykug & Kapanezsus s.n.	E	Unknown, 1956
<i>Alyssum gehamense</i> (2)	Balls 1313A	E	Vilayet, Turkey, 1934
<i>Alyssum giosnanum</i> (1)	Kühne 1384	E	Adana, Turkey, 1957
<i>Alyssum giosnanum</i> (2)	Davis & Hedge 27013	E	Hatay, Turkey, 1957
<i>Alyssum handelii</i>	Ackroyd 77	E	Olympus, Greece, 1973
<i>Alyssum harputicum</i>	Coode & Jones 2263	E	Eskisehir, Turkey, 1965
<i>Alyssum haussknechtii</i>	Coode & Jones 1349	E	Kayseri, Turkey, 1965
<i>Alyssum heldreichii</i>	Reeves 2461	E*	Pindus Mts., Greece, 2002
<i>Alyssum heterotrichum</i>	Isanhalus 30679E	E	Jadg, Iran, 1974

Table A2-1 Herbarium specimens from which DNA was obtained.

Species (& Accession No.)	Collector & Specimen No.	Herbarium¹	Collection Location & Date
<i>Alyssum huber-morathii</i>	Gavaz 33	OXF	Muğla, Turkey, 2006
<i>Alyssum huetii</i>	Dudley 35230	E	Antalya, Turkey, 1962
<i>Alyssum iranikum</i>	Frey & Kürschner 4974	E	Kerak, Jordan, 1980
<i>Alyssum lanceolatum</i>	Rechinger & Renz 48775	E	West Azerbaijan, Iran, 1974
<i>Alyssum lanigerum</i>	Foroughi & Assadi 18064	E	Estafhan, Iran, 1975
<i>Alyssum lycaonicum</i>	Archibald 6674	E	Malatya, Turkey, 1985
<i>Alyssum macropodium</i>	Dudley 35231	E	Antalya, Turkey, 1962
<i>Alyssum masmenaeum</i>	Gavaz 31	OXF	Muğla, Turkey, 2006
<i>Alyssum montanum</i> (1)	Jasiewicz & Palkowa s.n.	OXF	Olezki, Poland, 1970
<i>Alyssum montanum</i> (2) var. <i>clausonis</i>	Davis & Davis 49207	E	Ifrane, Morocco, 1969
<i>Alyssum mouradicum</i>	Reeves 1983	E*	Seyhan, Turkey, 1998
<i>Alyssum muelleri</i>	Noussavi et al. 40493E	E	Zanjan, Iran, 1983
<i>Alyssum murale</i> (1)	Reeves 2410	E*	Euboea, Greece, 2002
<i>Alyssum murale</i> (2)	Gavaz 116	OXF	Akseki-Beyşehir, Turkey, 2006
<i>Alyssum murale</i> (3)	Reeves 3306	E*	Librazhd, Albania, 2005
<i>Alyssum niveum</i>	Duman 5021	E	Eskisehir, Turkey, 1993
<i>Alyssum oxycarpum</i> (1)	Reeves 1989	E*	Seyhan, Turkey, 1998
<i>Alyssum paphlagonicum</i>	Rix et al. 1545	E	Ankara, Turkey, 1970
<i>Alyssum penjwinense</i>	Rechinger 11361	E	Erbil, Iraq, 1957
<i>Alyssum persicum</i>	Transhahs 20141-E	E	Lorestan, Iran, 1969
<i>Alyssum pinifolium</i>	Reeves 1658	E*	Çanakkale, Turkey, 1996
<i>Alyssum polycladum</i>	Sharif s.n.	E*	Khvoy, Azerbaijan prov., Iran, 1955
<i>Alyssum praecox</i>	Edmonson 511	E	Kastamonu, Turkey, 1971
<i>Alyssum propinquum</i>	Albury et al. 3361	E	Erzurum, Turkey, 1967
<i>Alyssum pseudomouradicum</i>	Davis & Coode D388913	E	Zonguldak, Turkey, 1962
<i>Alyssum rostratum</i>	C12167	E	Cultivated: Royal Botanic Garden Edinburgh, UK, 1977
<i>Alyssum samariferum</i>	Reeves 2213	E*	Turkey, 2001

Table A2-1 Herbarium specimens from which DNA was obtained.

Species (& Accession No.)	Collector & Specimen No.	Herbarium¹	Collection Location & Date
<i>Alyssum scardicum</i>	C11388	E	Cultivated: Royal Botanic Garden Edinburgh, UK, 1977
<i>Alyssum sibiricum</i>	Reeves 2035	E*	Kütahya, Turkey, 1998
<i>Alyssum simplex</i> (1)	Davis 59027	E	Ain Lellou, Algeria, 1975
<i>Alyssum singarense</i>	Archibald E 2201	E	Lake Razaiyeh, Iran, 1966
<i>Alyssum smyrnaeum</i>	Dudley 34877a	E	Izanci, Turkey, 1977
<i>Alyssum strictum</i>	Reeves 1957	E*	Niğde, Turkey, 1998
<i>Alyssum stylare</i>	Huber-Morath 17040	E	Çankiri, Turkey, 1964
<i>Alyssum subspinosum</i>	Collenette 9292	E	Badiah Harrat Rahah, Saudi Arabia, 1995
<i>Alyssum sulphureum</i>	Davis 43975	E	Doğubayazıt, Turkey, 1966
<i>Alyssum szovitsianum</i>	Edmonson & Miller 1529	E	Mehrız, Iran, 1977
<i>Alyssum thymops</i>	Reeves & Adiguzel 2307	E	Kayseri, Turkey, 2001
<i>Alyssum tortuosum</i> (1)	Termeh s.n.	E*	Khorassan, Iran
<i>Alyssum turgidum</i>	Wendelbo et al. 12280	E	Zanjan, Iran, 1974
<i>Alyssum turkestanicum</i>	Elias et al. 8245	E	Lake Chagiti, Tuva, Siberia, 1983
<i>Alyssum umbellatum</i>	Meikle 2709	E	Stavros tis Prokas, Cyprus, 1962
<i>Alyssum virgatum</i>	Davis & Coode 36685	E	Kütahya, Turkey, 1962
<i>Alyssum xanthocarpum</i>	Davis 45918	E	Van, Turkey, 1966
<i>Aurinia uechtritiziana</i> (1)	Baytop & Baytop 11873	E	Kasatura, Turkey, 1967
<i>Aurinia uechtritiziana</i> (2)	Harley & Kuzmanor 11983	E	Burgas, Bulgaria, 1973
<i>Berteroa incana</i>	Halliday 365/69	OXF	Hautes Alpes, France, 1969
<i>Berteroa mutabilis</i>	Gardner & Gardner 2987	E	Varna, Bulgaria, 1985
<i>Berteroa obliqua</i>	Jury & Thornton-Wood 9545	E	Burgas, Bulgaria, 1988
<i>Bornmuellera angustifolia</i>	Davis & Polunin 24431	E	Kara Dag, Turkey, 1954
<i>Bornmuellera baldaccii</i>	Reeves & Massoura 2470	E	Mavrovouni, Turkey, 2002
<i>Bornmuellera cappadocica</i>	Davis 45978	E	Van, Turkey, 1966
<i>Bornmuellera kiyakii</i>	Gavaz 142	OXF	Konya, Turkey, 2006

Table A2-1 Herbarium specimens from which DNA was obtained.

Species (& Accession No.)	Collector & Specimen No.	Herbarium¹	Collection Location & Date
<i>Bretschneidera sinensis</i>	Flynn 68	OXF	Cultivated: University of British Colombia Botanic Garden, Canada, 2010
<i>Carica papaya</i>	Flynn 69	OXF	Cultivated: Oxford Botanic Garden, UK, 2010
<i>Chorispura purpurascens</i>	Jallad 207	E	Amman, Jordan, 1974
<i>Clastopus erubescens</i>	Archibald 2160	E	Bijar, Iran, 1966
<i>Clastopus vestitus</i>	Archibald 2477	E	Kandevan Pass, Iran, 1966
<i>Clypeola aspera</i>	Rechinger 51124	E	East Khorasan, Iran, 1975
<i>Clypeola ciliata</i>	Vural 1707	E	Konya, Turkey, 1979
<i>Clypeola dichotoma</i>	Lamond 3858	E	Khvoy, Iran, 1971
<i>Clypeola jonthlaspi</i>	Uotila 16150	E	Khorasan, Iran, 1972
<i>Clypeola lappacea</i>	Lambinon 76/256	E	De Shiraz, Iran, 1976
<i>Fibigia lunarioides</i>	Buna 11054	E	Rhodes, Greece, 1971
<i>Fibigia membranacea</i>	Uotila 18598	E	Bamiyan, Afghanistan, 1972
<i>Fibigia multicaulis</i>	Wendelbo & Cobham 12299	E	Tehran, Iran, 1974
<i>Fibigia spathulata</i>	Botschantzev & Botschantzeva 1058	ALTB	Arkaly mountains, Kazakhstan
<i>Fibigia suffruticosa</i>	Archibald 2224	E	Kurdistan, Iran, 1966
<i>Fibigia thesigeri</i>	Moussavi et al. 40494E	E	Zanjan, Iran, 1983
<i>Fibigia umbellata</i>	Aryavand et al. 1421	E	Yazad, Iran, 1977
<i>Galitzkya spathulata</i>	German B163a	LE	Altai, Russia
<i>Hormathophylla halimifolia</i>	Chaipus 13902	E	Alpes Maritimes, France, 1977
<i>Hormathophylla lapeyrousiana</i>	Brummitt et al. 638	E	Tragacete, Spain, 1962
<i>Hormathophylla longicaulis</i>	Harley & Harley s.n.	OXF	Siera Navada, Spain, 1966
<i>Hormathophylla reverchonii</i>	Heywood & Davis 256	E	Jaen, Spain, 1948
<i>Hormathophylla spinosa</i>	Harley & Harley 637	OXF	Beni Mellal, Morocco, 1967
<i>Ladakiella klimesii</i>	Klimeš 2070	ALTB	Ladakh, India
<i>Leptoplax emarginata</i>	Reeves & Massoura 2455	E	Metzovo, Greece, 2002
<i>Physocardamum davisii</i>	Tatli 5806	E	Erzurum, Greece, 1977

Table A2-1 Herbarium specimens from which DNA was obtained.

Species (& Accession No.)	Collector & Specimen No.	Herbarium ¹	Collection Location & Date
<i>Physoptychis caspica</i>	Renz 56968	E	Kiyamaki Dagħ, Turkey, 1977
<i>Pseudocamelina glaucophylla</i>	Termeh et al. s.n.	E	Fars, Iran, 1987
<i>Stevenia tenuifolia</i>	Popov & Bardunov s.n.	E	Lake Biakal, Russia, 1955
<i>Straussiella purpurea</i>	Furse 2065	E	Sanandaj, Iran
<i>Thlaspi arvense</i>	Flynn 61	OXF	Ross-on-Wye, UK, 2010
¹ Herbarium codes: ALTB DNA supplied by D. A. German, obtained from specimens at the Altai State University Herbarium, Barnaul, Russia. E Herbarium at the Royal Botanic Garden Edinburgh, 20A Inverleith Row, Edinburgh, EH3 5LR, UK. E* Material supplied by R. D. Reeves, taken from specimens prior to depositing at E. OXF Fielding-Druce Herbarium, Department of Plant Sciences, University of Oxford, South Parks Road, Oxford, OX1 3RB. LE DNA supplied by D. A. German, obtained from specimens at the Herbarium at the Komarov Botanical Institute, St. Petersburg, Russia.			

Seed Sources

Table A2-2 Seeds and plant-grown leaf tissue from which DNA was obtained.

Species (& Accession No.)	Tissue Description	Seed Source ¹	Seed Accession	Voucher Specimen ²	Collection Location & Date
<i>Alyssoides utriculata</i> (2)	seed	AGS	1250	N/A	Unknown
<i>Alyssum aizoides</i>	silica-dried leaf tissue	P	J. Jurasek 2005: 341	<i>Flynn</i> 1	Bolkar Dag, Turkey, 2005
<i>Alyssum alyssoides</i>	fresh leaf tissue	BGTH	633054	<i>Flynn</i> 37	Cultivated: Lublin Botanic Garden, Poland
<i>Alyssum argenteum</i>	silica-dried leaf tissue	BGTH	697	<i>Flynn</i> 2	Unknown
<i>Alyssum argyrophyllum</i>	silica-dried leaf tissue	P	J. Jurasek 2005: 342	<i>Flynn</i> 38	Bolkar Dag, Turkey, 2005
<i>Alyssum baldaccii</i>	silica-dried leaf tissue	P	A. Baker 00233a	<i>Flynn</i> 43	Crete, Greece, 1995
<i>Alyssum bracteatum</i>	silica-dried leaf tissue	UPM	3697-75	<i>Flynn</i> 39	Tehran, Iran, 1975
<i>Alyssum caricum</i>	silica-dried leaf tissue	P	S. Gavaz 23	N/A	Muğla, Turkey, 2006
<i>Alyssum corsicum</i> (1)	silica-dried leaf tissue	UPM	8004-90	<i>Flynn</i> 59	Corsica, France, 1990
<i>Alyssum corsicum</i> (2)	silica-dried leaf tissue	P	S. Gavaz 16	N/A	Muğla, Turkey, 2006
<i>Alyssum cuneifolium</i>	seed	AGS	Weekers 28	N/A	Mont Ventoux, France, 2009
<i>Alyssum cypricum</i>	silica-dried leaf tissue	P	S. Gavaz 63	N/A	Burdur, Turkey, 2006
<i>Alyssum damascenum</i>	silica-dried leaf tissue	MSB	151924	<i>Flynn</i> 40	Ma'an, Jordan, 2001
<i>Alyssum dasycarpum</i>	fresh leaf tissue	UPM	3715-75	<i>Flynn</i> 29	Karadj, Iran, 1975
<i>Alyssum desertorum</i>	silica-dried leaf tissue	UPM	3769-76	<i>Flynn</i> 42	Karadj, Iran, 1976
<i>Alyssum discolor</i>	silica-dried leaf tissue	P	S. Gavaz 2	N/A	Muğla, Turkey, 2006
<i>Alyssum flahaultianum</i>	silica-dried leaf tissue	UPM	2184-74	<i>Flynn</i> 44	Jbel Anggour, Morocco, 1974

Table A2-2 Seeds and plant-grown leaf tissue from which DNA was obtained.

Species (& Accession No.)	Tissue Description	Seed Source ¹	Seed Accession	Voucher Specimen ²	Collection Location & Date
<i>Alyssum floribundum</i>	silica-dried leaf tissue	P	S. Gavaz 73	Flynn 32	Burdur, Turkey, 2006
<i>Alyssum granatense</i>	silica-dried leaf tissue	UPM	0877-66	Flynn 46	Madrid, Spain, 1977
<i>Alyssum hirsutum</i>	fresh leaf tissue	P	S. Gavaz site 16	Flynn 5	Konya, Turkey
<i>Alyssum lenense</i>	silica-dried leaf tissue	USDA	PI633058	Flynn 6	Mongolia
<i>Alyssum lepidoto-stellatum</i>	silica-dried leaf tissue	P	M. Pavelka 2006: 53	Flynn 47	Sivas, Turkey, 2006
<i>Alyssum lesbiacum</i>	silica-dried leaf tissue	P	A. Baker 00150n	Flynn 7	Lesvos, Greece, 1993
<i>Alyssum linifolium</i>	silica-dried leaf tissue	UPM	0893-68	Flynn 48	Murcia, Spain, 1968
<i>Alyssum loiseleurii</i>	silica-dried leaf tissue	UPM	Lesouef 3456-76	Flynn 8	Cape Breton, France, 1976
<i>Alyssum macrocalyx</i>	silica-dried leaf tissue	MSB	105363	Flynn 49	Ouarzazate, Morocco, 1995
<i>Alyssum malacitanum</i>	fresh leaf tissue	P	A. Baker AB00207d	Flynn 26	Sierra Bermejia, Spain, 1994
<i>Alyssum minutum</i>	silica-dried leaf tissue	UPM	1198-67	N/A	Sierra Nevada, Spain, 1967
<i>Alyssum montanum</i> (3)	silica-dried leaf tissue	UPM	1170-67	Flynn 31	Albacete, Spain, 1976
<i>Alyssum montanum</i> (4)	silica-dried leaf tissue	USDA	PI633061	N/A	Hungary
<i>Alyssum murale</i> (4)	fresh leaf tissue	P	A. Baker 00067a	N/A	Thessaloniki, Greece, 1988
<i>Alyssum murale</i> (5)	silica-dried leaf tissue	UPM	9477-98	N/A	Macedonia, 1998
<i>Alyssum murale</i> (6)	silica-dried leaf tissue	UPM	0985-66	Flynn 27	South Larissa, Greece, 1966
<i>Alyssum murale</i> (7)	silica-dried leaf tissue	USDA	AMES27613	Flynn 28	Cultivated: Elbasan Botanic Garden, Albania
<i>Alyssum nebrodense</i>	silica-dried leaf tissue	UPM	6950-85	Flynn 9	Sicily, Italy, 1985
<i>Alyssum obovatum</i>	silica-dried leaf tissue	USDA	Ames24118	Flynn 10	Altay, Russia
<i>Alyssum obtusifolium</i>	silica-dried leaf tissue	UPM	1340-68	Flynn 3	Cultivated: Rostoc Botanic Garden, 1964
<i>Alyssum ochroleucum</i>	silica-dried leaf tissue	P	M. Pavelka 2005: 55	Flynn 51	Tortum, Turkey, 2005
<i>Alyssum ovirens</i>	silica-dried leaf tissue	UPM	1214-67	Flynn 52	Unknown, 1967
<i>Alyssum oxycarpum</i> (2)	seed	P	J. Jurasek 2006: 201	Flynn 60	Salda, Turkey, 2006
<i>Alyssum pateri</i>	silica-dried leaf tissue	UPM	6299-83	Flynn 11	Eskisehir, Turkey, 1983

Table A2-2 Seeds and plant-grown leaf tissue from which DNA was obtained.

Species (& Accession No.)	Tissue Description	Seed Source ¹	Seed Accession	Voucher Specimen ²	Collection Location & Date
<i>Alyssum peltarioides</i>	silica-dried leaf tissue	P	S. Gavaz 155	N/A	Akseki-Beyşehir, Turkey, 2006
<i>Alyssum pintodasilvae</i> (1)	fresh leaf tissue	P	A. Baker 00337a	N/A	Basadre, Melide, Spain, 1999
<i>Alyssum pintodasilvae</i> (2)	fresh leaf tissue	P	A. Baker 00338f	Flynn 58	Bragança, Portugal, 1999
<i>Alyssum pterocarpum</i>	silica-dried leaf tissue	P	S. Gavaz 107	Flynn 12	Muğla, Turkey, 2006
<i>Alyssum repens</i>	silica-dried leaf tissue	P	J. Jurasek 540	N/A	Bucegi Mountains, Romania
<i>Alyssum robertianum</i>	silica-dried leaf tissue	UPM	8002-90	Flynn 13	Corsica, France, 1990
<i>Alyssum scutigerum</i>	fresh leaf tissue	UPM	1130-67	Flynn 34	Midelt, Morocco, 1967
<i>Alyssum serpyllifolium</i>	fresh leaf tissue	P	J. Pollard	Flynn 14	Madrid, Spain, 2002
<i>Alyssum simplex</i> (2)	fresh leaf tissue	UPM	3793-75	Flynn 53	Chalus, Iran, 1975
<i>Alyssum stapfii</i>	silica-dried leaf tissue	UPM	3774-75	Flynn 54	Chalus, Iran, 1975
<i>Alyssum stribryni</i>	fresh leaf tissue	AGS	1255	Flynn 15	Unknown
<i>Alyssum strigosum</i>	silica-dried leaf tissue	UPM	0893-69	Flynn 55	Athens, Greece, 1969
<i>Alyssum tenium</i>	silica-dried leaf tissue	MSB	12348	Flynn 36	Cyclades & Sporades, Greece, 1978
<i>Alyssum tetrastemon</i>	silica-dried leaf tissue	P	M. Pavelka 2008: 57	Flynn 56	Sultan Dag, Turkey, 2008
<i>Alyssum tortuosum</i> (2)	silica-dried leaf tissue	P	J. Jurasek 539	N/A	Turkey
<i>Alyssum troodi</i>	silica-dried leaf tissue	MSB	12289	Flynn 57	Cyprus, Greece, 1978
<i>Alyssum wulfenianum</i>	fresh leaf tissue	P	A. Baker AB00160b	Flynn 16	Leute, Kosch, Austria, 1993
<i>Arabidopsis thaliana</i>	fresh leaf tissue	P	Ecotype Col 0	N/A	Cultivated stock
<i>Aurinia corymbosa</i>	silica-dried leaf tissue	UPM	7413-86	Flynn 17	Mount Olympos, Greece, 1986
<i>Aurinia petraea</i>	silica-dried leaf tissue	UPM	1560-68	Flynn 18	1968
<i>Aurinia saxatilis</i> (1)	fresh leaf tissue	UPM	6030-82	Flynn 20	Attica, Greece, 1982
<i>Aurinia saxatilis</i> (2)	silica-dried leaf tissue	BGTH	700	N/A	Unknown
<i>Batis maritima</i>	fresh leaf tissue	MSB	405650	N/A	Texas, USA, 2007
<i>Bornmuellera tymphaea</i>	fresh leaf tissue	P	M. Pavelka 187	Flynn 21	Pindos, Greece, 2004
<i>Capparis spinosa</i>	seed	CS	272	N/A	Cultivated stock

Table A2-2 Seeds and plant-grown leaf tissue from which DNA was obtained.

Species (& Accession No.)	Tissue Description	Seed Source ¹	Seed Accession	Voucher Specimen ²	Collection Location & Date
<i>Degenia velebitica</i>	silica-dried leaf tissue	SRGC	1128	<i>Flynn</i> 36	Unknown
<i>Farsetia stylosa</i>	silica-dried leaf tissue	MSB	85243	N/A	Mauritania, 1987
<i>Fibigia clypeata</i>	silica-dried leaf tissue	SRGC	1464	<i>Flynn</i> 22	Unknown
<i>Fibigia macrocarpa</i>	seed	P	295	<i>Flynn</i> 23	Ala Dag, Turkey, 2001
<i>Fibigia triquetra</i>	seed	AGS	2876	N/A	Unknown
<i>Hormathophylla purpurea</i>	silica-dried leaf tissue	UPM	2164-72	<i>Flynn</i> 24	Sierra Nevada, Spain, 1972
<i>Iberis saxatilis</i>	silica-dried leaf tissue	P	J. Jurasek 255	N/A	Unknown
<i>Lobularia maritima</i>	silica-dried leaf tissue	UPM	N/A	N/A	1918-67, 1967
<i>Phyllolepidum cyclocarpum</i>	silica-dried leaf tissue	P	J. Jurasek 256	<i>Flynn</i> 19	Bolkar Dag, Turkey
<i>Ptilotrichum canescens</i>	silica-dried leaf tissue	USDA	AMES23252	<i>Flynn</i> 4	Mongolia
<i>Tropaeolum majus</i>	silica-dried leaf tissue	CS	1260M 'Tom Thumb'	<i>Flynn</i> 67	Cultivated Variety

¹ **Seed Source Codes:**

AGS	Alpine Gardening Society Seed Exchange, Alpine Gardening Society, AGS Centre, Avon Bank, Pershore, Worcestershire, WR10 3JP, UK.
BGTH	Botanischer Garten der Technischen Hochschule, Aachen, Germany.
SRGC	Scottish Rock Garden Club Seed Exchange, c/o Prof. S. Pawley, Acres of Keillour, Methven, Perth, PH1 3RA, UK.
CS	Chilterns Seeds Ltd., Bortree Stile, Ulverston, Cumbria, LA12 7PB, UK.
MSB	Millennium Seed Bank, Wakehurst Place, Ardingly, Haywards Heath, West Sussex, RH17 6TN, UK.
P	Private Seed Collections (various).
UPM	Universidad Politécnica de Madrid Crucifer Seed Bank, Dep. Biología Vegetal, Escuela T.S. Ing. Agrónomos, Universidad Politécnica de Madrid, Madrid, 28040, Spain.
USDA	United States Department for Agriculture North Central Regional Plant Introduction Station, Plant Introduction Unit, Iowa State University, Ames, Iowa, 50011, USA.

² All vouchers specimens have been deposited at Fielding-Druce Herbarium, Department of Plant Sciences, University of Oxford, South Parks Road, Oxford, OX1 3RB.

Appendix 3:

Trait and Geographic Data

This Appendix contains nickel hyperaccumulation, serpentine endemism and life history strategy data (see Table A3-1) and geographic distribution data (see Table A3-2) for the species listed in Table A1-1 in Appendix 1.

Notes to Accompany Tables A3-1 and A3-2

Missing data

Where data have not been obtained, this is indicated by a dash (-).

^A Alysseae Clades:

Clades A1a, A1b, A1c, A2, A3, A4, A5, A6, A7 and A8 are described in the main text of the thesis. Parentheses indicate that a species was not sampled here and it has been assigned to a clade as detailed in Appendix 4.

^B Nickel Hyperaccumulator Status Codes:

- H Nickel hyperaccumulator species (i.e., species for which reported shoot Ni content is always $>1000 \text{ mg kg}^{-1}$ dry weight in wild-collected plants).
- N Non-accumulator species (i.e., species for which reported shoot Ni content is always $<1000 \text{ mg kg}^{-1}$ dry weight in wild-collected plants).
- P Polymorphic species (i.e., species for which shoot Ni contents of both >1000 and $<1000 \text{ mg kg}^{-1}$ dry weight have been reported in wild-collected plants).

NB The definition of nickel hyperaccumulation is fully discussed in *Chapter 1 Introduction*.

^C Maximum Nickel Content Data Sources:

- | | | | |
|---|------------------------------------|---|--------------------------|
| 1 | Minguzzi and Vergnano (1948) cited | 7 | Adigüzel & Reeves (2002) |
| | in Reeves (1992) | | |

2	Brooks & Radford (1978)	8	Fritas (2004)
3	Brooks <i>et al.</i> (1979)	9	Ghaderian <i>et al.</i> (2007a)
3b	Reeves (1980)	10	Ghaderian <i>et al.</i> (2007b)
4	Brooks, Shaw & Marfil (1981)	11	Reeves & Adigüzel (2008)
5	Reeves <i>et al.</i> (1983)	12	Reeves <i>et al.</i> (2009)
6	R. D. Reeves <i>et al.</i> (2001)	13	This study: see <i>Chapter 3 Results</i>

NB. Where necessary, values were rounded to the nearest 1 mg kg⁻¹

^D Serpentine Endemic Status Codes:

- E Strict serpentine endemic species (i.e., species for which wild specimens have not been reported outside serpentine habitats).
- NE Serpentine non-endemic species (i.e., species for which wild specimens have been reported outside serpentine habitats).

NB Where no native substrate information has been obtained this is indicated by a dash (-) (ie. missing data).

^E Serpentine Endemic Status Data Sources

1	Tutin <i>et al.</i> (1993) and Jalas & Suominen (1994)	11b	Reeves <i>et al.</i> (1997)
2	Strid & Tan (2002)	12	R. D. Reeves <i>et al.</i> (2001)
3	Castroviejo (1993)	13	Mengoni <i>et al.</i> (2003b)
4	Săvulescu <i>et al.</i> (1955)	14	Stevanović <i>et al.</i> (2003)
5	Davis (1965)	15	Özhatay and Kultur (2006)
6	Komarov <i>et al.</i> (1939)	16	Ghaderian <i>et al.</i> (2007a)
7	Dudley (1964b)	17	Ghaderian <i>et al.</i> (2007b)
8	Brooks <i>et al.</i> (1979)	18	Reeves and Adigüzel (2008)
9	Reeves (1980)	19	Reeves, Adigüzel & Baker (2009)
10	Brooks <i>et al.</i> (1981)	20	Orcan (2005)
11	Reeves <i>et al.</i> (1983)		

^F Life History Strategy Codes:

- A Annual
- B Biennial
- P Perennial

Note that where sources give conflicting information, multiple codes may be shown.

^G Data Sources for Life History Strategy and Geographic Range Descriptions

1	Quézel & Santa (1962)	25	Orcan (2006)
2	Meikle (1977)	26	Yilmaz (2012)
3	Tutin <i>et al.</i> (1993)	27	This study: see <i>Chapter 3 Results</i>
4	Strid & Tan (2002)	28	Dudley (1965b)
5	Castroviejo (1993)	29	Colla (1833)
6	Parsa (1978)	30	Burt & Davis (1949)
7	Townsend & Guest (1980)	31	Quézel (1953)
8	Maire <i>et al.</i> (1967)	32	Greuter & Raus (1985)
9	Post & Dinsmore (1932)	33	An (1981)
10	Davis (1965)	34	Orcan (2006)
11	Komarov <i>et al.</i> (1939)	35	<i>Flora of China</i> (online)
12	Global Biodiversity Information Facility (http://data.gbif.org/)		(http://www.efloras.org/flora_page.aspx?flora_id=2)
13	Dudley (1964a)	36	Wesche <i>et al.</i> (2006)
14	Hedge (1968)	37	Cecchi (2011)
15	Brooks, Shaw & Marfil (1981)	38	Herbarium specimen label: <i>Davis</i> 46018 (at Royal Botanic Garden Edinburgh)
16	Rodríguez-Oubiña & Ortiz (1991)	39	Herbarium specimen label: <i>Davis</i> 44623 (at Royal Botanic Garden Edinburgh)
17	Aytac & Aksoy (2000)		
18	Aytaç and Duman (2000)	40	<i>Flora of Israel</i> (online) (http://flora.huji.ac.il/)
19	Kavousi (2001)	41	<i>Flora of Pakistan</i> (online)
20	Adigüzel & Reeves (2002)		(http://www.tropicos.org/Project/Pakistan)
21	Ančev & Goranova (2006)	42	Herbarium specimen label: <i>Chandargy</i> &
22	Ghaderian <i>et al.</i> (2007b)		<i>Thomas</i> 12545 (at Royal Botanic Garden Edinburgh)
23	Orcan & Binzet (2009)	43	Dudley (1964c)
24	A. J. Pollard (pers. comm., 2011)	44	Hedge (1968)

^H Geographic Range Descriptions

Note that distribution descriptions for Turkey use the grid system employed in the *Flora of Turkey* (Davis, 1965). In this grid, the vertical sectors A, B, and C correspond to latitudes of 42°–40° N, 40°–38° N, and 38°–36° N, respectively; the horizontal sectors 1 to 9 correspond to longitudes of 26°–28° E, 28°–30° E, etc., to 42°–44° E, respectively.

^{I,J} Western and Eastern Points

These are the two points most distant from one another (as measured by the shortest distance across the surface of the Earth) which lie within the distribution of a species (they necessarily lie on the perimeter of that distribution). These points do not necessarily represent the westernmost and easternmost edges of the distribution; they are referred to as eastern and western points merely in order to distinguish them from one another.

^K Latitude Data

Latitude is expressed in decimal degrees north.

^L Longitude Data

Longitude is expressed in decimal degrees east, with locations in the Western Hemisphere indicated by negative values (i.e., 2.5 decimal degrees west of Greenwich is expressed as -2.5 decimal degrees east).

^M Linear Range Estimator

The linear range estimator is the shortest distance (as measured across the surface of the Earth) between the Western and Eastern points. It represents the longest of all possible minimum surface distance lines across the range of a species. It is employed here as an alternative to species range area where the limits of a species distribution are known, but where detailed distribution information (such as herbarium specimen collection localities) is lacking.

^N Range Area (*Atlas Flora Europaeae*)

Range Area was obtained by counting the total number of 50 × 50 km grid squares in which a species is recorded as present in the species distribution maps of the *Atlas Flora Europaeae* Vol. 10 (Jalas & Suominen, 1994). It was only possible to obtain Range Area data for species included in that publication. Where the range of a species has been subject to significant anthropogenic introductions beyond its native range, or its native range includes areas outside the limits of coverage of the *Atlas Flora Europaeae*, Range Area will be over- and under-estimated, respectively by this measure. In such cases the Range Data figure is shown in square brackets and has been excluded from analyses.

Table 3-1. Table showing Alyseae clade membership, nickel hyperaccumulator status, maximum reported nickel content, serpentine endemic status and life history strategy for species of Alyseae. See accompanying notes on page A3-1 to A3-4.

Name	Alyseae Clade ^A	Ni Hyper-accumulator Status ^B	Max Ni Content ^C (mg kg ⁻¹)	Serpentine Endemic Status ^{D,E}	Life history Strategy ^{F,G}
<i>Alyssoides cretica</i>	A3	N	36 ⁵	N ²	p ⁴
<i>Alyssoides utriculata</i>	A3	N	36 ^{3,5}	N ²	p ⁴
<i>Alyssum aizoides</i>	A1a	N	10 ³	-	p ²⁷
<i>Alyssum akamasicum</i>	A1a	H	9090 ³	E ⁸	p ³⁰
<i>Alyssum alpestre</i>	A1a	P	3640 ^{2,3}	N ³	p ^{3,5}
<i>Alyssum alyssoides</i>	A2	N	5 ²	N ^{2,3,6,7}	A/B ^{3,4,5,10}
<i>Alyssum anatolicum</i>	A1a	P	8170 ³	N ¹⁸	p ³⁸
<i>Alyssum andinum</i>	(A2)	-	-	N ⁶	p ¹¹
<i>Alyssum antiatlanticum</i>	(A2)	-	-	-	-
<i>Alyssum arabicum</i>	-	-	-	-	-
<i>Alyssum argenteum</i>	A1a	H	29400 ^{2,3}	-	p ³
<i>Alyssum argyrophyllum</i>	A2	N	4 ³	-	p ¹⁰
<i>Alyssum armenum</i>	A2	N	3 ³	-	p ^{10,11}
<i>Alyssum artwinense</i>	(A2)	N	9 ³	-	p ^{10,11}
<i>Alyssum aurantiacum</i>	A2	N	0 ³	-	-
<i>Alyssum aureum</i>	A1b	N	3 ³	N ⁷	A ²⁸
<i>Alyssum austrodalmaticum</i>	-	-	-	-	-
<i>Alyssum baldaccii</i>	A1a	H	3960 ^{2,3}	E ^{11b}	p ³
<i>Alyssum bargalense</i>	-	-	-	-	-
<i>Alyssum baumgartnerianum</i>	A2	N	65 ^{3,10}	N ^{5,17}	p ¹⁰
<i>Alyssum bertolonii</i>	A1a	H	13400 ^{1,2,3}	E ¹³	p ³

Table 3-1. Table showing Alyseae clade membership, nickel hyperaccumulator status, maximum reported nickel content, serpentine endemic status and life history strategy for species of Alyseae. See accompanying notes on page A3-1 to A3-4.

Name	Alyseae Clade ^A	Ni Hyper-accumulator Status ^B	Max Ni Content ^C (mg kg ⁻¹)	Serpentine Endemic Status ^{D,E}	Life history Strategy ^{F,G}
<i>Alyssum blancheanum</i>	(A1a)	-	-	-	-
<i>Alyssum blepharocarpum</i>	A1b	N	9 ³	N ⁷	A ²⁸
<i>Alyssum bornmuelleri</i>	A2	N	3 ²	N ⁵	p ^{(10), 27}
<i>Alyssum bracteatum</i>	A1a	P	2300 ¹⁰	N ¹⁶	p ^{6, 11}
<i>Alyssum brughieri</i>	(A1a)	-	-	-	p ^{(3), 29}
<i>Alyssum bulbotrichum</i>	(A2)	N	10 ³	-	A ¹⁰
<i>Alyssum caespitosum</i>	A2	N	10 ³	N ⁵	p ³⁹
<i>Alyssum callichroum</i>	A1a	P	18730 ^{3, 6, 11}	N ¹⁸	p ¹⁰
<i>Alyssum calycocarpum</i>	A2	N	1 ^{2, 3}	N ^{5, 6}	p ^{10, 11}
<i>Alyssum caricum</i>	A1a	H	16500 ^{3, 5}	E ^{5, 18}	p ¹⁰
<i>Alyssum cassium</i>	A1a	H	26100 ^{3, 6}	E ¹⁸	B ¹⁰
<i>Alyssum cephalotes</i>	(A2)	N	3 ³	-	A ¹⁰
<i>Alyssum chondrogynum</i>	A1a	H	16300 ³	E ¹⁸	p ³⁰
<i>Alyssum cilicicum</i>	A1a	H	14840 ³	E ¹⁸	p ¹⁰
<i>Alyssum condensatum</i>	A1a	P	4990 ³	N ¹⁸	p ¹⁰
<i>Alyssum constellatum</i>	A1a	P	18130 ^{3, 6}	N ¹⁸	p ¹⁰
<i>Alyssum contemptum</i>	A2	N	3 ³	N ⁵	A ¹⁰
<i>Alyssum corningii</i>	(A2)	N	3 ³	N ⁵	p ¹⁰
<i>Alyssum corsicum</i>	A1a	H	21500 ^{2, 3}	E ^{8, 18}	p ¹⁰
<i>Alyssum corymbosoides</i>	(A1a)	N	2 ^{2, 3}	N ²	p ⁴
<i>Alyssum crenulatum</i>	A1a	H	21010 ³	E ¹⁸	B ¹⁰

Table 3-1. Table showing Alyseae clade membership, nickel hyperaccumulator status, maximum reported nickel content, serpentine endemic status and life history strategy for species of Alyseae. See accompanying notes on page A3-1 to A3-4.

Name	Alyseae Clade ^A	Ni Hyper-accumulator Status ^B	Max Ni Content ^C (mg kg ⁻¹)	Serpentine Endemic Status ^{D,E}	Life history Strategy ^{F,G}
<i>Alyssum cuneifolium</i>	A2	N	2 ^{2,3}	N ³	P ^{4,5}
<i>Alyssum cypricum</i>	A1a	H	23600 ^{3,6}	E ^{8,18}	P ¹⁰
<i>Alyssum damascenum</i>	A2	N	4 ³	N ⁷	A ²⁸
<i>Alyssum dasycarpum</i>	A2	N	6 ^{2,3}	N ⁷	A ^{11,28}
<i>Alyssum davisianum</i>	A1a	H	19600 ³	E ¹⁸	P ¹⁰
<i>Alyssum debarensae</i>	-	-	-	-	-
<i>Alyssum densistellatum</i>	A2	N	40 ^{2,3}	E ^{2,14}	P ⁴
<i>Alyssum desertorum</i>	A2	N	83 ^{2,3}	N ⁵	A ^{10,11}
<i>Alyssum diffusum</i>	A2	N	3 ^{2,3}	-	P ³
<i>Alyssum discolor</i>	A1a	H	11700 ^{3,6}	E ¹⁸	P ¹⁰
<i>Alyssum djurdjurae</i>	(A2)	-	-	-	-
<i>Alyssum doerfleri</i>	(A2)	N	2 ^{2,3}	N ²	P ⁴
<i>Alyssum dubertretii</i>	A1a	H	19250 ³	E ¹⁸	P ¹⁰
<i>Alyssum dudleyi</i>	A1a	H	22100 ^{6,7}	E ¹⁸	P ²⁰
<i>Alyssum embergeri</i>	(A2)	-	-	-	P ^{(4),31}
<i>Alyssum eriophyllum</i>	A1a	H	11500 ³	E ¹⁸	B/P ¹⁰
<i>Alyssum erosulum</i>	A2	N	2 ³	-	P ¹⁰
<i>Alyssum euboicum</i>	A1a	P	4550 ^{2,3,5}	E ^{2,8,14}	P ⁴
<i>Alyssum fedtschenkoanum</i>	(A1a)	N	7 ³	N ⁶	P ¹¹
<i>Alyssum filiforme</i>	A1a	N	814 ³	-	P ¹⁰
<i>Alyssum flahaultianum</i>	A2	N	57 ¹³	-	P ³²

Table 3-1. Table showing Alyseae clade membership, nickel hyperaccumulator status, maximum reported nickel content, serpentine endemic status and life history strategy for species of Alyseae. See accompanying notes on page A3-1 to A3-4.

Name	Alyseae Clade ^A	Ni Hyper-accumulator Status ^B	Max Ni Content ^C (mg kg ⁻¹)	Serpentine Endemic Status ^{D,E}	Life history Strategy ^{F,G}
<i>Alyssum floribundum</i>	A1a	P	22350 ^{3,6}	E ¹⁸	P ¹⁰
<i>Alyssum foliosum</i>	A2	N	<1 ^{2,3}	N ²	A ⁴
<i>Alyssum fragillimum</i>	A1a	N	<1 ^{2,3}	N ²	P ⁴
<i>Alyssum fulvescens</i>	A2	N	<1 ³	N ²	A ⁴
<i>Alyssum gadorensense</i>	(A1a)	-	-	N ³	P ⁵
<i>Alyssum gehamense</i>	A1a	N	<1 ³	-	P ¹⁰
<i>Alyssum gevgelicense</i>	-	-	-	-	-
<i>Alyssum giosnanum</i>	A1a	H	7390 ³	E ¹⁸	P ¹⁰
<i>Alyssum globosum</i>	-	-	-	-	-
<i>Alyssum granatense</i>	A2	N	6 ^{2,3}	N ^{3,7}	A ^{5,28}
<i>Alyssum gustavssonii</i>	(A2)	-	-	N ²	P ⁴
<i>Alyssum hajastanum</i>	-	-	-	-	-
<i>Alyssum handelii</i>	A2	N	5 ^{2,3}	N ²	P ⁴
<i>Alyssum harputicum</i>	A2	N	6 ³	-	P ¹⁰
<i>Alyssum haussknechtii</i>	A1a	N	274 ³	N ⁵	P ¹⁰
<i>Alyssum heideri</i>	-	-	-	-	-
<i>Alyssum heldreichii</i>	A1a	H	12500 ^{2,3}	E ¹⁴	P ¹⁰
<i>Alyssum heterotrichum</i>	A1b	N	12 ³	N ⁷	A ²⁸
<i>Alyssum hirsutum</i>	A2	N	9 ²	N ⁵	A ^{10,11}
<i>Alyssum homalocarpum</i>	(A2)	N	7 ³	N ⁷	A ²⁸
<i>Alyssum huber-morathii</i>	A1a	H	13500 ³	N ¹⁸	P ¹⁰

Table 3-1. Table showing Alyseae clade membership, nickel hyperaccumulator status, maximum reported nickel content, serpentine endemic status and life history strategy for species of Alyseae. See accompanying notes on page A3-1 to A3-4.

Name	Alyseae Clade ^A	Ni Hyper-accumulator Status ^B	Max Ni Content ^C (mg kg ⁻¹)	Serpentine Endemic Status ^{D,E}	Life history Strategy ^{F,G}
<i>Alyssum huetii</i>	A1b	N	1 ³	N ⁷	A ²⁸
<i>Alyssum idaeum</i>	(A2)	N	3 ^{2,3}	N ²	p ⁴
<i>Alyssum iljinskae</i>	-	-	-	-	-
<i>Alyssum iranicum</i>	A2	N	19 ^{3,5}	-	p ⁴⁰
<i>Alyssum kavadsense</i>	-	-	-	-	-
<i>Alyssum kaynakiae</i>	A2	-	-	-	B ²⁶
<i>Alyssum kurdicum</i>	-	-	-	-	-
<i>Alyssum lanceolatum</i>	A2	N	3 ³	-	p ⁴¹
<i>Alyssum lanigerum</i>	A1a	N	42 ³	-	p ⁶
<i>Alyssum lassiticum</i>	(A2)	N	10 ^{2,3}	N ²	p ⁴
<i>Alyssum lenense</i>	A1a	P	1030 ^{2,3}	N ⁶	p ³
<i>Alyssum lepidoto-stellatum</i>	A2	N	3 ³	N ⁵	p ¹⁰
<i>Alyssum lepidotum</i>	(A2)	N	7 ³	N ⁵	p ¹⁰
<i>Alyssum lesbiacum</i>	A1a	H	22400 ³	E ^{2,5,8,}	p ^{4,10}
<i>Alyssum libanoticum</i>	(A1a)	N	<1 ³	-	-
<i>Alyssum linifolium</i>	A2	N	16 ^{2,3}	N ⁷	A ²⁸
<i>Alyssum loiseleurii</i>	A2	N	9 ^{2,3}	N ^{1,3}	p ^{3,5}
<i>Alyssum luteolum</i>	(A2)	-	-	-	-
<i>Alyssum lycaonicum</i>	A2	N	33 ³	N ⁵	p ¹⁰
<i>Alyssum macrocalyx</i>	A2	N	5 ³	-	-
<i>Alyssum macropodum</i>	A2	N	5 ³	N ⁵	A ¹⁰

Table 3-1. Table showing Alyseae clade membership, nickel hyperaccumulator status, maximum reported nickel content, serpentine endemic status and life history strategy for species of Alyseae. See accompanying notes on page A3-1 to A3-4.

Name	Alyseae Clade ^A	Ni Hyper-accumulator Status ^B	Max Ni Content ^C (mg kg ⁻¹)	Serpentine Endemic Status ^{D,E}	Life history Strategy ^{F,G}
<i>Alyssum magicum</i>	(A2)	-	-	-	p ³³
<i>Alyssum malacitanum</i>	A1a	H	5000 ⁴	E ¹⁰	p ⁵
<i>Alyssum masmenaeum</i>	A1a	H	24300 ^{3,6}	E ¹⁸	p ¹⁰
<i>Alyssum menioides</i>	(A1b)	N	90 ¹⁰	N ⁷	A ²⁸
<i>Alyssum microphylliforme</i>	-	-	-	-	-
<i>Alyssum microphyllum</i>	(A2)	N	152 ³	-	-
<i>Alyssum minutum</i>	A2	N	22 ^{2,3}	N ^{2,3}	A ^{4,5,11}
<i>Alyssum misirdalianum</i>	(A2)	-	-	-	p ²³
<i>Alyssum moellendorffianum</i>	(A2)	N	3 ^{2,3}	-	p ³
<i>Alyssum montanum</i>	A2	N	22 ^{3,5}	N ^{2,3,6}	p ^{3,4,11}
<i>Alyssum mouradicum</i>	A2	N	12 ¹²	N ¹⁹	p ¹⁰
<i>Alyssum mozaffarianii</i>	(A1a)	-	-	-	p ¹⁹
<i>Alyssum muelleri</i>	A2	N	11 ³	-	p ¹¹
<i>Alyssum mughlaei</i>	(A1a)	-	-	E ²⁰	p ³⁴
<i>Alyssum murale</i>	A1a	P	13700 ^{2,3,6}	N ^{2,18,}	p ^{4,11}
<i>Alyssum musilii</i>	-	-	-	-	-
<i>Alyssum nebrodense</i>	A1a	N	11 ^{2,3}	N ²	p ⁴
<i>Alyssum nezaketiae</i>	(A2)	-	-	N ¹⁵	p ¹⁸
<i>Alyssum niveum</i>	A2	N	5 ³	N ⁵	p ¹⁰
<i>Alyssum obovatum</i>	A1a	P	4590 ^{2,3}	N ¹⁶	p ^{3,11}
<i>Alyssum obtusifolium</i>	A1a	N	60 ^{2,3}	N ⁶	p ^{3,11}

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Name	Alyseae Clade ^A	Ni Hyper-accumulator Status ^B	Max Ni Content ^C (mg kg ⁻¹)	Serpentine Endemic Status ^{D,E}	Life history Strategy ^{F,G}
<i>Alyssum ochroleucum</i>	A2	N	2 ³	N ⁵	p ¹¹
<i>Alyssum orbelicum</i>	(A1a)	-	-	-	-
<i>Alyssum ovirense</i>	A2	N	2 ^{2,3}	-	p ³
<i>Alyssum oxycarpum</i>	A1a	P	16250 ³	N ¹⁸	p ¹⁰
<i>Alyssum paphlagonicum</i>	A2	N	60 ³	-	p ¹⁰
<i>Alyssum pateri</i>	A1a	P	17090 ^{3,11}	N ¹⁸	p ¹⁰
<i>Alyssum peltarioides</i>	A1a	P	34690 ^{3,11}	N ¹⁸	p ^{10,11}
<i>Alyssum penjwinense</i>	A1a	P	7860 ^{3,10}	N ¹⁶	p ⁷
<i>Alyssum persicum</i>	A2	N	3 ³	-	p ⁶
<i>Alyssum pinifolium</i>	A1a	H	21800 ^{3,6}	E ¹⁸	p ¹⁰
<i>Alyssum pintodasilvae</i>	A1a	H	38105 ⁸	E ¹⁰	p ⁵
<i>Alyssum pirinicum</i>	(A2)	-	-	-	p ^{4,5,21}
<i>Alyssum pogonocarpum</i>	(A2)	-	-	E ²	-
<i>Alyssum polycladum</i>	A1a	N	127 ³	-	-
<i>Alyssum praecox</i>	A2	N	<1 ³	N ⁵	p ¹⁰
<i>Alyssum propinquum</i>	A2	N	67 ³	-	p ¹⁰
<i>Alyssum pseudomouradicum</i>	A2	N	9 ³	N ⁵	p ¹⁰
<i>Alyssum pterocarpum</i>	A1a	H	22200 ^{3,6}	E ¹⁸	p ¹⁰
<i>Alyssum pulvinare</i>	(A2)	N	9 ^{2,3}	N ²	p ⁴
<i>Alyssum repens</i>	A2	N	6 ^{2,3}	N ⁵	p ^{3,10,11}
<i>Alyssum robertianum</i>	A1a	H	12500 ^{2,3}	-	p ³

Table 3-1. Table showing Alyseae clade membership, nickel hyperaccumulator status, maximum reported nickel content, serpentine endemic status and life history strategy for species of Alyseae. See accompanying notes on page A3-1 to A3-4.

Name	Alyseae Clade ^A	Ni Hyper-accumulator Status ^B	Max Ni Content ^C (mg kg ⁻¹)	Serpentine Endemic Status ^{D,E}	Life history Strategy ^{F,G}
<i>Alyssum rostratum</i>	A2	N	10 ^{2,3}	N ⁶	A/B ^{3,11}
<i>Alyssum samariferum</i>	A1a	H	24350 ³	E ¹⁸	P ¹⁰
<i>Alyssum samium</i>	(A1a)	-	-	N ²	P ⁴
<i>Alyssum scardicum</i>	A2	N	26 ^{2,3}	-	P ³
<i>Alyssum scutigerum</i>	A2	N	9 ³	-	A ¹
<i>Alyssum serpentinum</i>	-	-	-	-	-
<i>Alyssum serpyllifolium</i>	A1a	N	4 ^{2,3}	N ^{3,10}	P ^{3,5}
<i>Alyssum sibiricum</i>	A1a	P	8810 ^{2,3,6,11}	N ^{2,18}	P ⁴
<i>Alyssum siculum</i>	(A2)	-	-	N ²	A ⁴
<i>Alyssum simplex</i>	A2	N	5 ^{2,3,10}	N ^{2,3}	A ^{4,5}
<i>Alyssum simulans</i>	(A2)	-	-	-	A ⁴
<i>Alyssum singarense</i>	A1a	H	1280 ³	-	P ⁷
<i>Alyssum skopjense</i>	-	-	-	-	-
<i>Alyssum smolikanum</i>	(A1a)	H	6600 ^{2,3}	E ^{2,14}	P ⁴
<i>Alyssum smyrnaeum</i>	A2	N	1 ^{2,3}	N ²	A ⁴
<i>Alyssum sphacioticum</i>	(A2)	N	2 ^{2,3}	N ²	P ⁴
<i>Alyssum stapfii</i>	A2	N	70 ^{3,10}	-	A ^{10,11}
<i>Alyssum stipitatum</i>	(A1a)	-	-	-	P ¹⁹
<i>Alyssum stribryni</i>	A1a	N	15 ^{2,3}	N ⁵	P ¹⁰
<i>Alyssum strictum</i>	A2	N	75 ^{2,10}	N ⁵	A ¹⁰
<i>Alyssum strigosum</i>	A2	N	5 ^{2,3}	N ²	A ⁴

Table 3-1. Table showing Alyseae clade membership, nickel hyperaccumulator status, maximum reported nickel content, serpentine endemic status and life history strategy for species of Alyseae. See accompanying notes on page A3-1 to A3-4.

Name	Alyseae Clade ^A	Ni Hyper-accumulator Status ^B	Max Ni Content ^C (mg kg ⁻¹)	Serpentine Endemic Status ^{D,E}	Life history Strategy ^{F,G}
<i>Alyssum stylare</i>	A1b	N	14 ³	N ⁷	A ²⁸
<i>Alyssum subbaicalicum</i>	-	-	-	-	-
<i>Alyssum subspinosum</i>	A1a	N	7 ³	-	p ⁴²
<i>Alyssum sulphureum</i>	A2	N	34 ³	N ⁵	p ¹⁰
<i>Alyssum syriacum</i>	(A1a)	H	10200 ^{3,11}	E ¹⁸	p ¹⁰
<i>Alyssum szarabiacum</i>	(A1a)	-	-	-	-
<i>Alyssum szovitsianum</i>	A2	N	6 ³	N ⁵	A ^{10,11}
<i>Alyssum taygeteum</i>	(A2)	N	2 ^{2,3}	N ²	p ⁴
<i>Alyssum tenium</i>	A1a	H	3420 ^{2,3}	E ⁸	p ⁴
<i>Alyssum tetrastemon</i>	A1a	N	75 ³	N ⁵	p ¹⁰
<i>Alyssum thymops</i>	A2	N	16 ³	N ⁵	p ¹⁰
<i>Alyssum tortuosum</i>	A1a	P	3700 ^{2,3,9}	N ^{6,17}	p ^{10,11}
<i>Alyssum trapeziforme</i>	(A1a)	H	11930 ³	E ¹⁸	p ¹⁰
<i>Alyssum trichocarpum</i>	(A2)	N	14 ³	-	A
<i>Alyssum troodi</i>	A1a	H	17100 ³	E ⁸	p ²
<i>Alyssum turgidum</i>	A1a	N	36 ³	-	p ⁴³
<i>Alyssum turkestanicum</i>	A2	N	5 ³	N ²	A ⁴
<i>Alyssum umbellatum</i>	A2	N	893 ^{2,3}	N ²	A ^{4,11}
<i>Alyssum virgatum</i>	A1a	H	6230 ³	E ¹⁸	p ¹⁰
<i>Alyssum wierzbickii</i>	(A2)	N	1 ^{2,3}	-	B/P ³
<i>Alyssum wulfenianum</i>	A2	N	1 ^{2,3}	-	p ³

Table 3-1. Table showing Alysseae clade membership, nickel hyperaccumulator status, maximum reported nickel content, serpentine endemic status and life history strategy for species of Alysseae. See accompanying notes on page A3-1 to A3-4.

Name	Alysseae Clade ^A	Ni Hyper-accumulator Status ^B	Max Ni Content ^C (mg kg ⁻¹)	Serpentine Endemic Status ^{D,E}	Life history Strategy ^{F,G}
<i>Alyssum xanthocarpum</i>	A2	N	20 ³	-	A ¹⁰
<i>Aurinia corymbosa</i>	A6	N	24 ^{2,3}	N ²	P ⁴
<i>Aurinia gionae</i>	(A6)	-	-	N ²	P ⁴
<i>Aurinia leucadea</i>	(A6)	N	24 ^{2,5}	-	P ³
<i>Aurinia moreana</i>	(A6)	-	-	N ²	P ⁴
<i>Aurinia petraea</i>	A6	N	24 ^{2,5}	N ⁴	P ³
<i>Aurinia saxatilis</i>	A6	N	24 ^{2,5}	N ^{2,4}	P ^{4,11}
<i>Aurinia sinuata</i>	(A6)	N	0 ⁵	-	P ³
<i>Aurinia uechtritiziana</i>	A6	N	24 ⁵	N ^{1,5}	P ¹⁰
<i>Berteroa gintlilii</i>	(A6)	N	0 ⁵	-	A/B ³
<i>Berteroa incana</i>	A6	N	14 ⁵	N ²	A/B ^{4,11}
<i>Berteroa mutabilis</i>	A6	N	14 ⁵	N ²	B/P ^{4,10,11}
<i>Berteroa obliqua</i>	A6	N	14 ⁵	N ²	B/P ⁴
<i>Berteroa orbiculata</i>	(A6)	N	14 ⁵	N ²	B/P ⁴
<i>Bornmuellera angustifolia</i>	A5	N	57 ³	N ¹¹	P ⁵
<i>Bornmuellera baldaccii</i>	A5	H	27300 ⁵	E ^{2,14}	P ⁴
<i>Bornmuellera cappadocica</i>	A5	N	48 ⁵	N ¹¹	P ¹⁰
<i>Bornmuellera dieckii</i>	(A5)	-	-	-	P ³
<i>Bornmuellera glabrescens</i>	(A5)	H	19200 ^{5,11}	E ¹¹	P ¹⁰
<i>Bornmuellera kiyakii</i>	A5	H	12590 ^{11,12}	E ¹¹	P ¹⁷
<i>Bornmuellera tymphaea</i>	A5	H	31200 ⁵	E ^{1,2}	P ⁴

Table 3-1. Table showing Alysseae clade membership, nickel hyperaccumulator status, maximum reported nickel content, serpentine endemic status and life history strategy for species of Alysseae. See accompanying notes on page A3-1 to A3-4.

Name	Alysseae Clade ^A	Ni Hyper-accumulator Status ^B	Max Ni Content ^C (mg kg ⁻¹)	Serpentine Endemic Status ^{D,E}	Life history Strategy ^{F,G}
<i>Clastopus erubescens</i>	A3	N	5 ⁵	-	p ⁶
<i>Clastopus vestitus</i>	A3	N	5 ⁵	-	p ⁶
<i>Clypeola aspera</i>	A1c	N	36 ⁵	N ⁵	A ¹⁰
<i>Clypeola ciliata</i>	A8	-	-	-	A ¹⁰
<i>Clypeola cyclodontea</i>	(A1c)	N	36 ⁵	N ³	A ⁵
<i>Clypeola dichotoma</i>	A1c	N	36 ⁵	-	A ⁶
<i>Clypeola elegans</i>	(A1c)	-	-	-	A ¹⁰
<i>Clypeola eriocarpa</i>	(A1c)	N	36 ⁵	N ^{3,6}	A ¹¹
<i>Clypeola jonthlaspi</i>	A1c	N	41 ⁵	N ^{2,6}	A ^{4,10}
<i>Clypeola lappacea</i>	A1c	N	36 ⁵	N ⁵	A ¹⁰
<i>Clypeola raddeana</i>	(A1c)	N	36 ³	N ⁶	A ¹⁰
<i>Degenia velebitica</i>	A3	N	6 ⁵	-	p ³
<i>Fibigia clypeata</i>	A3	N	72 ⁵	N ^{2,3}	p ^{4,5,11}
<i>Fibigia compacta</i>	(A3)	N	10 ⁵	-	-
<i>Fibigia heterophylla</i>	(A3)	-	-	-	-
<i>Fibigia lunarioides</i>	A3	N	10 ⁵	N ²	p ⁴
<i>Fibigia macrocarpa</i>	A3	N	6 ⁵	-	p ^{10,11}
<i>Fibigia membranacea</i>	A3	N	10 ⁵	-	-
<i>Fibigia multicaulis</i>	A3	-	-	-	p ⁶
<i>Fibigia pendula</i>	(A3)	-	-	-	p ⁶
<i>Fibigia spathulata</i>	A3	-	-	-	p ¹¹

Table 3-1. Table showing Alysseae clade membership, nickel hyperaccumulator status, maximum reported nickel content, serpentine endemic status and life history strategy for species of Alysseae. See accompanying notes on page A3-1 to A3-4.

Name	Alysseae Clade ^A	Ni Hyper-accumulator Status ^B	Max Ni Content ^C (mg kg ⁻¹)	Serpentine Endemic Status ^{D,E}	Life history Strategy ^{F,G}
<i>Fibigia suffruticosa</i>	A3	N	10 ⁵	-	p ^{10,11}
<i>Fibigia thesigeri</i>	A3	-	-	-	-
<i>Fibigia triquetra</i>	A3	N	10 ⁵	-	p ³
<i>Fibigia umbellata</i>	A3	N	20 ¹³	-	p ⁶
<i>Galitzkya macrocarpa</i>	(A6)	N	0 ⁵	-	p ³⁶
<i>Galitzkya potaninii</i>	(A6)	-	-	-	p ³⁵
<i>Galitzkya spathulata</i>	A6	-	-	-	p ³⁵
<i>Hormathophylla baetica</i>	(A4)	N	3 ³	N ¹	p ³
<i>Hormathophylla cadevalliana</i>	(A4)	-	-	N ³	p ³
<i>Hormathophylla halimifolia</i>	A4	N	24 ⁵	-	p ³
<i>Hormathophylla lapeyrousiana</i>	A4	N	7 ³	N ^{1,3}	p ³
<i>Hormathophylla longicaulis</i>	A4	N	9 ⁵	N ^{1,3}	p ³
<i>Hormathophylla macrocarpa</i>	(A4)	-	-	N ¹	p ³
<i>Hormathophylla purpurea</i>	A4	-	5 ³	-	p ³
<i>Hormathophylla pyrenaica</i>	(A4)	N	9 ⁵	N ¹	p ³
<i>Hormathophylla reverchonii</i>	A4	N	9 ⁵	N ^{1,3}	p ³
<i>Hormathophylla spinosa</i>	A4	N	9 ³	N ³	p ³
<i>Leptoplax emarginata</i>	A5	H	34400 ^{3b,4b}	E ^{2,9,14}	p ³
<i>Phyllolepidum cyclocarpum</i>	A7	N	24 ⁵	N ^{1,2}	p ^{3,4}
<i>Phyllolepidum cyclocarpum</i>	(A7)	-	24 ⁵	-	p ³⁷
<i>Physocardamum davisii</i>	A5	N	165 ¹³	-	p ⁴⁴

Table 3-1. Table showing Alysseae clade membership, nickel hyperaccumulator status, maximum reported nickel content, serpentine endemic status and life history strategy for species of Alysseae. See accompanying notes on page A3-1 to A3-4.

Name	Alysseae Clade ^A	Ni Hyper-accumulator Status ^B	Max Ni Content ^C (mg kg ⁻¹)	Serpentine Endemic Status ^{D,E}	Life history Strategy ^{F,G}
<i>Physoptychis caspica</i>	A3	N	581 ⁵	-	p ¹⁰
<i>Physoptychis haussknechtii</i>	(A3)	N	147 ⁵	-	p ¹⁰
<i>Straussiella purpurea</i>	A3	N	6 ⁵	-	p ⁶

Table A3-2. Table showing geographic distribution description, most distant points in the distribution, Linear Range Estimator and Range Area for species of Alysseae. See accompanying notes on Page A3-1 to A3-4.

Name	Geographic Range Description	Range Data Sources ^G	Western point ^I latitude (decimal degrees North ^K)	Western point ^I longitude (decimal degrees East ^L)	Eastern point ^J latitude (decimal degrees North ^K)	Eastern point ^J longitude (decimal degrees East ^L)	Linear Range Estimator (km) ^M	Range Area (Atlas Flora Europaeae) (No. 50×50 km grid cells) ^N
<i>Alyssoides cretica</i>	Central Crete to Karpathos & Astapalaya. Distribution map in <i>Flora Hellenica</i> .	4	35.42	24.24	35.90	27.23	279	-
<i>Alyssoides utriculata</i>	SC Alps to NE Turkey.	3, 10	46.15	9.13	48.12	43.12	2753	102
<i>Alyssum aizoides</i>	Endemic to Turkey: C4 to A7.	10	36.50	31.98	41.04	40.51	741	-
<i>Alyssum akamasicum</i>	Locally common on serpentine in Akamas peninsular, Cyprus.	2	35.10	32.28	35.01	32.35	36	-
<i>Alyssum alpestre</i>	NW Italy.	3	43.88	7.89	46.45	8.31	283	13
<i>Alyssum alyssoides</i>	Widespread introductions obscure native range. Excluded from analysis.	3, 4, 5	-	-	-	-	-	[1228]
<i>Alyssum anatolicum</i>	Endemic to Turkey: C6 & B6.	10	36.00	36.00	40.00	38.00	478	-
<i>Alyssum andinum</i>	Endemic to Dagestan, Caucasus.	11	45.00	45.72	41.23	47.83	450	-
<i>Alyssum antiatlanticum</i>	Insufficient distribution data available.	-	-	-	-	-	-	-
<i>Alyssum arabicum</i>	Insufficient distribution data available.	-	-	-	-	-	-	-
<i>Alyssum argenteum</i>	SW Alps, Italy.	3	44.27	6.86	45.94	8.13	212	6
<i>Alyssum argyrophyllum</i>	Endemic to Turkey: Taurus C2 to C5.	10	38.00	28.00	36.00	36.00	740	-

Table A3-2. Table showing geographic distribution description, most distant points in the distribution, Linear Range Estimator and Range Area for species of Alyseae. See accompanying notes on Page A3-1 to A3-4.

Name	Geographic Range Description	Range Data Sources ^G	Western point ^I latitude (decimal degrees North ^K)	Western point ^I longitude (decimal degrees East ^L)	Eastern point ^I latitude (decimal degrees North ^K)	Eastern point ^I longitude (decimal degrees East ^L)	Linear Range Estimator (km) ^M	Range Area (Atlas Flora Europaeae) (No. 50×50 km grid cells) ^N
<i>Alyssum armenum</i>	N Anatolia A3 to B10. Caucasia: Armenia & Kurdistan. (I.e. A3 in Turkey to Caspian Sea.)	10, 11	41.15	30.00	39.95	49.39	1644	-
<i>Alyssum artwinense</i>	Endemic to Turkey: A8.	10	40.00	40.00	41.50	42.00	211	-
<i>Alyssum aurantiacum</i>	Endemic to Turkey. SW Anatolia. C1 & C2.	10	37.06	27.24	38.00	30.00	266	-
<i>Alyssum aureum</i>	SC Turkey & Syria. B7 to S Syria.	10	32.33	36.84	40.00	40.00	879	-
<i>Alyssum austrodalmaticum</i>	W Balkans.	3	43.66	16.92	42.76	16.88	112	2
<i>Alyssum baldaccii</i>	CE Greece & Crete.	3, 4	39.43	22.52	34.98	25.06	545	3
<i>Alyssum bargalense</i>	Insufficient distribution data available.	-	-	-	-	-	-	-
<i>Alyssum baumgartnerianum</i>	Turkey: Taurus: C3 to C6. W Syria, N Iraq. Khoy, Iran. I.e. C6, Turkey to Khoy, Iran.	7, 10, 22	38.00	36.00	38.55	44.95	797	-
<i>Alyssum bertolonii</i>	Strongly disjunct distribution (N Italy & Balkans). Excluded from analysis.	3	44.70	8.62	41.28	24.36	[1332]	[28]
<i>Alyssum blancheanum</i>	Insufficient distribution data available.	-	-	-	-	-	-	-
<i>Alyssum blepharocarpum</i>	Endemic to Turkey. Scattered in inner Anatolia: B4 to A8.	10	38.00	32.00	41.50	42.00	936	-

Table A3-2. Table showing geographic distribution description, most distant points in the distribution, Linear Range Estimator and Range Area for species of Alysseae. See accompanying notes on Page A3-1 to A3-4.

Name	Geographic Range Description	Range Data Sources ^G	Western point ^I latitude (decimal degrees North ^K)	Western point ^I longitude (decimal degrees East ^L)	Eastern point ^I latitude (decimal degrees North ^K)	Eastern point ^I longitude (decimal degrees East ^L)	Linear Range Estimator (km) ^M	Range Area (Atlas Flora Europaeae) (No. 50×50 km grid cells) ^N
<i>Alyssum bornmuelleri</i>	Endemic to Turkey: A5 to B6.	¹⁰	42.00	34	38.00	38.00	559	-
<i>Alyssum bracteatum</i>	S Caucasus & W, N, & C Iran.	^{6, 11}	41.48	44.88	31.58	54.60	1395	-
<i>Alyssum brughieri</i>	Insufficient distribution data available.	-	-	-	-	-	-	-
<i>Alyssum bulbotrichum</i>	Endemic to Turkey: A5 to B7.	¹⁰	42.00	34.00	38.00	40.00	675	-
<i>Alyssum caespitosum</i>	Endemic to Turkey: C5 to B6.	¹⁰	36.28	33.99	40.00	37.98	539	-
<i>Alyssum callichroum</i>	Endemic to Turkey: C5 to A9.	¹⁰	36.28	34.00	41.57	42.82	963	-
<i>Alyssum calycocarpum</i>	Turkey (C9), Crimea, Caucasia, N Iran, i.e. Crimea to N Iran.	^{3, 10}	45.00	33.76	35.15	54.18	2033	[6]
<i>Alyssum caricum</i>	Endemic to Turkey: C2.	¹⁰	36.56	27.98	38.00	30.00	237	-
<i>Alyssum cassium</i>	SC Turkey & N Syria. I.e. C6, Turkey to Lebanon.	¹⁰	38.00	38.00	34.65	36.01	411	-
<i>Alyssum cephalotes</i>	Insufficient distribution data available.	-	-	-	-	-	-	-
<i>Alyssum chondrogynum</i>	Endemic to Cyprus, Division 3.	²	34.73	32.45	34.94	33.45	93	-
<i>Alyssum cilicicum</i>	Endemic to Turkey: C5 & C6.	¹⁰	36.31	34.00	38.00	38.00	403	-
<i>Alyssum condensatum</i>	Widespread in C & S Turkey (A3 to C6), Syria, Lebanon & N Iraq. A3 to N Iraq.	¹⁰	41.17	30.00	36.29	43.62	1294	-
<i>Alyssum constellatum</i>	S Anatolia (C2) to N Iraq.	¹⁰	38.00	28.00	36.67	43.01	1348	-
<i>Alyssum contemptum</i>	C Anatolia (C5) to Iran.	¹⁰	38.00	34.00	32.35	60.77	2506	-

Table A3-2. Table showing geographic distribution description, most distant points in the distribution, Linear Range Estimator and Range Area for species of Alysseae. See accompanying notes on Page A3-1 to A3-4.

Name	Geographic Range Description	Range Data Sources ^G	Western point ^I latitude (decimal degrees North ^K)	Western point ^I longitude (decimal degrees East ^L)	Eastern point ^I latitude (decimal degrees North ^K)	Eastern point ^I longitude (decimal degrees East ^L)	Linear Range Estimator (km) ^M	Range Area (Atlas Flora Europaeae) (No. 50×50 km grid cells) ^N
<i>Alyssum corningii</i>	Endemic to Turkey (B4 to C5).	¹⁰	40.00	32.00	36.00	36.00	568	-
<i>Alyssum corsicum</i>	Endemic to Turkey (only introduced to Corsica): B1 to C3.	¹⁰	40.00	26.19	36.54	32.00	635	-
<i>Alyssum corymbosoides</i>	Bulgaria, N Greece, Macedonia & Serbia.	³	40.44	21.71	42.54	27.48	546	23
<i>Alyssum crenulatum</i>	C6 & Syrian part of Mt. Cassius.	¹⁰	36.00	36.00	38.00	38.00	281	-
<i>Alyssum cuneifolium</i>	Pyrenees, SE France & Central Italy.	³	43.19	-1.69	43.42	13.65	1247	15
<i>Alyssum cypricum</i>	Turkey: C2 & Cyprus: Division 2.	^{2, 10}	38.00	28.00	34.98	33.20	574	-
<i>Alyssum damascenum</i>	Syria & Israel.	⁹	29.54	34.95	36.79	36.74	820	-
<i>Alyssum dasycarpum</i>	Widespread in Turkey, Caucasus, all of Iran, Afghanistan. Istanbul to E Afghanistan.	^{3, 6, 10, 11}	41.14	29.07	35.46	71.68	3740	[2]
<i>Alyssum davisianum</i>	Endemic to Turkey: B2.	¹⁰	40.00	28.00	38.00	30.00	280	-
<i>Alyssum debarense</i>	Insufficient distribution data available.	-	-	-	-	-	-	-
<i>Alyssum densistellatum</i>	C Evvoia, Greece.	³	38.75	23.36	38.42	24.07	81	1
<i>Alyssum desertorum</i>	C Europe to E Siberia.	¹¹	48.65	17.20	48.39	139.43	7905	-
<i>Alyssum diffusum</i>	Pyrenees & S Italy.	³	42.06	-3.46	39.83	16.42	1690	21
<i>Alyssum discolor</i>	Endemic to Turkey: C2.	¹⁰	36.56	27.98	38.00	30.00	237	-
<i>Alyssum djurdjurae</i>	Insufficient distribution data available.	-	-	-	-	-	-	-

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Name	Geographic Range Description	Range Data Sources ^G	Western point ^I latitude (decimal degrees North ^K)	Western point ^I longitude (decimal degrees East ^L)	Eastern point ^J latitude (decimal degrees North ^K)	Eastern point ^J longitude (decimal degrees East ^L)	Linear Range Estimator (km) ^M	Range Area (Atlas Flora Europaeae) (No. 50×50 km grid cells) ^N
<i>Alyssum doerfleri</i>	C mainland Greece & Macedonia.	³	41.84	21.13	38.36	22.58	405	8
<i>Alyssum dubertretii</i>	Endemic to Turkey: C6	¹⁰	38.00	36.00	36.84	38.00	218	-
<i>Alyssum dudleyi</i>	Endemic to Turkey, found between 28.9 & 29.5 degrees East at 39.6 deg N.	²⁰	39.59	28.90	39.59	29.50	52	-
<i>Alyssum embergeri</i>	Insufficient distribution data available.	-	-	-	-	-	-	-
<i>Alyssum eriophyllum</i>	Endemic to Turkey: B6 & C6.	¹⁰	36.00	36.00	40.00	38.00	477	-
<i>Alyssum erosulum</i>	Endemic to Turkey: A2 to C3.	¹⁰	42.00	28.00	36.54	32.00	696	-
<i>Alyssum euboeum</i>	C & N Evvoia, Greece.	³	38.85	22.82	38.24	24.26	142	2
<i>Alyssum fedtschenkoanum</i>	Insufficient distribution data available.	¹¹	-	-	-	-	-	-
<i>Alyssum filiforme</i>	Endemic to Turkey: B4 to B9.	¹⁰	40.00	32.00	38.00	44.00	1060	-
<i>Alyssum flahaultianum</i>	Insufficient distribution data available.	-	-	-	-	-	-	-
<i>Alyssum floribundum</i>	Endemic to Turkey: B2 to C5.	¹⁰	40.00	28.00	36.00	36.00	830	-
<i>Alyssum foliosum</i>	Greece. W Greece to Rhodes.	⁴	38.87	20.77	36.14	28.10	713	19
<i>Alyssum fragillimum</i>	Endemic to Levka Ori in W Crete.	⁴	35.37	24.04	35.22	24.04	17	1
<i>Alyssum fulvescens</i>	Endemic to Turkey: B1 to C2.	¹⁰	40.00	26.20	36.20	30.00	540	-
<i>Alyssum gadorensense</i>	Sierra de Gádor, Almeria, Spain.	³	36.97	-2.95	36.86	-2.48	44	1

Table A3-2. Table showing geographic distribution description, most distant points in the distribution, Linear Range Estimator and Range Area for species of Alysseae. See accompanying notes on Page A3-1 to A3-4.

Name	Geographic Range Description	Range Data Sources ^G	Western point ^I latitude (decimal degrees North ^K)	Western point ^I longitude (decimal degrees East ^L)	Eastern point ^I latitude (decimal degrees North ^K)	Eastern point ^I longitude (decimal degrees East ^L)	Linear Range Estimator (km) ^M	Range Area (Atlas Flora Europaeae) (No. 50×50 km grid cells) ^N
<i>Alyssum gehamense</i>	Turkey: A8 & A9; Armenia. A8 to E Armenia.	¹⁰	40.93	40.00	38.88	46.53	588	-
<i>Alyssum gevgelicense</i>	Insufficient distribution data available.	-	-	-	-	-	-	-
<i>Alyssum giosnanum</i>	Endemic to Turkey: C2 to C6.	¹⁰	38.00	28.00	36.88	38.00	892	-
<i>Alyssum globosum</i>	Insufficient distribution data available.	-	-	-	-	-	-	-
<i>Alyssum granatense</i>	SW Spain to C Pyrenees. Morocco to NW Libya. NE Portugal to NW Libya.	³	41.50	-6.63	31.00	13.00	2125	[38]
<i>Alyssum gustavssonii</i>	High mountains of C. Sterea Ellas, Greece	⁴	38.89	21.99	38.39	22.64	78.74	1
<i>Alyssum hajastanum</i>	Insufficient distribution data available.	-	-	-	-	-	-	-
<i>Alyssum handelii</i>	Endemic to Mt. Olympus. Greece.	⁴	40.09	22.20	40.03	22.46	24	1
<i>Alyssum harputicum</i>	Insufficient distribution data available.	¹⁰	-	-	-	-	-	-
<i>Alyssum haussknechtii</i>	Endemic to Turkey: C6 & B6.	¹⁰	36.00	36.00	40.00	36.00	477	-
<i>Alyssum heideri</i>	Insufficient distribution data available.	-	-	-	-	-	-	-
<i>Alyssum heldreichii</i>	NW Greece.	⁴	39.81	20.86	40.71	21.80	127	8

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<i>Alyssum heterotrichum</i>	Insufficient distribution data available.	-	-	-	-	-	-	-
<i>Alyssum hirsutum</i>	NE Bulgaria to Caspian Sea	3, 10	44.00	22.71	42.16	48.14	2076	[117]
<i>Alyssum homalocarpum</i>	Sinai (Egypt) to W Pakistan. Deserts of Iraq.	7, 9, 13	29.60	32.75	28.58	66.98	3351	-
<i>Alyssum huber-morathii</i>	Endemic to Turkey: C2 & C3.	10	38.00	28.00	36.53	32.00	391	-
<i>Alyssum huetii</i>	Endemic to Turkey: A3 to B8.	10	41.14	30.00	38.00	42.00	1087	-
<i>Alyssum idaeum</i>	Restricted to Mt. Psiloritis, Crete.	4	35.29	24.71	35.17	24.91	23	1
<i>Alyssum iljinskiae</i>	Insufficient distribution data available.	-	-	-	-	-	-	-
<i>Alyssum iranicum</i>	Insufficient distribution data available.	-	-	-	-	-	-	-
<i>Alyssum kavadarsense</i>	Insufficient distribution data available.	-	-	-	-	-	-	-
<i>Alyssum kaynakiae</i>	Insufficient distribution data available.	-	-	-	-	-	-	-
<i>Alyssum kurdicum</i>	Insufficient distribution data available.	-	-	-	-	-	-	-
<i>Alyssum lanceolatum</i>	Insufficient distribution data available.	-	-	-	-	-	-	-
<i>Alyssum lanigerum</i>	Widespread in Iran.	6	39.48	44.56	25.56	61.40	2197	-
<i>Alyssum lassiticum</i>	Endemic to SE Crete.	3, 4	35.15	25.40	35.06	25.65	25	1

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<i>Alyssum lenense</i>	From NE of the Black Sea to E Siberia.	3, 11	51.17	37.53	65.10	-172.69	6749	[7]
<i>Alyssum lepidostellatum</i>	Endemic to Turkey: B6 to B7.	10	40.00	36.00	38.00	40.00	410	-
<i>Alyssum lepidotum</i>	Endemic to Turkey: C1 to B9.	10	36.67	27.41	40.00	44.00	1494	-
<i>Alyssum lesbiacum</i>	Endemic to SE part of Lesbos, Greece.	4	39.17	26.29	39.02	26.61	32	-
<i>Alyssum libanoticum</i>	Insufficient distribution data available.	13	-	-	-	-	-	-
<i>Alyssum linifolium</i>	C Spain to W Siberia / Central Asia & Iran.	3	40.18	-4.39	25.30	61.43	6244	[170]
<i>Alyssum loiseleurii</i>	SW France & N Spain coast.	3	43.06	-9.28	43.45	-1.50	630	7
<i>Alyssum luteolum</i>	Insufficient distribution data available.	-	-	-	-	-	-	-
<i>Alyssum lycaonicum</i>	Endemic to Turkey: C4 & C5.	10	36.50	32.00	38.00	36.00	389	-
<i>Alyssum macrocalyx</i>	Insufficient distribution data available.	-	-	-	-	-	-	-
<i>Alyssum macropodum</i>	Endemic to Turkey: C2 to B6.	10	36.53	28.00	40.00	38.00	951	-
<i>Alyssum magicum</i>	Insufficient distribution data available.	-	-	-	-	-	-	-
<i>Alyssum malacitanum</i>	Sierra Bermeja & Sierra de Aguas mountains in SE Spain.	15, 24	36.55	-5.28	36.64	-4.55	66	-

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<i>Alyssum masmenaeum</i>	Endemic to Turkey: B1 to C5.	¹⁰	40.01	26.21	36.95	35.97	917	-
<i>Alyssum meniocoides</i>	SE Turkey (C6 to C8), Syria, Iran, Afghanistan. C6 to C Afghanistan.	¹⁰	38.00	36.00	33.97	65.67	3144	-
<i>Alyssum microphylliforme</i>	Insufficient distribution data available.	-	-	-	-	-	-	-
<i>Alyssum microphyllum</i>	Insufficient distribution data available.	-	-	-	-	-	-	-
<i>Alyssum minutum</i>	Insufficient distribution data available.	³	-	-	-	-	-	[87]
<i>Alyssum misirdalianum</i>	Endemic to Mersin Province, Turkey.	²³	36.10	32.55	37.43	34.78	245	-
<i>Alyssum moellendorffianum</i>	E Bosnia & Herzegovina	³	43.78	17.02	43.40	18.34	112	2
<i>Alyssum montanum</i>	SW Spain to 5 degrees N of Caspian Sea.	^{3, 11}	36.83	-5.96	53.95	48.83	4564	[401]
<i>Alyssum mouradicum</i>	Endemic to Turkey: B2 to C5.	¹⁰	40.00	28.00	36.00	36.00	830	-
<i>Alyssum mozaffarianii</i>	Insufficient distribution data available.	-	-	-	-	-	-	-
<i>Alyssum muelleri</i>	Armenia to C Iran (S Yazd).	^{6, 11}	41.14	43.50	29.91	54.54	1600	-
<i>Alyssum mughlaei</i>	SW Turkey: C2.	²⁵	36.56	27.98	38.00	30.00	237	-
<i>Alyssum murale</i>	NW Balkans to NW Iran.	^{3, 6, 7, 10, 11}	44.70	14.38	33.44	48.78	3194	[84]

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<i>Alyssum musilii</i>	Insufficient distribution data available.	N/A	-	-	-	-	-	
<i>Alyssum nebrodense</i>	C. Sicily to C. Greece.	3,4	37.54	13.96	39.00	21.95	724	2
<i>Alyssum nezaketiae</i>	Endemic to Turkey. NW of Çankırı Province only.	18	40.10	32.79	40.76	33.24	43.64	-
<i>Alyssum niveum</i>	Insufficient distribution data available.	10	-	-	-	-	-	-
<i>Alyssum obovatum</i>	Caspian Sea area to NW Canada.	11	46.36	53.05	63.35	-129.2	7827	-
<i>Alyssum obtusifolium</i>	W Bulgaria to Caucasus.	3	42.46	22.49	40.93	49.40	2230	[22]
<i>Alyssum ochroleucum</i>	Endemic to Turkey: A7 to B9.	10	40.97	38.00	38.00	44.00	609	-
<i>Alyssum orbelicum</i>	Insufficient distribution data available.	-	-	-	-	-	-	-
<i>Alyssum ovirens</i>	Italian Alps to SW Serbia.	3	46.10	10.95	42.31	20.45	866	14
<i>Alyssum oxycarpum</i>	Endemic to Turkey: B1 to C6.	10	40.00	26.21	36.86	38.00	1088	-
<i>Alyssum paphlagonicum</i>	Endemic to Turkey: A4 to B7	10	41.50	32.00	38.00	40.00	787	-
<i>Alyssum pateri</i>	Endemic to Turkey: A3 to B10	10	41.17	30.00	38.00	44.30	1277	-
<i>Alyssum peltarioides</i>	Endemic to Turkey: A4 to C10.	10	41.10	33.17	37.20	44.50	1098	-
<i>Alyssum penjwinense</i>	N Iraq, W Iran.	7	37.12	42.42	35.81	48.13	530	-
<i>Alyssum persicum</i>	N Iraq, W Iran.	7	37.12	42.42	35.81	48.13	530	-
<i>Alyssum pinifolium</i>	Endemic to Turkey: A1	10	40.00	26.20	40.00	28.00	271	-

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<i>Alyssum pintodasilvae</i>	Melide in Galicia to Bragança in Portugal.	¹⁶	41.17	-7.30	43.02	-7.93	212	-
<i>Alyssum pirinicum</i>	Restricted to the alpine area of the N Pirin mountains, Bulgaria.	²¹	41.73	23.15	41.56	23.28	21	-
<i>Alyssum pogonocarpum</i>	Insufficient distribution data available.	-	-	-	-	-	-	-
<i>Alyssum polycladum</i>	Insufficient distribution data available.	-	-	-	-	-	-	-
<i>Alyssum praecox</i>	Endemic to Turkey: B2 to B9.	¹⁰	40.00	28.00	38.00	44.00	1402	-
<i>Alyssum propinquum</i>	Endemic to Turkey: C2 to C5.	¹⁰	38.00	28.00	36.00	36.00	740	-
<i>Alyssum pseudomouradicum</i>	Endemic to Turkey: A4 to A7.	¹⁰	41.50	32.00	40.00	40.00	697	-
<i>Alyssum pterocarpum</i>	Endemic to Turkey: C1 to C3.	¹⁰	37.00	28.00	38.00	32.00	315	-
<i>Alyssum pulvinare</i>	S Mainland Greece to E Bulgaria.	³	44.42	22.62	42.28	26.50	847	9
<i>Alyssum repens</i>	E Italy to N Iran	^{3, 10}	46.11	13.27	37.71	48.70	3044	[98]
<i>Alyssum robertianum</i>	NW Corsica.	³	41.82	9.31	39.91	9.35	308	2
<i>Alyssum rostratum</i>	SW Romania to NE Ukraine.	³	44.54	22.72	49.61	39.90	1419	63
<i>Alyssum samariferum</i>	Turkey B7 to SW Syria.	^{9, 10}	40.00	40.00	33.14	36.17	879	-
<i>Alyssum samium</i>	Endemic to E part of Samos, Greece.	⁴	37.72	26.58	37.73	26.67	9	-

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<i>Alyssum scardicum</i>	SW Bulgaria to W Bosnia & Herzegovina	3	44.32	16.64	41.40	23.30	629	22
<i>Alyssum scutigerum</i>	Algeria, Morocco, Atlas to Laghouat in NC Algeria. Not listed in Flora of Libya.	1	30.10	-9.69	33.72	2.87	1233	-
<i>Alyssum serpentinum</i>	Insufficient distribution data available.	-	-	-	-	-	-	-
<i>Alyssum serpyllifolium</i>	SE France to S Spain.	3, 8	33.00	-5.86	44.52	-5.95	1607	[96]
<i>Alyssum sibiricum</i>	Albania to E Siberia. Widespread in Turkey & Russia.	3, 11, 10	40.74	19.44	54.67	135.19	7806	[12]
<i>Alyssum siculum</i>	NC Sicily to Crete.	3	37.70	13.43	35.07	25.75	1142	22
<i>Alyssum simplex</i>	SE Iberia to E Siberia & Iran.	3, 10, 11	37.09	-8.92	54.99	135.08	9241	[449]
<i>Alyssum simulans</i>	SW mainland Greece to SE Crete.	4	38.53	22.38	35.04	25.56	479	-
<i>Alyssum singarense</i>	NW & NC Iraq.	7	37.18	42.62	36.44	45.07	234	-
<i>Alyssum skopjense</i>	Insufficient distribution data available.	-	-	-	-	-	-	-
<i>Alyssum smolikanum</i>	S Albania & NW Greece.	3, 4	40.27	19.40	39.91	21.22	160	6
<i>Alyssum smyrnaeum</i>	Crete & Peloponnese to C Turkey & Crimea.	3, 10	34.90	24.75	45.17	35.50	1463	[6]
<i>Alyssum sphacioticum</i>	Restricted to Levka Ori in W Crete.	4	35.30	23.89	35.27	24.22	30	2
<i>Alyssum stapfii</i>	Turkey (B5) to Afghanistan.	7, 10	40.00	34.00	36.69	71.62	3285	-

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<i>Alyssum stipitatum</i>	Insufficient distribution data available.	19	-	-	-	-	-	-
<i>Alyssum stribryni</i>	SW Romania to CE Turkey (C7).	3, 10	44.04	22.93	36.85	39.00	1366	[13]
<i>Alyssum strictum</i>	Turkey (B4) to Iran (Harsin).	6, 10, 22	40.00	32.00	34.27	47.59	1522	-
<i>Alyssum strigosum</i>	Insufficient distribution data available.	-	-	-	-	-	-	[74]
<i>Alyssum stylare</i>	Endemic to Turkey: A8 to C5.	10	36.30	34.00	41.52	42.00	779	-
<i>Alyssum subbaicalicum</i>	Insufficient distribution data available.	-	-	-	-	-	-	-
<i>Alyssum subspinosum</i>	Insufficient distribution data available.	13	-	-	-	-	-	-
<i>Alyssum sulphureum</i>	NE Syria. N Iraq. C & SE Turkey (B6 & C9). I.e. B6, Turkey to N Iraq.	7	40.00	36.00	35.21	46.19	1047	-
<i>Alyssum syriacum</i>	Insufficient distribution data available.	13	-	-	-	-	-	-
<i>Alyssum szarabiacum</i>	Insufficient distribution data available.	13	-	-	-	-	-	-
<i>Alyssum szovitsianum</i>	Turkey (C2) to W Pakistan.	7, 10	38.00	28.00	28.40	66.75	3735	-
<i>Alyssum taygeteum</i>	S mainland Greece & Peloponnese.	3	35.20	22.11	36.74	22.41	223	3
<i>Alyssum tenium</i>	E Tinos, Greece.	4	37.61	25.11	37.54	25.25	12	1
<i>Alyssum tetrastemon</i>	Endemic to Turkey: C4 to B6.	10	36.52	32.00	40.00	38.00	649	-

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<i>Alyssum thymops</i>	Endemic to Turkey: B4 to C5.	¹⁰	40.00	32.00	36.00	36.00	568	-
<i>Alyssum tortuosum</i>	N Peloponnese, Greece to Caspian Sea, C Asia & Siberia.	^{3, 11}	38.22	21.81	54.97	135.07	7896	[93]
<i>Alyssum trapeziforme</i>	Endemic to Turkey: C5.	¹⁰	36.00	34.00	38.00	36.00	284	-
<i>Alyssum trichocarpum</i>	Endemic to Turkey: B5 & B6.	¹⁰	38.00	34.00	40.00	38.00	414	-
<i>Alyssum troodi</i>	Endemic to Mt. Olympus on Cyprus.	²	34.91	32.85	34.97	32.90	8	-
<i>Alyssum turgidum</i>	Insufficient distribution data available.	¹³	-	-	-	-	-	-
<i>Alyssum turkestanicum</i>	Insufficient distribution data available.	^{3, 11}	-	-	-	-	-	[442]
<i>Alyssum umbellatum</i>	CN Greece to Crimea.	^{3, 10}	40.88	21.70	45.07	35.41	1209	-
<i>Alyssum virgatum</i>	Endemic to Turkey: B2 to B6.	¹⁰	40.00	28.00	38.00	38.00	894	-
<i>Alyssum wierzbickii</i>	SW Bulgaria to E Romania.	³	41.43	23.08	45.28	28.46	615	13
<i>Alyssum wulfenianum</i>	E Italy & S C Austria.	³	46.03	11.68	46.88	14.48	233	4
<i>Alyssum xanthocarpum</i>	Turkey (A9) to W Syria.	^{9, 10}	32.75	36.85	41.50	42.00	1150	-
<i>Aurinia corymbosa</i>	NE Bosnia & Herzegovina to Thessaloniki in Greece.	³	44.84	18.00	39.98	23.95	729	30
<i>Aurinia gionae</i>	SE Albania to S mainland Greece.	^{3, 4}	40.37	20.48	38.47	22.37	268	6
<i>Aurinia leucadea</i>	SW Croatia to SE Italy.	³	43.73	16.22	39.81	18.35	471	8
<i>Aurinia moreana</i>	N Peloponnese, Greece.	⁴	38.19	21.77	37.73	22.57	86	3

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<i>Aurinia petraea</i>	NW N Romania to NW Greece.	³	39.57	20.15	47.86	25.29	996	60
<i>Aurinia saxatilis</i>	NE Germany to SW Turkey	^{3, 10, 11}	53.00	12.43	26.24	30.03	1685	203
<i>Aurinia sinuata</i>	N Croatia to NW Greece.	³	45.54	14.54	40.86	20.99	702	28
<i>Aurinia uechtritziana</i>	NE Bulgaria to NE Greece.	^{3, 10}	40.78	26.07	43.40	27.99	329	8
<i>Berteroa gintlilii</i>	Montenegro.	³	43.52	19.06	42.08	19.16	159	3
<i>Berteroa incana</i>	NW Italy to E Siberia.	^{3, 11}	45.44	7.17	54.88	135.06	8132	-
<i>Berteroa mutabilis</i>	NW Balkans to NE Turkey.	^{3, 10}	45.38	14.35	41.31	41.62	2248	[57]
<i>Berteroa obliqua</i>	CW Italy to NE Bulgaria.	³	42.08	11.88	43.72	28.52	1363	56
<i>Berteroa orbiculata</i>	W Albania to Thessaloniki, Greece.	³	41.93	20.60	39.96	24.01	359	13
<i>Bornmuellera angustifolia</i>	Insufficient distribution data available.	¹⁰	-	-	-	-	-	-
<i>Bornmuellera baldaccii</i>	W Albania & NW Greece.	³	42.14	19.99	39.85	21.67	291	7
<i>Bornmuellera cappadocica</i>	Turkey: E. Anatolia: B6 to B9.	¹⁰	40.00	36.00	38.00	44.00	726	-
<i>Bornmuellera dieckii</i>	S Kosovo & NW Macedonia.	³	42.17	20.43	41.75	21.68	112	2
<i>Bornmuellera glabrescens</i>	Insufficient distribution data available.	¹⁰	-	-	-	-	-	-
<i>Bornmuellera kiyakii</i>	Endemic to Turkey: C3.	¹⁷	36.22	30.00	38.00	32.00	265	-
<i>Bornmuellera tymphaea</i>	NC Greece.	⁴	39.50	21.10	40.27	21.67	98	3

Table A3-2. Table showing geographic distribution description, most distant points in the distribution, Linear Range Estimator and Range Area for species of Alysseae. See accompanying notes on Page A3-1 to A3-4.

Name	Geographic Range Description	Range Data Sources ^G	Western point ^I latitude (decimal degrees North ^K)	Western point ^I longitude (decimal degrees East ^L)	Eastern point ^I latitude (decimal degrees North ^K)	Eastern point ^I longitude (decimal degrees East ^L)	Linear Range Estimator (km) ^M	Range Area (Atlas Flora Europaeae) (No. 50×50 km grid cells) ^N
<i>Clastopus erubescens</i>	Insufficient distribution data available.	-	-	-	-	-	-	-
<i>Clastopus vestitus</i>	N Iraq, N & W Iran.	6, 7	37.07	42.40	33.63	50.08	800	-
<i>Clypeola aspera</i>	C Turkey to Afghanistan.	6, 10	39.23	36.39	33.08	69.21	3012	[234]
<i>Clypeola ciliata</i>	Endemic to Turkey: C2.	10	36.56	27.98	38.0	30.00	237	-
<i>Clypeola cyclodonteia</i>	South of France to Lower Ebro.	3, 5	-	-	-	-	-	[1]
<i>Clypeola dichotoma</i>	Armenia to NE Iran.	11, 6	41.16	43.40	33.45	60.96	1759	-
<i>Clypeola elegans</i>	Turkey (A8), Armenia, Kurdistan. A8 to SE Armenia.	10, 11	40.94	40.05	38.88	46.50	597	-
<i>Clypeola eriocarpa</i>	C & SE Spain.	3, 5	40.47	-3.86	36.86	-3.58	402	6
<i>Clypeola jonthlaspi</i>	S Spain to Iran.	3, 10, 11	36.20	-5.79	31.85	60.58	3918	[117]
<i>Clypeola lappacea</i>	W Syria, Iran, Lebanon, Palestine, Iraq, Turkey (B7 to C8). W Lebanon to E Iran.	10	34.60	35.99	31.09	61.83	2440	-
<i>Clypeola raddeana</i>	Endemic to Turkey: A8.	10	40.00	40.00	41.43	42.00	238	-
<i>Degenia velebitica</i>	W Croatia.	3	43.99	15.44	44.63	15.55	436	1
<i>Fibigia clypeata</i>	N C Italy to C Iran.	3, 10, 11	44.04	11.09	31.59	56.43	4166	[45]
<i>Fibigia compacta</i>	Insufficient distribution data available.	N/A	-	-	-	-	-	-
<i>Fibigia heterophylla</i>	Insufficient distribution data available.	N/A	-	-	-	-	-	-

Table A3-2. Table showing geographic distribution description, most distant points in the distribution, Linear Range Estimator and Range Area for species of Alysseae. See accompanying notes on Page A3-1 to A3-4.

Name	Geographic Range Description	Range Data Sources ^G	Western point ^I latitude (decimal degrees North ^K)	Western point ^I longitude (decimal degrees East ^L)	Eastern point ^J latitude (decimal degrees North ^K)	Eastern point ^J longitude (decimal degrees East ^L)	Linear Range Estimator (km) ^M	Range Area (Atlas Flora Europaeae) (No. 50×50 km grid cells) ^N
<i>Fibigia lunarioides</i>	Cyclades & Aegean Islands, Greece	3, 4, 10	35.00	24.59	37.47	26.99	356	10
<i>Fibigia macrocarpa</i>	Syria, C. Turkey (B6 to C9), Armenia, NW Iran south to Shiraz. Turkey B6 to W Iran.	6, 10	40.00	36.00	29.58	52.59	1903	-
<i>Fibigia membranacea</i>	Insufficient distribution data available.	N/A	-	-	-	-	-	-
<i>Fibigia multicaulis</i>	Insufficient distribution data available.	6, 7	-	-	-	-	-	-
<i>Fibigia pendula</i>	Insufficient distribution data available.	-	-	-	-	-	-	-
<i>Fibigia spathulata</i>	Insufficient distribution data available.	-	-	-	-	-	-	-
<i>Fibigia suffruticosa</i>	Turkey B7 to C9, S Caucasus, Assyria, N Iraq, Iran (to Shiraz). W Goergoa to Shiraz.	6, 11,	43.41	40.00	29.58	52.53	1895	-
<i>Fibigia thesigeri</i>	Insufficient distribution data available.	7	-	-	-	-	-	-
<i>Fibigia triquetra</i>	S Croatia.	3	44.06	15.81	43.18	17.36	157	5
<i>Fibigia umbellata</i>	Insufficient distribution data available.	-	-	-	-	-	-	-
<i>Galitzkya macrocarpa</i>	Insufficient distribution data available.	-	-	-	-	-	-	-

Table A3-2. Table showing geographic distribution description, most distant points in the distribution, Linear Range Estimator and Range Area for species of Alysseae. See accompanying notes on Page A3-1 to A3-4.

Name	Geographic Range Description	Range Data Sources ^G	Western point ^I latitude (decimal degrees North ^K)	Western point ^I longitude (decimal degrees East ^L)	Eastern point ^J latitude (decimal degrees North ^K)	Eastern point ^J longitude (decimal degrees East ^L)	Linear Range Estimator (km) ^M	Range Area (Atlas Flora Europaeae) (No. 50×50 km grid cells) ^N
<i>Galitzkya potaninii</i>	Insufficient distribution data available.	-	-	-	-	-	-	-
<i>Galitzkya spathulata</i>	Insufficient distribution data available.	-	-	-	-	-	-	-
<i>Hormathophylla baetica</i>	SE Spain. See map in <i>Flora Europaeae</i> .	³	38.09	-2.63	37.58	-2.16	71	1
<i>Hormathophylla cadevalliana</i>	SE Spain. See map in <i>Flora Europaeae</i> .	³	37.23	-2.85	38.31	-1.98	143	5
<i>Hormathophylla halimifolia</i>	SE France. See map in <i>Flora Europaeae</i> .	³	43.52	6.75	44.27	8.44	420	7
<i>Hormathophylla lapeyrousiana</i>	E Spain. See map in <i>Flora Europaeae</i> .	³	37.18	-3.48	42.40	2.03	746	60
<i>Hormathophylla longicaulis</i>	SE Spain. See map in <i>Flora Europaeae</i> .	³	36.88	-4.56	38.01	-1.56	291	8
<i>Hormathophylla macrocarpa</i>	S France. See map in <i>Flora Europaeae</i> .	³	44.71	1.69	42.37	2.91	270	19
<i>Hormathophylla purpurea</i>	Insufficient distribution data available.		-	-	-	-	-	-
<i>Hormathophylla pyrenaica</i>	E Pyrenees, France. See map in <i>Flora Europaeae</i> .	³	42.74	1.73	42.41	2.46	71	1
<i>Hormathophylla reverchonii</i>	SE Spain. See map in <i>Flora Europaeae</i> .	³	37.85	-2.86	38.72	-2.25	112	2

Table A3-2. Table showing geographic distribution description, most distant points in the distribution, Linear Range Estimator and Range Area for species of Alysseae. See accompanying notes on Page A3-1 to A3-4.

Name	Geographic Range Description	Range Data Sources ^G	Western point ^I latitude (decimal degrees North ^K)	Western point ^I longitude (decimal degrees East ^L)	Eastern point ^J latitude (decimal degrees North ^K)	Eastern point ^J longitude (decimal degrees East ^L)	Linear Range Estimator (km) ^M	Range Area (Atlas Flora Europaeae) (No. 50×50 km grid cells) ^N
<i>Hormathophylla spinosa</i>	S France to S Spain. See map in <i>Flora Europaeae</i> .	³	36.41	-6.05	43.14	6.09	1278	61
<i>Leptoplax emarginata</i>	NC Greece to C Evvoia.	³	40.61	21.30	38.54	23.96	323	1
<i>Phyllolepidum cyclocarpum</i>	NE Greece to CSW Turkey.	³⁷	40.85	26.01	38.12	40.03	2754	17
<i>Phyllolepidum cyclocarpum</i>	CE Italy only.	³⁷	42.34	12.88	42.28	14.49	132	-
<i>Physocardamum davisii</i>	E Turkey: B9 (Ağri: Murat Valley).	¹⁴	38.00	42.00	40.00	44.00	281	-
<i>Physoptychis caspica</i>	S Caucasus to Iran (S to Lorestan).	¹¹	43.40	40.05	32.86	49.48	1430	-
<i>Physoptychis haussknechtii</i>	Endemic to Turkey: B6.	¹⁰	38.00	36.00	40.00	38.00	280	-
<i>Straussiella purpurea</i>	Insufficient distribution data available.	⁶	-	-	-	-	-	-

Appendix 4:

Terminal Clade Assignment for Unsourced Species

Table A4-1 indicates the rationale for the assignment of unsourced species of Alyseae to terminal clades within the Alyseae tree. This assignment is based primarily on the taxonomy of unsourced species, and was necessary in order to carry out MEDUSA and BiSSE species diversification rate analyses, which take unsourced species into account.

Of the 21 *Alyssum* species for which sections have not been assigned in the literature (as listed in Table A1-1 in Appendix 1), one species (*Alyssum flahaultianum*) was sampled in this study (and hence clade membership was determined empirically) and four species could be assigned to Alyseae terminal clades based on the literature (see rationale in Table A4-1). A total of 16 *Alyssum* species therefore remain without an Alyseae terminal clade assigned (6.3% of the tribe).

Terminal clade assignment for the other unsourced species from tribe Alyseae was based on their placement within Alyseae genera or within *Alyssum* sections. This approach is justified by the congruence between the generic and sectional classification of Alyseae and clade membership for the sampled taxa that was revealed in this study. Further details for unsourced Alyseae species are included in Table A4-1. Clade assignments for all species are listed in table A3-1 in Appendix 3.

Table A4-1. Rationale for the assignment of unsampled *Alyseae* species to terminal clades for MEDUSA and BiSSE species diversification rate analyses, based on generic and sectional classifications. For species authorities see Table A1-1 in Appendix 1.

Taxon	No. of unsampled Species	Assigned terminal Clade	Rationale
<i>Alyssum</i> sect. <i>Alyssum</i>	22	A2	Of the 46 <i>Alyssum</i> sect. <i>Alyssum</i> species (51 accessions) sampled in this study, 43 are strongly supported in the A2 clade. (The three outside the A2 clade (<i>A. aizoides</i> Boiss., <i>A. lenense</i> and <i>A. stribryni</i>) are all strongly supported in the A1a clade.)
<i>Alyssum</i> sect. <i>Gamosepalum</i>	2	A2	Of the nine <i>Alyssum</i> sect. <i>Gamosepalum</i> species (nine accessions) sampled in this study, eight are strongly supported as members of the A2 clade. (The single exception is <i>A. tetrastemon</i> , which is strongly supported within the A1a clade.)
<i>Alyssum</i> sect. <i>Meniocus</i>	1	A1b	Of the five <i>Alyssum</i> sect. <i>Meniocus</i> species (six accessions) sampled in this study, four are strongly supported as members of the A1b clade. (The single exception is <i>A. linifolium</i> , which is nested in the A2 clade with strong support.)
<i>Alyssum</i> sect. <i>Odontarrhena</i>	13	A1a	All of the 59 <i>Alyssum</i> sect. <i>Odontarrhena</i> species (70 accessions) sampled in this study are strongly supported as members of the A1a clade.
<i>Alyssum</i> sect. <i>Psilonema</i>	2	A2	Of the four <i>Psilonema</i> species (four accessions) sampled in this study, all are strongly supported within the A2 clade.
<i>Alyssum brughieri</i>	1	A1a	In the protologue (Colla, 1833), this species is described as being allied to <i>A. argenteum</i> which is in <i>Alyssum</i> section <i>Odontarrhena</i> . See entry for <i>Alyssum</i> sect. <i>Odontarrhena</i> above.
<i>Alyssum embergeri</i>	1	A2	In the protologue (Quèzel, 1953) this species is described as being allied to <i>Alyssum montanum</i> and <i>Alyssum flahaultianum</i> . <i>A. montanum</i> is in <i>Alyssum</i> sect. <i>Alyssum</i> , and <i>A. flahaultianum</i> was found to be present in the A2 clade in this study. See entry for <i>Alyssum</i> sect. <i>Alyssum</i> above.

Table A4-1. Rationale for the assignment of unsampled *Alyseae* species to terminal clades for MEDUSA and BiSSE species diversification rate analyses, based on generic and sectional classifications. For species authorities see Table A1-1 in Appendix 1.

Taxon	No. of unsampled Species	Assigned terminal Clade	Rationale
<i>Alyssum gadorens</i>	1	A1a	The protologue (Küpfer, 1993) describes this species as a perennial having a single seed per locule which is consistent with placement in <i>Alyssum</i> sect. <i>Odontarrhena</i> . (According to Dudley's (1964a) key to the sections of <i>Alyssum</i> , no other <i>Alyssum</i> section contains any perennial plants having a single seed per locule). See entry for <i>Alyssum</i> sect. <i>Odontarrhena</i> above.
<i>Alyssum magicum</i>	1	A2	In the protologue (An, 1981), this species is put into a new <i>Alyssum</i> section: sect. <i>Magicum</i> . From the description, the species is perennial with 4 to 6 seeds per locule, and is therefore likely to be closely allied to sect. <i>Gamosepalum</i> or sect. <i>Alyssum</i> (according to Dudley's (1964) key to the sections of <i>Alyssum</i>). See entries for sect. <i>Alyssum</i> and sect. <i>Gamosepalum</i> above.
<i>Alyssum pirinicum</i>	1	A2	<i>Alyssum cuneifolium</i> subsp. <i>pirinicum</i> in sect. <i>Alyssum</i> is a synonym of this species. See entry for <i>Alyssum</i> sect. <i>Alyssum</i> above.
<i>Alyssum arabicum</i> , <i>Alyssum austrodalmaticum</i> , <i>Alyssum bargalense</i> , <i>Alyssum debarens</i> , <i>Alyssum gevgelicense</i> , <i>Alyssum globosum</i> , <i>Alyssum hajastanum</i> , <i>Alyssum heideri</i> , <i>Alyssum iljinskiae</i> , <i>Alyssum kavadarsense</i> , <i>Alyssum kurdicum</i> , <i>Alyssum microphylliforme</i> , <i>Alyssum musilii</i> , <i>Alyssum serpentinum</i> , <i>Alyssum skopjense</i> , <i>Alyssum subbailicum</i>	16	N/A	These species cannot be assigned to terminal clades and are therefore excluded from the species diversification analyses. They constitute 6.3% of species in the tribe Alyseae.

Table A4-1. Rationale for the assignment of unsampled *Alysseae* species to terminal clades for MEDUSA and BiSSE species diversification rate analyses, based on generic and sectional classifications. For species authorities see Table A1-1 in Appendix 1.

Taxon	No. of unsampled Species	Assigned terminal Clade	Rationale
<i>Aurinia</i>	4	A6	Of the four <i>Aurinia</i> species (five accessions) sampled in this study, all are strongly supported in the A6 clade.
<i>Berteroa</i>	2	A6	All of the three <i>Berteroa</i> species (three accessions) sampled in this study are strongly supported in the A6 clade.
<i>Bornmuellera</i>	2	A5	All of the five <i>Bornmuellera</i> species (five accessions) sampled in this study are strongly supported within the A5 clade.
<i>Clypeola</i>	4	A1c	Of the five <i>Clypeola</i> species (five accessions) sampled in this study, four are strongly supported in the A1c clade. (The single exception, <i>C. ciliata</i> , is sister to the A1c clade without support, though strongly supported within the A1 clade. In the diversification analyses, this single species forms the A8 terminal clade, which is sister to A1c.)
<i>Fibigia</i>	3	A3	All of the 10 <i>Fibigia</i> species (10 accessions) sampled in this study are strongly supported as members of the A3 clade.
<i>Galitzkya</i>	2	A6	The single <i>Galitzkya</i> species (one accession) sampled in this study is strongly supported as a member of the A6 clade.
<i>Hormathophylla</i>	4	A4	All of the five <i>Hormathophylla</i> species (five accessions) sampled in this study are strongly supported as members of the A4 clade.
<i>Phyllolepidum</i>	1	A7	The single <i>Phyllolepidum</i> species sampled in this study is strongly supported as sister to the A3 clade. (The monophyly of <i>Phyllolepidum</i> is supported by the phylogeny of Cecchi <i>et al.</i> , (2011).)
<i>Physoptychis</i>	1	A3	The single <i>Physoptychis</i> species (one accession) sampled in this study is strongly supported as a member of the A3 clade.
Total unsampled Alysseae species	84		

Appendix 5:

Re-Analysis of the *ITS* Dataset of Mengoni *et al.* (2003)

To date, the phylogenetic study with the greatest level of sampling of Alysseae species is the *ITS*-based phylogeny of Mengoni *et al.* (2003). However, the quality of the data in this study has been questioned (e.g., Bailey *et al.*, 2006; Warwick *et al.*, 2008) and these data have been excluded from other phylogenetic studies in the Alysseae or the Brassicaceae (e.g. Bailey *et al.*, 2006; Warwick *et al.*, 2008; Cecchi *et al.*, 2010). No re-analysis of the Mengoni *et al.* (2003) *ITS* dataset has been published, and it is not clear whether the alleged poor data quality is due to errors in sample labelling, sequencing, sequence alignment, phylogenetic analysis, or the presence of *ITS* pseudogenes (non-functional copies of *ITS* sequence which, unlike functional copies, do not undergo concerted evolution (see Bailey *et al.*, 2003), and therefore could introduce paralogy problems into analyses in which *ITS* copy-types are incompletely sampled).

A re-analysis of the Mengoni *et al.* (2003) dataset was carried out here in order to determine (1) whether the allegations of poor data quality are justified, and therefore (2) the extent to which the results of the results of Mengoni *et al.* (2003) can be accepted, and (3) whether the *ITS* sequence data of Mengoni *et al.* (2003) can usefully be included in new phylogenetic studies of the Alysseae.

This re-analysis included: (1) a qualitative evaluation of the Mengoni *et al.* (2003) *ITS* sequence alignment (hereafter ‘the published alignment’); (2) re-alignment of the sequences using modern alignment procedures (hereafter the ‘re-alignment’); (3) phylogenetic analysis of the original alignment and the re-alignment; and (4) evaluation of the individual *ITS* sequences using some of

the pseudogene screening approaches of Hughes *et al.* (2002) and Bailey *et al.* (2003) to attempt to detect the presence of non-functional copy types of *ITS* sequences.

Materials and Methods

ITS Data

The Mengoni *et al.* (2003) *ITS* DNA sequence alignment (accession ALIGN_000514) was obtained from the EMBL alignment database (<ftp://ftp.ebi.ac.uk/pub/databases/embl/align/>). The alignment format was converted from .aln to .fas format file in BioEdit (version 7.2.9) (Hall, 2007), and subsequently viewed and manipulated in Mega (version 4) (Tamura *et al.*, 2004). This alignment contained 38 sequences and had a total length of 765 bp. A total of 33 *Alyssum* species and *Iberis umbellata* were represented in the alignment. All taxon names used in this document are those used in Mengoni *et al.* (2003); any synonymy *sensu* Appendix 1 is indicated in parentheses.

Alignment Quality

The alignment was inspected visually for signs of incorrect sequence orientation and non-homologous regions.

Re-alignment

Sequences were aligned using MUSCLE (Edgar, 2004) followed by minor manual editing.

Phylogenetic Analysis

Parsimony trees were constructed using both the published alignment and the re-aligned *ITS* dataset of Mengoni *et al.* (2003) (hereafter the re-alignment) using Nona (version 2) (Goloboff, 1999) spawned from Winclada (version 1) (Nixon, 1999). A 1000-replicate tree search was performed, with five starting trees and 50 trees kept per replication. The TBR + TBR branch

swapping search strategy was employed. Branch support values were obtained via a 1000-replicate bootstrap analysis, with five starting trees and 10 trees searched per replication, with other settings in default positions. Where more than one maximum parsimony tree was found, results were summarized on a strict consensus tree indicating bootstrap support values.

Maximum likelihood trees were produced for the two alignments using RAxML (version 7.2.8 Black Box; Stamatakis, 2010) run on the Teragrid computer grid accessed via the CIPRES Science Gateway (<http://www.phylo.org>). Only the GTR model of nucleotide substitution is available in this online version. The Γ and I parameters were included in the model used, on the basis of jmodeltest (Posada, 2008) results for the larger *ITS* dataset described in the main text (see *Chapter 2 Materials and Methods* and *Chapter 3 Results*). Branch support values were estimated using the rapid bootstrapping procedure within RAxML. In order to facilitate comparison with the Mengoni *et al.* (2003) tree, branches with weak support were not collapsed. Bootstrap values of 50–70% were considered to represent weak clade support; 70–80%, moderate clade support and 80–100%, strong clade support. Clades with bootstrap values <50% were considered unsupported.

Pseudogene Screening

GenBank *ITS* sequences for *Arabidopsis thaliana* (sequence accession AJ232900.1) and *Adenothamnus validus* (Asteraceae) (sequence accession M93787.1) were added to the re-aligned alignment in order to facilitate the identification of *ITS1*, *ITS2* and 5.8S regions, using the annotated sequences published in Unfried & Gruendler (1990) and Baldwin (1992). The alignment was then re-aligned to include the two new sequences using MUSCLE.

The length of the 5.8S regions was measured for each sequence using the *Site # without gaps* function in Mega after deleting the *ITS1* and *ITS2* regions. Pair-wise variation (i.e. the number of substitutions or indel-based gap sites) between *Iberis umbellata* and each of the *Alyssum*

sequences in the alignment was determined by pairing full *ITS* sequences in separate alignment files in Mega and manually counting the number of non-conserved sites. This species was chosen for the comparison because it is the most basal of the sequences in the original alignment. Variation from the *I. umbellata* sequences for the 5.8S region alone was also determined in a similar way for each of the *Alyssum* sequences. For each *Alyssum* sequence, the observed *ITS* variation due to the 5.8S region was calculated by dividing the 5.8S variation from *I. saxatilis* by the total *ITS* variation from *I. saxatilis*. The expected *ITS* variation due to the 5.8S region, (assuming *ITS* non-functionality, and hence no sequence conservation in the 5.8S region) was calculated by dividing the 5.8S length by the total *ITS* length (Hughes *et al.*, 2002).

Results

Alignment Quality

Visual inspection of the Mengoni *et al.* (2003) *ITS* alignment showed no evidence of any major alignment errors such as misplaced large indels, incorrect sequence orientation or missing data. There were numerous minor differences between the published Mengoni *et al.* (2003) *ITS* alignment and the re-alignment produced here.

Trees based on the published alignment

The maximum parsimony tree produced from the published alignment (Figure A5-1) has eight clades (indicated A–H) with moderate (70–80%) or high (over 80%) bootstrap support. All of these clades also receive moderate or strong support in the maximum likelihood tree produced from the same dataset (Figure A5-2). An additional three clades (I–K) receive moderate or strong support in the maximum likelihood tree only; of these, two are present with low (50–69%) support in the maximum parsimony tree (I and J). The third (K) is absent in the maximum parsimony tree, where

its constituent clades (A and B) form a polytomy with a clade containing the remainder of the *Alysseae* species sampled.

Clade B corresponds to the A2 clade recognized by Warwick *et al.* (2005). Species from the A1 clade of Warwick *et al.* (2005) are not monophyletic in these trees since they form a polytomy with clade B in the maximum parsimony tree, and are paraphyletic in the maximum likelihood tree. These results are consistent with the parsimony and maximum likelihood trees of Mengoni *et al.* (2003).

A. malacitanum and *A. pintodasilvae* have previously been considered sub-species of *A. serpyllifolium* (Dudley 1967a,b; Goday, 1972), and these three species may be closely related. In both maximum likelihood and parsimony trees, the two *A. serpyllifolium* accessions form a clade with *A. guitanii* (= *A. pintodasilvae*) (indicated with an asterisk), which has weak support. However the single accessions of *A. pintodasilvae* and *A. malacitanum* are not within this clade; *A. pintodasilvae* is sister to *A. lesbiacum* (Clade J) with weak support in the parsimony tree and with strong support in the maximum likelihood tree. *A. malacitanum* is sister to *A. nebrodense* with weak support in both trees.

With the exception of clade J, clades A–K are all present within the maximum parsimony and maximum likelihood trees presented in Mengoni *et al.* (2003) (included here as Figures A5-3 and A5-4 respectively). The trees presented in Mengoni *et al.* are considerably better resolved than those obtained here, and their support values are generally higher: for the Mengoni *et al.* (2003) maximum parsimony tree, 31/34 nodes receive moderate or strong support (cf. 8/20 obtained here); for the maximum likelihood tree, the figures are 19/30 (cf. 10/26 obtained here).

Total branch lengths in the maximum likelihood tree obtained from the re-analysis are variable between species. Particularly long terminal branches were present for five accessions (*A.*

anatolicum, *A. caricum*, *A. pintodasilvae*, *A. malacitanum*, and *A. wulfenianum*). It is not possible to compare these values with the equivalent tree of Mengoni *et al.* (2003) because the latter tree is presented in ultrametric format. Total tree length for the parsimony tree obtained here was 640 steps. The length of the parsimony tree of Mengoni *et al.* (2003) is not stated in that publication.

Trees based on the re-alignment

Nine clades receive moderate (70—80%) or strong (>80%) support in both the maximum parsimony and maximum likelihood trees produced from the re-alignment (Figures A5-5 and A5-6 respectively). Of these clades, six (A, B, E, F, H and K) were also present in trees produced here from the published alignment of Mengoni *et al.* (2003), and three (L, M, N) were not. One additional clade (O) receives strong support in the maximum likelihood tree and weak support in the maximum parsimony tree based on the re-alignment.

A clade containing all five accessions of *A. serpyllifolium*, *A. pintodasilvae*, *A. quitianii* (= *A. pintodasilvae*) and *A. malacitanum* was present with weak support in both trees based on the re-alignment.

As in all four trees based on the published alignment, species from the A1 clade of Warwick *et al.* (2005) are not monophyletic in either of the two trees based on the re-alignment. Species from the A2 clade of Warwick *et al.* (2008) form a clade (clade B) in both these latter trees with moderate or strong support. The maximum parsimony and maximum likelihood trees based on the re-alignment have moderate or strong support for 8/19 nodes and 9/28 nodes respectively. These figures indicate a level of resolution and support that is approximately equal to that in the trees produced from the re-analysis of the published alignment, and considerably lower than in the published trees of Mengoni *et al.* (2003).

Total branch lengths in the maximum likelihood tree produced from the re-alignment are variable between species. Particularly long terminal branches were present for three accessions (*A. anatolicum*, *A. caricum*, and *A. wulfenianum*). The long terminal branches for *A. malacitanum* and *A. pintodasilvae* obtained in the re-analysis of the published alignment were not present here. Total tree length for the parsimony tree was 461 steps, i.e. 179 steps (28%) fewer than for the tree produced here from the published alignment.

Pseudogene Screening

Results of the 5.8S length analysis are presented in Table A5-1. The length of the 5.8S region lies outside the accepted functional range of 163–164 bp (Baldwin *et al.*, 1995) for twelve sequences, of *A. corsicum* (sequence accessions AY237950 and AY237949), *A. cypricum* (AY237948), *A. davisianum* (AY237947), *A. fallacinum* (= *A. baldaccii*) (AY237946), *A. malacitanum* (AY237941), *A. minus* (AY237939), *A. nebrodense* (AY237935), *A. oxycarpum* (AY237934), *A. pinifolium* (AY240871), *A. virgatum* (237925) and *A. wulfenianum* (AY237924).

Results of the analysis of 5.8S versus total *ITS* sequence variation cf. *Iberis umbellata* are presented in Table A5-1. The expected values relate to non-functional copy types of *ITS*; for functional copy types, conservation of the 5.8S region will reduce the variation of *ITS* due to the 5.8S region below this expected value. Observed variation values which approach or exceed the expected variation values therefore suggest the presence of non-functional copy types (pseudogenes). The observed value is very close to the expected value for *A. malacitanum* (26.4% and 26.9% respectively), and is >70% of the expected value for *A. wulfenianum* (19.9% and 26.3% respectively). Both of these sequences have 5.8S lengths outside the known functional range. For all other sequences the observed value is < 52 % of the expected value.

Conclusions

The qualitative evaluation of the published sequence alignment did not reveal any major alignment problems such as misplaced indels, incorrect sequence orientation or missing data. The alignment produced here using MUSCLE showed numerous minor differences to the published alignment, which had been produced using Multalign (Corpet, 1988). For a given alignment there were no supported incongruencies between trees produced here using maximum parsimony and maximum likelihood methods. The trees produced here from the published alignment had a similar topology to that of the published trees, but had lower levels of resolution and support. The trees produced from the re-alignment showed several differences in topology, and also had lower resolution and support. Trees produced here from the realignment had shorter branch lengths for some taxa, and contained a clade consisting of the possibly closely related taxa *A. serpyllifolium*, *A. pintodasilvae*, *A. malacitanum* and *A. quitianii* (= *A. pintodasilvae*), which was not present in the other trees produced from the Mengoni *et al.* (2003) dataset. The pseudogene screening carried out here found 5.8S sequence lengths outside the reported functional range in twelve *Alyssum* accessions (i.e. 32% of sequences in the alignment), and found a high proportion of sequence variation in the 5.8S region in two *Alyssum* accessions, for *A. malacitanum* and *A. wulfenianum*.

This study focused on the re-analysis of the Mengoni *et al.* (2003) *ITS* dataset, and was not a comprehensive review of all published *ITS* sequence data for *Alyssum* or the Alyseae. To this extent it was limited and was not able to assess the utility of the total published *ITS* dataset. An additional limitation was the lack of a statistical approach in the pseudogene screening procedure, meaning that the significance or otherwise of differences in observed and expected *ITS* variation could not be objectively determined. A tree-based approach which does allow statistical assessments has been proposed by Bailey *et al.* (2003), but was not employed here due to time constraints.

This analysis suggest that the allegations of poor data quality made against the Mengoni *et al.* (2003) *ITS* sequence data are not justified; there are no signs of poor sequencing or poor alignment potential in these sequences. However, re-alignment and re-analysis using phylogenetic methods has produced different results to those published by these authors, suggesting that errors may have occurred at the alignment, phylogenetic analysis or tree summary stage. The published results of Mengoni *et al.* (2003) should therefore not be accepted. Despite this, the Mengoni *et al.* (2003) sequence data should be regarded as a useful resource, despite pseudogene screening suggesting that some taxa may be represented by sequences for non-functional *ITS* copy types. The fact that the functional *ITS* sequence may be under-sampled in this dataset does mean, however, that the results obtained from any such future analysis should be interpreted with caution.

The lack of monophyly of species from the A1 clade of Warwick *et al.* (2008) is a notable result. In all cases the lack of monophyly was due to clade A (consisting of the two *A. murale* accessions, a species which is a member of clade A1 in Warwick *et al.* (2008)) being sister to clade B (consisting of species from section *Alyssum*, represented by clade A2 in Warwick *et al.* (2008)); all other *Alyssum* species in the tree were from section *Odontarrhena* (represented by Clade A1 in Warwick *et al.* (2008), and formed a separate clade, sister to the A + B clade here. This result also disagrees with the *ITS* trees of Cecchi *et al.* (2010), in which *A. murale* was nested within the A1 clade with strong support.

A wider phylogenetic analysis of the Alyseae to include all published *ITS* sequences would therefore be of considerable value in elucidating its phylogeny, as would the sequencing and phylogenetic analysis of other molecular markers such as chloroplast sequences or single-copy nuclear markers.

Figure A5-1. Strict consensus of 392 equally parsimonious trees produced from the published Mengoni *et al.* (2003) *ITS* alignment using Nona (version 2) spawned from Winclada (version 1). Tree rooted on *Alyssum–Iberis umbellata*. Branch numbers indicate % bootstrap support based on a 1000-replicate bootstrap analysis. Tree length = 640 steps; CI =0.52; RI = 0.54. Lettered clades or species groups are referred to in the main text.

Figure A5-2 Maximum likelihood tree produced from the published Mengoni *et al.* (2003) ITS alignment using RAxML (version 7.2.8 Black Box). Tree rooted on *Alyssum–Iberis umbellata*. Branch numbers indicate estimated bootstrap support. Lettered clades are referred to in the main text.

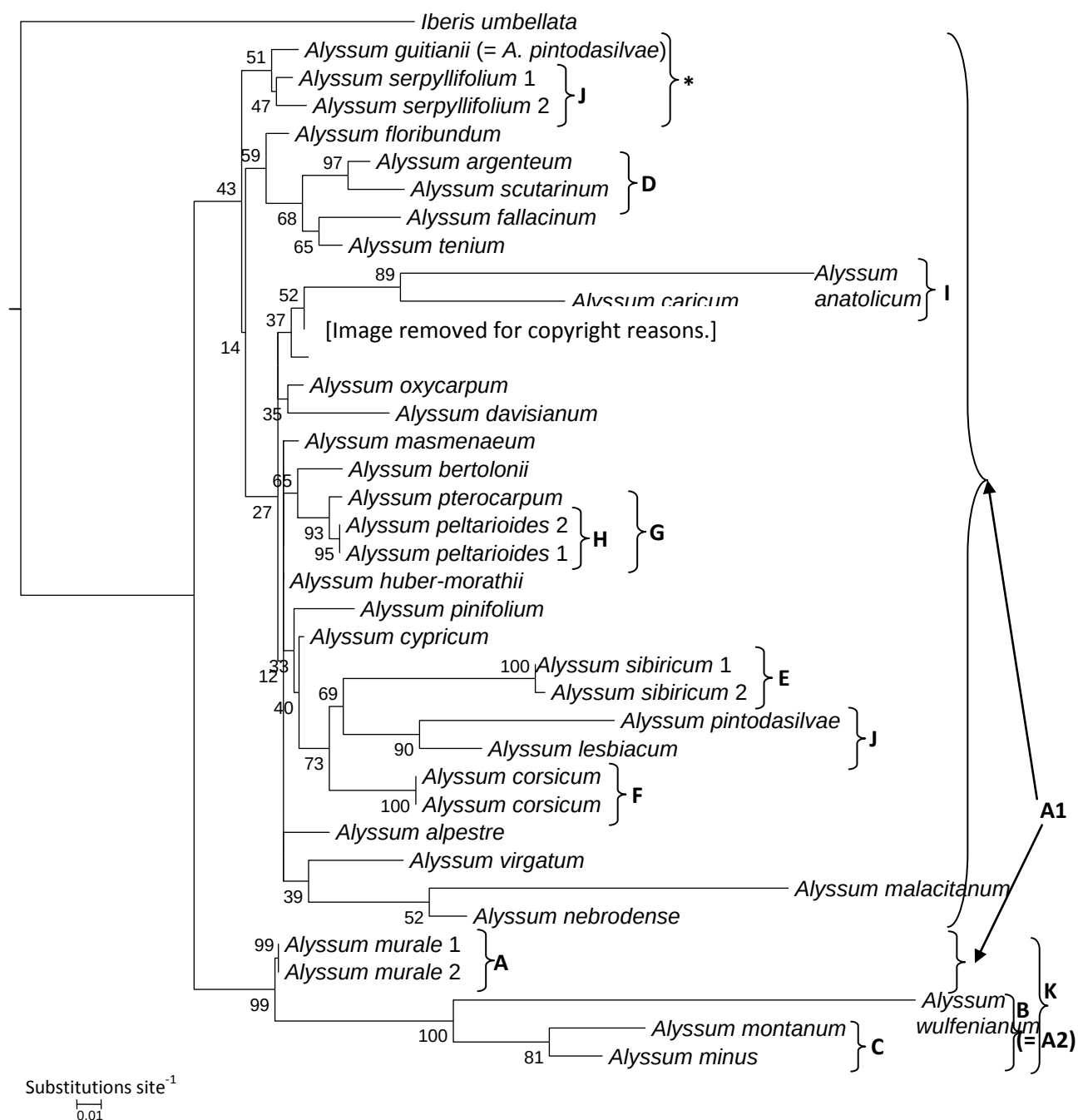


Figure A5-3 Consensus maximum parsimony tree of *Alyssum* species reproduced from Mengoni *et al.* (2003) (Figure 2 p.696 in that publication). Based on *ITS* sequence data aligned using Multialign (Corpet, 1988). Tree produced using Mega (version 3.2) (Kumar *et al.* 1993) and rooted on *Iberis umbellata*. Branch numbers indicate estimated percentage bootstrap support obtained from a 500-replicate analysis; value lower than 50% are not shown. Nickel hyperaccumulator species are shown in bold type. Lettered clades and groups are referred to in the text, these clades are not indicated in the published figure and have been added here. Number of equally parsimonious trees is not stated.

[Image removed for copyright reasons.]

Figure A5-4 Maximum likelihood tree of *Alyssum* species reproduced from Mengoni *et al.* (2003) (Figure 3, p.697 in that publication). Based on *ITS* sequence data aligned using Multialign (Corpet, 1988). Tree produced using PAUP (version 4.0b) (Swofford, 2000) and rooted on *Alyssum–Iberis umbellata*. Branch numbers indicate estimated percentage bootstrap support obtained from a 500-replicate analysis; value lower than 50% are not shown. Nickel hyperaccumulator species are shown in bold type. Lettered clades are referred to in the text, these clades are not indicated in the published figure and have been added here.

[Image removed for copyright reasons.]

Figure A5-5 Strict consensus of 392 maximum parsimony trees produced from the re-aligned Mengoni *et al.* (2003) ITS dataset using Nona (version 2) spawned from Winclada (version 1). Tree Rooted on *Alyssum–Iberis umbellata*. Branch numbers indicate % bootstrap support based on a 1000-replicate bootstrap analysis. Tree length = 461 steps; CI =0.52; RI = 0.60. Lettered clades are referred to in the text.

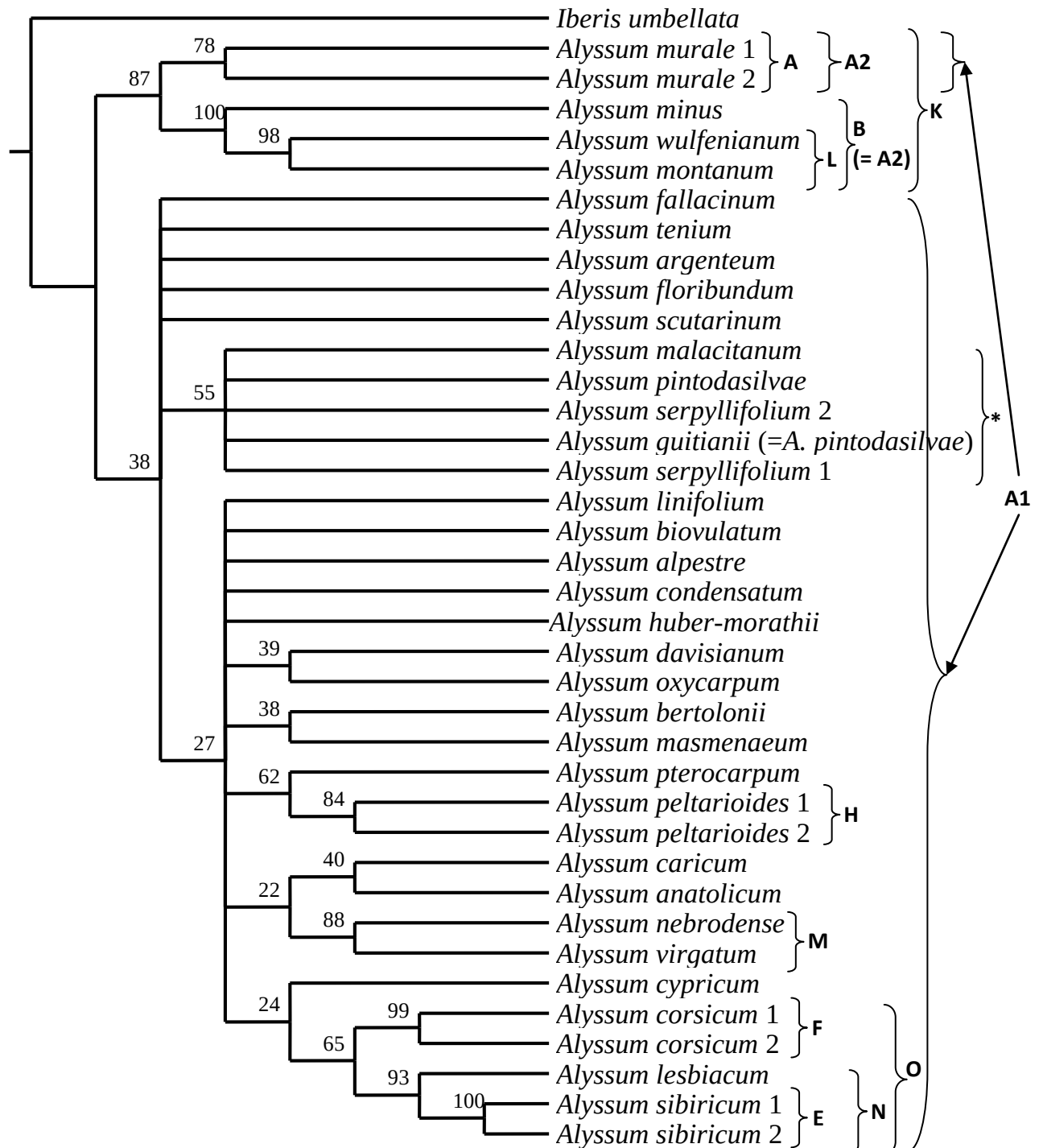


Figure A5-6 Maximum likelihood tree produced from the re-aligned Mengoni *et al.* (2003) ITS dataset produced using RAxML (version 7.2.8 Black Box). Tree rooted on *Alyssum–Iberis umbellata*. Branch numbers indicated estimated bootstrap support. Lettered clades are referred to in the text.

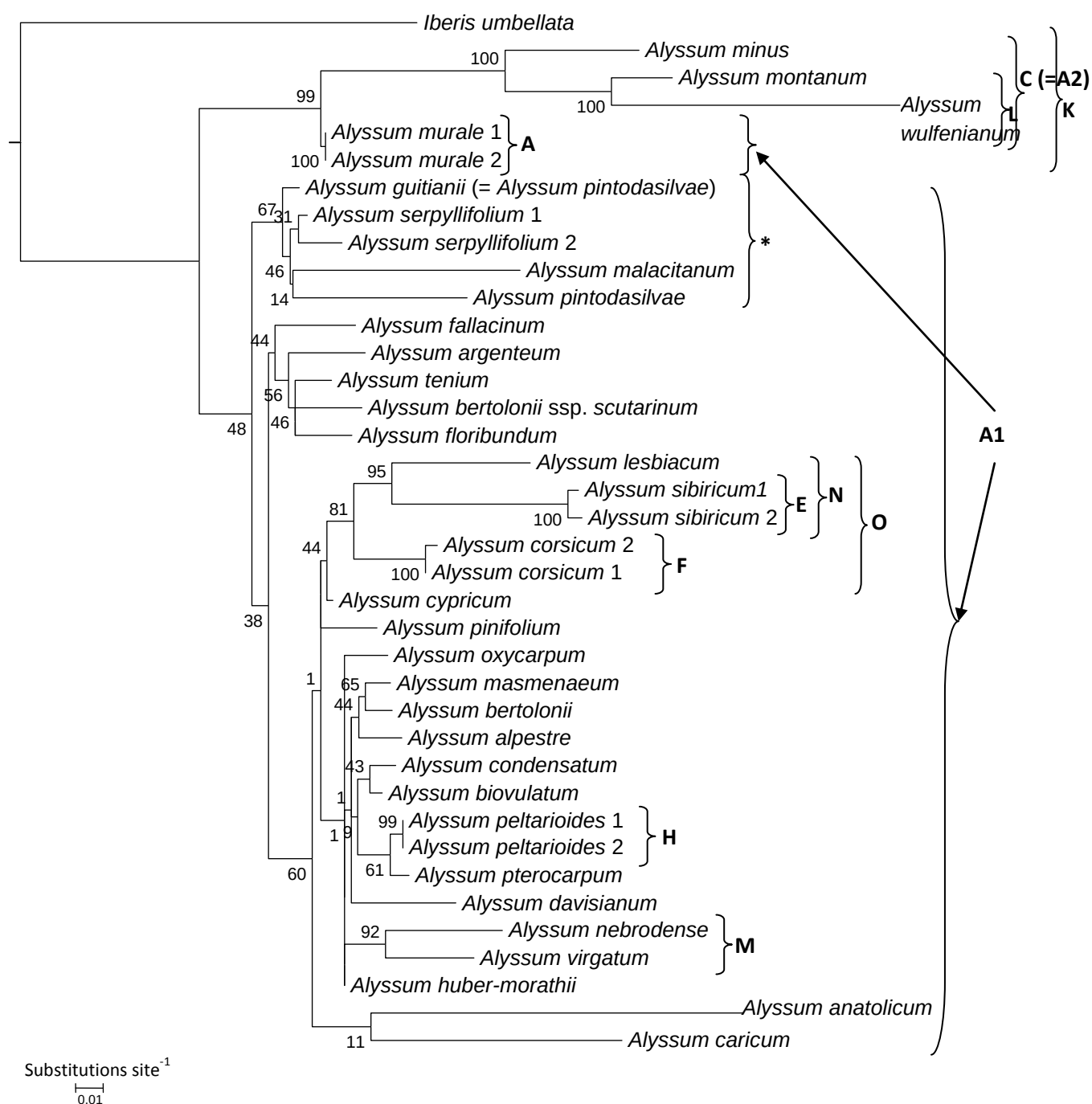


Table A5-1. Results of pseudogene screening for the Mengoni *et al.* (2003) *ITS* sequence data.

GenBank accession	Taxon ¹	5.8S length (bp) ²	% <i>ITS</i> variation (cf. <i>Iberis saxatilis</i>) due to 5.8S	
			Observed	Expected
AY237957	<i>Alyssum alpestre</i>	164	2.46	25.8
AY237956	<i>Alyssum anatolicum</i>	164	2.31	26.8
AY237955	<i>Alyssum argenteum</i>	164	1.56	25.2
AY237954	<i>Alyssum bertolonii</i>	164	1.61	25.7
AY237930	<i>Alyssum bertolonii</i> ssp. <i>scutarinum</i>	164	1.48	25.5
AY237953	<i>Alyssum biovulatum</i> (= <i>A. obovatum</i>)	164	2.36	25.7
AY237952	<i>Alyssum caricum</i>	164	1.30	25.4
AY237951	<i>Alyssum condensatum</i> ssp. <i>flexibile</i>	164	1.67	26.2
AY237950	<i>Alyssum corsicum</i> (1)	166	7.91	25.8
AY237949	<i>Alyssum corsicum</i> (2)	166	7.19	25.9
AY237948	<i>Alyssum cyprium</i>	165	3.36	25.8
AY237947	<i>Alyssum davisianum</i>	169	5.93	26.2
AY237946	<i>Alyssum fallacinum</i> (= <i>A. baldaccii</i>)	165	4.00	25.7
AY237945	<i>Alyssum floribundum</i>	164	1.56	25.8
AY237944	<i>Alyssum guitanii</i> (= <i>A. pintodasilvae</i>)	164	1.61	25.4
AY237943	<i>Alyssum huber-morathii</i>	164	1.68	25.8
AY237942	<i>Alyssum lesbiacum</i>	163	13.0	25.6
AY237941	<i>Alyssum malacitanum</i>	185	26.4	26.9
AY237940	<i>Alyssum masmenaeum</i>	164	3.25	25.8
AY237939	<i>Alyssum minus</i>	165	2.33	26.1
AY237938	<i>Alyssum montanum</i>	164	1.94	25.6
AY237937	<i>Alyssum murale</i> ssp. <i>murale</i> var. <i>murale</i> (1)	164	1.53	25.7
AY237936	<i>Alyssum murale</i> ssp. <i>murale</i> var. <i>murale</i> (2)	164	1.53	25.7
AY237935	<i>Alyssum nebrodense</i>	175	9.30	26.0
AY237934	<i>Alyssum oxycarpum</i>	165	3.28	25.7
AY237933	<i>Alyssum peltarioides</i> (1)	164	1.57	25.8
AY237932	<i>Alyssum peltarioides</i> (2)	164	1.56	25.8
AY240871	<i>Alyssum pinifolium</i>	167	3.15	26.3
AY237929	<i>Alyssum pintodasilvae</i>	164	9.52	25.6
AY239731	<i>Alyssum pterocarpum</i>	164	1.56	25.8
AY237923	<i>Alyssum serpyllifolium</i> ssp. <i>serpyllifolium</i> (1)	164	1.59	25.9
AY237922	<i>Alyssum serpyllifolium</i> ssp. <i>serpyllifolium</i> (2)	164	1.42	25.9
AY237928	<i>Alyssum sibiricum</i> (1)	164	13.2	25.6
AY237927	<i>Alyssum sibiricum</i> (2)	164	12.5	25.6
AY237926	<i>Alyssum tenium</i>	163	2.29	25.4
AY237925	<i>Alyssum virgatum</i>	176	10.3	27.1
AY237924	<i>Alyssum wulfenianum</i>	195	19.9	28.4

¹Taxon names are those listed in Mengoni *et al.* (2003). Synonymy *sensu* Appendix 1 is indicated in parentheses where appropriate. Accessions numbers are in parentheses.

²5.8S lengths in bold are those outside the reported functional range of 163–164 bp (Baldwin *et al.*, 1995) and hence indicative of potentially non-functional *ITS* copy types (pseudogenes).

Appendix 6:

Non Time-calibrated Trees

Maximum Likelihood Trees:	Page
Figure A6-1 RAxML tree based on <i>ITS</i>	A6-2
Figure A6-2 RAxML tree based on <i>matK</i>	A6-4
Figure A6-3 RAxML tree based on <i>ndhF</i>	A6-6
Figure A6-4 RAxML tree based on <i>PHYA</i>	A6-8
Figure A6-5 RAxML tree based on <i>rbcl</i>	A6-10
Figure A6-6 RAxML tree based on <i>rps16-trnK</i>	A6-11
Figure A6-7 RAxML tree based on <i>trnD-trnT</i>	A6-13
Figure A6-8 RAxML tree based on <i>trnL-trnF</i>	A6-15
Figure A6-9 RAxML tree based on Concatenated Alignment A	A6-17
Figure A6-10 RAxML tree based on Concatenated Alignment C	A6-19

Parsimony Trees

Figure A6-11 Winclada/NONA tree based on Concatenated Alignment A	A6-21
Figure A6-12 Winclada/NONA tree based on Concatenated Alignment B	A6-24

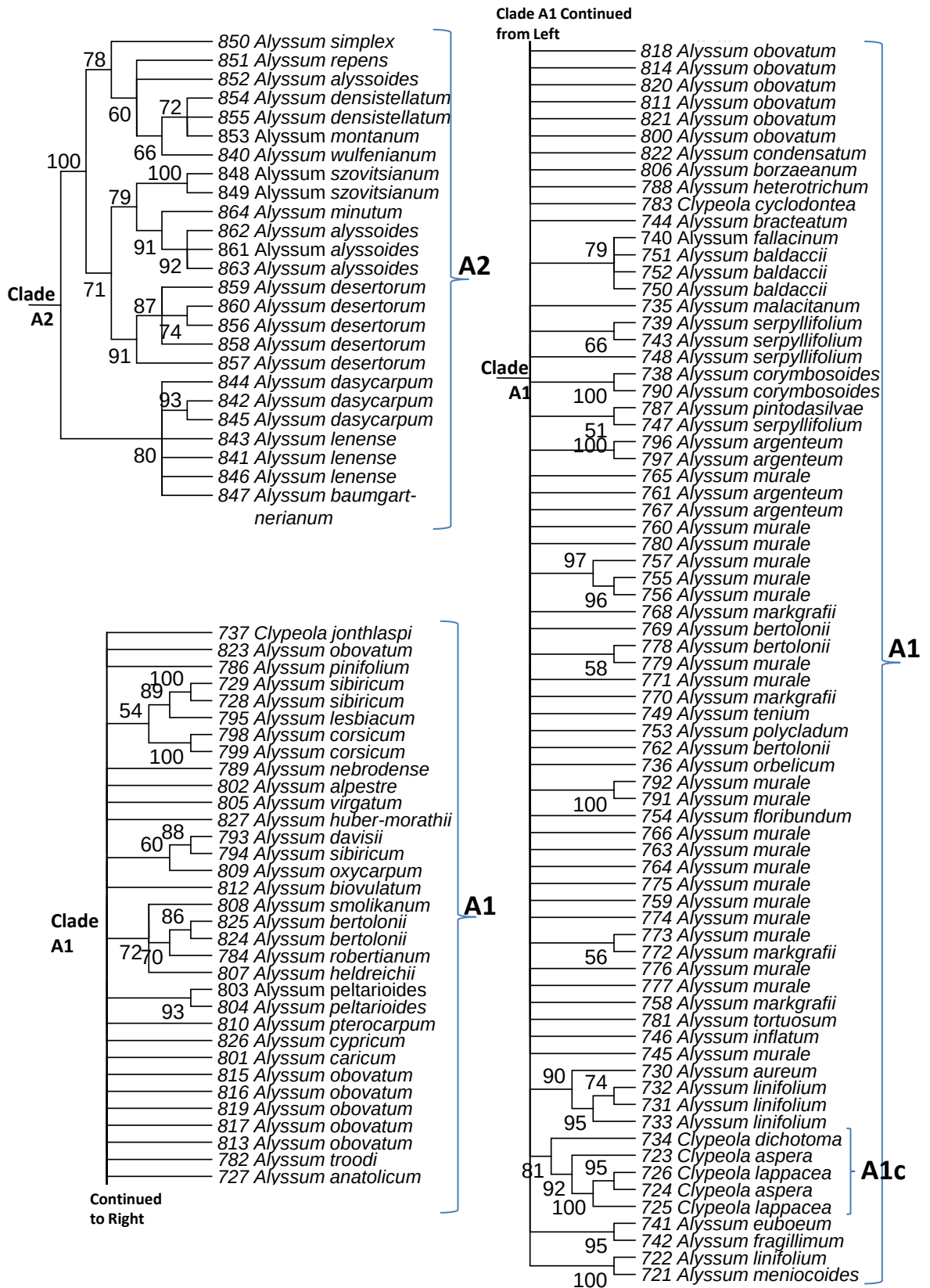


Figure A6-1b Detail of Clades A1 and A2 from Figure A6-1a (maximum likelihood tree for Alyseae based on *ITS*). Figures on branches indicate estimated bootstrap support. Nodes receiving < 50 % bootstrap support have been collapsed.

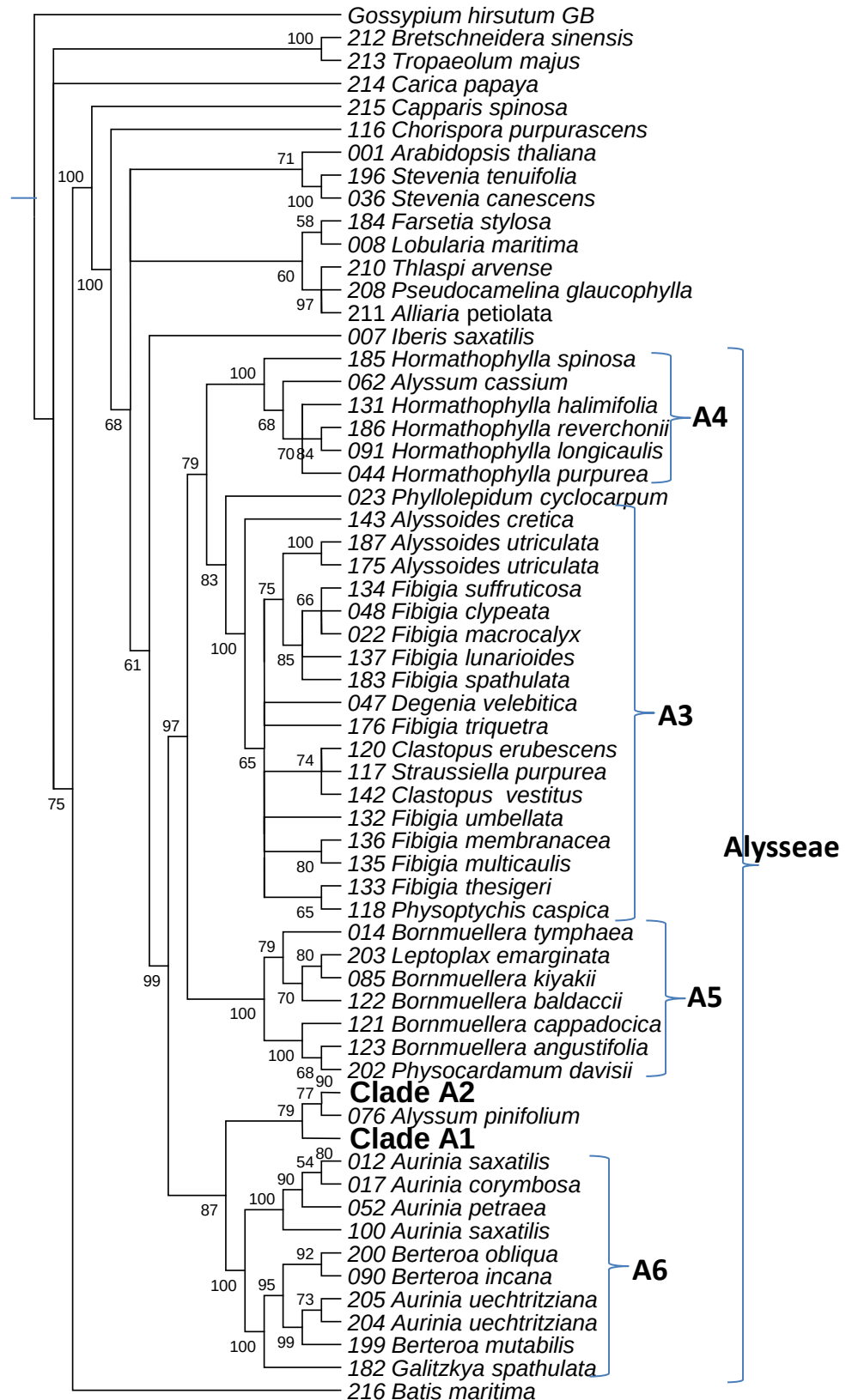
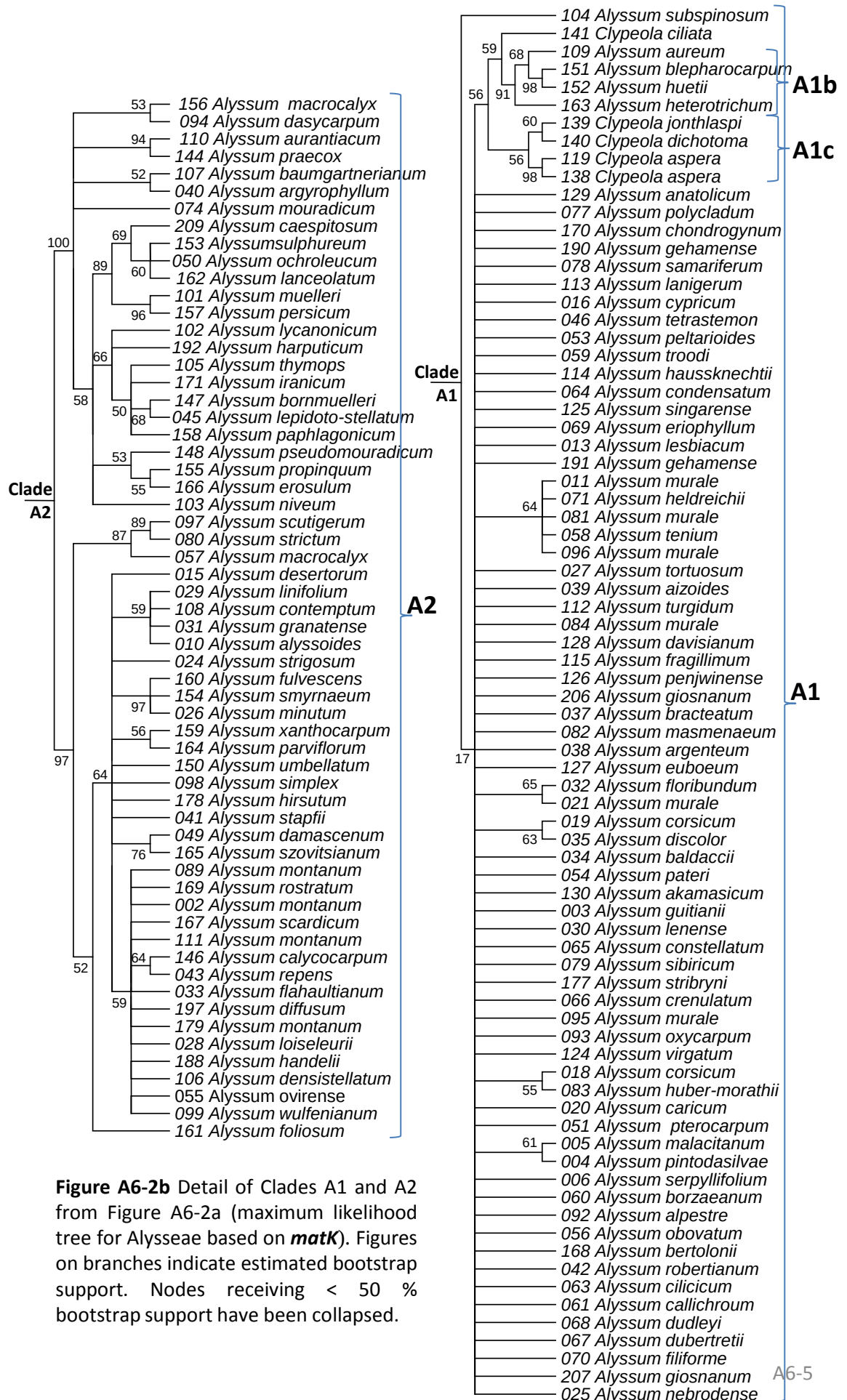
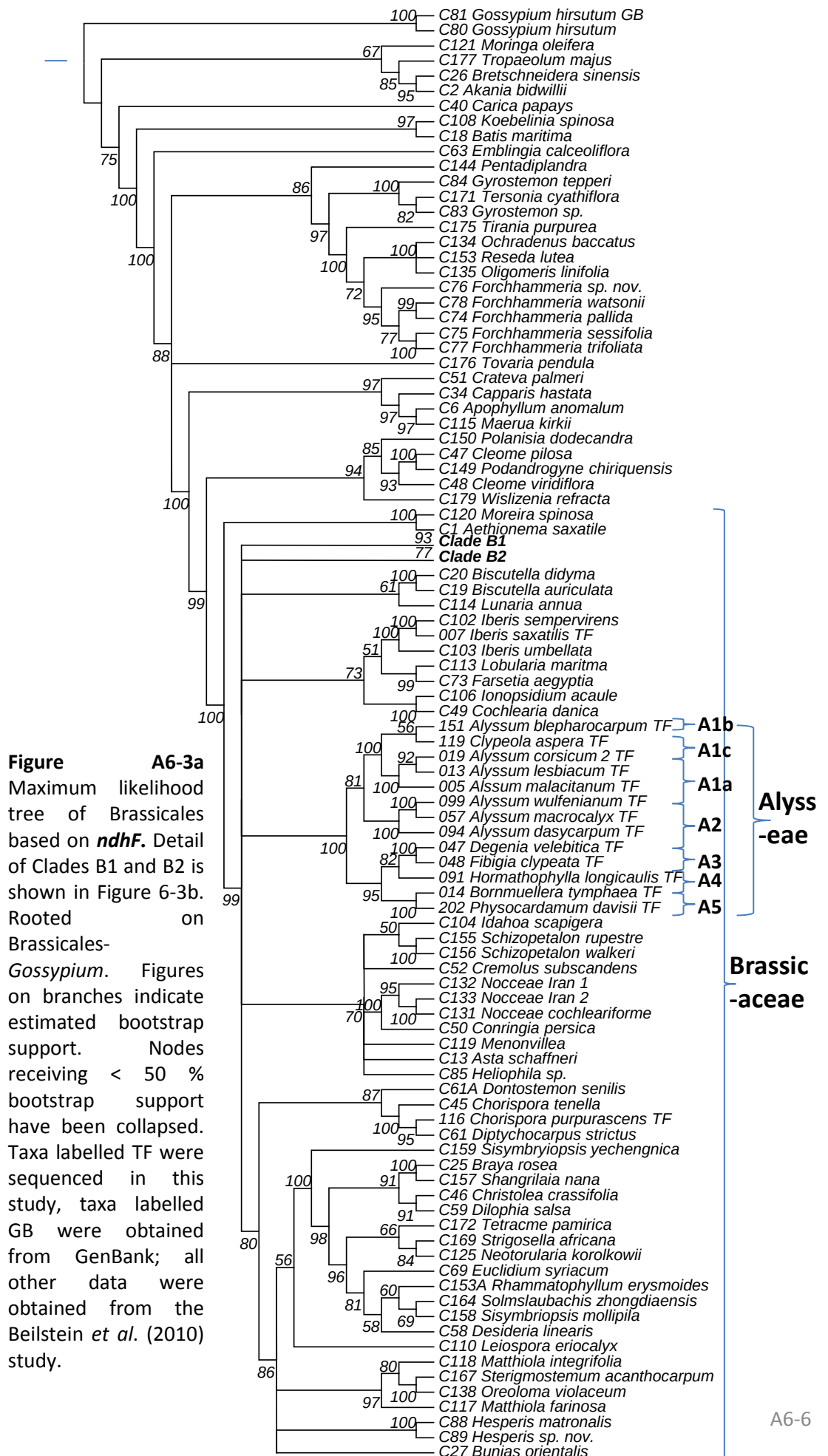


Figure A6-2a Maximum likelihood tree for Alysseae based on *matK*. Detail of Clades A1 and A2 is shown in Figure A6-2b. Rooted on Brassicales-*Gossypium hirsutum*. Figures on branches indicate estimated bootstrap support. Nodes receiving < 50 % bootstrap support have been collapsed.





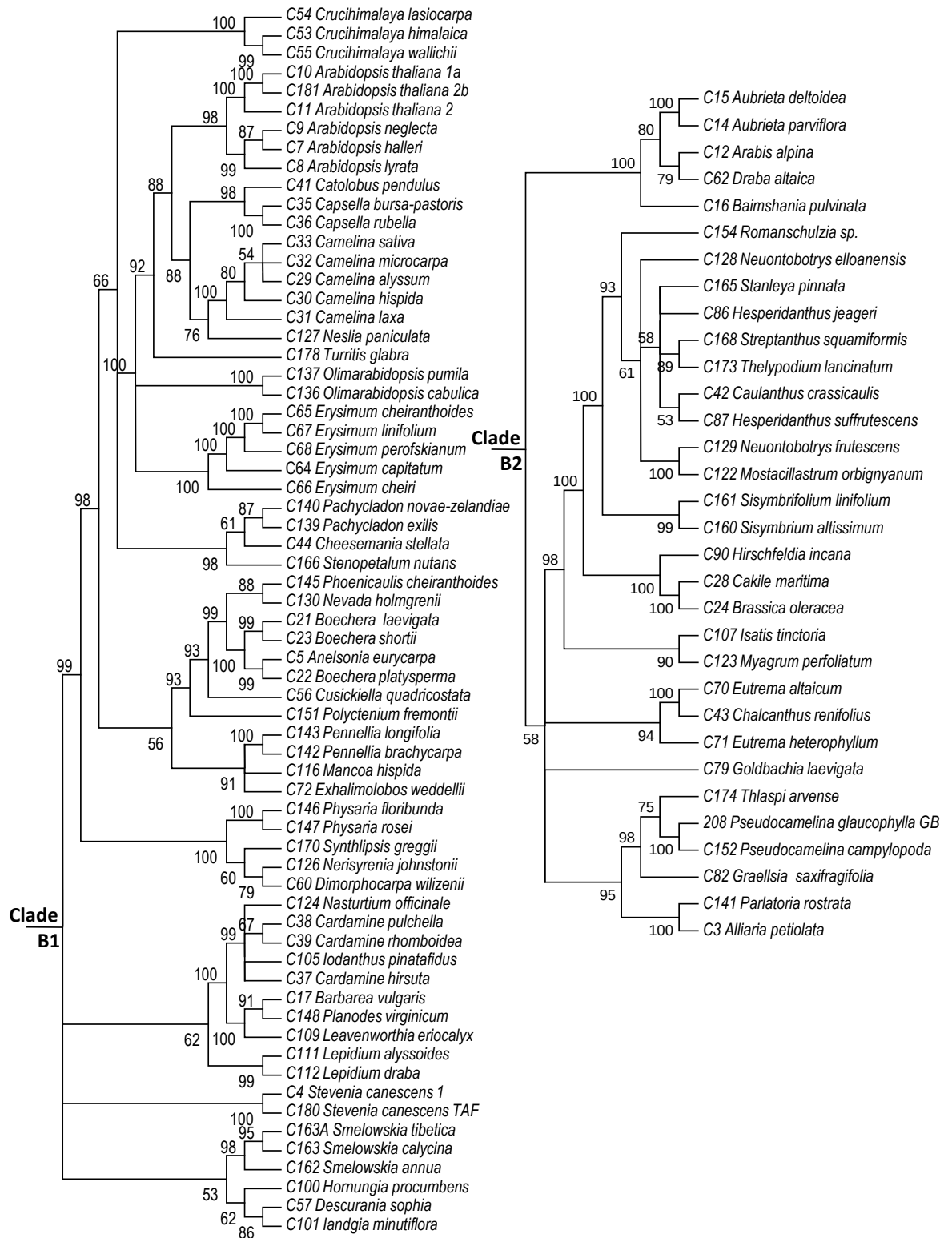
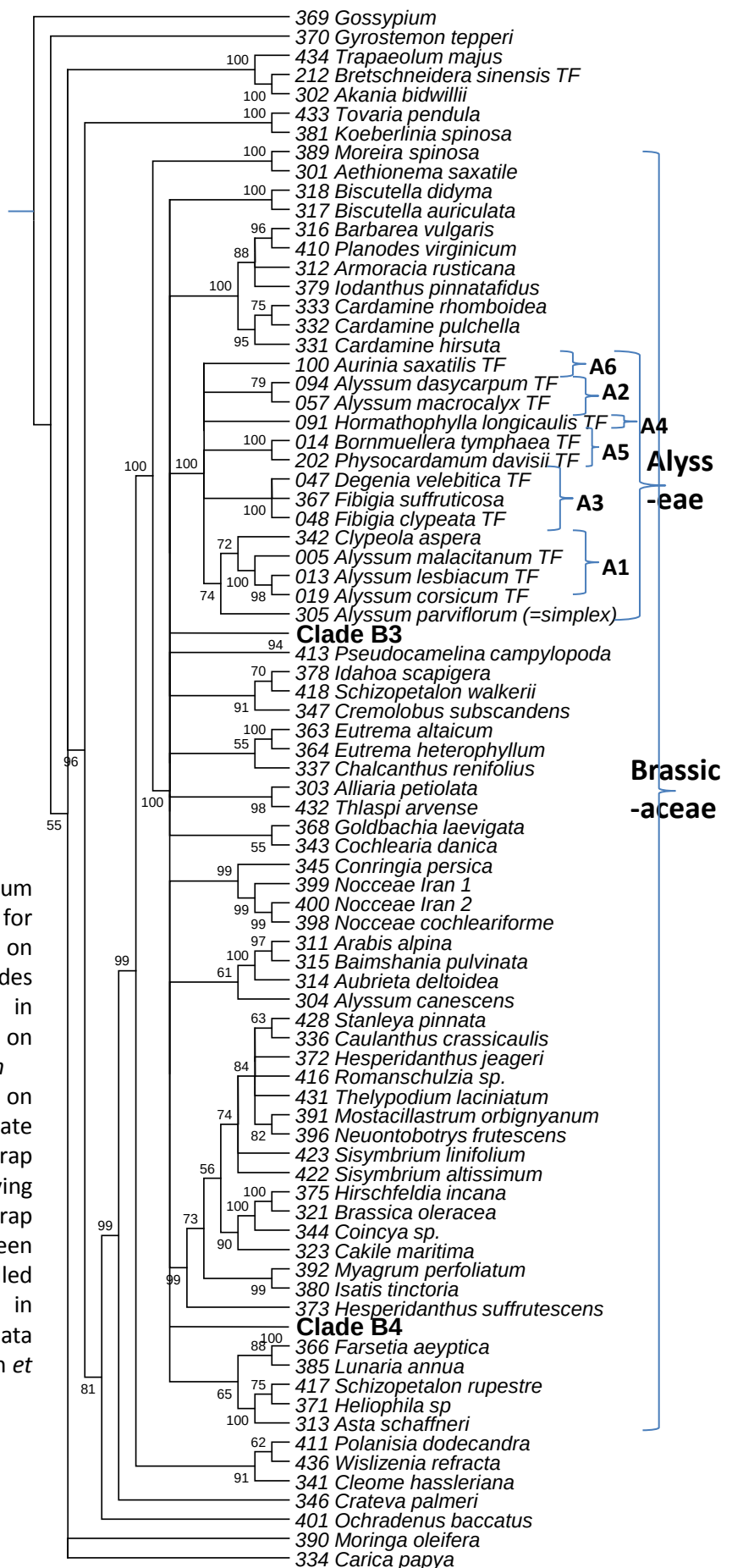


Figure A6-3b Detail of Clades B1 and B2 from Figure A6-3a (maximum likelihood tree for Alyseae based on *ndhF*). Figures on branches indicate estimated bootstrap support. Nodes receiving < 50 % bootstrap support have been collapsed. Taxa labelled TF were sequenced in this study, taxa labelled GB were obtained from GenBank; all other data were obtained from the Beilstein *et al.* (2010) study.

Figure A6-4a Maximum likelihood tree for Brassicales based on **PHYA**. Detail of Clades B3 and B4 is shown in Figure A6-4b. Rooted on Brassicales-*Gossypium gossypoides*. Figures on branches indicate estimated bootstrap support. Nodes receiving < 50 % bootstrap support have been collapsed. Taxa labelled TF were sequenced in this study, all other data was from the Beilstein *et al.* (2010) study.



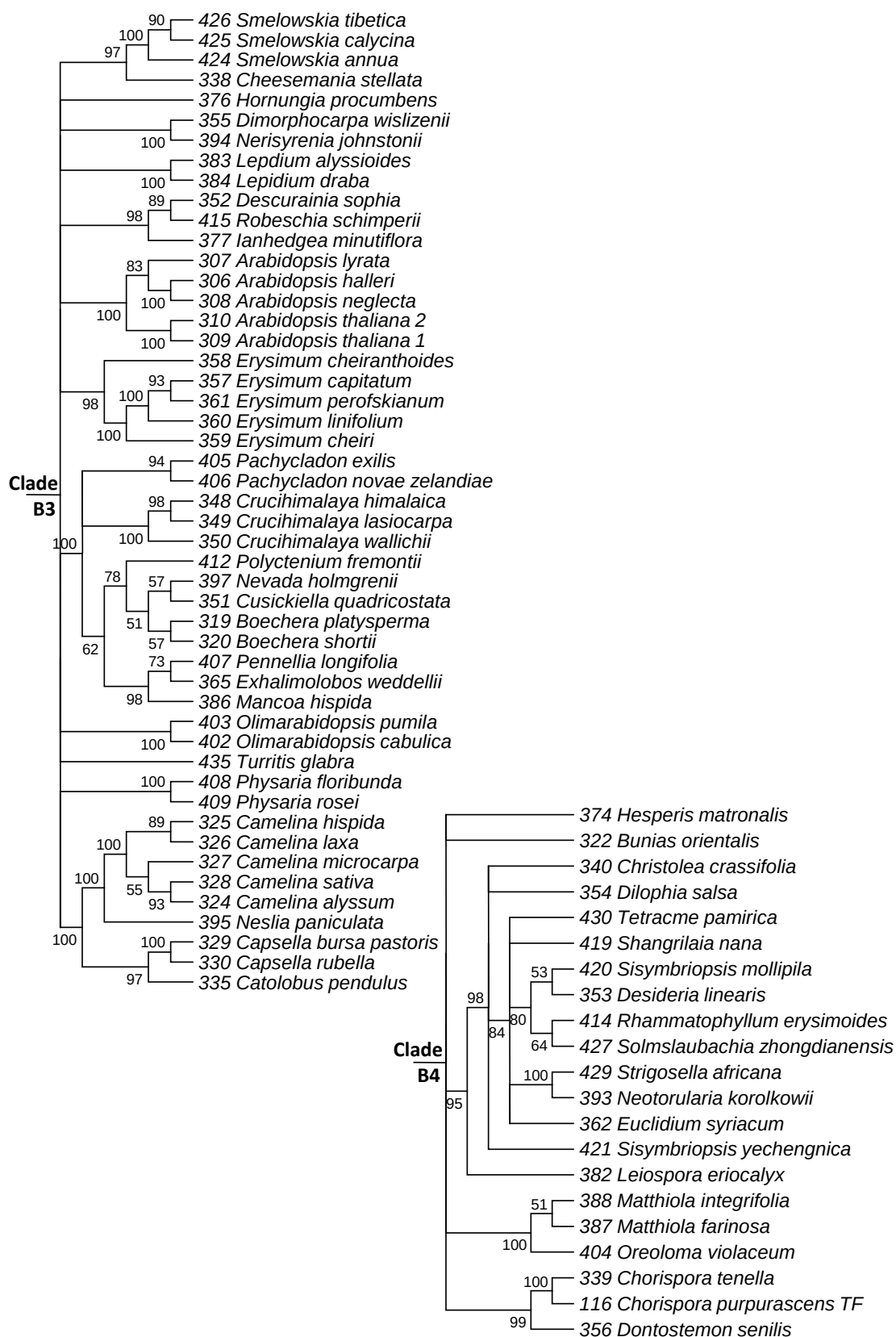


Figure A6-4b Detail of Clades B3 and B4 from Figure A6-4a (maximum likelihood tree for Brassicales based on *PHYA*). Figures on branches indicate estimated bootstrap support. Nodes receiving < 50 % bootstrap support have been collapsed. Taxa labelled TF were sequenced in this study, all other data were obtained from the Beilstein *et al.* (2010) study

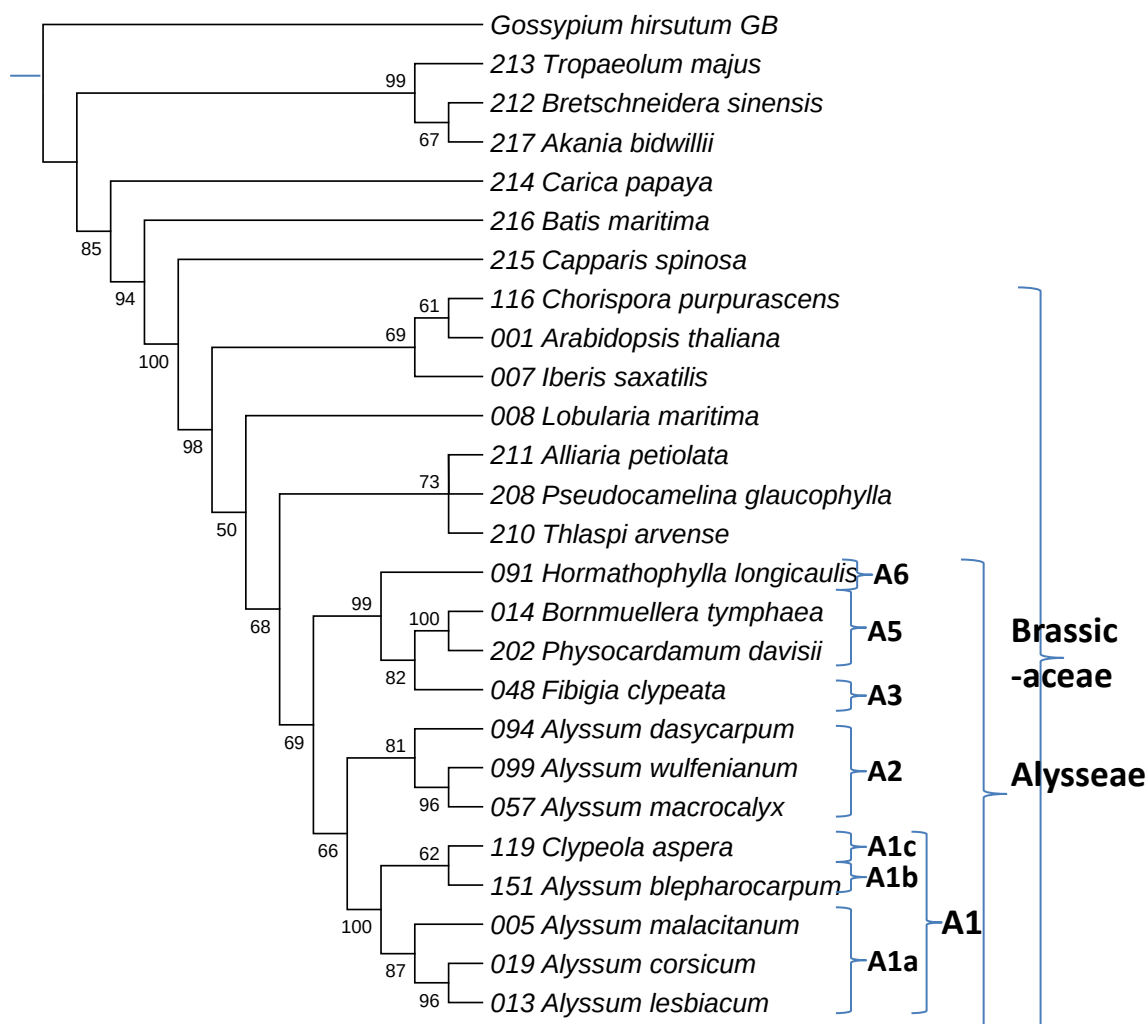


Figure A6-5 Maximum likelihood tree for Brassicales based on *rbcL*. Rooted on Brassicales-*Gossypium hirsutum*. Figures on branches indicate estimated bootstrap support. Nodes receiving < 50 % bootstrap support have been collapsed.

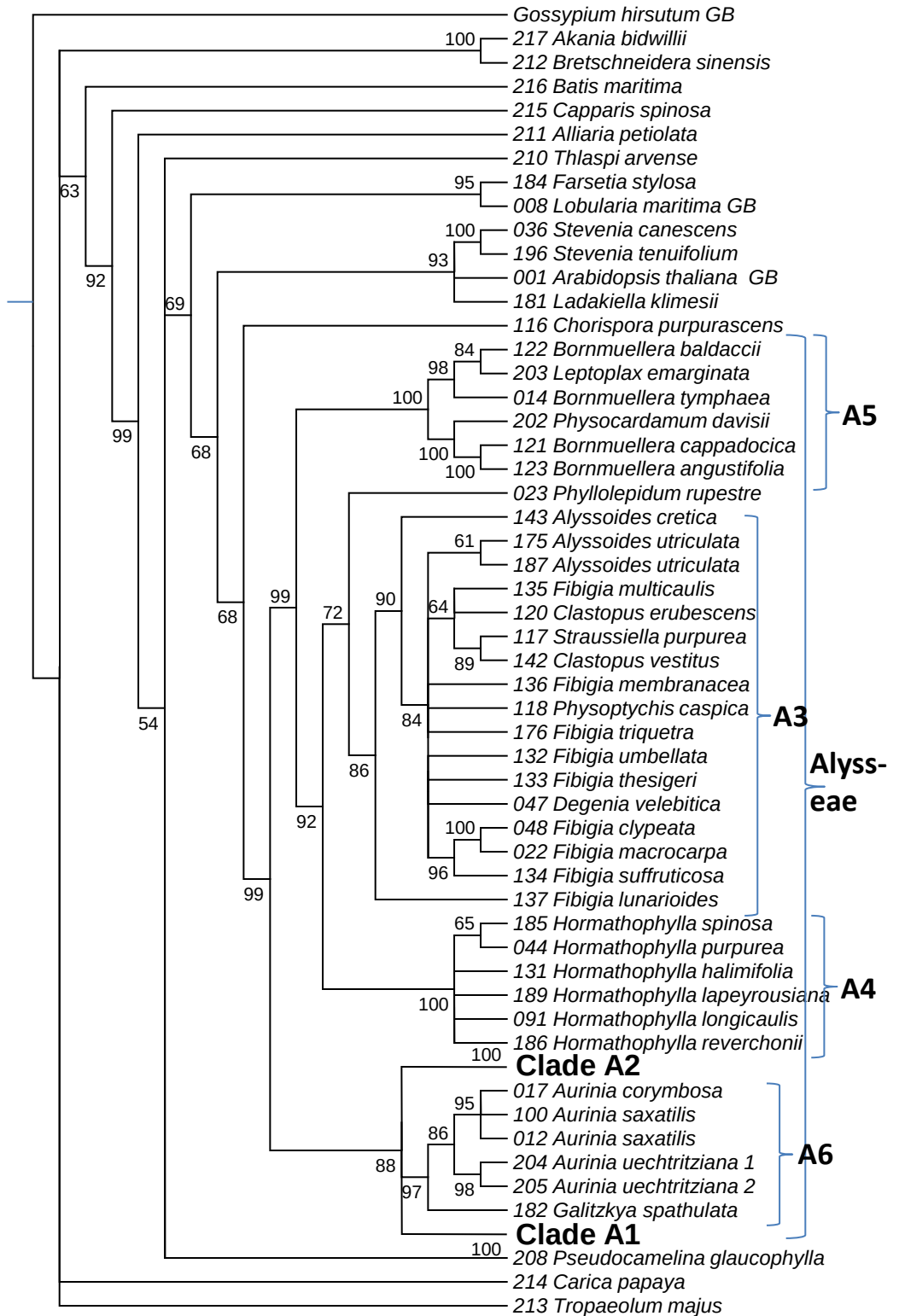


Figure A6-6a Maximum likelihood tree for Brassicales based on *rps16-trnK*. Detail of Clades A1 and A2 is indicated in Figure A6-6b. Rooted on Brassicales-*Gossypium hirsutum*. Figures on branches indicate estimated bootstrap support. Nodes receiving < 50 % bootstrap support have been collapsed. Taxa labelled GB were obtained from GenBank; all other data were sequenced in the present study

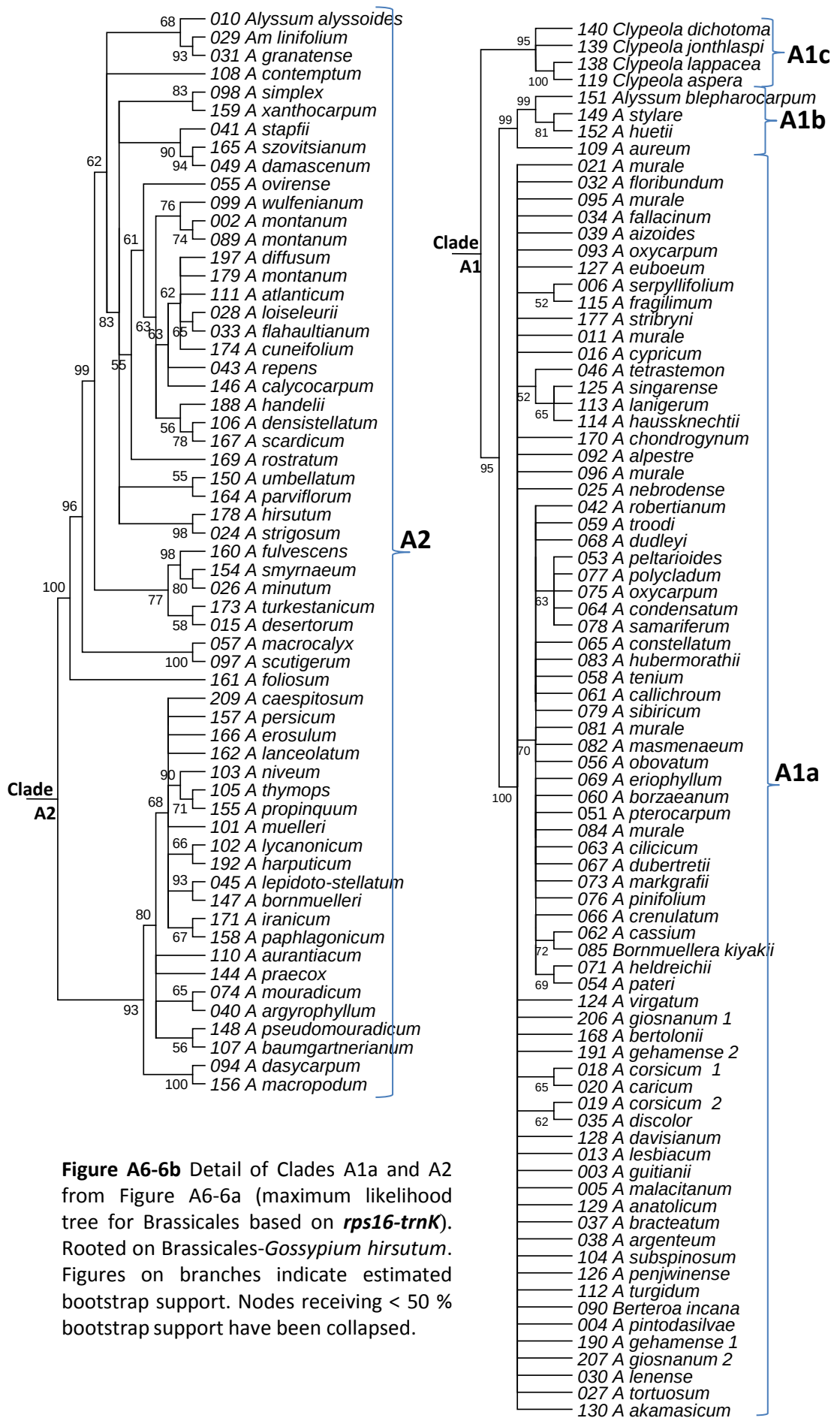


Figure A6-6b Detail of Clades A1a and A2 from Figure A6-6a (maximum likelihood tree for Brassicales based on *rps16-trnK*). Rooted on Brassicales-*Gossypium hirsutum*. Figures on branches indicate estimated bootstrap support. Nodes receiving < 50 % bootstrap support have been collapsed.

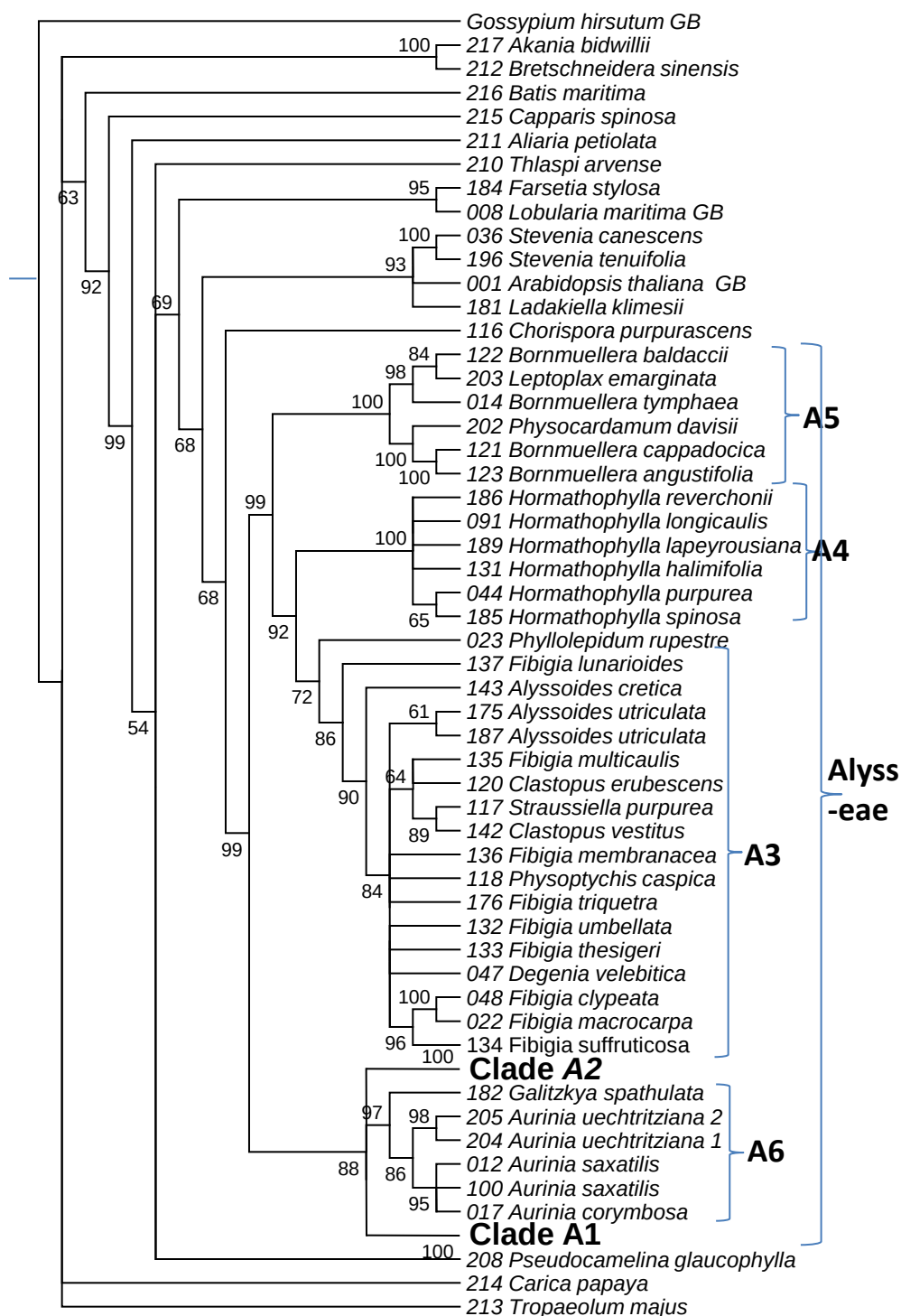


Figure A6-7a Maximum likelihood tree for Brassicales based on *trnD-trnT*. Detail of Clades A1 and A2 from as indicated in Figure A6-7b. Rooted on Brassicales-*Gossypium hirsutum*. Figures on branches indicate estimated bootstrap support. Nodes receiving < 50 % bootstrap support have been collapsed. Taxa labelled GB were obtained from GenBank; all other data were obtained from the Beilstein *et al.* (2010) study

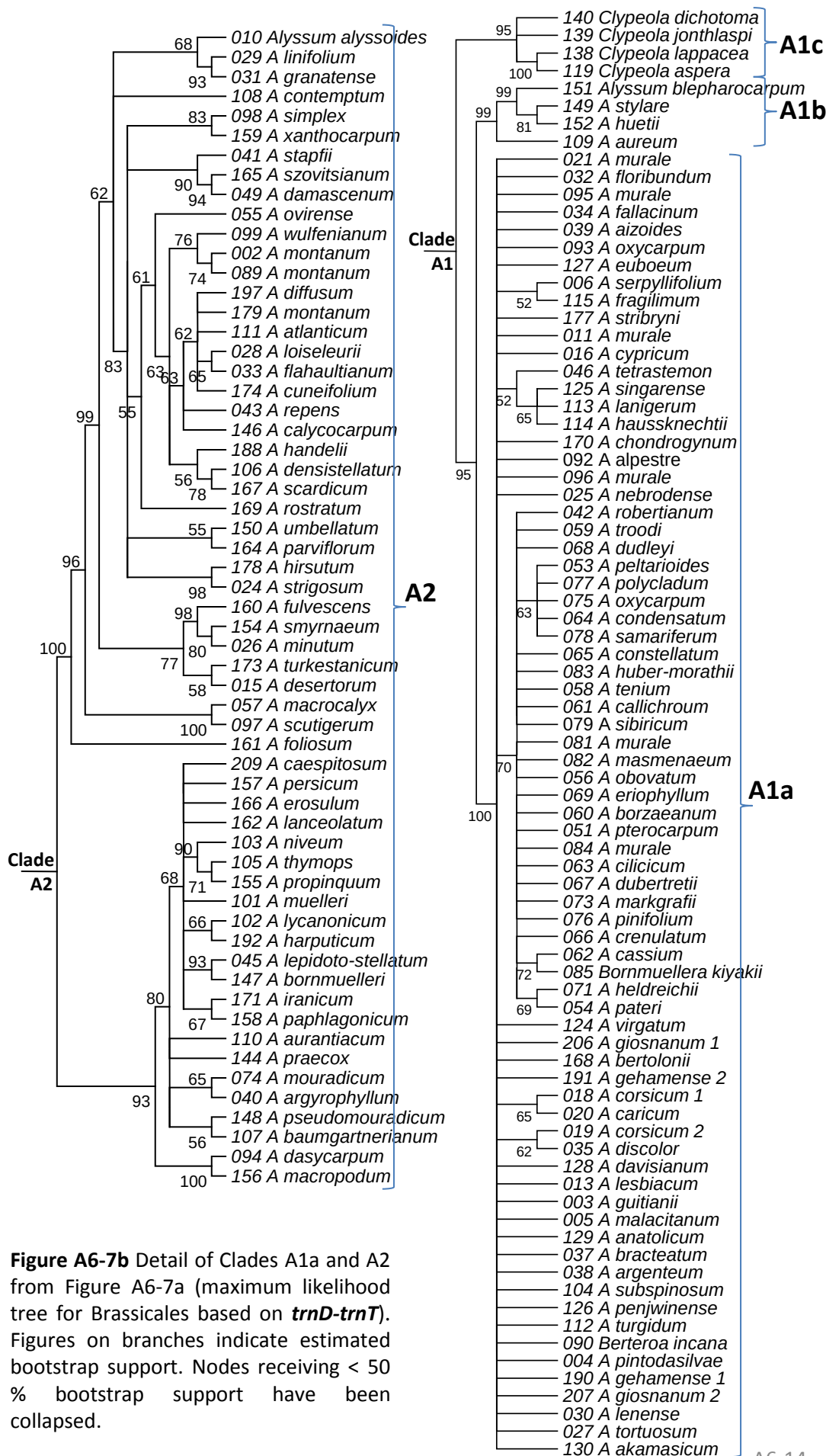


Figure A6-7b Detail of Clades A1a and A2 from Figure A6-7a (maximum likelihood tree for Brassicales based on *trnD-trnT*). Figures on branches indicate estimated bootstrap support. Nodes receiving < 50 % bootstrap support have been collapsed.

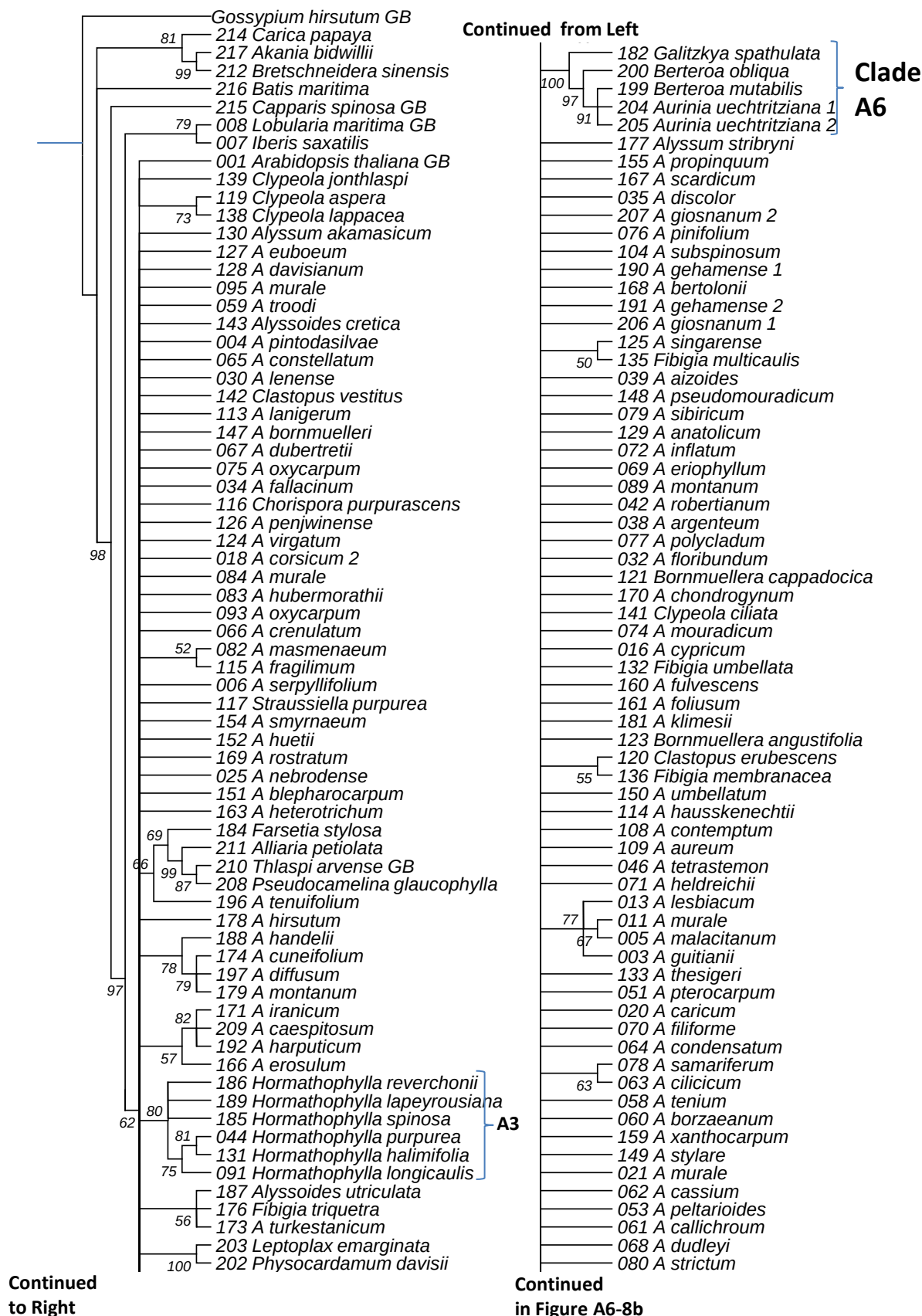


Figure A6-8a Maximum likelihood tree for Brassicales based on *trnL-trnF*. The tree continues in Figure A6-8b. Figures on branches indicate estimated bootstrap support. Tree rooted on *Gossypium*-Brassicales. Branches receiving < 50 % bootstrap support have been collapsed. Taxa labelled TF were sequenced in this study, taxa labelled GB were obtained from GenBank; all other taxa were sequenced in the present study.

Continued
From Figure A6-8a

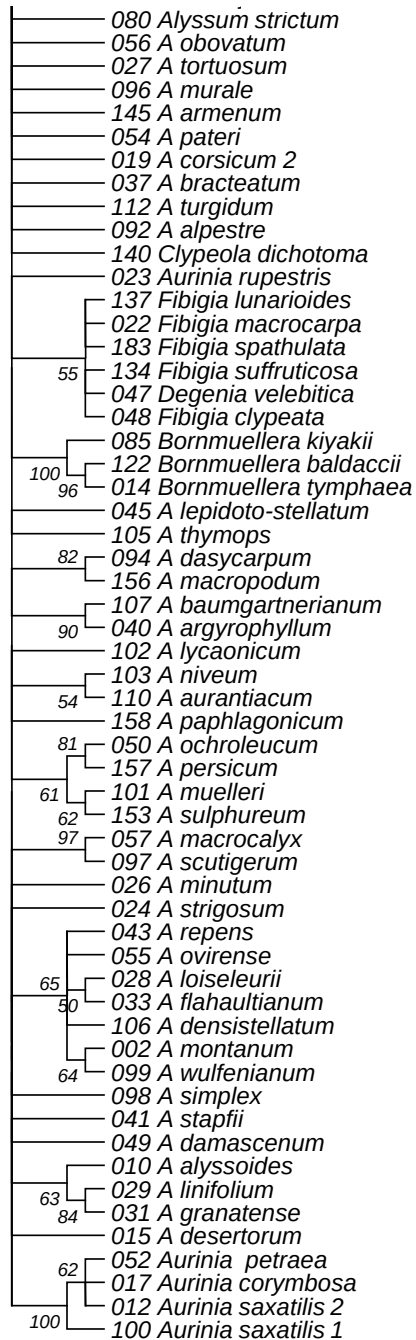


Figure A6-8b Figure continued from Figure A6-8a (maximum likelihood tree for *trnL-trnF*). Figures on branches indicate estimated bootstrap support. Tree rooted on *Gossypium*-Brassicales. Branches receiving < 50 % bootstrap support have been collapsed.

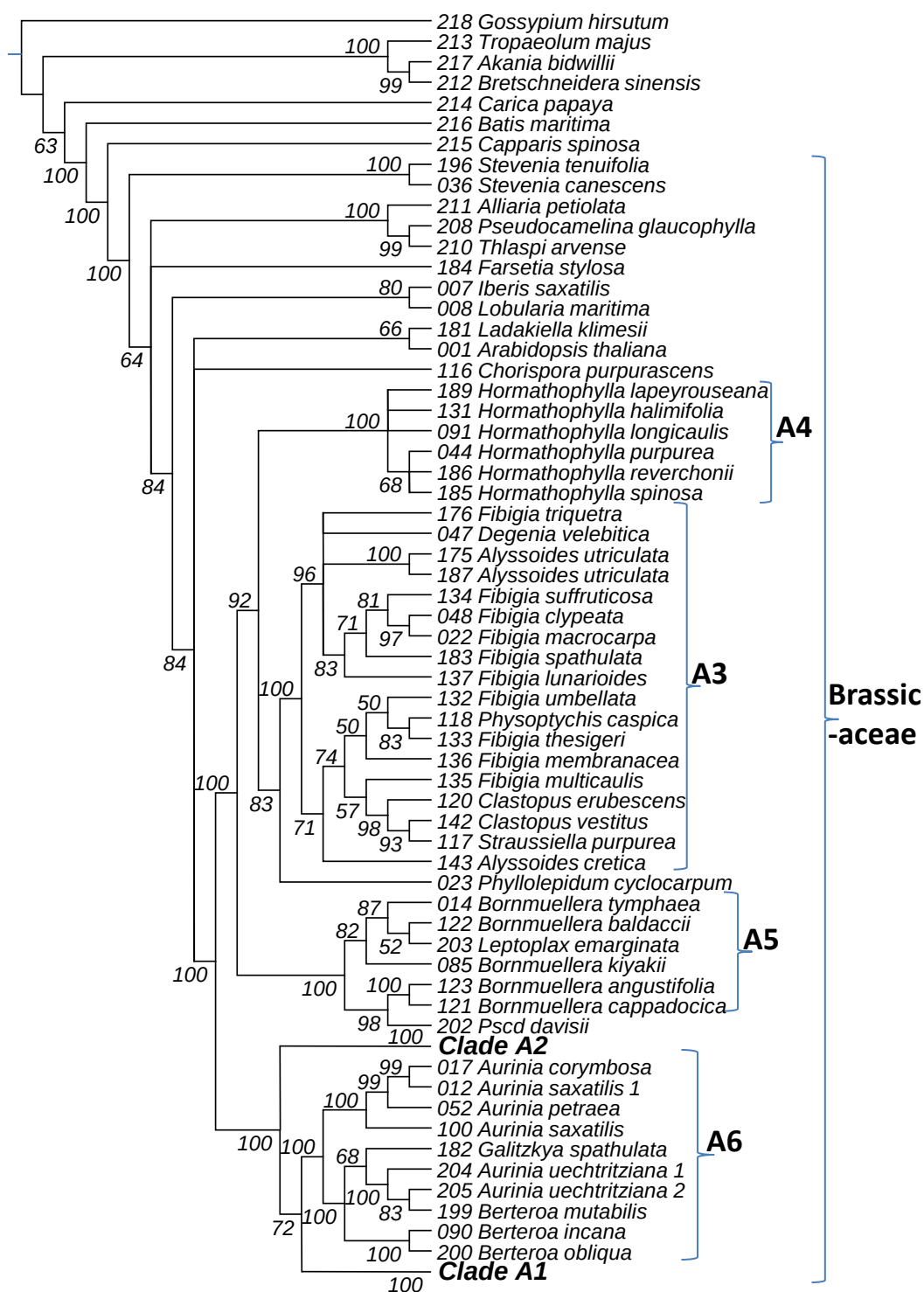


Figure A6-9a Maximum likelihood tree for Brassicales based on **Concatenated Alignment A**. Detail of Clades A1 and A2 is indicated in Figure 3-9b. Rooted on *Gossypium*-Brassicales. Figures on branches indicate estimated bootstrap support. Nodes receiving < 50 % bootstrap support have been collapsed.

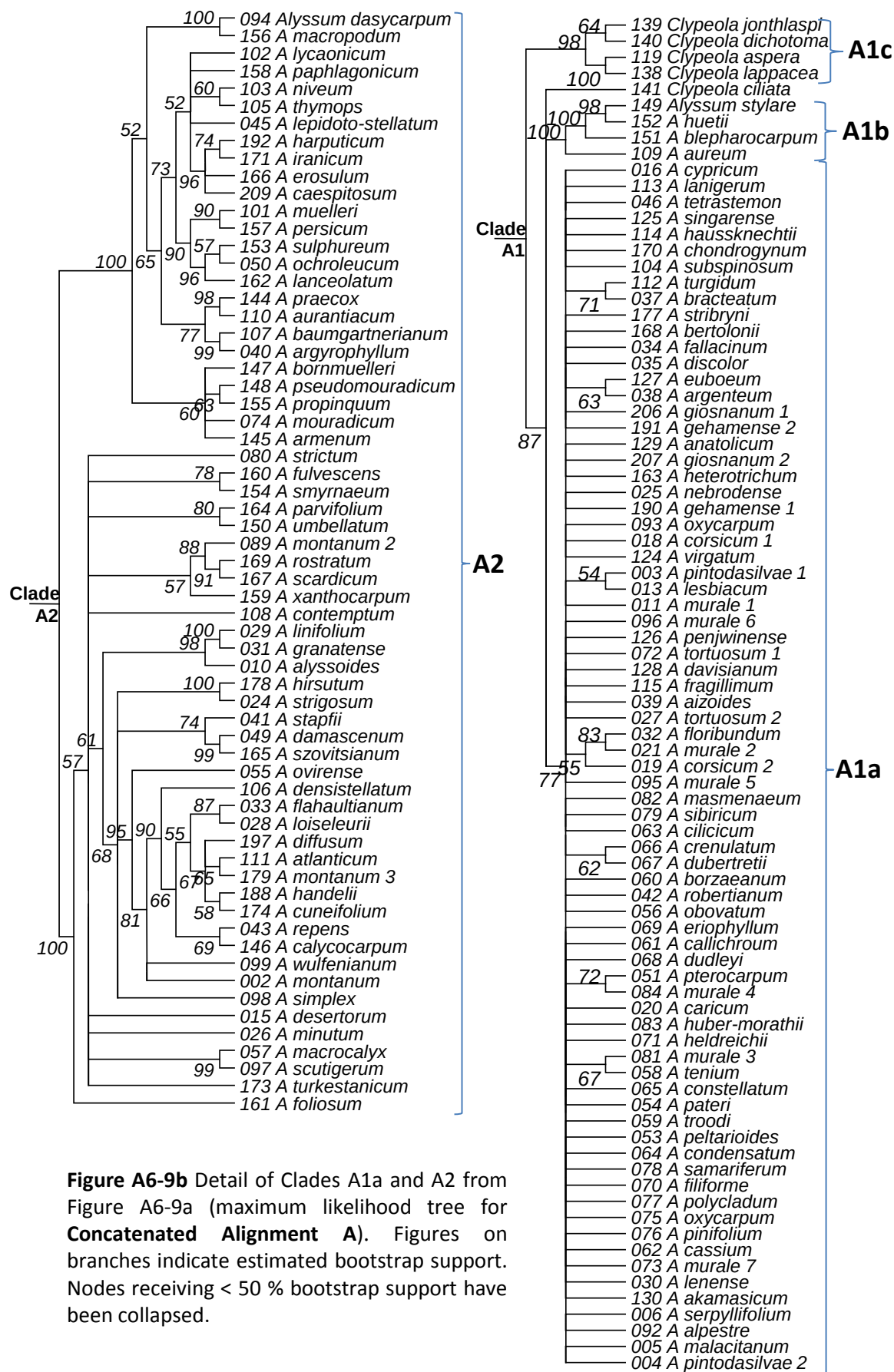


Figure A6-9b Detail of Clades A1a and A2 from Figure A6-9a (maximum likelihood tree for **Concatenated Alignment A**). Figures on branches indicate estimated bootstrap support. Nodes receiving < 50 % bootstrap support have been collapsed.

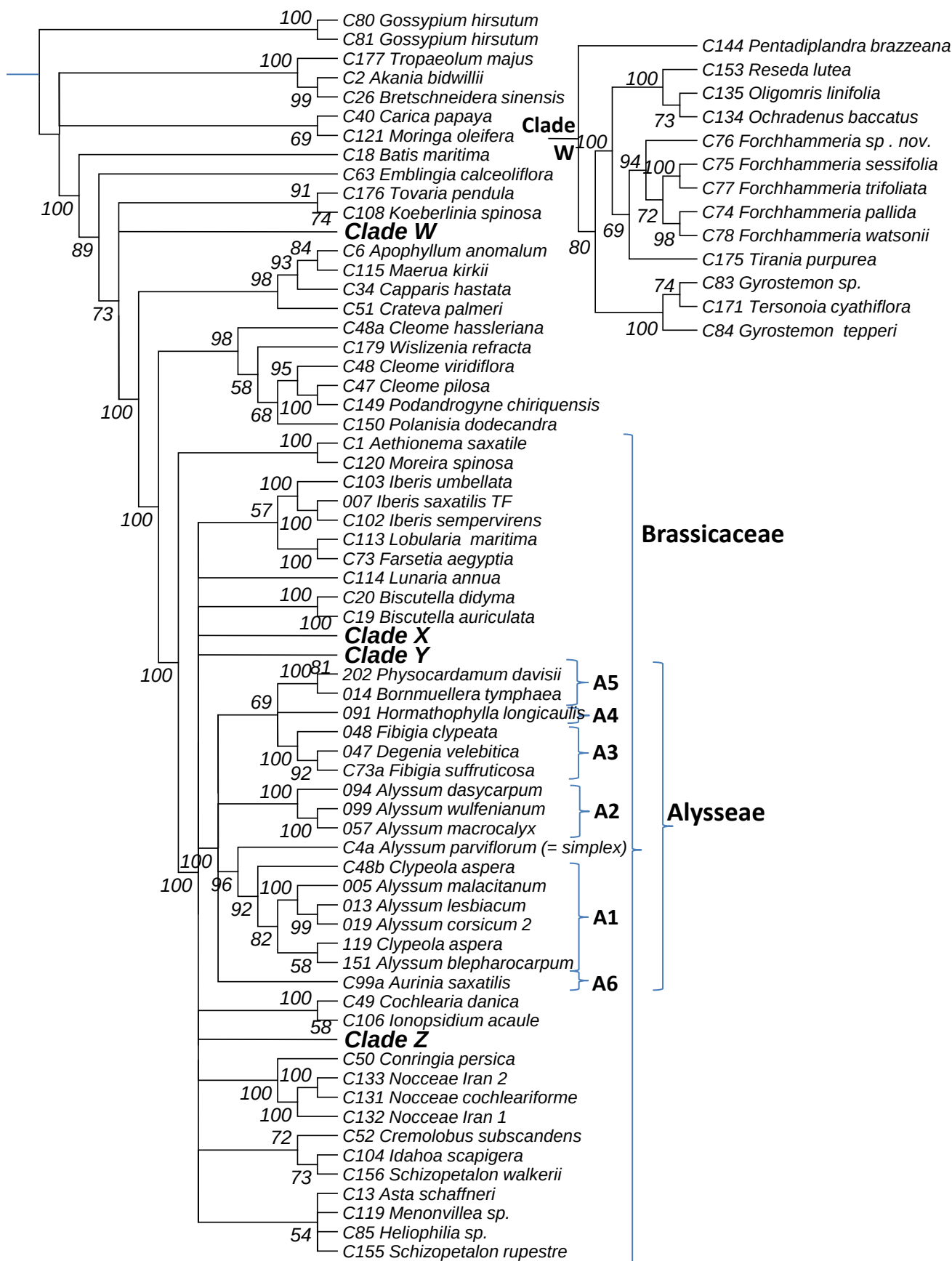


Figure A6-10a Maximum likelihood tree for Brassicales based on **Concatenated Alignment C**. Detail of Clades X, Y and Z is indicated in Figure 3-11b. Rooted on *Gossypium*-Brassicales. Figures on branches indicate estimated bootstrap support. Nodes receiving < 50 % bootstrap support have been collapsed.

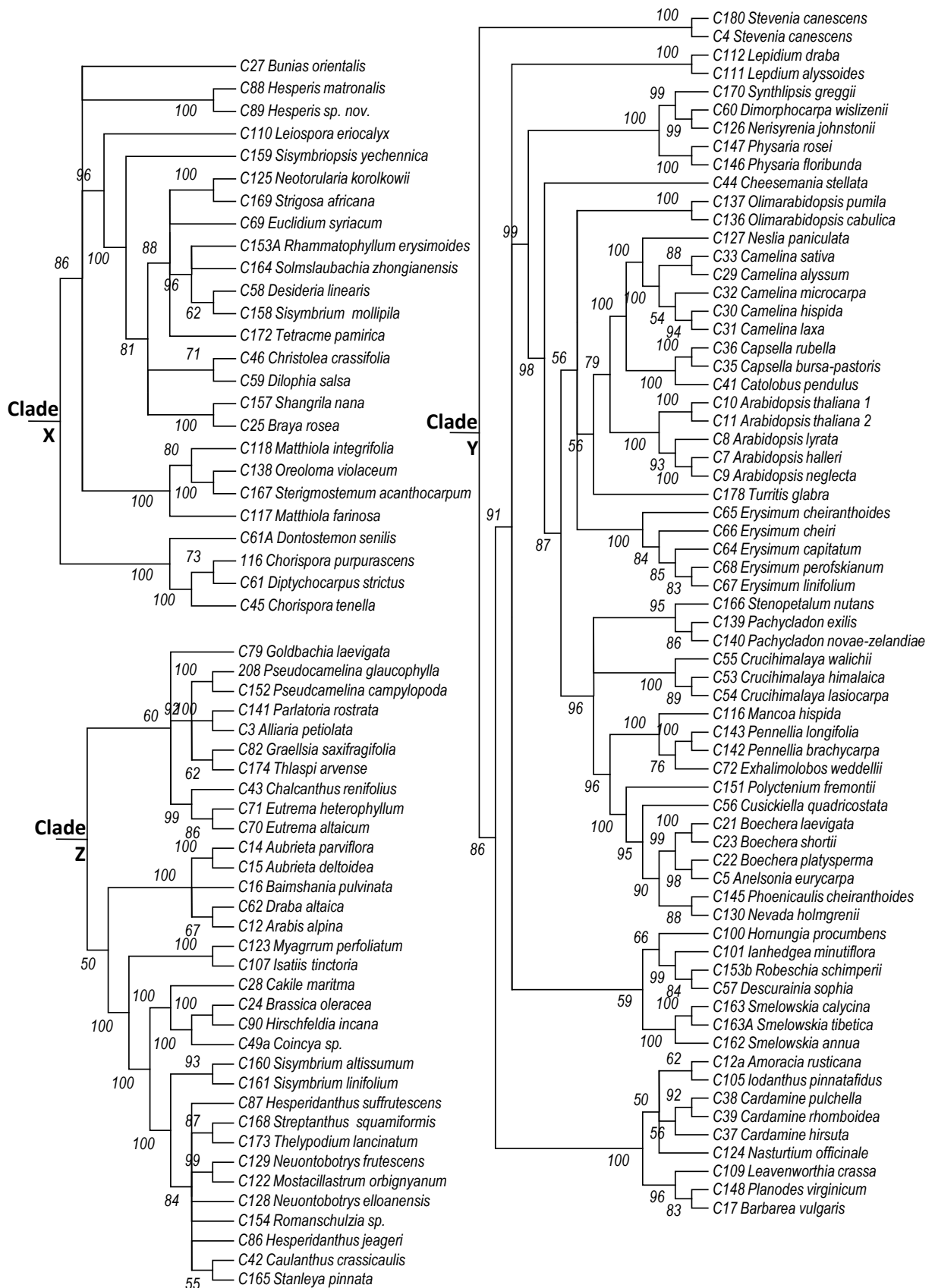


Figure A6-10b Detail of Clades X, Y and Z from Figure A6-10a (maximum likelihood tree for **Concatenated Alignment C**). Figures on branches indicate estimated bootstrap support. Nodes receiving < 50 % bootstrap support have been collapsed.

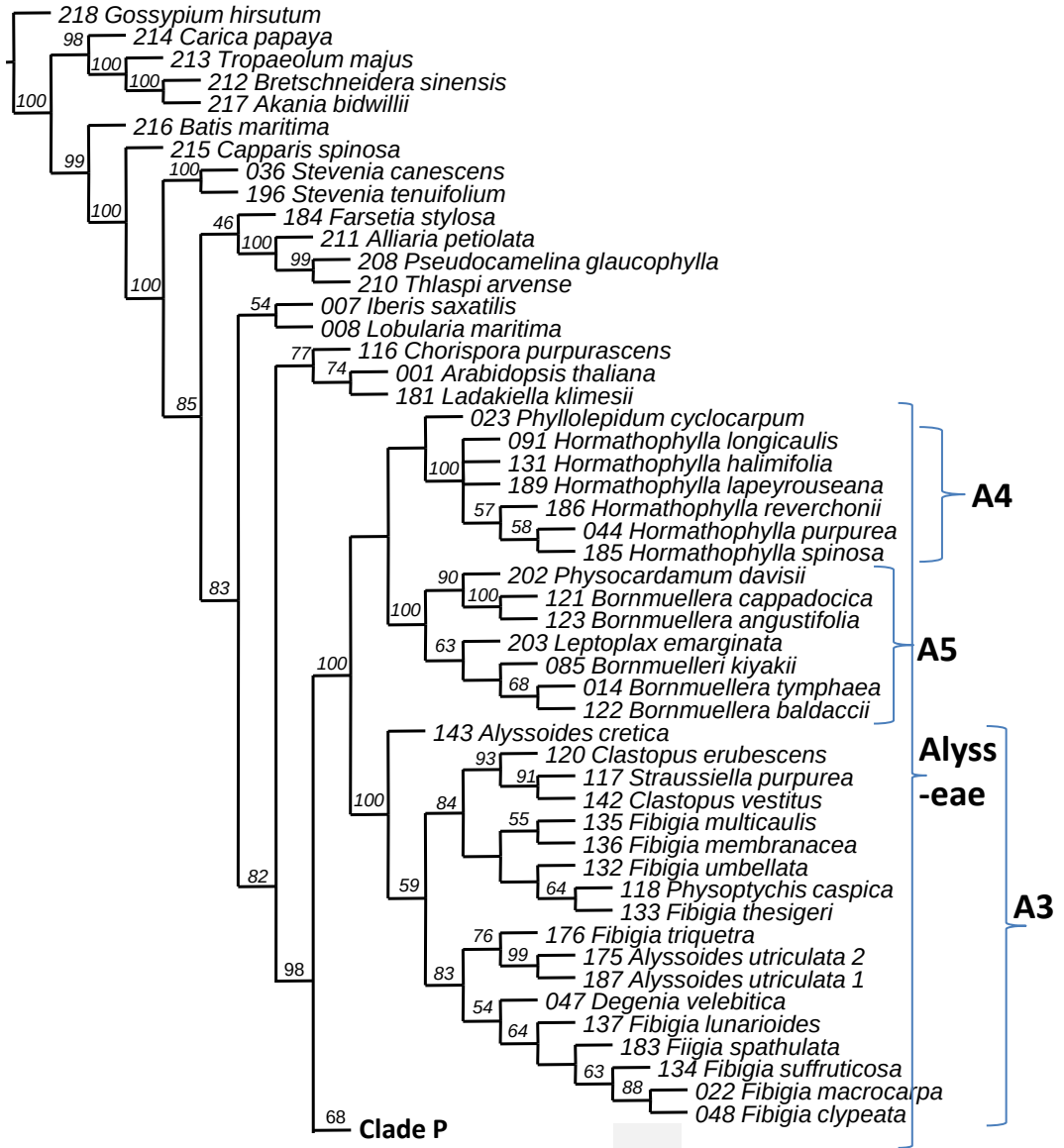
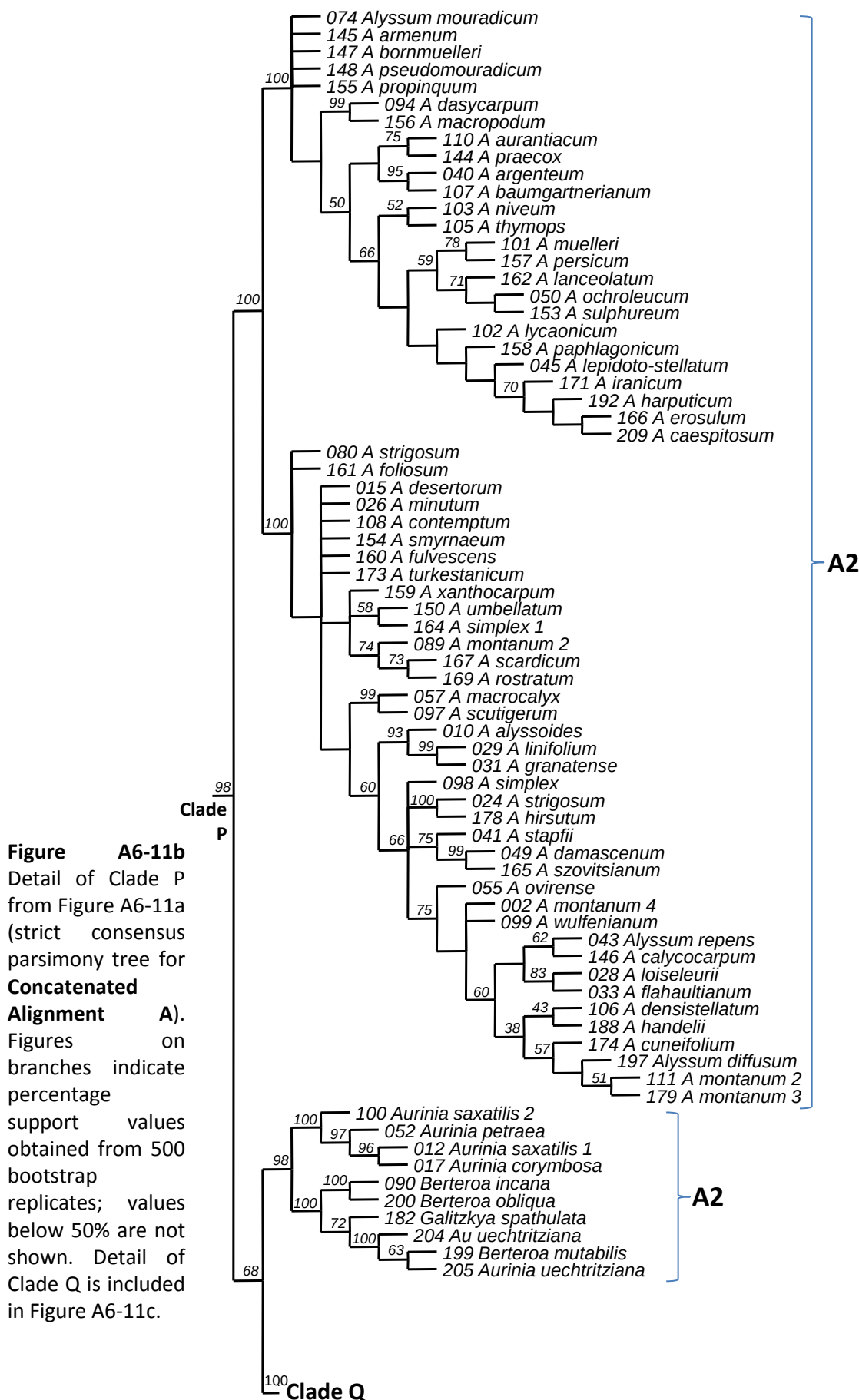


Figure A6-11a Strict consensus parsimony tree (of 5000 equally parsimonious trees) of Brassicaceae based on **Concatenated Alignment A**. Detail of Clades A1 and A2 is shown in Figures A6-11b and c. Figures on branches indicate percentage support values obtained from 500 bootstrap replicates; values below 50% are not shown. Total tree length: 9067 steps; CI: 0.48; RI: 0.82.



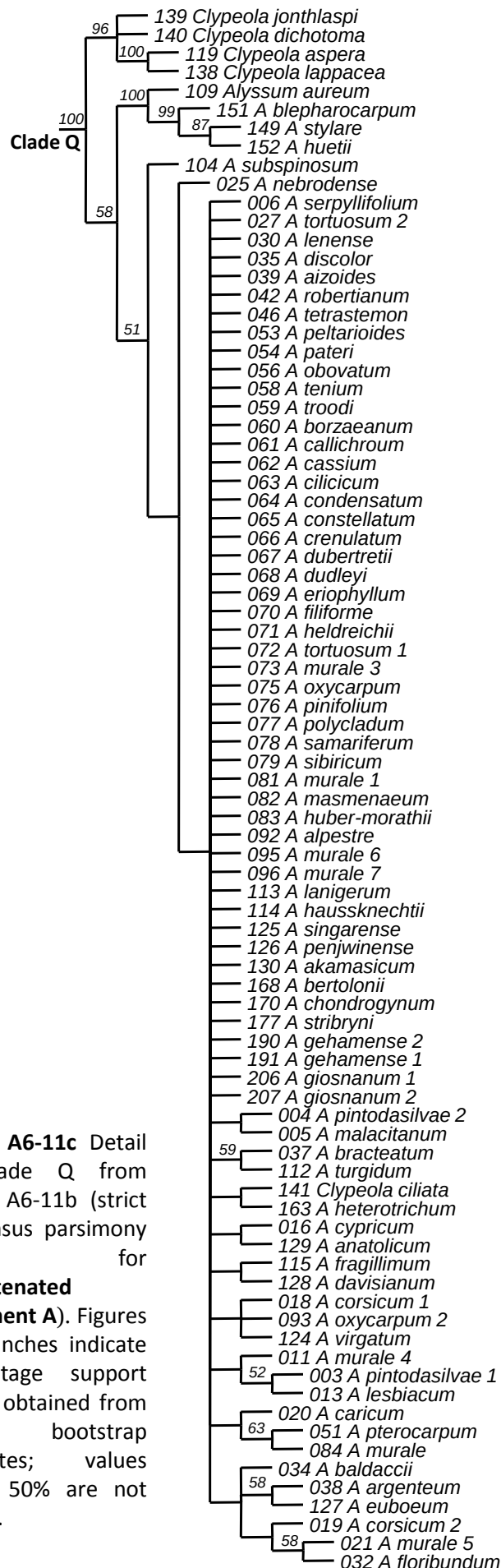


Figure A6-11c Detail of Clade Q from Figure A6-11b (strict consensus parsimony tree for **Concatenated Alignment A**). Figures on branches indicate percentage support values obtained from 500 bootstrap replicates; values below 50% are not shown.

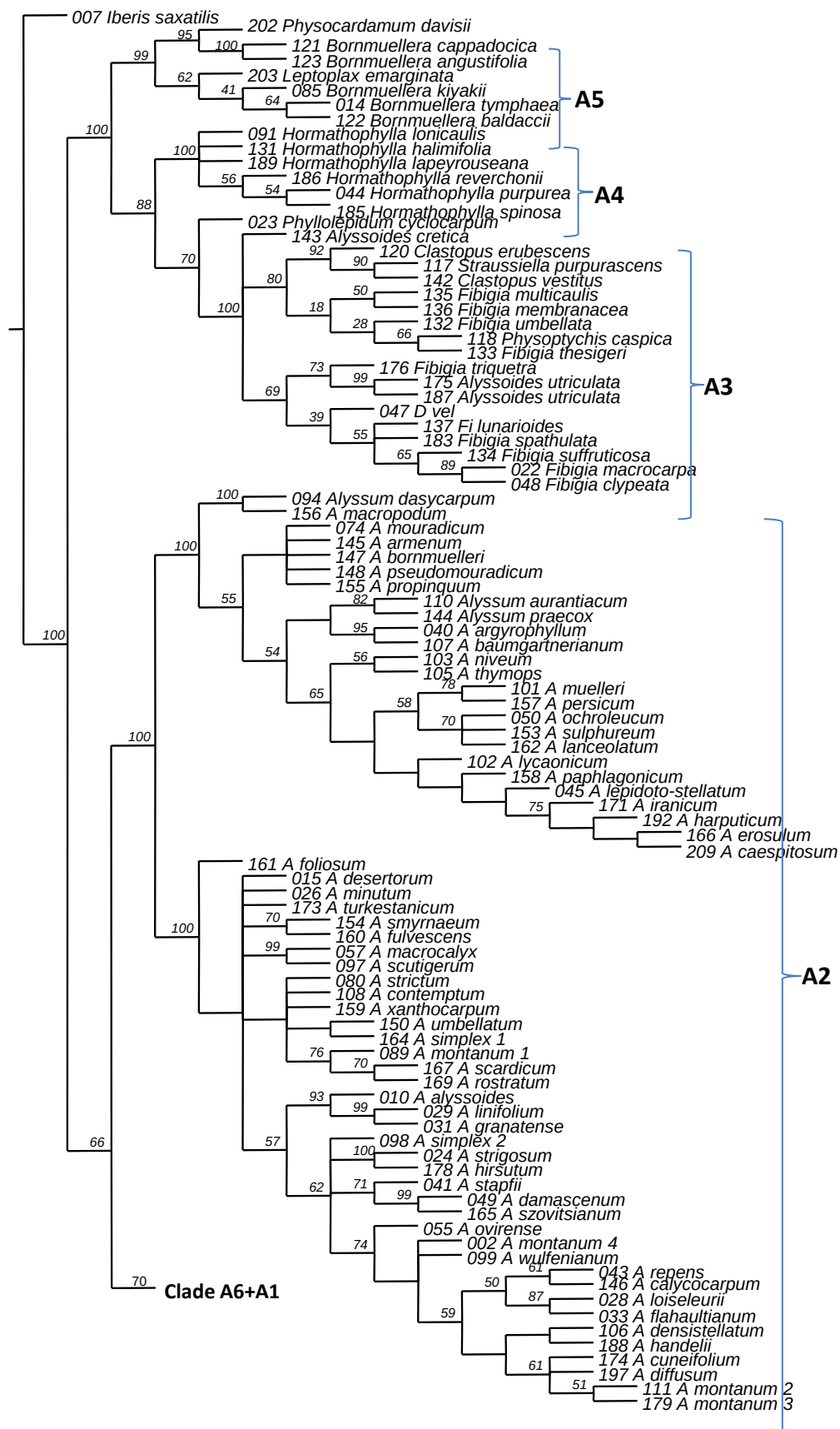


Figure A6-12a Strict consensus parsimony tree for **Concatenated Alignment B**. Detail of Clades A1 and A2 is included in Figure A6-12b. Figures on branches indicate percentage support values obtained from 500 bootstrap replicates; values below 50% are not shown. Total tree length: 4872 steps; CI: 0.45; RI: 0.87.

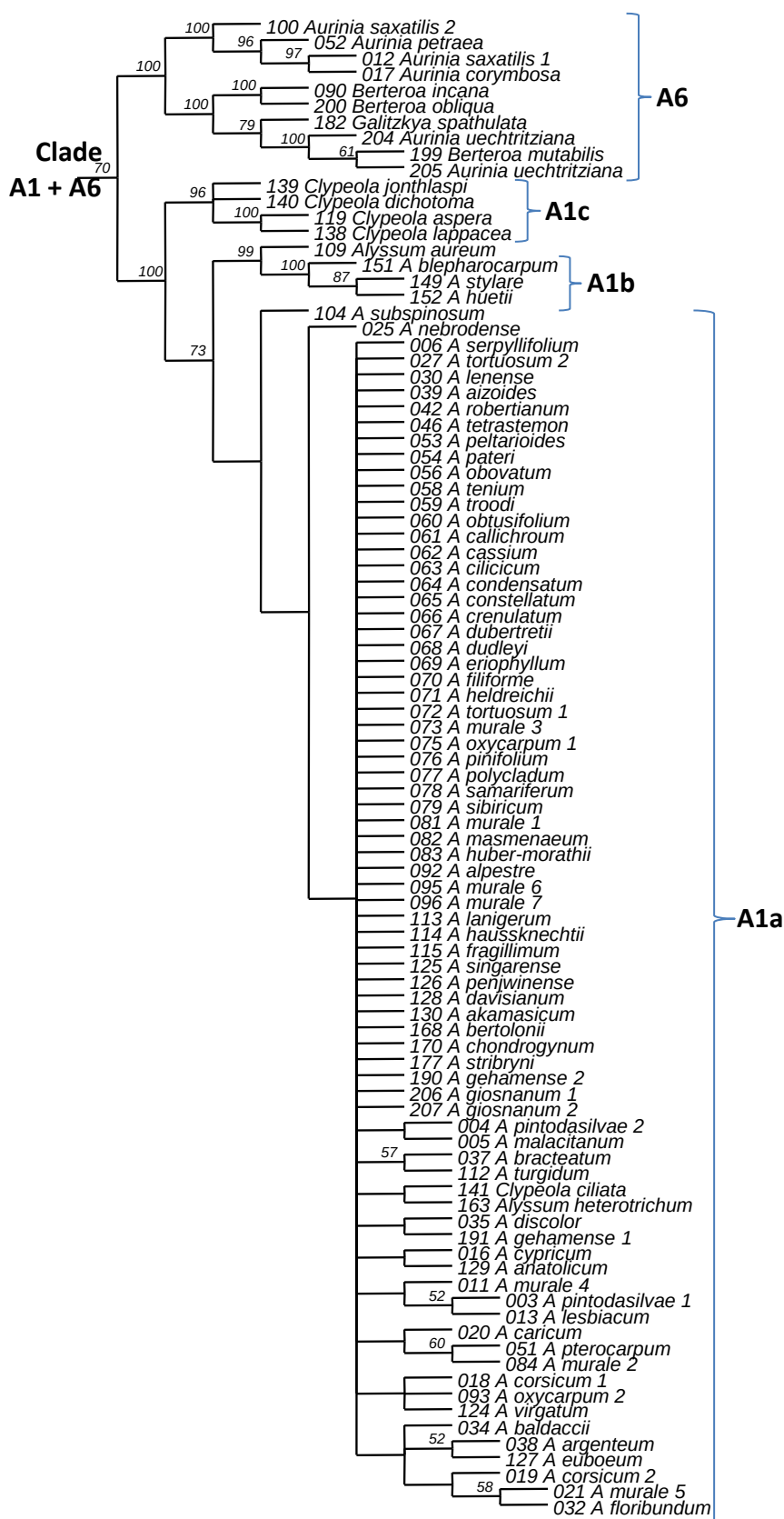


Figure A6-12b Detail of Clade A1+A6 from Figure A6-12a. Strict consensus parsimony tree for **Concatenated Alignment B**. Figures indicate percentage branch support based on 500 bootstrap replicates; values below 50% are not shown.

Appendix 7:

Time-calibrated Tree from Calibration Strategy 2

	Page
Figure A7-1 Tree based on Calibration Strategy 2	A7-2

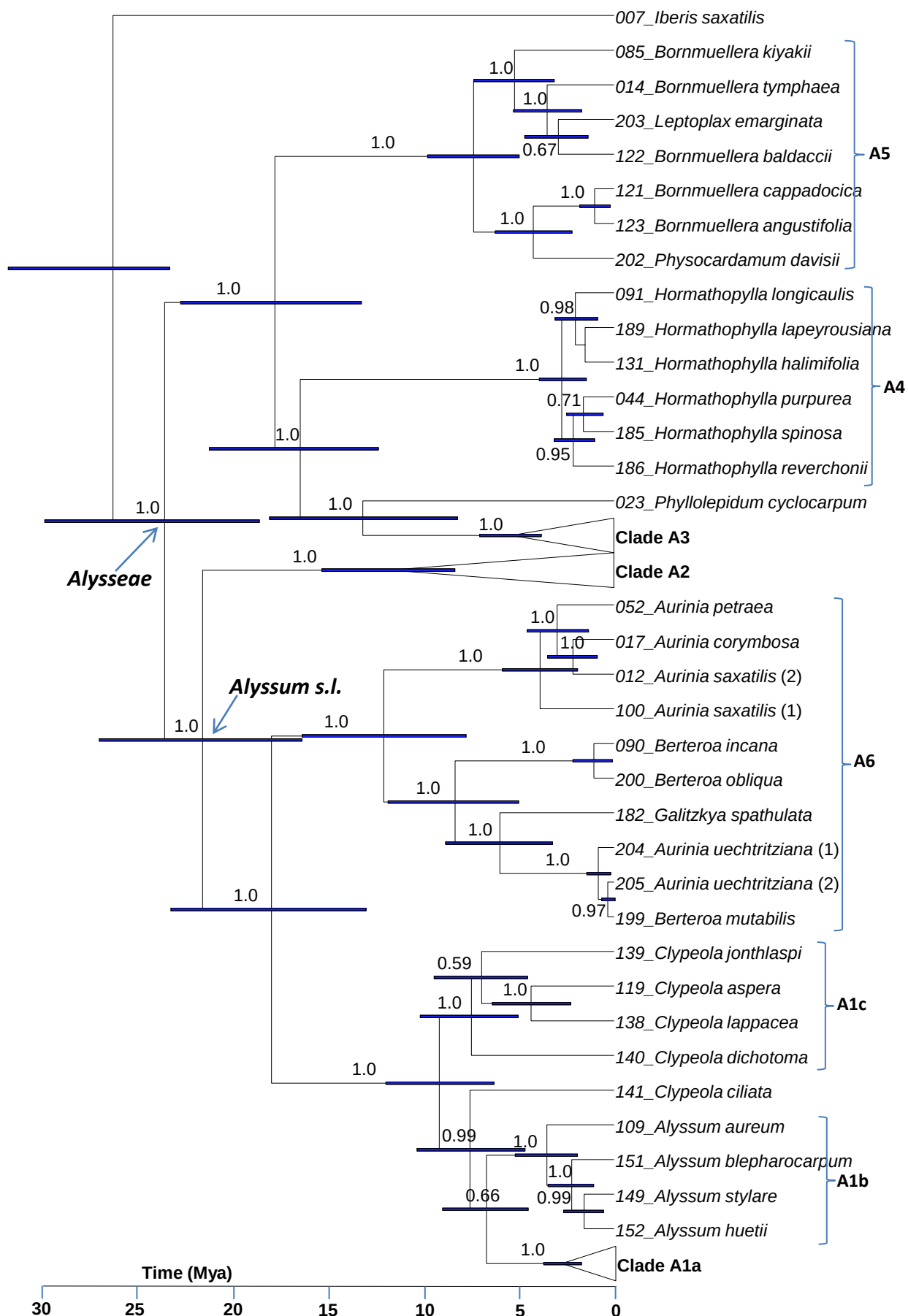


Figure A7-1a. Dated MCC tree produced in BEAST using Dating Strategy 2 (secondary calibration based on a published age estimate for the Alyseae crown node). Detail of Clades A1a, A2 and A3 is included in Figures A7-2b, c and d. Numbers indicate posterior probabilities of nodes. Bars indicate 95 % confidence interval of node age. These statistics are not shown for nodes with posterior probabilities < 0.5.

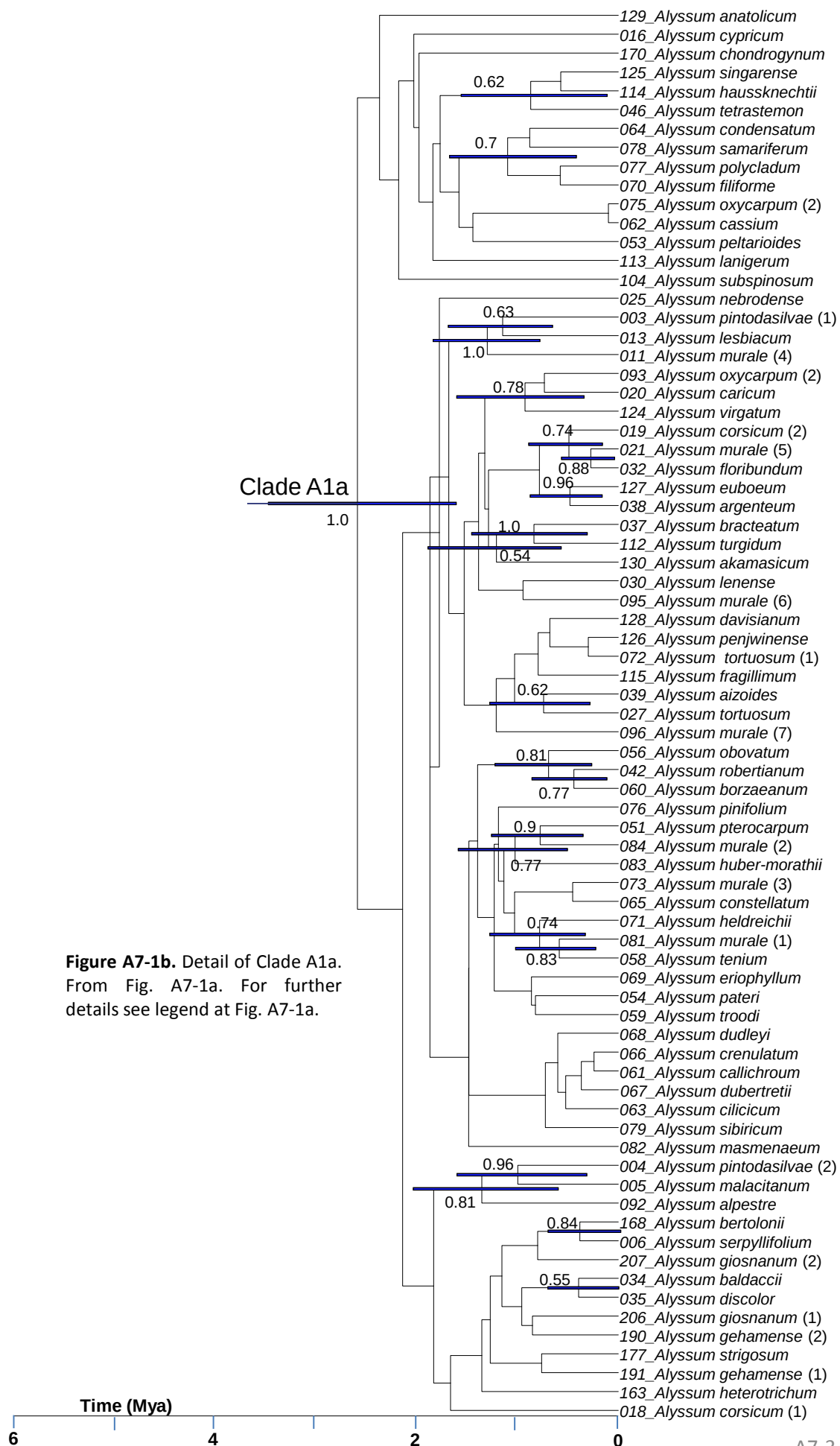


Figure A7-1b. Detail of Clade A1a. From Fig. A7-1a. For further details see legend at Fig. A7-1a.

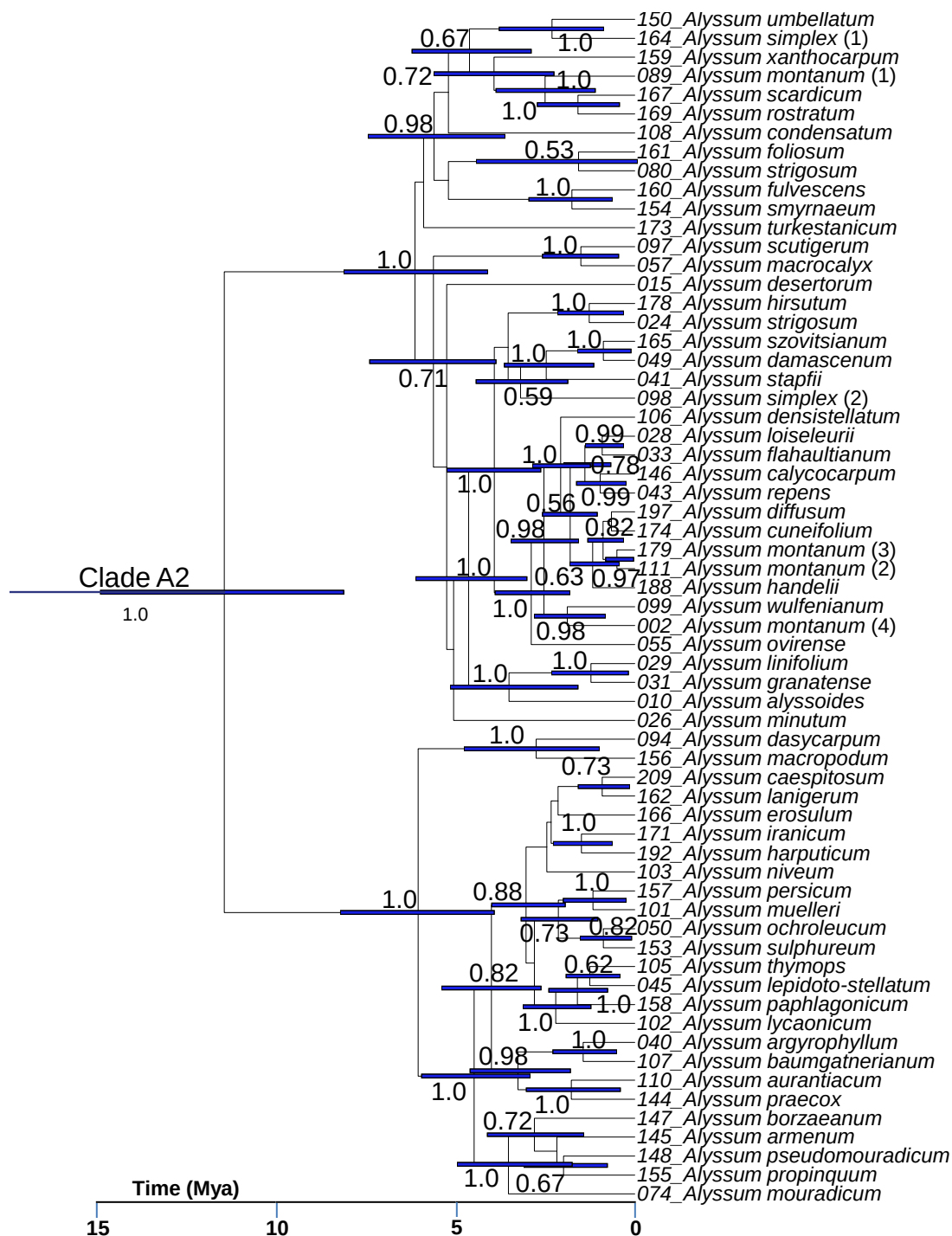


Figure A7-1c. Detail of Clade A2 from Fig. A7-1a. For further details see legend at Fig. A7-1a.

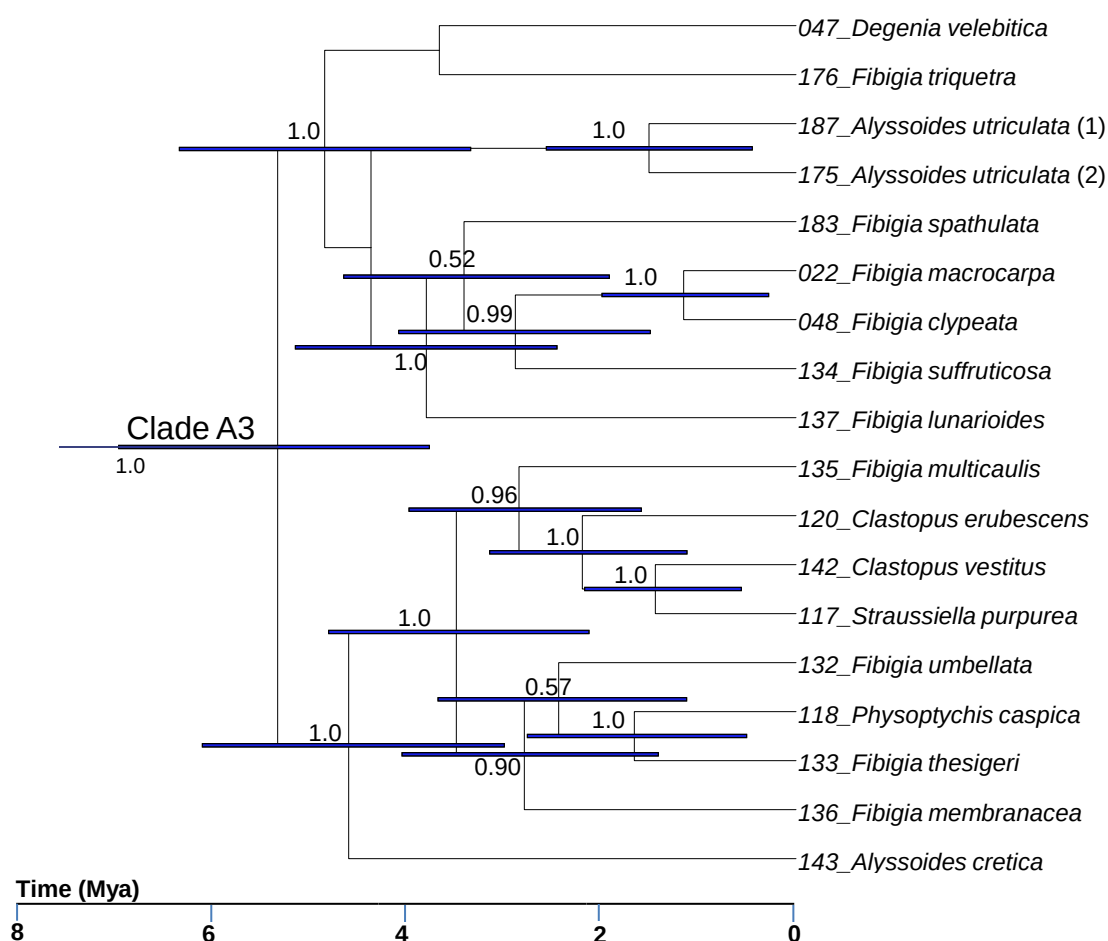


Figure A7-1d. Detail of Clade A3 from Figure A7-1a. For further details see legend at Fig. A7-1a.

Appendix 8:
Evolution of Nickel Hyperaccumulation
coded as a Three-state Character

	Page
Figure A8-1	A8-2

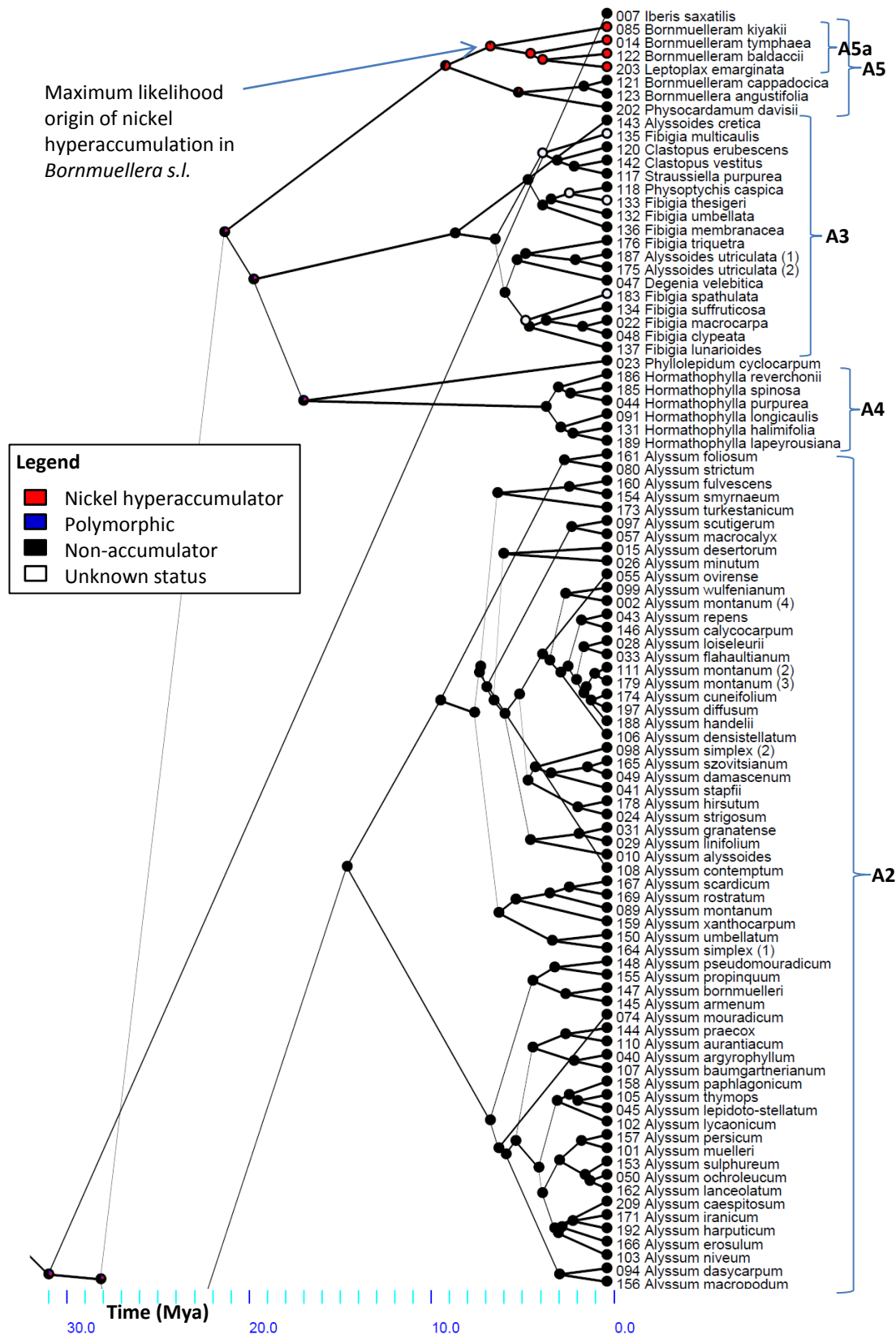


Figure A8-1a. Nickel hyperaccumulation optimized using maximum likelihood (using a 1-rate symmetrical model) and three character states onto the tree of Alyseae obtained using Concatenated Alignment B and Calibration Strategy 3.

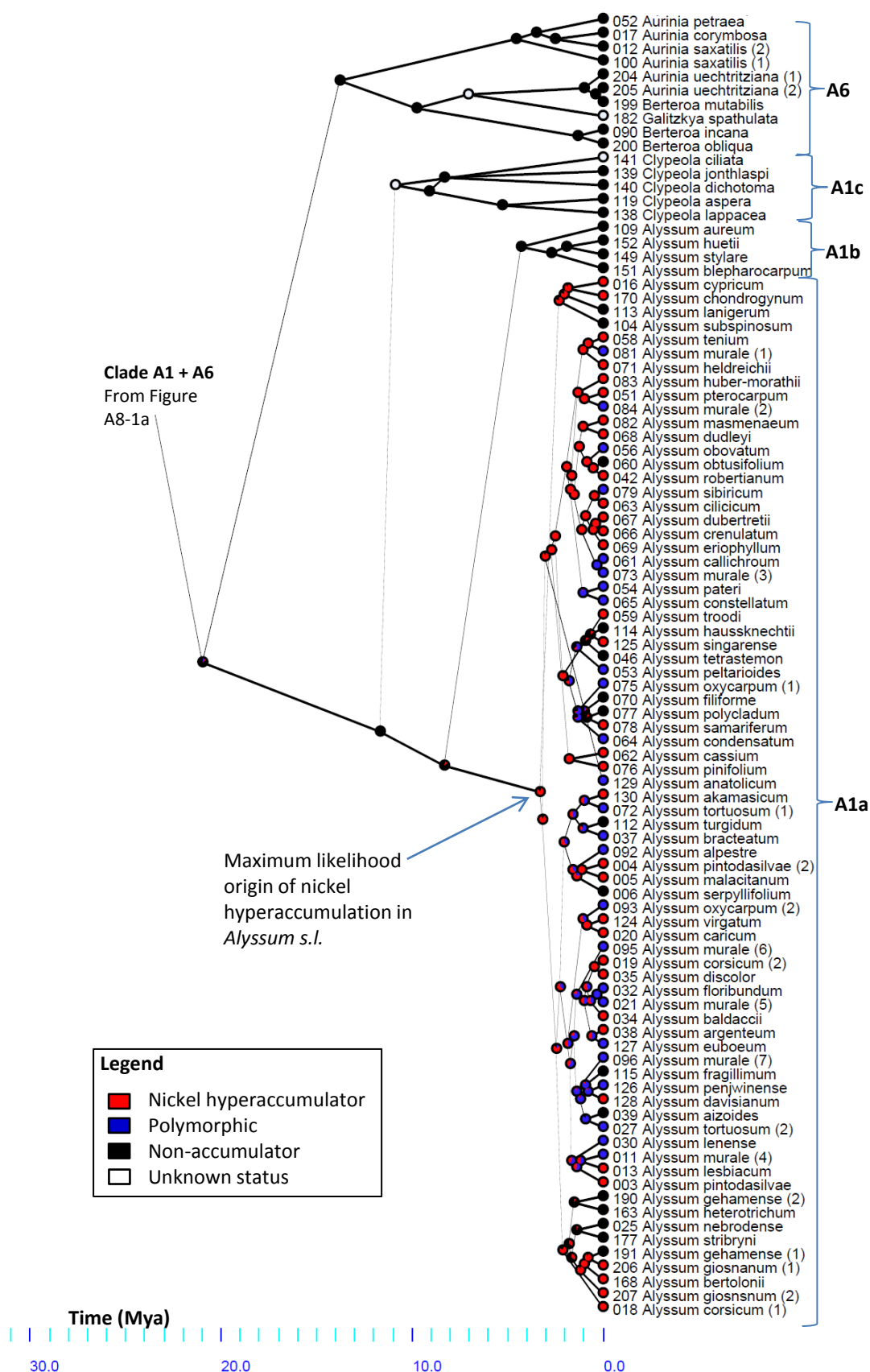


Figure A8-1b. Detail of Clade A1 + A6 from Figure A8-1a. Nickel hyperaccumulation optimized using maximum likelihood and three character states onto the tree of Alyseae obtained using Concatenated Alignment B and Calibration Strategy 3. For further details see Figure A8-1a.