

1 **Evolution of nickel hyperaccumulation and serpentine adaptation in the *Alyssum***
2 ***serpyllifolium* species complex**

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22 Abstract

23 Metal hyperaccumulation is an uncommon but highly distinctive adaptation found in certain
24 plants that can grow on metalliferous soils. Here we review what is known about evolution of
25 metal hyperaccumulation in plants and describe a population-genetic analysis of the
26 *Alyssum serpyllifolium* (Brassicaceae) species complex, which includes populations of
27 nickel-hyperaccumulating as well as non-accumulating plants growing on serpentine (S) and
28 non-serpentine (NS) soils, respectively. To test whether the S and NS populations belong to
29 the same or separate closely related species, we analysed genetic variation within and
30 between four S and four NS populations from across the Iberian peninsula. Based on
31 microsatellites, genetic variation was similar in S and NS populations (average $H_o = 0.48$).
32 The populations were significantly differentiated from each other (overall $F_{ST} = 0.23$), and the
33 degree of differentiation between S and NS populations was similar to that within these two
34 groups. However, high S versus NS differentiation was observed in DNA polymorphism of
35 two genes putatively involved in adaptation to serpentine environments, *IREG1* and
36 *NRAMP4*, while no such differentiation was found in a gene (*ASIL1*) not expected to play a
37 specific role in ecological adaptation in *A. serpyllifolium*. These results indicate that S and
38 NS populations belong to the same species and that nickel hyperaccumulation in *A.*
39 *serpyllifolium* appears to represent a case of adaptation to growth on serpentine soils.
40 Further functional and evolutionary genetic work in this system has the potential to
41 significantly advance our understanding of the evolution of metal hyperaccumulation in
42 plants.

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45 INTRODUCTION

46 **Metal hyperaccumulation in plants**

47 One of the most extraordinary adaptations known in the plant kingdom is the ability of certain
48 plants to hyperaccumulate trace elements in their above-ground biomass. This trait is
49 present in only about 500 species, representing less than 0.2% of all angiosperm species
50 (Reeves and Baker, 2000; Verbruggen *et al.*, 2009; Krämer, 2010; Van der Ent *et al.*, 2013).
51 In contrast to metal excluders, whose strategy is to control the uptake of metals into the root
52 and prevent metal translocation to aerial organs, hyperaccumulators accumulate metals in
53 the shoot to levels toxic to most other plants (Baker, 1981; Baker and Brooks, 1989; Baker *et al.*,
54 2000; Pollard *et al.*, 2002; Krämer, 2010; Rascio and Navari-Izzo, 2011). This is
55 remarkable since the photosynthetic apparatus is one of the major targets of metal
56 phytotoxicity, typically resulting in severe symptoms such as chlorosis and necrosis, wilting,
57 abnormal development and reduced growth (Pandey and Sharma, 2002; Rahman *et al.*,
58 2005; Marschner and Marschner, 2012). These toxic effects are a product of numerous
59 harmful interactions at the cellular level (Haydon and Cobbett, 2007), including non-specific
60 binding of metals to enzyme functional groups and displacement of other metals from their
61 binding sites, generation of reactive oxygen species by redox-active metals, which can lead
62 to disruption of the electron-transport chain (Qadir *et al.*, 2004), lipid peroxidation and
63 subsequent impairment of membrane integrity (Pandolfini *et al.*, 1992; Ros *et al.*, 1992;
64 Gonnelli *et al.*, 2001; Haydon and Cobbett, 2007; Krämer, 2010; Hanikenne and Nouet,
65 2011).

66 Of various hypotheses proposed to explain the possible adaptive advantage conferred
67 by hyperaccumulation of metals (Boyd and Martens, 1992), only the herbivore/pathogen
68 “elemental defence” hypothesis has gathered plentiful supporting experimental evidence
69 (Boyd, 2004, 2007). There have been many reports of decreased herbivory or reduced
70 pathogen infection on plants hyperaccumulating metals (Poschenrieder *et al.*, 2006; Boyd,
71 2007; Fones *et al.*, 2010; Rascio and Navari-Izzo, 2011). However, in some studies no
72 protective effect of metal hyperaccumulation against herbivory was observed (Noret *et al.*,
73 2007), and designing trials to demonstrate such an effect in the field is particularly
74 challenging, so further work is required to substantiate the selective advantage offered by
75 metal hyperaccumulation. Nonetheless, it remains broadly true that hyperaccumulator
76 species show a very high degree of metal tolerance (otherwise the accumulation of such
77 exceptional concentrations of metals in the shoot would be suicidal), and that the degree of
78 metal tolerance of different species or populations tends to be quite closely correlated with
79 the metal content of the natural substrates on which they grow (Antonovics *et al.*, 1971;
80 Roossens *et al.*, 2003; de la Fuente *et al.*, 2007; Pollard *et al.*, 2014).

Two broad hypotheses have been considered in the literature regarding the origin and spread of metalicolous populations, i.e. those adapted to soils containing an elevated concentration of a particular metal (Pauwels *et al.*, 2005). One is based on the observation that populations inhabiting metalliferous outcrops are often separated by large geographic distances; this would limit dispersal and genetic exchange between metalicolous populations, and instead would favour evolution of locally adapted metalicolous populations from nearby non-metalliferous sites (Schat *et al.*, 1996) driven by ecological speciation (Rundle and Nosil, 2005). The other hypothesis proposes a single origin of a genetic adaptation to metalliferous substrates, its spread across outlying metalliferous sites and subsequent differentiation between more recently established metalicolous populations due to genetic drift. These alternative views resonate with earlier debates as to whether local populations of metal-tolerant plants occurring on metalliferous outcrops represent 'neoendemics' or 'palaeoendemics', respectively (equivalent to the 'insular' and 'depleted' species of Stebbins (1942), as discussed by Kazakou *et al.* (2008)). Correspondingly, non-metallicolous populations of such species could be either ancestral, as in the first scenario, or locally derived from metalicolous populations, as in the second.

Evolution of metal tolerance in plants

The question whether metal tolerance is a constitutive trait across all populations of a species, or the degree to which ecotypic differentiation has occurred in response to different substrates, has been one of the most important issues underpinning hypotheses concerning the evolutionary origins of this trait. Two of the most intensively studied species of hyperaccumulator plants, *Arabidopsis halleri* (L.) O'Kane & Al-Shehbaz (formerly *Cardaminopsis halleri* (L.) Hayek) and *Noccaea caerulescens* (J.Presl & C.Presl) F.K.Mey. (formerly *Thlaspi caerulescens* J.Presl & C.Presl), display a basal, constitutive level of zinc tolerance and hyperaccumulation in both metalicolous and non-metallicolous populations (Meerts and Van Iacker, 1997; Bert *et al.*, 2000, 2002; Escarré *et al.*, 2000; Frérot *et al.*, 2003; Pauwels *et al.*, 2006; Meyer *et al.*, 2010). However, across the geographic range of these species, there is a significant positive correlation between the degree of tolerance and substrate metal concentration, indicative of local adaptation to the natural habitat (Roosens *et al.*, 2003; Pauwels *et al.*, 2005). The relationship between metal tolerance and hyperaccumulation is rather less clear, and whilst the highest shoot metal concentrations observed in the field are found in populations growing on metalliferous soils, there is some evidence that the level of metal hyperaccumulation shown by these populations may be inversely related to their degree of metal tolerance (Bert *et al.*, 2000; Roosens *et al.*, 2003).

While it is generally believed that the emergence of the hyperaccumulation trait in these two model species was driven by, and coincident with, the appearance of

anthropogenic metal-polluted sites in the mining regions of Europe (Pauwels *et al.*, 2006; Jiménez-Ambriz *et al.*, 2007; Besnard *et al.*, 2009), recent research on *A. halleri* supports a much more ancient appearance of the trait – or at least selection on major genes responsible for the trait – during the speciation process giving rise to this lineage hundreds of thousands of years ago, and thus pre-dating any human industrial activity (Roux *et al.*, 2011). It has been suggested that major loci involved in metal tolerance and accumulation, such as the *HMA4* gene (encoding the plasma membrane ATPase responsible for transporting Zn^{2+} out of root cells for loading into the xylem and translocation to the shoot), were targets of selection early in the history of the species (Pauwels *et al.*, 2005; Roux *et al.*, 2011). This would have established a basal level of zinc tolerance and accumulation in *A. halleri*, with local selection subsequently acting on additional genes in each established metallophyte population to enhance metal tolerance and possibly hyperaccumulation capacity still further (cf. Macnair, 2003). This has been supported by the results of genome-scan analyses, which have shown the presence of outliers specific to different metallicolous populations of *A. halleri* (Meyer *et al.*, 2009). Large variation in the degree of zinc tolerance within non-metallicolous populations and individuals can then be explained by either local gene flow from metallicolous to non-metallicolous populations, or as a result of ancestral standing genetic variation present within the non-metallicolous populations, which may have been exploited as the initial basis for metal tolerance in metallicolous populations.

Nickel hyperaccumulation

Metal hyperaccumulator plants are known to accumulate zinc, cadmium, copper, cobalt, manganese, and the metalloids arsenic, thallium and selenium, but the largest number (~80%) of hyperaccumulator species described are known to accumulate nickel (Reeves and Baker, 2000; Verbruggen *et al.*, 2009; Krämer, 2010), possibly due to its prevalence in ultramafic rocks across the continents (Van der Ent *et al.*, 2013). Ultramafic substrates are derived from igneous rocks with a low silica content but rich in mafic minerals such as magnesium, iron and nickel. Weathering of ultramafic bedrock creates serpentine soils with distinctive physical and chemical characteristics, including particularly high contents of nickel, cobalt and chromium, high Mg/Ca ratio, and low levels of the essential macronutrients nitrogen, phosphorus and potassium (Brooks, 1987; Kazakou *et al.*, 2008). Most types of soil show nickel concentrations typically between 7 and 50 mg/kg, while in serpentine soils the nickel content usually ranges from 700 to 8000 mg/kg (Reeves, 1992; Reeves and Baker, 2000). This characteristic geochemistry, combined with a characteristically thin soil cover and granular texture, poor water-holding capacity, ready erosion and exposure to high light intensity, makes serpentine soils a notoriously difficult environment for plant growth (Proctor, 1975; Freitas *et al.*, 2004). As a result, serpentine outcrops are treated as ecological islands

inhabited by specialized floras. Indeed, the discontinuity between serpentine outcrops, with their sparse vegetation, and neighbouring soils can often be clearly delimited from afar (Kruckeberg, 2004; Brady *et al.*, 2005).

Despite the greater taxonomic abundance of nickel hyperaccumulator species (>400), much more research has so far been conducted on the molecular basis and evolution of zinc and cadmium hyperaccumulation in the *de facto* model organisms *Arabidopsis halleri* and *Noccaea caerulescens*. However, only 15 described species are known to display zinc hyperaccumulation (Meerts and Van Isacker, 1997; Bert *et al.*, 2000; Krämer, 2010), and nickel hyperaccumulation warrants further research as part of attempts to understand the evolutionary basis of plant adaptation to serpentine (Brady *et al.*, 2005; Kazakou *et al.*, 2008; Turner *et al.*, 2010). The present paper thus focuses on evolution of nickel hyperaccumulation in the genus *Alyssum* (Brassicaceae), which contains 51 known hyperaccumulator taxa out of about 190 species, making this the largest number of hyperaccumulating species found within a single genus (Brooks, 1998; Burge and Barker, 2010).

Evolution of nickel hyperaccumulation in genus *Alyssum*

Hyperaccumulator species within the genus *Alyssum* are widely distributed on serpentine sites across the entire Mediterranean basin, from the Iberian peninsula in the west to the Irano-Turanian region in the east (Brooks, 1987). It is not clear whether hyperaccumulation ability arose at each serpentine site independently, or whether the trait has evolved just once in an ancestral population and then spread by dispersal and range expansion to serpentine sites scattered over a broad geographical area, but this represents an excellent study group given the history of detailed taxonomic treatments of the genus (Ball and Dudley, 1993).

Several taxa within the genus *Alyssum* are classed as facultative hyperaccumulators (Pollard *et al.*, 2014), providing an opportunity to study evolution of this trait by comparing hyperaccumulating and non-accumulating ecotypes. *Alyssum serpyllifolium* represents the best-studied example of facultative nickel hyperaccumulation in the genus. *Arabidopsis halleri* and *Noccaea caerulescens* also belong to the group of facultative metallophytes, since they occur both on and off metalliferous substrates. However, *A. serpyllifolium* is likely to have a different evolutionary history compared to *A. halleri* and *N. caerulescens*, which are most characteristically found on metal-contaminated sites created in the last two to three millennia as a result of anthropogenic disturbance (such as mining activities). Serpentine populations of *Alyssum*, in contrast, typically occupy isolated ultramafic outcrops that have been exposed for many millions of years, at least since the Miocene. Additionally, most of the current range of *A. serpyllifolium* is beyond the maximum advance of the main polar ice cap during the Pleistocene glaciations (Reeves, 1992; Hewitt, 1999), unlike the ranges of *A.*

192 *halleri* and *N. caerulescens*, possibly offering a more stable habitat and leading to a different
193 evolutionary trajectory. However, glacial ice covered parts of the current range of *A.*
194 *serpyllifolium*, including southern Spain, certain regions in central Spain, as well as the
195 Pyrenees and the south of France (Levin, 2013), so the Last Glacial Maximum cannot be
196 entirely ruled out as a factor in the phylogenetic history of this taxon. Indeed, an influence of
197 climatic fluctuations can be important for the population dynamics of serpentine species
198 (Kolář *et al.*, 2012). One scenario envisages formerly widespread metallophyte species
199 being excluded from non-serpentine sites during post-glacial reforestation due to their low
200 competitiveness on non-metalliferous soils; the serpentine populations would become
201 separated and disjunct, and because of reduced gene flow progressively differentiate due to
202 drift and selection. This 'depleted' (or palaeoendemic) species scenario proposed by
203 Stebbins (Stebbins, 1942; Stebbins and Major, 1965), and considered in the serpentine
204 context by Kruckeberg (Kruckeberg, 1954), has been shown to have taken place in the
205 serpentine subspecies *Minuartia laricifolia* ssp. *ophiolitica* in the Alps (Moore *et al.*, 2013)
206 and in the *Streptanthus glandulosus* complex (Mayer *et al.*, 1994). In a subsequent phase,
207 non-serpentine progenitors can re-invade and come into secondary contact with serpentine
208 populations (depleted species-recolonization scenario).

209 The population history of *Alyssum serpyllifolium* is likely to have been complicated by
210 various factors. Serpentine substrates inhabited by this species represent relatively small
211 edaphic 'islands' up to tens of kilometres in diameter (Brooks, 1987; Flynn, 2013), separated
212 by vast stretches of a non-serpentine 'sea' with different geochemistry. As serpentine-
213 adapted plants may not be successful competitors on non-serpentine substrates (Brooks,
214 1987; Elmendorf and Moore, 2007; Anacker and Harrison, 2012; Anacker, 2014), the
215 populations occupying such serpentine islands would become isolated and could eventually
216 diverge into separate species. Landscape and topography may also contribute to isolation of
217 populations of *A. serpyllifolium*, as they often occur in mountain ranges such as Sierra de
218 Aguas (population Carratraca-S), Sierra Bermeja de Estapona (Sierra Bermeja-S), and
219 Serra do Careón (Barazón-S). Furthermore, ploidy difference is likely to act as an additional
220 isolating mechanism between some populations of this species. All the Iberian populations
221 analysed in this study are diploid, with $n = 8$ (Fernandes and Queirós, 1973; Küpfer, 1974;
222 Cecchi *et al.*, 2013), but both diploid $n = 8$ (Küpfer, 1974) and tetraploid $n = 16$ (Bonnet,
223 1963; Puech, 1963) populations have been reported in France. All these factors may
224 contribute to gradual divergence between individual populations, and the plants from
225 different populations show some morphological differences. This has led to earlier proposals
226 to raise the serpentine populations of *A. serpyllifolium* to the status of distinct subspecies, or
227 even separate species (Dudley, 1986a,b). However, analyses based on chloroplast DNA
228 sequences have been unable to establish clearly resolved relationships between the

different *A. serpyllifolium* populations (Cecchi *et al.*, 2013; Flynn, 2013). In this paper, we have therefore reinvestigated the relationships between serpentine and non-serpentine populations of *A. serpyllifolium* using population-genetic approaches.

MATERIALS AND METHODS

Plant material

In this study, four serpentine and four non-serpentine populations (Figure 1) were sampled from across the main range of *Alyssum serpyllifolium* in the Iberian peninsula (Ball and Dudley, 1993). The four serpentine populations originate from the three serpentine areas inhabited by *A. serpyllifolium*: Trás-os-Montes province in northeastern Portugal (Samil-S population), Melide, Galicia (Barazón-S population) and the western Baetic Cordilleras in Andalucía (Carratraca-S and Sierra Bermeja-S populations) in Spain. The four remaining non-serpentine populations (Alhaurín-NS, León-NS, Morata-NS and Rubiá-NS) were collected from across the Iberian peninsula in varying proximity to the serpentine outcrops. Seeds were collected from fruiting plants in years between 1999 and 2012.

DNA extraction

Genomic DNA from dried plant shoots was extracted using a modification of the CTAB method (Rogers and Bendich, 1985; Porebski *et al.*, 1997) from up to 40 individuals in each of the eight sampled *A. serpyllifolium* populations. The concentration of DNA in each sample was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, USA) following extraction.

EST-SSR detection and PCR amplification of EST-SSR fragments

Perfect microsatellites were detected in the consensus *A. serpyllifolium* transcriptome assembly (manuscript in preparation) with SciRoKo version 3.4 (Kofler *et al.*, 2007) using the following default settings: di-nucleotide pattern – minimum 7 repeats; tri-nucleotide – minimum 5 repeats; tetra-, penta-, hexa-nucleotide – minimum 4 repeats. Out of 3942 microsatellites detected, 32 were selected based on repeat size (3 or 6 nucleotides, i.e. no introduction of frame-shift mutation), as well as the function of the target gene being considered unlikely to be important for adaptation to serpentine. Primers were then designed around each locus, and amplification success along with level of polymorphism was tested in 3–4 individuals in each population.

Each individual plant was then genotyped for 8 developed microsatellites. The PCR mixture for each individual amplification reaction was assembled with the following reagents: 1.5 µl of 10× buffer (New England BioLabs, Hitchin, UK), 0.3 µl of 10 mM total dNTPs (Thermo Scientific, Hemel Hempstead, UK), 1.2 µl of 25 mM MgCl₂ (New England BioLabs),

0.06 µl of 10 µM forward primer, 0.3 µl of 10 µM reverse primer, 0.3 µl of FAM/HEX/NED labelled universal primer, 0.1 µl of *Taq* Polymerase (New England BioLabs), 1–2 µl of DNA solution (depending on the concentration, 10–100 ng contained in the reaction), and double-distilled water to 15 µl total volume. Oligonucleotide primers were supplied by Eurofins MWG Operon (Ebersberg, Germany), and in addition to the gene-specific sequences listed in Table S2, forward primers contained a 5' overhang fragment of 5'-CACGACGTTGTAAAACGAC-3' to facilitate incorporation of the dye-labelled universal primer in subsequent PCR cycles. PCR cycling was carried out using the following conditions: (1) initial denaturation (3 min at 95°C), (2) 10 cycles of touch-down PCR (30 s at 95°C, 30 s at 65°C [reduced by one degree per cycle], 1 min at 68°C), (3) 30 cycles of standard amplification (30 s at 95°C, 30 s at 55°C, 1 min at 68°C), and (4) final extension (5 min at 68°C).

Genotype scoring and data analysis

PCR-amplified fragments containing target microsatellite repeats were run on an Applied Biosystems Capillary Genetic Analyzer at the Department of Zoology, University of Oxford, UK, with GeneScan 500 LIZ Size Standard added as a reference and results collected using a DS-30 filter. Microsatellite genotypes were scored manually using GeneMarker version 2.6.3 (SoftGenetics, Pennsylvania, USA) following the default settings for plant microsatellite scoring. In cases where a genotype could not be established with confidence, PCR products were re-run a second time. Micro-checker version 2.2.3. (Van Oosterhout *et al.*, 2004) was then employed to test for genotyping artefacts – null alleles, stuttering, and high allele drop-out levels. All of the individual genotypes were exported from MS Excel into CREATE version 1.37 (Coombs *et al.*, 2008), which was then used to convert the data matrix into formats required for various programs.

Firstly, departures from HWE and linkage disequilibrium (LD) were tested in Arlequin version 3.5 (Excoffier *et al.*, 2005) and FSTAT version 2.9.3.2 (Goudet, 1995). Subsequently, two programs were used in calculating basic population genetics parameters: allele frequency, F -statistics, heterozygosity, as well as AMOVA (Arlequin). The presence of an isolation-by-distance pattern (Wright, 1943) was investigated using the Mantel test with 1000 permutations (Mantel, 1967) in Genepop version 4.2 (Raymond and Rousset, 1995) by examining the correlation between log-transformed Euclidean distances between pairs of populations and linearized pairwise genetic distances ($F_{ST} / (1 - F_{ST})$). The PHYLIP package version 3.69 (Felsenstein, 2005) was employed to calculate various inter-population genetic distance metrics with bootstrapping (1000 replicates), while Factorial Correspondence Analysis was carried out in Genetix version 4.05 using the default settings (<http://www.genetix.univ-montp2.fr/>).

Bayesian analysis in Structure version 2.3.3 (Falush *et al.*, 2003) was used to assign individuals to genetic clusters (K). A model was chosen in which individuals had admixed ancestries and correlated allele frequencies to allow detection of more ancient admixture events (Falush *et al.*, 2003). Choosing independent allele frequencies made no significant difference to the structure detected, and so results based on correlated allele frequencies are reported here. The LocPrior clustering method implemented in the latest version of Structure was used, which is not only based on the individual multilocus genotypes but also takes into account the sampling locations. The model is recommended by the authors when the genetic data are not highly informative in order to detect population structure, and was chosen because of a moderate number of microsatellite markers employed in the present study.

The number of genetic clusters (K) was set from a minimum of 2 to a maximum of 10, and five simulations were run for each K value with a burn-in of 100,000 and a main run of 1,000,000 MCMC iterations. To define the most probable value of K present in the data, the method proposed by Evanno *et al.* (Evanno *et al.*, 2005) was initially used, which is based on an *ad hoc* measure ΔK that depends on the rate of change in the log probability of the data between successive values of K . Secondly, the number of true clusters was also inferred following $L(K)$ over a number of clusters, and then looking for the signature of $L(K)$ starting to plateau with diminished variance in $L(K)$ (Pritchard *et al.*, 2000). These calculations were carried out by Structure Harvester Web version 0.6.94 (Earl and vonHoldt., 2012), which also generated CLUMPP input files. Subsequently, cluster assignments from across the replicate runs were aligned and averaged using CLUMPP version 1.1 (Jakobsson and Rosenberg, 2007) run with the Greedy algorithm and 1000 permutations of randomized input order. Resulting final cluster assignments were visualized using the program DISTRUCT version 1.1 (Rosenberg, 2004).

Locus selection and primer development

For sequence analyses of selection we chose two loci involved in metal hyperaccumulation and homeostasis in other plant species: NATURAL RESISTANCE ASSOCIATED MACROPHAGE PROTEIN 4 (*NRAMP4*) and IRON-REGULATED PROTEIN 1 (*IREG1*). On the other hand, 6B-INTERACTING PROTEIN 1-LIKE 1 (*ASIL1*) was selected as a reference gene for comparisons with *NRAMP4* and *IREG1*, as *ASIL1* is not expected to play a specific role in ecological adaptation in *Alyssum* as it is a transcriptional repressor of seed maturation genes in germinating seeds and seedlings in *Arabidopsis* (Gao *et al.*, 2009). Primers for these genes were developed based on *A. serpyllifolium* transcriptome sequences of these genes using the PrimerDesignM web server (Yoon and Leitner, 2015) with default settings. For both *IREG1* and *NRAMP4* genes, amplification primers were also used as sequencing

primers (Table S3), but in the neutral gene *ASIL1* a different reverse primer was used for amplification (*ASIL1* R1) and sequencing (*ASIL1* INTF1). Sequenced regions in both *IREG1* and *NRAMP4* were found to contain putative introns: 82 bp long in *IREG1* and 145 bp long in *NRAMP4*, which were excluded from the analyses described below, unless otherwise stated.

Gene fragment amplification and Sanger sequencing

PCR mixture (30 µl) for each sample was assembled with the following reagents: 3 µl of 10× buffer (New England BioLabs), 0.6 µl of 10 mM total dNTPs (Thermo Scientific), 2.4 µl of 25 mM MgCl₂ (New England BioLabs), 0.6 µl of 10 µM forward primer, 0.6 µl of 10 µM reverse primer, 0.2 µl of *Taq* Polymerase (New England BioLabs), 1 µl of DNA solution (25–250 ng), and 21.6 µl of double-distilled water. In the case of *ASIL1*, 4.8 µl of 5 M betaine was added to a final concentration of 0.8 M, with concomitant reduction of the water volume, to increase primer annealing specificity and fragment amplification rate (Ralser *et al.*, 2006). PCR cycling was carried out under the following conditions: (1) initial denaturation (3 min at 95°C); (2) 10 cycles of touch-down PCR (30 s at 95°C, 30 s at 65°C [reduced by one degree per cycle], 1 min at 68°C); (3) 33 cycles (*IREG1*) or 40 cycles (*NRAMP4*) of amplification (30 s at 95°C, 1 min at 45–55°C, 4 min at 68°C), or in the case of *ASIL1* the first 10 cycles carried out at 42°C and the final 30 cycles with 50°C annealing temperature to facilitate permissive primer binding and target region amplification in the initial steps of the PCR reaction; and (4) final extension step (5 min at 68°C). PCR products of correct size, high purity and concentration were then sequenced at the DNA Sequencing Unit, Department of Zoology, University of Oxford, on an ABI 3730xl DNA Analyzer.

Data analysis

Raw ABI output chromatograms were examined and exported to the FASTA format using FinchTV software version 1.4 (Perkin-Elmer, Beaconsfield, UK). Consensus DNA contigs from combined forward primer and reverse primer sequencing results were created manually in MEGA (Tamura *et al.*, 2013) from sequences trimmed on base call quality. DnaSP version 5 (Librado and Rozas, 2009) was employed to calculate the relevant population-genetic parameters, carry out tests for selection, and phase the diploid sequences with the PHASE algorithm (Stephens *et al.*, 2001). AMOVA was performed in Arlequin version 3.5 (Excoffier *et al.*, 2005) and gene trees based on Nei's D_A (Nei *et al.*, 1983) were prepared in POPTREE2 (Takezaki *et al.*, 2010) with 1000 bootstrap replicates.

RESULTS

Genetic diversity and population structure

To analyse population structure and degree of isolation between the sampled populations of *Alyssum serpyllifolium*, we genotyped eight microsatellite loci in 272 individuals from four serpentine (Barazón-S, Carratraca-S, Samil-S, Sierra Bermeja-S) and four non-serpentine populations (Alhaurín-NS, León-NS, Morata-NS, Rubiá-NS; see Figure 1). This revealed between 4 and 12 alleles per locus, with a mode of 5 alleles. No evidence for genotyping artefacts, such as null alleles, was found with Micro-checker (Van Oosterhout *et al.*, 2004). All microsatellite loci were in linkage equilibrium.

Within-population gene diversity (H_o) ranged from 0.33 to 0.62 (average H_o = 0.48) and did not differ significantly between serpentine (average H_o = 0.50) and non-serpentine (average H_o = 0.46) populations (Table 1). F_{IS} values ranged from 0.07 to 0.35, and for most populations were significantly different from 0, based on 1000 permutations (Table 1), which points to a non-negligible degree of inbreeding in *A. serpyllifolium*. These results are in line with expectations for serpentine endemics, reflecting small effective population sizes and small distribution ranges and, as a consequence, low genetic diversity and high levels of inbreeding (Anacker *et al.*, 2011). On the other hand, we found significant F_{IS} only in two of the four serpentine *A. serpyllifolium* populations studied (Table 1).

The results show relatively high levels of population differentiation, with overall F_{ST} = 0.23 (95% CI 0.17 – 0.29). AMOVA results indicated that 23.7% of all genetic variation was partitioned between populations, while 16.7% and 59.6% partitioned within populations among and within individuals, respectively. When populations are grouped into serpentine (S) and non-serpentine (NS) ecotypes, only a small proportion of variation is accounted for by differentiation between these groups in microsatellite genetic diversity (Table 2).

Population-genetic structure analysis with Structure software (Falush *et al.*, 2003), revealed two optimal numbers of genetic clusters (K): $K = 4$ (as per ΔK : Figure S1) and $K = 8$ (as per $\text{LnP}(D)$: Figure S2). $K = 4$ results show a trend towards grouping together geographically close populations of the same ecotype, while $K = 8$ simply place each population in its own separate cluster (Figure 2). Admixture proportions of individuals in different populations varied in the $K = 4$ solution. Individuals in non-serpentine populations (northern Rubiá-NS and southern Alhaurín-NS) belonged to a single cluster each (pink and green clusters, respectively), and so did the serpentine Sierra Bermeja-S and Carratraca-S populations in the south (blue cluster). The other four populations showed varying degrees of admixture. The northern Barazón-S and Samil-S populations mostly belonged to the fourth cluster (brown), with more (Samil-S) or less (Barazón-S) admixture from Alhaurín-NS's green cluster. The northern and central non-serpentine populations Morata-NS and León-NS contained the highest admixture from Alhaurín-NS's green cluster, in addition to smaller admixture from Samil-S's and Barazón-S's brown cluster and Rubiá-NS's pink cluster (only in León-NS).

To investigate whether the pattern of isolation-by-distance (IBD) could be detected over all populations, a Mantel test (Mantel, 1967) was carried out on the Weir and Cockerham (Weir and Cockerham, 1984) F_{ST} pairwise distance matrix (Table S4). This did not reveal any direct correlation, and the number of populations within each ecotype was too small to allow IBD testing for serpentine and non-serpentine populations separately within them. No consensus neighbour-joining tree could be obtained with reliable branch support values based on Nei's D_{ST} distance metric (Nei, 1987), and no population structure could be detected using factor analysis in GENETIX (data not shown). Taken together, our data indicate that the analysed *A. serpyllifolium* populations appear to be more-or-less equidistant from each other and do not cluster according to serpentine and non-serpentine ecotypes.

Patterns of sequence diversity at loci putatively involved in metal hyperaccumulation

Genetic diversity across serpentine and non-serpentine populations of *A. serpyllifolium* was analysed in three genes (Table 3), one of which, *ASIL1* (*6B-INTERACTING PROTEIN 1-LIKE 1*), was used as a reference locus unlikely to be involved in evolution of the nickel hyperaccumulation trait, while the two other loci, *NRAMP4* (*NATURAL RESISTANCE ASSOCIATED MACROPHAGE PROTEIN 4*) and *IREG1* (*IRON-REGULATED PROTEIN 1*), have established roles in metal hyperaccumulation and homeostasis in other plant species.

In individual *A. serpyllifolium* populations, between 5 and 13 individuals were sequenced for *ASIL1*, 10 to 12 individuals for *IREG1*, and 6 to 11 individuals for *NRAMP4*, with the exception of the León-NS population, for which only 2 individuals were successfully sequenced (Table 3). For this reason, the León-NS population was excluded from most of the analyses focusing on *NRAMP4*. Overall, similar levels of sequence diversity were obtained for the two candidate genes and the reference gene (Table 3). Within the populations, genetic diversity varied widely in all three genes, with no clearly discernible pattern. Tests for selection did not detect any departures from neutrality in any of the populations (Table 3).

Interestingly, the two genes putatively involved in metal hyperaccumulation revealed striking differentiation between serpentine and non-serpentine populations, as measured by F_{ST} and D_A , in contrast to low differentiation at *ASIL1* and the microsatellites (Tables 2, S4 and S5). For both *NRAMP4* and *IREG1*, two strongly supported subclades grouping serpentine and non-serpentine populations were reconstructed based on Nei's (1983) D_A (Figures 3 and S3). Conversely, no well supported population tree (all bootstrap support values < 60 %) could be recovered based on *ASIL1*, with serpentine and non-serpentine populations intermixed on the tree (Figures 3 and S3).

Pairwise F_{ST} values for *ASIL1* did not vary significantly across population pairs regardless of their serpentine or non-serpentine origin (Table S5). Conversely, for *NRAMP4*,

pairwise comparisons of the serpentine and non-serpentine populations resulted in $F_{ST} > 0.9$, whereas for pairwise comparisons within serpentine populations much lower differentiation was found, with F_{ST} typically < 0.125 , with the exception of Barazón-S, where *NRAMP4* appears to have undergone additional longer sequence evolution than in other serpentine populations (Table S5).

AMOVA results (Table 2) confirm the high degree of shared genetic variation in the two candidate genes in populations of the same ecotype. When populations are grouped into serpentine and non-serpentine ecotypes, the overwhelming percentage (89.7% in *NRAMP4* and 64.5% in *IREG1*) of genetic variation is split between the two groups, while the rest is evenly distributed among populations within groups and within populations. The opposite pattern is found for the reference gene *ASIL1* as well as for microsatellites, where serpentine versus non-serpentine population grouping explained a very small proportion of overall genetic variation (Table 2).

Both candidate genes also contained a significant proportion of SNPs fixed or nearly fixed between the two ecotypes, with serpentine populations often featuring fixed derived genetic variants (Table 4). In particular, *NRAMP4* contains eight SNPs fixed for distinct alleles in serpentine and non-serpentine populations. Four of these SNPs encode non-synonymous changes, and in all cases the serpentine alleles are derived relative to the non-accumulators *Arabidopsis thaliana* and *Clypeola jonthlaspi* (the latter a member of the same clade within the tribe Alysseae as *A. serpyllifolium*: Rešetnik *et al.*, 2013; Španiel *et al.*, 2015), and so could be of adaptive importance. In particular, the Asp→Asn amino acid replacement at position 1190 leads to a change from an amino acid with acidic to neutral side-chain properties in the serpentine populations, which may influence protein function. Two further synonymous *NRAMP4* SNPs are nearly fixed between the ecotypes: at one SNP (position 343) the serpentine allele is segregating in Rubiá-NS, and at another SNP (position 1222) the serpentine allele is not fixed in Barazón-S. Similarly, *IREG1* contained six SNPs fixed or nearly fixed between the serpentine and non-serpentine ecotypes, four of which were non-synonymous (Table 4).

DISCUSSION

Our analyses revealed considerable genetic diversity in *Alyssum serpyllifolium* populations regardless of their serpentine (S) or non-serpentine (NS) origin. Such a relatively even distribution of genetic diversity is not expected if either S or NS populations were founded only recently. In particular, our results rule out a scenario of recent colonisation of serpentine environments by local migrants from non-serpentine populations, and indicate that both S and NS populations of *A. serpyllifolium* have existed for a long time. This is consistent with a relatively minor impact of glaciations on *A. serpyllifolium* populations in parts of south-

western Europe beyond the maximum advance of the main polar ice cap during the Pleistocene glaciations (Reeves, 1992; Hewitt, 1999).

Previous studies of the population genetics of species harbouring metalicolous and non-metallicolous populations have often speculated that, given the scenario of local evolution of metalicolous populations from relatively metal-tolerant, low-frequency genotypes present in non-metallicolous populations, a founder effect may have been present in metalicolous populations when they originally diverged from the source populations (Pauwels *et al.*, 2005), resulting in lower genetic diversity in the metalicolous populations. However, no differences in genetic diversity between metalicolous and non-metallicolous populations have been consistently found in the species investigated to date (Vekemans and Lefèbvre, 1997; Quintela-Sabaris *et al.*, 2010), such as *Arabidopsis halleri* (Pauwels *et al.*, 2005), *Alyssum bertolonii* (Mengoni *et al.*, 2003) and *Minuartia laricifolia* ssp. *ophiolitica* (Moore *et al.*, 2013), and neither have they been found here (Table 2). However, the bottlenecks in question would need to have been quite recent to stand out in this way, and gene flow and accumulation of new mutations would later erode the signal of any putative bottlenecks (Vekemans and Lefèbvre, 1997).

Our results reveal relatively high levels of population differentiation in *Alyssum serpyllifolium*, with overall $F_{ST} = 0.23$ (95% CI 0.17 – 0.29). Moderate-to-high values of F_{ST} in *A. serpyllifolium* are typical of values from other taxa endemic to specific substrates, such as *Primulina tabacum* (0.3936: Ni *et al.*, 2006), *Jurinea pinnata* (0.374: Salmerón-Sánchez *et al.*, 2014a), or *Convolvulus boissieri* (0.395: Salmerón-Sánchez *et al.*, 2014b). An even higher F_{ST} range was encountered between western Swiss populations of the model hyperaccumulator *Noccaea caerulescens*, in which F_{ST} reached an average of 0.591 (Besnard *et al.*, 2009). The values observed here provide evidence for moderate to low gene flow between the populations of *A. serpyllifolium*, probably determined by both ecological (serpentine “islands” of endemism) and topographical barriers, such as mountains and canyons, resulting in fragmentation of the species’ range. Consistent with this, AMOVA indicated that population divergence accounts for about one-quarter of all genetic variation in *A. serpyllifolium*. Similar between-population partitioning of genetic variation (22%) was found in an ISSR (Inter-Simple Sequence Repeat) study of four populations of the nickel hyperaccumulator *Alyssum lesbiacum* on the island of Lesbos (Adamidis *et al.*, 2014). In contrast, another *Alyssum* hyperaccumulator, *A. bertolonii*, had over a half (51%) of its genetic variation partitioned between populations in a study of 9 populations in Italy genotyped with chloroplast SSRs (Mengoni *et al.*, 2003). Between-population variation found in a chloroplast RFLP (Restriction Fragment Length Polymorphism)-based study of 28 populations *Arabidopsis halleri* (Pauwels *et al.*, 2005) was even higher and amounted to 68%. Thus, the levels of between-population genetic variation revealed in our study are not

dissimilar to those found in other metal hyperaccumulator species, indicating that evolution of metal hyperaccumulation in *A. serpyllifolium* is likely to be representative of the processes driving the evolution of the trait in other hyperaccumulator species as well.

Our analyses based on microsatellites and DNA sequence data from the *ASIL1* reference locus demonstrate that differentiation between serpentine and non-serpentine ecotypes accounts for very little genetic diversity in *A. serpyllifolium* populations, indicating that there is little differentiation between the ecotypes across the genome. In contrast, two genes putatively involved in metal hyperaccumulation, *NRAMP4* and *IREG1*, show far higher differentiation between S and NS ecotypes. *NRAMP4* expression appears to correlate positively with Ni tolerance (Oomen *et al.*, 2009; Halimaa *et al.*, 2014) in *Noccaea caerulescens*, one of the metal-hyperaccumulating species of Brassicaceae that can accumulate Ni as well as Zn and Cd. In both *Arabidopsis thaliana* and *N. caerulescens*, *NRAMP4* has been shown to encode a tonoplast-localised Zn, Fe, Mn and Cd transporter (Thomine *et al.*, 2000, 2003; Lanquar *et al.*, 2005, 2010; Pottier *et al.*, 2015). In particular, ectopic overexpression of *NRAMP4* in transgenic *A. thaliana* promotes Zn and Cd remobilisation from root vacuoles and decreased metal accumulation in the root (Pottier *et al.*, 2015), thus acting in a process expected to operate at a high level in hyperaccumulator species that preferentially translocate metals to the shoot. The *NRAMP4* homologue from the serpentine Ni hyperaccumulator *Thlaspi japonicum* has been shown to transport Ni specifically when heterologously expressed in yeast (Mizuno *et al.*, 2005), so this transporter may be directly implicated in Ni transport and/or sequestration in plants specifically adapted to serpentine substrates. Expression of *IREG1*, another membrane-localised metal-ion transporter, has also been shown to be important for nickel and cobalt detoxification in *A. thaliana* (Kirchner, 2009), consistent with increased nickel tolerance in *IREG1*-overexpressing transgenic lines and increased nickel sensitivity in *IREG1* knockout lines. Additionally, a newly characterised IREG family member in the nickel hyperaccumulator *Psychotria gabrielliae* (Rubiaceae) appears to confer increased nickel tolerance, possibly by enhancing vacuolar accumulation of Ni (Merlot *et al.*, 2014). Taken together, we hypothesise that both *NRAMP4* and *IREG1* are likely to be of high adaptive significance for the nickel-hyperaccumulation ability of *A. serpyllifolium*. Conversely, *ASIL1*, selected as a reference gene for comparisons with *NRAMP4* and *IREG1*, is a transcriptional repressor of seed maturation genes in germinating seeds and seedlings in *Arabidopsis* (Gao *et al.*, 2009), and is thus unlikely to play a significant role in nickel tolerance and hyperaccumulation in *Alyssum*.

Stronger differentiation between S and NS ecotypes at *NRAMP4* and *IREG1*, compared to *ASIL1* and microsatellites, is consistent with diversifying selection in *NRAMP4* and *IREG1* genes driven by local adaptation to serpentine and non-serpentine environments.

Furthermore, local adaptation at these genes is supported by the presence of derived SNPs, including amino acid replacements that are fixed or nearly fixed in serpentine populations, while non-serpentine populations are fixed for the ancestral alleles (Table 4). The question when and how these putatively adaptive alleles spread across the serpentine populations of *A. serpyllifolium* is of considerable interest, as this may shed light on the evolution of nickel hyperaccumulation. If the spread were recent ($<2N_e$ generations ago), it would be expected to leave characteristic footprints of a selective sweep, such as reduced variation in and around the loci concerned in serpentine populations. However, no deviations from neutrality were detected in any of the populations, indicating that local adaptation to serpentine conditions in *NRAMP4* and *IREG1* genes may have occurred relatively early in the history of the species complex. This is consistent with the distribution of serpentine *A. serpyllifolium* populations on relatively old ultramafic outcrops that have been exposed for many millions of years.

In summary, the present results from the population-genetic analysis of *Alyssum serpyllifolium* across the Iberian peninsula suggests that the serpentine and non-serpentine ecotypes can be regarded as belonging to the same species complex and do not warrant description as distinct taxonomic entities. Although earlier work has demonstrated that the serpentine populations show particular morphological characteristics when growing on these substrates *in situ*, and that they display a much higher degree of nickel tolerance than the non-serpentine populations when tested under common-garden conditions, the distinctiveness of these ecotypes is not supported by the results of the microsatellite analysis. Rather, all eight populations tested were differentiated from each other to an approximately equal extent, with evidence of a significant degree of inbreeding probably indicative of a relatively long history of isolation of these populations. At the level of DNA polymorphism, however, two candidate genes putatively involved in nickel hyperaccumulation showed signatures of adaptive evolution that may represent an integral part of the mechanism of local adaptation to serpentine substrates.

CONCLUSIONS

Our results demonstrate that divergence between serpentine and non-serpentine ecotypes of *A. serpyllifolium* is no greater than among populations of the same ecotype. This does not support earlier suggestions that serpentine and non-serpentine *A. serpyllifolium* populations represent separate species (Dudley, 1986a,b). Although earlier work has demonstrated that the serpentine populations show particular morphological characteristics when growing on these substrates *in situ* (Dudley, 1986a,b), and that they display a much higher degree of nickel tolerance than the non-serpentine populations when tested under common-garden conditions (Brooks *et al.*, 1981), the distinctiveness of these ecotypes is detectable only at

the loci putatively involved in the metal hyperaccumulation trait. Selective pressure at these loci to adapt to highly distinct conditions at serpentine and non-serpentine sites has resulted in strong differentiation, with many synonymous and non-synonymous mutations fixed between the two ecotypes. Contrary to this, no such differentiation was detected elsewhere in the genome; all eight populations tested were differentiated from each other to an approximately equal extent and contained similar levels of genetic diversity, providing no support to the hypothesis that populations of one ecotype originated from local populations of the other ecotype. We conclude that adaptation to serpentine and non-serpentine environments in *A. serpyllifolium* populations probably had a long history, but affected only the genes involved in evolution of metal tolerance and hyperaccumulation, while patterns of polymorphism in the rest of the genome have been dominated by geographic isolation of these populations. Expanding this analysis to a greater number of genes and populations in the future will help to identify more genes involved in evolution of metal hyperaccumulation and more precisely reconstruct the history of evolution of this peculiar trait.

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Conflict of Interest

The authors declare no conflict of interest.

Data Archiving

The DNA sequence data in this paper are available under GenBank accession numbers KX592209–KX592426.

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Figure legends:

Figure 1. Distribution of the sampled populations of *Alyssum serpyllifolium* on the Iberian peninsula. Serpentine (S) and non-serpentine (NS) populations are shown as filled and open circles, respectively.

Figure 2. Assignment of genotypes of individuals in the eight *Alyssum serpyllifolium* populations to either 4 (*top*) or 8 (*bottom*) genetic clusters as defined to be most likely by Structure. Serpentine populations are indicated in blue and non-serpentine in red. *n*, number of sampled individuals in each population.

Figure 3. Gene trees based on net population divergence (D_A) at all sites for *NRAMP4*, *IREG1* and *ASIL1* genes. For trees based on silent sites see Figure S3. Numbers on the branches show percentage support over 1000 bootstrap replicates. Serpentine populations are in blue and non-serpentine populations in red.

Table 1. Population diversity and inbreeding coefficient (F_{IS}) in samples of n individuals genotyped for eight microsatellite loci in eight populations of *Alyssum serpyllifolium*. F_{IS} values deviating significantly ($P < 0.05$) from 0 are labelled with an asterisk (*). SD = standard deviation.

Population	n	F_{IS}	H_o	SD	H_e	SD
Alhaurín-NS	36	0.35*	0.33	0.21	0.51	0.22
León-NS	22	0.22*	0.51	0.26	0.65	0.23
Morata-NS	30	0.22*	0.55	0.14	0.70	0.09
Rubiá-NS	41	0.24*	0.44	0.21	0.58	0.13
<i>Average for NS</i>	32	<i>0.2575</i>	<i>0.4575</i>	<i>0.205</i>	<i>0.61</i>	<i>0.1675</i>
Barazón-S	35	0.07	0.40	0.27	0.43	0.25
Carratraca-S	33	0.27*	0.48	0.24	0.65	0.15
Samil-S	36	0.16*	0.62	0.14	0.73	0.11
Sierra Bermeja-S	36	0.08	0.51	0.20	0.55	0.18
<i>Average for S</i>	35	<i>0.145</i>	<i>0.5025</i>	<i>0.2125</i>	<i>0.59</i>	<i>0.1725</i>

Table 2. Percentage of variation in microsatellites and three genes accounted for by differentiation between ecotypes and populations, as revealed by AMOVA analyses. Eight populations of *Alyssum serpyllifolium* were split into two groups: serpentine (S) and non-serpentine (NS), with four populations in each group, as defined in Table 1.

	Among S and NS	Among populations	Within
	ecotypes	within ecotypes	populations
Microsatellites	4.97	20.59	74.45
<i>ASIL1</i>	4.5	41.1	54.4
<i>IREG1</i>	64.5	16.8	18.7
<i>NRAMP4</i>	89.7	4.6	5.6

Table 3. Summary statistics for DNA sequence polymorphism analysis in three nuclear genes.

		Serpentine populations				Non-serpentine populations				
		All	Barazón	Carratraca	Sierra		Alhaurín	León	Morata	Rubiá
					Samil	Bermeja				
<i>ASIL1</i> (length = 735 bp)										
sample size	71	10	13	8	6	10	9	10	5	
haplotype diversity	0.965	0.189	0.963	0.958	0.894	0.695	0.993	0.968	0.800	
nucl. diversity (nonsynonym.)	0.0032	0.033	0.047	0.175	0.282	0.301	0.253	0.139	0.253	
nucl. diversity (synonymous)	0.0123	0	1.562	0.651	0.541	0.287	1.449	1.315	0.349	
Tajima's <i>D</i> (all sites)	-1.85*	-0.59	0.86	-0.16	0.93	0.94	0.97	0.86	0.53	
Fay & Wu's <i>H</i> (all sites)	-0.911	0.17	-0.37	-0.77	-0.70	0.59	-1.83	-0.02	0.89	
<i>IREG1</i> (length = 668 bp)										
sample size	86	10	12	10	10	12	10	11	11	
haplotype diversity	0.834	0	0.236	0.958	0.911	0.772	0.363	0.005	0	
nucl. diversity (nonsynonym.)	0.0033	0	0.019	0.244	0.142	0.055	0.112	0.079	0	
nucl. diversity (synonymous)	0.0117	0	0.069	0.846	1.050	0.601	0.044	0.525	0	
Tajima's <i>D</i> (all sites)	-1.49	-	-1.20	1.12	1.76	-0.93	-1.78	0.01	-	
Fay & Wu's <i>H</i> (all sites)	-1.77	-	0.22	-2.52	-2.47	-3.75	0.55	-0.48	-	
<i>NRAMP4</i> (length = 1180 bp)										
sample size	65	6	11	8	11	10	2	10	7	
haplotype diversity	0.809	0.848	0.574	0.400	0.363	0.484	-	0.233	0.615	
nucl. diversity (nonsynonym.)	0.0035	0.122	0.024	0	0	0.024	-	0.030	0.056	
nucl. diversity (synonymous)	0.0134	0.314	0.269	0.105	0.102	0.084	-	0	0.067	
Tajima's <i>D</i> (all sites)	0.42	0.32	-0.95	0.33	-1.14	-0.16	-	-0.45	0.32	
Fay & Wu's <i>H</i> (all sites)	-0.07	0.67	-1.01	0.29	0.26	0.42	-	0.20	-1.05	

* $P < 0.05$

Table 4. Summary of SNPs fixed or nearly fixed among S (serpentine) and NS (non-serpentine) populations of *Alyssum serpyllifolium* in *IREG1* and *NRAMP4* genes. SB = Sierra Bermeja. Ancestral status of the alleles was determined based on *Clypeola jonthlaspi* and *Arabidopsis thaliana* outgroups.

Position of candidate SNP	Fixed in all S populations?	Present in NS populations?	Substitution (NS→S)	Substitution effect	S allele derived?
<i>IREG1</i>					
493*	Segregating in SB and Samil	No	C→A	Ser→Arg	No
504	Absent in SB, segregating in Samil	No	A→G	-	Yes
509	Segregating in SB and Samil	No	T→C	Val→Ala	Yes
516	Segregating in SB and Samil	No	G→T	Arg→Ser	No
556	Segregating in SB and Samil	No	C→A	Leu→Ile	No
681*	Segregating in SB and Samil	No	G→A	-	Yes
<i>NRAMP4</i>					
343	Yes	Segregating in Rubiá	C→G	-	Yes
511	Yes	No	T→G	-	Yes
519	Yes	No	C→G	Ala→Gly	Yes
527	Yes	No	G→A	Val→Ile	Yes
571	Yes	No	C→T	-	No
976	Yes	No	T→C	-	No
1019	Yes	No	A→C	Ile→Leu	Yes
1060	Yes	No	C→T	-	Yes
1190	Yes	No	G→A	Asp→Asn	Yes
1222	Segregating in Barazón	No	T→C	-	No

* outlier SNP not detected in the original RNA-Seq dataset

100 km



Barazon-S



Rubia-NS



Leon-NS

Samil-S



Morata-NS

Carratraca-S

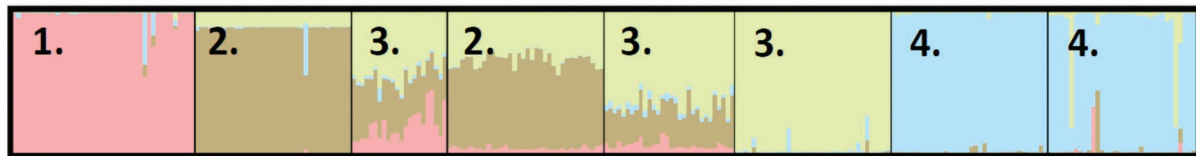


Alhaurin-NS

Sierra Bermeja-S



$K = 4$



Rubiá

Barazón

León

Samil

Morata

Alhaurín

**Sierra
Bermeja**

Carratraca

n

41

35

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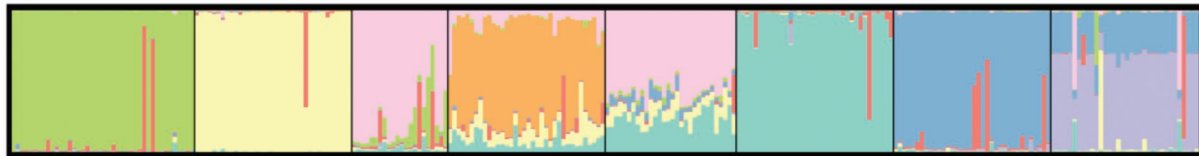
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$K = 8$

