ECOLOGICAL GENETICS OF *TRIFOLIUM REPENS*

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ABSTRACT

The theory of Evolution by Natural Selection deals substantially with events now past, and with processes too slow for contemporary study. Experimental studies of evolution are possible, but are liable to the criticism that they deal with artificial, or at least extreme and atypical situations, involving rapid evolutionary change. Such criticism can be avoided to a degree by studying situations now in equilibrium.

It is suggested that interesting results may be obtained by studying polymorphisms, and comparing the equilibria reached in populations of an organism in different environments. The chosen approach was to find a case of polymorphism convenient to study, followed by an attempt to interpret the pattern of its variation in terms of features of environments where a morph was common selecting in its favour, and vice versa.

The material presented falls into three parts.

1) Material and Methods: Background and Preliminary Studies.

The system chosen for study was the white V-shaped leaf markings of Trifolium repens. The species is widespread, abundant, and variable; the character is expressed on vegetative organs, and is therefore always accessible; and the known variation in this character had already been interpreted in terms of the action of a set of several alleles at one locus.

The results of crosses indicate general agreement with previous reports. One heterozygous plant apparently largely failed to transmit one allele, $\underline{V}^{b}$, through the pollen. Segregations indicate that a new phenotype, 'smeared', is determined by a particular $\underline{V}^{h}$ allele. Two more new phenotypes, 'shaded' and 'marginal' are described. The places where these and other rare phenotypes have been found are listed. New reports and the author's other experience are combined with previous reports in a new general account of leaf marking in the species, which also deals with at least three distinct classes of red markings. There appears to be more variation, implying more alleles, than previous accounts allow for, and reportedly distinct types, for example, $\underline{V}^{h}$, $\underline{V}^{l}$ and $\underline{V}^{f}$, seem to be linked by a series of intermediates, among which clear dividing lines are hard or impossible to draw.

Experimental studies relating to the techniques of population sampling are reported. Scarification by 10 to 20 minutes exposure to concentrated sulphuric acid was chosen to deal with hard seed. The effect of a restricted number of mother plants on the accuracy of a seed sample is discussed; predictions concerning the frequencies of different types in progeny from single heads grown separately were roughly consistent with observations. Possible differences between vegetative and seed samples are discussed. Morph frequencies in a sample of plants bearing inflorescences were not found to be significantly different from those of the whole vegetative sample. The consequences of this species' ability to reproduce vegetatively are discussed, and a brief clone-mapping project is described.

The procedures used to obtain and score material, and to obtain information about its background are described. The amounts of material of different types from different sources is summarised in Table 6.2. Problems involved in estimating gene frequencies are discussed, and the derivation of the values used to represent the different morph frequencies is outlined; they are basically phenotypic frequency figures, gene frequency calculations being used only where it was found to be necessary.

2) Observations on Population Samples.

Polymorphism in respect of white leaf marks was found to be present in all except 15 out of 624 samples. The commonest group of phenotypes are the 'simple V-marks', referred to as 'L'. The next commonest is the unmarked type, '0', present in all except 72 out of 624 samples, with an overall frequency among the plants scored of 17 per cent.

Study of the sample data reveals a deficiency of the double marked phenotypes expected to be showed by $\underline{V}^{by}\underline{V}^{l}$ plants. This is explained as the result of a degree of dominance of $\underline{V}^{by}$ in such combinations; the effect of such dominance is allowed for in the frequency figures representing the frequency of 'L' marks, and of double-marks containing two members of the 'L-series' of simple-V-producing alleles.

The possibility of demonstrating interaction between the frequencies of different morphs is discussed. There are indications of lower frequencies of 'By', 'B', and 'F' marks when the unmarked phenotype is common.

The possibilities for the main object of the work, discovering associations between morph frequencies and environmental factors are shown in Table 8.1, giving the sets of data presentable for particular methods of analysis.

Data on 148 British samples, scored in the field by the author, were treated by Multiple Regression Analysis (using the KDF 9 Computer of the Oxford Computing Laboratory), to test for association of morph frequencies with geographical location, altitude, and soil pH. Significant increases in the proportion of unmarked plants are shown with greater distance north and higher altitude.

Frequency data for all the classes of morphs described is shown in the form of maps, of the British Isles, Western Europe, and the whole of the species' natural range. These maps confirm the northwards increase in frequency of the unmarked form found in Britain. Maps for the rare morphs show various patterns, most of which seem to involve central regions of higher frequency north of the Mediterranean (France - Alps - Greece) with lower frequencies elsewhere.

Regression analysis of a set of seed samples from Spain supports the conclusion from British vegetative samples of an increase in unmarked frequency with higher altitude. Examination of the information about the background of other samples suggests higher unmarked frequencies in pastures than on waste ground, meadows being intermediate.

British data suggest an association with wet, and particularly with badly-drained sites, with trodden paths, and with dense vegetation. There are patterns of response to water regime elsewhere but (e.g. Polish data) they tend to suggest the opposite association, of high unmarked frequency with dry conditions.

3) Comparison with other species.

New observations are reported on some other related species. Available information on marking in other species of *Trifolium* is reviewed, and it is pointed out that it is scattered, difficult of access (much unpublished), and sometimes ambiguous or contradictory.

Summary of this data indicates the presence of white V-markings in nearly 30 species of *Trifolium*, and its absence in at least as many more. Marks are probably entirely absent in subgenus *Chronosemium* and perhaps in part of subgenus *Trifolium*, but seem to occur in most of the other subgenera for which there is information. When marks are present, it

appears that they are nearly always variable. Only three species are definitely reported as always marked, and in two of these there is variation between different types of marking. This suggests that the factors producing or preserving polymorphism in T. repens act also in other marked species. The presence of red leaf marks of various kinds is reported in 21 species of Trifolium.

Also in the tribe Trifolieae, both red and white V-markings are found in Parochetus communis. Material grown by the author showed great variability within each plant in leaf mark, but no clear differences between plants. Red leaf marks, variable, and in some cases approaching a V-shape, are also found in Medicago.

Several lines of evidence indicate relationships between the white markings, and the various red marking systems in T. repens. It is suggested that the white and red V-markings have a common evolutionary origin.

Some examples of leaf marking with analogous properties in genera unrelated to T. repens are also briefly reviewed.

In discussion some possible challenges to the validity of the results claimed are discussed, and evidence is presented suggesting that the reported genetic clines in unmarked frequency are real. Selective factors affecting unmarked frequency are tentatively suggested to be temperature and water regime. The problem of relating these to markings on leaves is discussed, and also the possibility that the phenomena of leaf marking are by-products of unknown processes, and are of no intrinsic importance. It is suggested that the interactions between red and white markings support the hypothesis that leaf markings themselves are of selective importance; and some possibilities as to what form this selective importance might take are mentioned.

Possibilities for further work indicated in the course of the studies presented here are discussed. They include studies on important problems of population dynamics, which affect the design of techniques for sampling for leaf markings, but in which also observations on leaf markings could be used as means to ends of wider significance.

Ways in which the methods used in the present study could be improved in a repeated study are suggested; however, it is felt that the clines observed provide a starting point for experimental work, and that this might be more rewarding than further descriptive work.

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PART I

INTRODUCTION

CHAPTER I

SIGNIFICANCE AND VALUE OF MORPH-FREQUENCY SURVEYS

1. Methods of Studying Natural Selection.

It is believed that evolutionary change is brought about by the selective effect of the environment on the organisms living in it. Over very long periods, members of fossil sequences show continued changes in almost all characters. If, however, one desires to show evolution in action by contemporary studies on living organisms, it seems necessary to choose rather unusual situations of rapid evolutionary change.

Such changes can most easily be seen as the readjustment which follows a change of environment and selective pressures, or also following the introduction by mutation or gene flow of a favourable mutation not previously present. Situations of altered selection are on the whole rare in nature, and those studied include very many artificial ones concerning populations in more or less completely controlled laboratory conditions. The natural cases are rare but well known; for example, the spread of melanism in moths in industrially polluted areas, affecting over eighty species in Britain (Ford, 1964, p. 248), and the evolution of resistance to myxomatosis by European and Australian rabbit populations, following the introduction of virulent strains of myxoma virus (Fenner, 1959). There are also cases of cyclical selection which may contribute nothing directly to long term evolutionary change; for example, seasonal fluctuations in the frequencies of different chromosome arrangements in *Drosophila pseudoobscura* (Dobzhansky, 1951, pp. 118-9); irregular fluctuations in the frequency and selective value of the *medionigra* gene in the Cothill population of *Panaxia dominula* (Ford, 1964, pp. 113-7). Such examples must be atypical when compared with the greater part of the selection experienced by natural populations in the wild. Most selection may be expected to be relatively steady, and most populations may be expected to be close to equilibrium with regard to characters thus selected. It is in many ways easier to study cases of response to catastrophically altered selection, but if steadier selection which has already produced an approach to equilibrium is felt to be more typical of what is actually happening in nature, a special interest attaches to any investigation that may be possible of such selection and its consequences.

One way in which such an investigation can be attempted is by comparisons between populations of the same species established in different environments, and each presumably adapted to its own environment. Differences between the genetic constitution of the different populations should reflect differences in the selective experience to which these populations have been and are being exposed. Such an investigation can be begun, on the one hand, by observing genetic variation, and attempting to relate it to environmental variation. This is the approach adopted here in studying leaf marking in Trifolium repens, and by Daday (1954a,b; 1958) in his work on cyanogenesis in the same species. On the other hand, one can choose an aspect of environmental variation, and look for genetic differences in adaptation to it. Examples of this approach, again in Trifolium repens, are studies demonstrating adaptation to particular day-length regimes (Gibson, 1957), or soil-types (Bradshaw and Snaydon, 1959).

By the first approach we choose an aspect of genetic variation, and look for possible selective factors to explain the pattern of variation. We aim to portray the variation in selection which determines the pattern of genetic variation affecting a particular facet of the genotype. To do this we must try to compose a new and special picture of the environment, in which some apparently conspicuous features may be neglected, while other obscure ones may become important. Further, we must try to distinguish between the few environmental features which actually cause the selection studied, and those, perhaps many and perhaps more apparent, which are only correlated, more or less closely, with the actual selective agency. We may use the genetic make-up of the populations in each environment and the difference between those in different environments as a coded record of the different selective pressures each population has experienced. When we have observed that genetic variation is demonstrable, we can next attempt to discover differences in the underlying selection which is acting, and to understand the mechanism of the code by which the organism displays the presence and pattern of variations in this selection.

The second type of approach referred to above enables one to choose an environmental factor which is easy, or important, to study, and perhaps one whose extreme variation is felt certain to provide results in the form of genetic differences; the first approach involves choosing the type of genetic factor, one which is easily accessible for study, and perhaps one which is easy to describe. Discontinuous variation may offer the opportunity to describe rather than to measure, and may simplify all mathematical work by one order of complexity. In both cases a species must be chosen; requirements one can suggest for a successful study of this kind are that the species chosen should be distributed in a sufficiently numerous and variable range of environments, that each local population should be sufficiently isolated to have diverged from the others, and that sufficient variation should be available in each population in the character studied, so that one can feel confident that the genetic situation expresses the selection acting, that a different balance of selective forces could have achieved different results.

2. Balanced Polymorphisms.

a. Suitability for Studies of Evolution.

Balanced polymorphisms are systems which meet these requirements well; variation is discontinuous and qualitative, and can generally be seen to be present, however the frequencies may be modified, in all populations. Ford (1940) has defined polymorphic situations as those in which two or more forms of a species coexist in the same area at frequencies too great to be explained by recurrent mutation. Such a situation may be a state of transition towards fixation of one form, or it may be an accidental expression of characters too trivial to experience selection strong or consistent enough to bring about fixation. If however it is widespread and persistent it would seem extremely likely that the variability is maintained by some mechanism such as heterozygous advantage. This would make it very difficult for any selective fluctuation to eliminate variation and fix one homozygous form. Commonly such an equilibrium involves at least two selective factors, the heterozygote being superior to one homozygote in one respect, to the other in another. The frequency of the three types would vary according to the relative importance of the two factors, and if one of them has a predominant effect, one homozygote may be very rare. However, fixation would only follow if one selective factor vanished completely.

Such a polymorphic situation is characteristically sensitive and responsive to selection over a middle range of gene frequency, but more and more resistant to greater pressures which might produce fixation. It provides a system which may be very responsive to variations in the selective action of the environment. It should also be remembered that it may respond to other environmental variation as well as to variations in the selective pressures which maintain its stability.

In the investigation of a particular example of polymorphism, the questions to which it is appropriate to seek answers fall into two groups, though the content of the answers may overlap. The first group concern the preservation of the polymorphism against selective pressure towards fixation. This aspect is of interest since strong selective pressures may be seen in continuous action, yet producing no change. The consequences on the rest of the gene pool of persistent variation at one locus are also of importance. The second group of questions involve the use of the polymorphic system as a responsive but robust measure expressing part of the variations in selection actually experienced by populations in different environments. This may or may not provide access to the basic selective pressures protecting the existence of the polymorphism, but is also of interest in producing a picture, through the organisms' own eyes as it were, of the variation and action of some of the selective pressures affecting the population.

This work has been mainly concerned with an attempt to map variations in the frequency of different forms in a polymorphic system, in an attempt to provide answers to these questions, particularly the second group.

b. Achievements of the Study of Polymorphism.

It is interesting to refer to some of the cases of polymorphisms which have been studied already, particularly those where the method of study or the sequence of discovery, or the nature of the material, have some connection with those in the work to be described below. More extensive general reviews can be found in Huxley (1955), Ford (1964).

The classic example of the appropriateness of the attempt to relate morph frequencies to environmental variables is that of the sickle cell allele in man. The high frequency of the anaemic homozygotes for this allele in certain areas can be understood when it is realised that the heterozygotes, who are not hampered by symptoms of anaemia, are in fact highly favoured by resistance to the subtertian form of malaria which is common in these areas (Allison, 1954).

The approach projected for the work described below consists of comparing morph frequency and environmental variables between a number of populations, searching for correlations between the two, and an attempt to determine which environmental variables are most closely related to the environmental factors which actually act selectively, and perhaps to identify the factors which have a selective action discriminating between the genetic factors whose variation was studied. Precisely this sequence has been successfully followed for another character in the same organism as that chosen for this work.

Certain genotypes confer on leaf tissue of *Trifolium repens* the ability to release HCN when detached from the plant. This requires the presence of a dominant allele controlling the presence of the HCN-producing substrates, and of a second allele controlling the presence of the enzyme by which HCN production from them is catalysed (Corkill, 1942; Atwood and Sullivan, 1943). It can be shown that in this, and in another species (*Lotus corniculatus*) with the same property, certain predators - voles, rabbits, slugs, snails - avoid the cyanogenic plants (Jones, 1962). Why then are some plants not cyanogenic? Several papers by Daday (1954a, b, 1958) showed that the frequency of non-cyanogenic forms rose in the North-East of Europe, and at higher altitudes in the Alps. This implied counter-selection, favouring the non-cyanogenic types, by a factor acting particularly strongly in these areas, and the obvious interpretation was that this was some expression of winter temperature. Working from this clue, it was possible for Daday (1962, 1965) to show that the growth of cyanogenic plants was retarded, relative to that of non-cyanogenic plants, when exposed to frost, and that this was the result of the release of HCN in vivo and consequent metabolic inhibition. The release of HCN was produced by activation of the enzyme by low temperatures, which could also be demonstrated in vitro.

In this case selective forces acting against each phenotype have been identified, one - the effect of cold - as a result of the indications provided by a geographical mapping of the variations in gene frequency. The two known factors together offer a logically sufficient explanation of the known situation, except that the reason for the prevalence (and therefore presumed stability) of polymorphism, rather than uniformity at most individual sites, is not clear. In some other systems the prevalence of polymorphism is explained by demonstrable heterozygous advantage, though disruptive selection or possibly gene flow could also contribute.

The predator-selection effect, in contrast to that of cold, was first demonstrated by comparisons within populations and by experiment. If there were grounds for predicting variations in the intensity of selective predator pressure in particular environments, it would be possible to make and test predictions of gene frequencies above or below those predicted on the basis of the local winter temperature regime.

c. Survey of Examples of Plant Polymorphism.

Examples of plant polymorphism apart from those involving reproductive compatibility or major structural heterozygosity are relatively few in number, ~~and~~ or at least less completely explained than that quoted above (cf. examples referred to by Turrill (1948) and Huxley (1955)). It seems worth while to survey and compare the cases for which information could be discovered. One may contrast two types of 'case history'. In the first, observations of frequency distribution patterns were the starting point, leading to some degree towards the identification of selective factors (as in the case of the cold effect in the cyanogenesis system described above, and as hoped for in the work to be described below). In the other type, selective factors were first demonstrated by within-population comparisons, observational and experimental, and predictions about natural distribution pattern were derived from these before any comparison between natural populations was attempted. The cases to be described will be arranged with clear cases of the first type at the beginning, and clear cases of the second type at the end.

In a second respect, the polymorphisms described below fall into three groups. Firstly, chromosomal polymorphisms, mostly involving supernumerary chromosomes. Secondly, polymorphisms where there are only two viable phenotypes, the homozygous dominant and the heterozygote, the homozygous recessive being lethal. In both of these types one of the selective factors necessary to explain the balance of the polymorphism would be easily recognisable under any conditions, the factor being lethality or sublethality, substantially immune to adjustment by differences in environment. The third class includes cases where the more easily distinguished (recessive) homozygote is viable and probably has a selective advantage under some conditions, at least when compared with the other homozygote.

In *Nicandra physalioides* the selective pressures acting on a polymorphism based on the presence of either one or two isochromosomes, and controlling seed dormancy, were identified because the dormant form was absent in seedlings emerging in the year of planting new seed, but frequent in a population which had reappeared from seed in the soil after several years' absence (Darlington and Janaki-Ammal, 1945). An increase with increased altitude of the frequency of glaucous forms in three species of *Eucalyptus* was attributed by Barber (1955) to differences in frost activity. In *Pisum arvense*, morph frequencies for thin or thick waxy layers on the leaves are correlated with variation in humidity, as for instance between the British Isles and Central Europe. The frequency of thick wax layers was shown to rise over four generations of reproduction in a dry environment (Scheibe, 1955).

In other cases, geographical patterns which can be identified as those of some particular environmental factor are known, although one cannot come so close to identifying an actual selective agent. In *Dactylis glomerata* ssp *judaica* the frequency of heterozygotes for albino recessives, unexpectedly high in all populations, was found to be higher on terra rossa than on rendzina soils (Apirion and Zohary, 1961). Two patterns of distribution of supernumerary chromosomes should also be mentioned. Supernumeraries are more frequent in populations of *Festuca pratensis* on clay than on non-clay soils (Bosemark, 1956), and in *Centaurea scabiosa* they appear to be less common in oceanic climates and more so in continental climates (Fröst, 1958). A cline in the frequency of red leaf-flecks has been described in *Arum maculatum*, flecked forms being commoner in the south (Prime, 1955b). In *Atropa belladonna*, homozygous recessives with yellow-green flowers are commoner as one moves eastward in Britain; they are less hardy, and are restricted to sheltered sites (Burnett, unpublished, quoted in Huxley, 1955).

Geographical heterogeneity in morph frequencies has been recorded in some other cases, suggesting heterogeneity in selection, though without any pattern of environmental correlation being identified. Fruiting head structure in *Aegilops speltoides* was polymorphic, with widely varying frequencies of two alleles at the locus concerned, in nearly all the populations studied in Israel and Turkey (Zohary and Imber, 1963). Variation in the frequency of a hairy recessive was widespread in *Digitalis purpurea* (Saunders, 1918). Distinct heterogeneity was found in supernumerary chromosome frequency between a small number of populations of *Lilium callosum* (Kimura and Kayano, 1961).

In other cases there have been discovered appreciable frequencies of heterozygotes for lethals, or frequencies of heterozygotes higher than could be predicted from overall gene frequencies, or heterozygotes occurring in the wild for alleles at loci controlling major characters, which seem unlikely to be of trivial selective importance. Such evidence suggests that further investigation would demonstrate a balanced polymorphic system. Examples include high frequencies of carriers of albino recessives in *Dactylis glomerata* ssp *woronowii* (Curran, 1961), of albino and other lethal recessives in *Trifolium pratense* (R.D. Williams, 1937, 1939); high frequencies of heterozygotes for fruit colour, for albino recessives, and for one particular *S*-allele in *Prunus avium* (W. Williams and Brown, 1956); and the occurrence of heterozygotes for the distribution of heterochromatic chromosome segments in *Trillium* and *Paris* (Darlington and Shaw, 1959), in *Fritillaria*, *Tulbaghia*, *Cestrum* and *Hyacinthus* (Dyer, 1963) and for different nucleolar chromosomes in *Allium paniculatum* (Ved Brat, 1965).

The second type of case history of discovery is well exemplified by the work on the distribution of waxy bloom on the stems of populations of *Ricinus communis* naturalised in Peru. It was observed that plants carrying the dominant allele producing wax failed to fruit under the foggy conditions common at coast level, while the wax-less recessive homozygote fruited freely. Subsequently it was shown that there was a gene-frequency cline between all-waxy populations at high altitudes exposed to bright light, and all-wax-less populations at sea level (Harland, 1947). The case of anthocyanin pigment variation in *Chloris gayana* (Bogdan, 1963) could have been mentioned above, as an example of observation of geographical pattern followed by experimental demonstration of differences in fitness. However it does not seem to be clearly established that the observed variation in nature is controlled by the same genetic system as that studied experimentally. The experiments concerned a rare phenotype completely lacking anthocyanin, determined by recessive homozygosity at two duplicate loci; it was shown that the double recessive phenotype had reduced growth rate and viability, but increased resistance to drought and *Helminthosporium* attack. These selective factors may also discriminate between the more or less intensely pigmented forms whose quantitative variation was observed in collections from the wild. Further study might show that the recessives do have appreciable frequencies in wild populations. If either of these possibilities turns out to be true and the experimental observations are shown to be relevant to natural situations in the field, then this would be the first case, after cyanogenesis in *Trifolium repens*, where different and opposing environment-dependent selective factors have been identified in a plant polymorphism.

3. The Example Chosen.

The work presented below concerns the polymorphism for the presence of various forms, or the absence, of white leaf markings in Trifolium repens, white clover.

This species seems suitable for this kind of work since it is an outbreeder, is known to be variable in many respects, and is widespread. (These points and those which follow will be expanded and documented in following chapters.) It is also the species in which the two-locus polymorphism for cyanogenesis described above has been so successfully investigated. Because of its agricultural importance a considerable amount of published work is available to answer many of the questions one would ask as a background to this work.

The character chosen is one which is expressed by the plant in the vegetative condition. Coexistence of different leaf-mark phenotypes in the same population has already been reported, and two studies describing a simple mechanism of inheritance of this character have been published.

The main difficulties involve the expression of the character and the diagnosis of the different phenotypes, which is not as simple as the previous work would suggest, and the abundance of deliberate sowings of cultivated seed, which requires care in the choice of populations sampled.

Other complications are associated with the perennial and vegetatively spreading habit of the species, rendering individual genotypes potentially infinite in age and size. Selection may indeed be acting largely or exclusively in some communities in the vegetative phase, as the establishment of new genotypes as seedlings in an undisturbed closed grassland community may be very rare.

The principal concern of this work has been the description and comparison of the frequencies of different mark phenotypes in different natural populations, and the attempt to relate any variation in frequencies to variation in environment.

This method is particularly appropriate for investigating this situation since no hypothesis explaining the selective values of presence and absence of marking is readily available to the intuition, and one alternative is to look for a clue from the pattern of distribution of the different types.

CHAPTER II

THE SPECIES

1. Growth Form and Reproduction.

The primary shoot of a seedling of Trifolium repens is erect, but its growth is limited, and after the earliest seedling stages a plant of this species grows in the form of prostrate runners or stolons, from which leaves on erect petioles arise at regularly spaced nodes. Many of the nodes develop adventitious roots, especially when in contact with the soil. At each node a vegetative branch or an inflorescence may arise, any development from the axil normally becoming visible soon after the leaf at that node is fully developed. Once rooted, each stolon is more or less self-sufficient; older parts of the stems commonly die off, leaving the runners to grow and branch more or less indefinitely. In some larger-growing cultivated strains there is a drop in vigour and yield once contact with the deeper primary root has been lost (Westbrooks and Tesar, 1955), and then the survival of isolated runners in competition is limited (cf. Carnahan, 1960, but also Gibson and Trautner, 1965). The achievement of flowering may further limit survival (Gibson, 1957), and these forms are used as annuals or biennials. For them, the 'persistence' the breeder considers consists of persistence of contact between the stolons and the primary root system (Crowder and Craigmiles, 1960). The British wild form, on the other hand, is truly perennial, and perhaps potentially immortal. Harberd (1961) has deduced clonal ages of the order of centuries in *Festuca rubra* from the distance apart at which apparently identical phenotypes were recovered. In *T. repens* he found smaller total extent of clones, which he attributed to greater sensitivity to local variations in the environment, but he suggests (Harberd, 1963) that there is no reason to assume any shorter lifetime for clones in *T. repens* than in *F. rubra*.

Flowering heads are perhaps most abundant in May and June, but can also be found in plenty in July and August. This suggests that the species requires Long-Day conditions for inflorescence initiation and development, and this has been confirmed experimentally (Roberts and Struckmeyer, 1938; Hollowell, 1952; McCloud and Cole, 1951 - quoted by Gibson, 1957; Laude *et al*. 1958; Haggar, 1961). Flowering may however stop before the period of long days is over (Haggar, 1961), and in my own experience, all mature plants in the greenhouse have stopped flowering by August (a month or so earlier than in the field) while summer sown seedlings may flower for the first time in September. This suggests that following flowering there is a temporary loss of the ability to respond to what would otherwise be an adequate flowering stimulus. This fits in with the finding that a period of cold or short days may be required before the return of the readiness to respond - again for a limited length of time -

to long days (Thomas, 1961). One clone has been found in Arizona which had only a short day requirement (Beatty and Gardner, 1961). Cold conditions (for instance at night) may also facilitate - or even replace - the Long Day requirement (Roberts and Struckmeyer, 1938; Laude et al. 1958; Britten, 1961; Beatty and Gardner, 1961). There is variation in the response of individual clones (Britten, 1960; Haggar, 1961), and variation between populations which appears to represent adaptation to local conditions, longer day-lengths being required by forms adapted to high latitudes (Gibson, 1957). Whyte (1960) suggests that the extra vegetative vigour of non-flowering plants may make strains adapted to the photoperiodic regime of high latitudes attractive for cultivation at low latitudes.

There is no obvious mechanism for seed dispersal, though Erith (1925) considers the size of the seeds small enough to be such an adaptation. They are usually retained in the pod when the head falls, and if the head then becomes damp, a cluster of germinating seedlings can often be seen emerging from it. Suckling (1952) showed that some of the seed can pass unharmed through the intestinal tract of sheep (which certainly do not avoid eating the heads, and may favour them), and a consequence may be deposition of seed in a new site. Man-made open or new habitats such as embankments and roadside verges which are apparently suitable for the species often have only scattered, though thriving, single patches. This suggests that at least in the absence of free access for larger grazing animals there is a bottleneck at the stage of dispersal into new habitats.

The seeds which can survive passage through a sheep are apparently the impermeable, dormant 'hard' seeds which constitute over half of many wild collections. Nearly all the seed in some seed collections of wild origin, if threshed by hand, require scarification by abrasion or by treatment with concentrated sulphuric acid before they will germinate. Samples commercially available have been somewhat scarified by friction during mechanical threshing, and bred strains will probably have been selected to some extent (if only unconsciously) for full and early germination. They may therefore contain a lower proportion of hard seed.

Dispersal in space can occur by vegetative means, when there is a continuous sequence of favourable microenvironments available, and this may be as important as seed as a space-dispersal mechanism. The main function of seed in the life cycle may rather be the achievement of persistence in a locality, or 'dispersal in time'. Disturbance of soil, especially when combined with the provision of lime and other fertilisers, can be followed by the appearance of a strong stand of 'volunteer' white clover, even if growing plants of clover have been absent from the site for some years (Hollowell, 1952). In some grassland the clover population may have to migrate continually, any one patch being dominated alternately by grass and clover (cf. Lieth, 1960).

The growth habit of the species protects the growing points from damage by grazing, and the rapid production of new leaves also adapts the plant to tolerate frequent loss of leaves by grazing or mowing. Large and irregular fluctuations in sward height are a feature of vegetation subjected to predation or cropping of this kind, and the remarkable plasticity of the process of petiole elongation (Kerner, 1895) serves as an adaptation to bring each leaf into a favourable position for light interception whatever the conditions. This species is considered an important component of established herbage communities exploited agriculturally (meadows and pastures), and its seed is included in seed mixtures intended to produce similar vegetation. Some of the cultivated varieties, especially the larger growing ones, are lacking in persistence, but because of their vigorous early growth they are favoured for use in short-term leys and for undersowing as a catch-crop in stubble. They are also included for the sake of their contribution in early years in seed mixtures intended for intermediate or long-term leys. The cultivated strains which first interested farmers all belonged more or less to this relatively short-lived and luxuriant type. More recently has been appreciated the value of more persistent plants closer to the wild type in sowings designed to produce long leys or new permanent pasture. Strains of this type also have been produced by the plant breeders (W. Williams, 1945).

2. Habitat, Natural Distribution, and Introduction by Man.

The species is found in semi-natural communities exploited agriculturally - pastures and meadows - and on similar natural short grassland communities, such as cliff-tops, dunes, and warrens. In Britain, it is rarely abundant above 1000 ft., though it is sometimes found up to 3000 ft. This limit is probably due to the generally low pH, and low base and phosphate content of heavily leached highland soils, rather than to any intrinsic effect of altitude (Snaydon and Bradshaw, 1962; Holding and King, 1963).

The natural distribution of the species extends throughout Europe, parts of North Africa, and much of Western Asia, as far east as Lake Baikal (according to Komarov, 1945, quoted by Daday, 1958), and as far south as upland areas in Palestine, Iraq, Iran, West Pakistan and India.

Deliberate introduction as an agricultural plant, or other more accidental introductions by man have now led to its establishment in natural communities throughout the North and South Temperate Zones: Asiatic USSR, China, Japan, North America, Australia, South Africa, and most recently South America, where according to Daday (1958) it is still quite rapidly being distributed into new areas. It also grows wild now in some tropical areas, especially at high altitudes; Hawaii (Britten, 1961), Costa Rica, Guatemala and Venezuela (Daday, 1958), and Colombia (Daday, 1958; Crowder, 1960);

and is in cultivation in Nigeria and Kenya (in litt., Bogdan, 1961), though no naturalised populations are (yet) reported.

The map of the species' distribution published by Daday (1958) shows that he regards its presence in Iceland as due to introduction. The samples described below however include two described by the sender as of native Icelandic origin. Daday regards its presence in Morocco and Algeria as natural, as also in the Canaries, where he notes that it has been recorded in undisturbed native vegetation. A sample from the Azores is included below, from material 'considered wild', though the collector suggests it may be descended at least in part from early introduction both from North America and Europe. Pickering (1879), quoted by Ware (1925), says that the white clover now found in the Azores and in Madeira was carried there by European colonists.

According to Gamble (1935) the species has run wild from introductions in high altitude pastures in the Nilgiri Hills in South India. Two samples from this area are recorded below. The presence of the species in the Nilgiri Hills was referred to by Hooker (1879). He also described as probably introduced plants of _T_. _repens_ occurring at high altitudes in Ceylon. The samples recorded below also include one from Ethiopia which might represent a previously unrecorded extension of its natural range.

Carrier and Bort (1916) (cf. also Ahlgren and Sprague (1940)) quote several interesting but inaccessible references about the early spread of this species in Eastern North America. Among them, Smyth (1887) quotes Benjamin Franklin writing of the presence of this species in the area in 1749. Christopher Gist, travelling in 1750, found the species in abundance as far inland as southern Ohio and Kentucky. Strickland (1801) describes his observations of the rapid spread of the species during the 1790's. Kalm (1749) describes the abundance of the species in Canada, and Carrier and Bort suggest that it was distributed by the French missionaries to the Indians in many of the areas they penetrated, including Ohio, and perhaps south to the Mississippi valley.

Seed was taken to Japan in 1846 (Daday, 1958), to Western North America rather later, e.g. Washington State 1850-1860, New Zealand about 1880 (in litt., Daly 1961), and Alaska 1920 (Daday, 1958). Polunin (1959) cites Lange (1887) referring to its presence in West Greenland as an introduction established in waste and pastured areas. It was found in Uruguay from 1915 onwards (Cortobarría, in litt. 1961), and reached Brazil more recently (Daday, 1958), and is also present now on Kerguelen Island in the South Indian Ocean (in litt., Bost, 1961). Many of these reports (Western North America,(Daday, 1958), Colombia (Crowder, 1960), Kerguelen) indicate that, as might be expected, the species is first found after introduction in areas disturbed by man, for example near buildings and roads.

3. Genetic Variability and the Mechanism of Outbreeding.

The great variability of this species has been demonstrated in a number of reports (Ware, 1925; R.D. Williams, 1931b, Ahlgren and Sprague, 1940; Dessureaux, 1949; Wolter, 1958; Britten, 1960, 1961; Dunn et al., 1962; Coombe, 1962; Beinhart et al., 1963) and is more briefly referred to elsewhere (Haller, 1768 (quoted by Ware, 1925); De Vries, 1907; Stapledon, 1924; W. Williams, 1945), and is accepted as one of the notable features of the species. The selection of improved strains has exploited this variation, and may appear to have added to it, if plants from introductions of selected strains, or their descendants, are included in what are supposed to be wild samples. However, the diversity in the wild species is well demonstrated by further studies which are concerned with variation between populations, interpreted as ecotypic adaptation (Gibson, 1957; Snaydon and Bradshaw, 1962; King, 1963). Indications of considerable intra-population variation also occur in several of these works, and the work of Harberd (1963) suggests that this variability may provide for adaptation to environmental differences on the most detailed local scale, between adjacent plants. This appears to be a greater degree of local adaptation than in the other species he studied; in other species, each clone had spread out more or less equally in all directions, but in clover the distributions of each clone suggested that spread in some directions was much harder than in others (interpreted as respectively up and down, and at right-angles to, ecological gradients).

Qualitative variation, dividing the plants into discrete classes, is also present with regard to cyanogenesis (Mirande, 1912; R.D. Williams, 1939; Corkill, 1942; Atwood and Sullivan, 1943), whose distribution has been studied by Daday, (1954a, b, 1955, 1958, 1962, and 1965); with regard to white and red leaf marking, which will be discussed below; with regard to flower colour, though most of this variation is restricted to a few populations (Coombe, 1961; Borg, 1927; Turrill, 1948, and cf. Brewbaker, 1962); and in the presence of particular members of the *S*-allele series controlling the incompatibility system.

This incompatibility system is of the type also described in *Oenothera*, *Nicotiana*, and *Prunus*; the pollen expresses the one *S*-allele in its own haploid genotype and is inhibited on any style containing that allele. The prevalent variability within populations, and much of that which made possible differentiation in adaptation between different populations, can be attributed to the presence of this system. The high level of heterozygosity to which the species is adjusted is indicated by a reduction of vigour of 30 per cent after one generation of enforced selfing (Atwood, 1938, quoted by Carnahan, 1960).

Unfertilised florets remain barren (Darwin, 1891; Ware, 1925). The incompatibility mechanism reduces seed set on artificial selfing considerably in comparison with seed set in crosses made by the same technique with foreign pollen. This reduction is reported as from 732 seed in 743 florets in outcrosses to 6 seed in 500 selfed florets, a factor of 82 (Ware, 1925); as reduction by a factor of 20 or more (R.D. Williams, 1931b); or even by a factor of about 350 (Zwingli, 1956, using Swiss wild material). The achievement of even these relatively low yields of selfed seed requires the physical event of pollen transfer; in the wild any pollinating insect would be very likely to carry some foreign pollen, and after pollen-tube competition the number of seeds derived from selfing might be lower still. Under artificial selfing, Williams found that only 26% of his plants showed any seed set by 'pseudo-self-compatibility', though Zwingli found it in 137 out of 153. This variation between plants in the ability to set seed on selfing may be heritable (Atwood, 1942a). There is also a much smaller proportion of plants which are fully self-compatible as a result of the presence of a 'fertility' allele, $\underline{S}^f$, in the $\underline{S}$ series (Atwood, 1942b) and these only produce 19% outcrossed seed when exposed to bee pollination (Atwood, 1945). The frequency of these self-fertile plants is low; not more than 1 or 2% (R.D. Williams, 1931b), 1 in 615 (Atwood, 1941), or 5 in 153 (Zwingli, 1956).

Crosses between unrelated plants are nearly always compatible; 1 incompatible out of 155 crosses (R.D. Williams, 1931a), 5 and 4 respectively incompatible out of two sets of 136 crosses each among plants from one wild population (Zwingli, 1956). This suggests the presence of large numbers of S-alleles. Detailed studies by Atwood (1942c, 1944) prove that nearly all the alleles sampled are different, at least 39 different alleles being present in one sample. Zwingli's method does not sample the alleles so thoroughly, but proves there must be at least fourteen different alleles in both his sets of 17 wild plants. In Atwood's work, the plants sampled were all crossed with an S-homozygous plant produced by selfing. One plant was taken from each progeny, and all these chosen progeny plants were crossed together. They all had one allele in common, from the homozygous parent, and therefore any identity between two of the sampled alleles would be revealed by incompatibility in one of the crosses. Only one allele was represented in this testing scheme from each parent plant, removing the possibility of bias in subsequent calculations resulting from the presumed absence of homozygotes in the sample. It is possible to calculate from Atwood's results best estimates and probability limits for the number of different alleles in the whole population from which the sample was drawn. This has been done, by the method described by Bateman (1947), and the results are set out below. These results point even more definitely than the observations reported above to the conclusion that this species has a highly efficient outbreeding system, which can prevent selfing, and a proportion of crosses between relatives, at the cost of quite infrequent infertility between unrelated plants. The calculations suggest that an estimate of the order of 100 different *S*-alleles is not at all unreasonable.

Table II.1. Estimate of the Number of *S*-alleles in *T. repens*.

Source of data: Origin of Material.	Numbers of alleles		Calculated numbers of alleles in 'whole population'		
	Sampled	Proved to be different	Minimum	Best	Maximum
Atwood, 1942c					
Various origins	26	25	59	325	13000
Various origins	34	27	38	78	194
" stock alleles added	41	34	55	114	285
Atwood, 1944					
Isolated population, between 1000 and 10,000 plants	49	36	41	65	110
100 acres, not isolated	49	39	64	118	245

(Probability = 0·95 that true value lies within these limits)

4. Cytology and Genetics.

Most species of *Trifolium* have a chromosome number 2n=14 or 16. (Bleier, 1925; Karpechenko, 1925; Wexelsen, 1928). However, as first reported by Wexelsen (1928), *Trifolium repens* is an exception with 2n = 32 (this report also claims that the large non-persistent Ladino varieties have larger chromosomes than other varieties). Although this chromosome number strongly suggests that the species is tetraploid, meiosis is regular and diploid-like with 16 bivalents (Atwood and Hill, 1940). Genetic evidence confirms this with about a dozen cases of disomic inheritance. A different type of evidence for a double structure of the T. repens genome derives from the discoveries of a pair of duplicate loci with complementary dominant alleles controlling a mottling of the leaves which resembles virus symptoms (Atwood and Kreitlow, 1946), of another pair of loci, dominant alleles at either of which suppress cyanidin pigmentation of the corolla, and possibly of a third pair controlling a condition of rudimentary corolla (Brewbaker, 1962). Recently W.E. Davies (1965) has observed segregations for a sterile unifoliate form which can, in contrast to these previous findings, most easily be explained as being produced by tetrasomic inheritance. This suggests that for at least one of the sets of homologous and homoeologous chromosomes divergence between the two presumed ancestors was slight, and not great enough to prevent pairing between the genomes. Small chromosome size may account for the absence of tetravalents or other distinctive features in meiosis. The conclusion would seem to be that this species is a segmental allopolyploid, close to the ideal allotetraploid but showing the properties of an autotetraploid in a few respects.

This implies that it originated by hybridisation, associated with a doubling of chromosome number, between two diploids, probably well separated as different species, but retaining some similarity in the structural organisation of their hereditary material.

Although the inheritance and expression of some characters in this species has been intensively studied, only 11 loci are definitely known. They were listed by Brewbaker (1962) as follows: the *S* locus controlling incompatibility; the *Ac* and *Li* loci controlling cyanogenesis; *V* and *R* loci controlling white and red leaf markings;. the pairs of duplicate loci controlling virus-like mottling and (recessive) cyanidin corolla pigmentation (pink corolla in Scilly Is. forms is dominant (Turrill, 1948)); a 'blush' locus controlling a fainter corolla pigment; and a locus controlling a rare non-clasping attitude of the bracts (? stipules). No linkage has been demonstrated between any of these loci (Carnahan *et al.*, 1955; Brewbaker, 1962; Chakravarty, 1963b).

5. Hybridisation, Interspecific Relationships, and Phylogeny.

Hybridisation is generally difficult in this genus, and for *Trifolium repens* has only been reported with three other species: with *T. nigrescens* (Trimble and Hovin, 1960), the F_1 being fertile if the cross was carried out with colchicine-treated 4n parents (Brewbaker and Keim, 1953); with *T. uniflorum* (Pandey, 1957; Evans, 1962a), and according to Starzycki (1959,

1961) with *T. pratense*, a surprising cross to achieve between what are regarded as different subgenera, followed by genetically and cytologically remarkable segregations in later generations. Some degree of embryo development also occurred in crosses with *T. hybridum*, *T. alexandrinum*, *T. arvense*, and *T. subterraneum* (Evans, 1962). The last three species are generally classified by taxonomists in positions remote from *T. repens*; the hybridisation criterion of affinity shows few parallels with judgments based on morphology.

T. nigrescens is a diploid, 2n = 16. The fertile hybrid from colchicine-treated *T. repens* (8x) and *T. nigrescens* (4x) is therefore an allohexaploid, 6x = 48, whose constitution could be represented $R_1R_1R_2R_2NN$. Studies of the genetics of these hybrids (Brewbaker and Keim, 1953; Brewbaker, 1962) revealed tetrasomic inheritance for the loci controlling incompatibility (*S*-locus), white leaf-marking (*V*-locus), red leaf-flecking (*R*-locus), and possibly also for the determinant of the property found in *T. repens* of rooting at the nodes of the creeping stems, which appeared to be recessive in the hybrid, but reappeared in a small proportion of the F_2. The evidence suggests a greater degree of pairing, and presumably of homology, between the genome of *T. nigrescens* and one of the genomes of the allotetraploid *T. repens* than between the two *T. repens* genomes themselves. It also suggests that the loci showing tetrasomic inheritance in these families are derived from that one of the supposed diploid ancestors of T. repens which is relatively closely related to T. nigrescens.

Morphologically, the best candidate as a close relative and perhaps ancestor of T. repens is T. occidentale. It is diploid (2n = 16), self-fertile, and practically uniform (Coombe, 1961). These peculiarities require that it should be set apart from T. repens, but the close morphological resemblance between the two species, and the apparent relative scarcity of T. occidentale, probably explain why it had not attracted notice earlier.

T. occidentale has so far only been recorded in the Channel Isles, the western-most part of Cornwall, and the Scilly Isles (Coombe, 1961), and in north-west France, on the coasts of western Brittany and the Cotentin peninsula (Géhu, 1963). T. nigrescens is found around the Mediterranean (Taubert, 1894), but not in Britain. It is not difficult to imagine that the origin of T. repens could have been from a species like T. nigrescens and one very like T. occidentale, the incompatibility system (and the V-locus controlling white leaf marks) being inherited from the former, and the node-rooting habit and a number of other morphological features from the latter.

CHAPTER III

LEAF MARKING PHENOTYPES AND GENOTYPES: RANGE OF VARIATION, AND GENETICAL BASIS

1. Reports of the Presence and Occasional Absence of Leaf-Markings.

In the Flora of the British Isles (Clapham, Tutin and Warburg, 1962) we find italicised as a distinguishing character of T. repens 'Leaflets ... usually with a whitish angled band towards the base.' The observations recorded below confirm this description.

The qualification that the mark is only 'usually' present is supported by many published references, as well as by the results newly presented here; in nearly all samples there is a minority of completely unmarked plants. The possession of the mark is in fact used as a key character in the work quoted; most other floras give it much less prominence and many do not mention it at all. In fact, the possibility of confusion would be somewhat less than might appear, since unmarked plants would often have closely similar neighbours, recognisable as the same species, but marked; the results presented below show that even a majority of unmarked plants is very rare. The species with which T. repens could most easily be confused are those sharing its growth-form, with prostrate stems rooting at the nodes. Of these, the recently distinguished and local T. occidentale is always unmarked, and T. fragiferum never has more than a faint yellowish-green mark (see below), and is often unmarked, and has often in fact been described as uniformly unmarked.

A number of quite early works as well as some contemporary floras and monographs mention or illustrate markings of the kind we are concerned with. Many of these references make neither the shape, nor the colour, nor the position of the marks clear, and most do not refer to their variability. The result is a degree of confusion with red marks, which will also be described here. The marks we are principally concerned with are whitish, placed in the centre of the leaflet, and crescent- or inverted V-shaped. They are sometimes absent, but marked plants are generally in the majority. In contrast, the commonest type of red markings consist of scattered flecks of irregular shape, only slightly more common in the region of the midrib, and rarely present at all, and certainly not conspicuously so, in a majority of any population.

Sowerby's English Botany (1794) contains no mention of the markings in the text, though the presence in T. pratense and the variability of similar markings in T. glomeratum are mentioned. However, an illustration of T. repens shows an exaggeratedly clear mark.

Gray (1821) first mentions the red marks and the variable presence (in this species) of the white marks, saying 'Leaves often spotted with brown and white', but makes no reference to the differences in shape, position, and frequency between the two kinds of marks.

Sir J.E. Smith (1829) is more explicit about the white marks: 'Leaflets ... mostly with pale, curved, transverse stripe'. This lacks only an indication of the position of the mark, and, in common with all except recent specialised genetical references, any mention of the variability between different types of marked phenotype. He also indicates that the frequency of the white stripe is greater than that of the red flecks: 'mostly' as against 'sometimes'. Two more early references worth mentioning are those of Lowe (1868) 'the leaflets are mostly pale- or dark-spotted', and a rather puzzling remark by Kirk (1899) 'leaflet ... often with a dark spot'.

Reichenbach (1903) illustrates both marked and unmarked plants, but without referring to marks in the text. De Vries (1907) refers to the abundance in clovers of 'elementary species' which can be discerned by criteria including the presence or absence of whitish spots on the leaflets.

2. Terms used to refer to the White V-shaped Markings on Leaflets.

The fullest description of these markings would be 'whitish inverted-V leaflet markings controlled by the V-locus'. Very many different phrases have been used, different authors stressing different aspects in the name they choose. There is no clear standard name, though the terms V-locus and V-alleles seem to be accepted, as arbitrary labels rather than as descriptive names. White V-markings (Stanford et al., 1960) or leaflet V-markings would seem the best short descriptive phrases, or simply V-markings or V-marks (Carnahan et al., 1955; Stanford et al., 1960), although some important publications have used the typographically strange-looking 'V-leaf markings' (Brewbaker, 1955; Brewbaker and Carnahan, 1956; Zwingli, 1956; W.E. Davies, 1963). Elsewhere have appeared simply 'leaf markings' (Dolan, 1962), which is confusing, since not intended to cover red flecking; 'patterned leaf markings' (Brown, 1947), 'light coloured V marking' (Brigham and Wilsie, 1955), 'light leaf markings' (Coombe, 1961) and 'genetical leaf markings' (Harberd, 1963). Another unusual description is the 'water-mark' (Hollowell, 1952). The description of the shape as a 'V' seems firmly established in genetical literature, though descriptions like crescent-shaped or horseshoe-shaped (Bentham and Hooker, 1924) appear especially in earlier taxonomic works. If the standard position of a leaflet is thought of as stalk downward, the V is strictly speaking inverted, and Hossain (1961) actually prints an inverted V, referring to 'Λ -shaped spots'. Especially perhaps in the smaller growing wild forms, the mark is in fact often curved or even reentrant at the tip, and these would be better described as inverted U- or W-marks. Other marks are restricted to a small triangle, the 'V-point', or to a pair of irregular spots either side of the midrib, and would not at once be associated with a description of V-marks.

I shall refer simply to leaf markings, or to white leaf-markings, when it is necessary to distinguish them from 'red' markings (also described as brown, black, or purple), which differ in shape, position and physical basis. A more correct epithet, as used in the Flora and quoted above, would be 'whitish', since the colour is rarely if ever white, except by contrast with the darker green background. Compared to a norm of a pale green colour in the mark, particular marks can be described, relatively, as chalky-white, yellowish, silvery-metallic, or bluish.

3. White Leaf Markings: Structural Basis.

Kajanus (1912) concluded that the similar V-mark in _T. pratense_ results from a reduced growth rate of the palisade cells relative to the epidermis, and that because the palisade tissue is looser than normal, the cells are exposed to stronger light and their chlorophyll is bleached; this would explain his finding that there is either less or paler chlorophyll in the tissue in the marked area. Hector (1936) suggested that there is a reduction in the number of chloroplasts in marked parts of leaflets in _Trifolium_.

Carnahan _et_ _al_. (1955) studied the structure of the marked tissue and show a picture of a transverse section in the region of the mark. They found 'that the palisade cells in the leaf V-mark area are less elongated, smaller, more irregularly shaped and that there are more intercellular spaces between them than in the mesophyll of no-mark leaves'. Other leaf tissues appear to be normal.

Two main factors producing the paleness of the mark seem to be clear, both resulting presumably from the occurrence of abnormally few cell divisions in the developing tissue. These are an increase in light reflection from an increased number of intercellular spaces filled with air, and an overall reduction in chlorophyll content caused by the reduction in the number of cells. It is also possible that the cells of the modified palisade, which are certainly less distinctive in shape than normal ones, may be defective in other aspects of palisade cell differentiation, such as the number and content of the chloroplasts.

The leaflets develop folded, with the adaxial faces which bear the mark pressed together, and remain folded until they reach almost their full size. I have opened by hand folded leaflets that were almost ready to open themselves, and find that the mark is clearly visible, though the background colour of the leaf is not quite so dark, and provides slightly less contrast. The fact that the mark can develop when there is always at least one leaflet's thickness of spongy mesophyll between it and any light implies that the bleaching suggested by Kajanus is of little or no importance, at least in this species; if the spongy mesophyll remains green, it is difficult to see how a layer of cells beyond it could be bleached.

4. Discovery of the Mechanism of Genetic Control of White Leaf Markings.

The first report on the inheritance of white leaf-markings in *T. repens* is attributed by Brewbaker (1955) to Atwood (1937-1944: cf. Atwood and Sullivan, 1943). Before this time the inheritance of leaf markings in *T. pratense* had been studied by several workers (Kajanus, 1912; Gmelin, 1914; 1916; R.D. Williams, 1931b, 1937; 1939; Wexelsen, 1932) and later by Smith (1950). Atwood's results agree with the finding in the other species that the absence of white mark is recessive to its presence. (For *T. pratense* details, cf pp 227-230.)

In *T. repens*, crosses between unmarked plants never give marked progeny; crosses between unmarked and marked plants give progeny of which either half or none are unmarked; and crosses among the marked progeny of unmarked plants produce a segregation of three marked to one unmarked (Brewbaker, 1955, Table I; Carnahan *et al*., 1955, Tables II, V). These results provide a firm foundation for the conclusion that absence of marking can be attributed to the homozygous presence of a recessive allele for no-mark at one particular locus.

The recessivity of no-mark, indicated by segregations of 3 marked to 1 unmarked plants, has been confirmed by data published on crosses carried out for other purposes by Brigham and Wilsie (1955), Zwingli (1956) and Chakravarty (1963b).

According to Brewbaker (1955), Atwood (1937-1944) 'reported that several leaf marking types were useful as genetic markers in white clover investigations'. This seems to be the first mention of the existence of more than one kind of mark. Brown (1947, also as quoted by Brewbaker, 1955), concluded that these V-markings were controlled by members of a multiple allelic series, of which he listed eight members. Brewbaker and Anderson (1952) mention seven alleles, and make the point that heterozygotes between different positive mark alleles express both marks. Detailed accounts followed, with illustrations, of this work (Brewbaker, 1955), and of similar work carried out by Carnahan *et al*. (1955). Their conclusions and terminology were combined in a joint paper (Brewbaker and Carnahan, 1956) in which eleven different alleles were named, all at the one V-locus, including a new one discovered by Pandey (unpublished). Both alleles were expressed in most, though not all, of the heterozygotes between these alleles other than the recessive for no mark.

The mark-types listed by Brewbaker and Carnahan (1956) are listed in Table 3.1. below. The phenotypic code is an extension of that used by Brewbaker (1955), extended according to manuscript notes in the margin of the copy he sent me of the joint paper. The alleles and descriptions are as in the joint paper, though the order has been changed. I have added my own phenotypic code, which I think is simpler, showing generally the same letter as the superscript in the allele name; and also some comments on the application of the descriptions.

Table 3.1. Description of leaf-marking types, based on Brewbaker and Carnahan, 1956.

Phenotypic Code (JLB)	Allele	Description	Comment (JGP)	Phenotypic Code (JGP)
O	v	No mark	Recessive	O
A^H	V^h	Full V - high	Possibly parts of a continuous range; certainly there are more than four alleles. For most purposes I have grouped them together under V^l and L, as 'simple marks', in contrast to those below.	H
A^i	V^i	Full V - intermediate		
A	V^l	Full V - low		L
C	V^p	V-point		(P)
E	V^{ba}	Basal V	Clearly distinct from the 'L-series' of 'simple V-marks'. Touches margin very low, but very acute, and can be confused with some high V's.	Ba
D	V^f	Filled V	Often only an ordinary V-mark shape is really white - within it, only a paler green than the upper half of the leaflet; 'Lf'.	F Lf
G	V^{fg}	Filled V, green base (Pandey)	Unless this is the mark described as Lf, I have not seen this.	
B	V^b	Broken V	Wide, abrupt, central 'break' - mark bluish-silvery. Brewbaker illustrates a heterozygote between these two, but I have no evidence for considering more than one broken-V allele.	B
B^d	V^{bd}	Broken V dominant		
F	V^{by}	Broken V yellow tip	I would include here all cases of ± broken marks with faint or undetectable yellow tip if the mark is chalky white and tapers towards the break.	Y, By B(y) B( )

5. Mechanism of Expression and Interaction of Leaf-marking Alleles.

Dominance is described in three situations. (1) All other alleles are dominant to the recessive $\underline{v}$ for no mark. According to Brewbaker (1955), heterozygotes with $\underline{v}$ could not be distinguished from homozygotes for each allele and they can therefore be considered fully dominant. Wexelsen (1932) records a similar observation in *T. pratense*: However, Zwingli (1956) suggests only partial dominance by $\underline{V}^{bs}$ over $\underline{v}$ (2) When a larger mark overlaps a smaller, it is only possible to distinguish one mark; thus, $\underline{V}^f\underline{V}^p$ and $\underline{V}^f\underline{V}^{ba}$ may simply appear as F, and for some types of $\underline{V}^l$, $\underline{V}^f\underline{V}^l$ may appear only as F, and $\underline{V}^l\underline{V}^p$ as L. This may depend on the size of the leaves; small-leaved field-grown $\underline{V}^l\underline{V}^p$ and $\underline{V}^f\underline{V}^l$ plants appeared with L and F phenotypes respectively, while the larger-growing greenhouse cuttings from these plants showed clear separation so that a double mark phenotype could be recognised. (3) In the combination $\underline{V}^{bd}\underline{V}^p$ only a broken mark phenotype appears, and the area where the $\underline{V}$-point could have appeared remains green.

Generally, however, it seems as though each mark expresses itself independently; in a heterozygote, all areas which can be marked by the action of either allele are so marked; a single dose being enough (full dominance, as mentioned above, over $\underline{v}$) to produce full intensity of marking. Brewbaker (1955) describes this situation as mosaic dominance, which seems to imply that each allele has both a property of making some area whitish, and of keeping other areas green; but that in a heterozygote, the allele which would act to make any area whitish always is expressed in that area, showing dominance, although in some other part of the leaf the positions may be reversed, and the allele would be recessive in areas which it would tend to keep green.

Brewbaker (1955) also introduces a suggestion which could explain the similar V-shape of the different marks. He publishes a diagram showing the positions of the different marks superimposed as a 'concentric' set of V's; and suggests that these 'represent time-zones in leaflet ontogeny at which V alleles operate to prevent the normal elaboration of chlorophyll in the tissues'.

6. Red Leaf-Markings in *T. repens*.

The red leaf-markings in *T. repens* fall into three classes. The first is a group of marks occurring only in combination with the $\underline{V}^{by}$ allele at the locus controlling the white marks (W.E. Davies, 1963). The second is a group under genetic control at a distinct *R*-locus, the commonest being sporadic red flecking, mentioned above and referred to in many descriptions of the species (Carnahan *et al*., 1955; W.E. Davies, 1963). Both show considerable dependence on favourable environmental conditions for clear expression. This is even more true of the remainder of the marks, a heterogeneous group whose genetic basis, and some may have none, has not been investigated.

The red markings associated with $\underline{V}^{by}$ may only depend on the presence of $\underline{V}^{by}$ to permit their expression, but for one at least it has been shown that the red mark is controlled by a genetic factor completely linked to the $\underline{V}^{by}$ allele (Hovin and Gibson, 1961). This being so, it seems possible that the other marks may be under similar control - linked to $\underline{V}^{by}$, as well as limited to $\underline{V}^{by}$-containing plants.

The most familiar of these in the wild is a red marking affecting the apical sector of the leaf, largely confined to the terminal leaflet, with a sharp boundary running parallel to veins on the base of the terminal leaflet or the distal side of a lateral leaflet, and often a diffuse distal boundary so that the colour does not extend to the leaflet tip. The expression of this mark depends on favourable environmental conditions which seem to be bright light and relatively low temperature. In the greenhouse it rarely appears at all. It would seem that there is some variability in its expression from plant to plant, and it would not be possible at present to say that any $\underline{V}^{by}$ plants completely lack the ability to express this phenotype.

There are three other rare marks in this group, also only appearing in $\underline{V}^{by}$ derivatives.

The second is a dark purple mark, usually a thin line following precisely all the irregularities of the inside edge of the white V, or of both white V's in a double-marked plant. Under favourable conditions, other spots may appear away from the white V's on the leaflet base. The colour is distinct from that of all the other 'red' markings discussed here; when faintly expressed it appears to be no more than a dark green line along the white V.

The third mark is a red V-shaped mark, fairly acute and straight sided. It does not occupy exactly the area of any of the white marks I have seen combined with it. On the different leaves of a plant, and the different progeny of a cross, it may vary in expression from a thin red line, to a heavy V with a lighter red dusting over all the leaf; but unlike the other red marks in this class, it is only on exceptionally small leaves that it disappears altogether under any conditions. It seems that this mark has only been found in one area in the wild. All the specimens seen in Britain are derived from a plant sent to the Welsh Plant Breeding Station by Dr. P.B. Gibson, and presumably that was related to the plants found in a garden and in a pasture near Spartanburg, South Carolina, by Mr. Paul Tabor and Dr. Gibson respectively (Hovin and Gibson, 1961; personal communication, Gibson, 1964). It seems remarkable that this mark should have been found only in an area in which the species is not native. Genetical studies have shown that this mark is completely linked to the $\underline{V}^{by}$ allele.

The fourth red mark associated with $\underline{V}^{by}$ is a red speckling confined to the tip of the white and yellow V, halfway up the midrib. Like the first two types, it requires favourable conditions for its expression.

A second class of red markings are those controlled by the *R*-locus (Carnahan *et al*., 1955). The first reference to the genetics of these marks is probably that of Frith (1928) who reported dominance of the red leaf character of a var. *purpureum*.

The system Carnahan describes is based on four alleles, each dominant to those lower in the series. The basal recessive, *r*, produces no pigmentation. The next allele, $\underline{R}^{f}$, produces scattered flecks whose intensity and perhaps frequency is influenced by the environment. The other alleles are $\underline{R}^{m}$, which produces an irregular stripe of red along the midrib, and $\underline{R}^{l}$, which produces red pigment over the whole upper surface of the leaf except very close to the margin. Carnahan reports that the pigment is confined to the epidermal cells, and that pigmented areas are much commoner in the upper epidermis.

Brewbaker (1962), listing known genetic factors in the species, refers to the *R* locus controlling red leaf, and to an *Fl* locus controlling red leaf flecks, and says that all the factors he lists are independent in inheritance. If the *Fl* locus in his own work is independent, and if Carnahan's report of $\underline{R}^{l}$-$\underline{R}^{f}$ allelism is correct, there must be two separate loci with alleles which can produce red flecking. Other reports of the dominance of red flecking (Stanford *et al*., 1960, Bula *et al*., 1964) might refer to either of these, but describe them as $\underline{R}^{f}$.

These marks seem to represent a system completely unrelated to the V-mark system, and without interaction with it, except that Carnahan reports that the red colour in a $\underline{R}^{m}$ phenotype was appreciably less intense on the basal part of the leaf, after it crossed the white V, in a $\underline{V}^{by}$ plant.

This system of marks is described and illustrated by W.E. Davies (1963) who uses Carnahan's terminology of one $\underline{R}$-locus. He describes a new allele $\underline{R}^{fa}$ with a higher frequency of red flecks than other $\underline{R}^{f}$ alleles.

The third class of markings, without established genetic basis, include types of bronzing, perhaps a pathogenic symptom; and also some cases where red pigment develops in the white mark. The existence of types (referred to as 'mutants' presumably because of their scarcity) with red pigment in the white mark is briefly mentioned by W. Williams (1964). New observations on these markings will be described below, but one earlier reference which may concern them is that of Allan (1940) who describes 'white _to_ purple blotches or flecks' (my underline), and may be referring to something more complex than the white V-marks and $\underline{R}$-locus red flecks simply superimposed.

PART 2

PRELIMINARY AND GENERAL STUDIES

CHAPTER IV

VARIATION AND INHERITANCE OF LEAF MARKINGS

1. Artificial Crossing.

Stock plants showing the range of leaf-mark phenotypes, and new plants with interesting phenotypes which had been collected in the field, were kept in the greenhouse and at an early stage in the work a number of crosses were carried out. Later, the field and seed-sample work prevented any further development of the crossing programme beyond the first generation. The results of the crosses from which reasonably large progenies were obtained are shown below.

Crosses were carried out without emasculation, and without testing for self-fertility, and selfing is therefore a possible explanation of some unexpected progeny. Nevertheless, all that is known about the species indicates that a large majority of progeny in all crosses can be attributed to crossing. The recipient heads were thinned down to less than twenty florets, and the keel petals removed by grasping them with forceps just below their tip. This would often remove the anthers as well, leaving the style completely exposed. Pollen was picked up on a folded V of stiff card, whose point was scooped into the keel of a number of florets of the pollen parents. Seed was collected when the peduncle began to turn brown, between four and six weeks after the cross was made.

The plants used were of various origins, distinguished by the first letter of the label. A and B were plants received at the Oxford Botany Department at various times from the Welsh Plant Breeding Station as leaf marking phenotype stocks, to demonstrate the allele combinations as teaching material. The B group had been received before 1960, and had been kept primarily as stock plants for the different phenotypes for cyanogenesis. The A group were collected from the W.P.B.S. in autumn 1960 as leaf-marking specimen plants. The W plants were wild growing plants collected on the old pastures beside the river Thames at the Oxford University Farm, Wytham.

Crosses

(1) Crosses between simply marked and unmarked plants

Cross	Female	Male	Progeny			Comment and Conclusion
a 59	S12	W30	0	L		S12 is V^1v. Possibly some
	L	0	16	7		V^1v progeny scored as 0.
b 96	W18	W1-2	0	L	B	W1-2 is V^1v. B plant by
	0	L	22	37	1	contamination?
c 98	W23	W18	0	L		W23 is V^1v.
	L	0	8	11		

		0		L	
Total for L x 0 ;	Observed	46	:	55	
1:1	Expected	50.5		50.5	$P > 0.1$

(2) Crosses between plants with simple mark phenotypes.

Cross	Female	Male	Progeny				Comment and Conclusion
d 66	S12	D2	0	L			D2 also V^1v.
	L	L	9	21			
	(V^1v, cf. (a))						
e 78	B6	W7	0	L	LL		LL by contamination, or by slight difference between parent V^1 alleles. W7 also V^1v heterozygote.
	L	L	4	29	1		
f 91	B6	W21	0	L			W21 also heterozygous.
	L	L	2	5			
	(V^1v, cf. (e))						
g 115	W98	B6		L			W98 does not contain v.
	L	L		19			
		(V^1v, cf. e, f)					
h 48	W14	B6		L			W14 does not contain v.
	L	L		25			
		(V^1v, cf. e, f)					
i 116	B6	W97	0	L	H	LH	W97 contains v; its mark allele is different enough to produce a clear double mark, but not for certain recognition as a single.
	L	L	5	29	3	13	
	(V^1v, cf. e,f)(highish)						

Here compare also:

Cross	Female	Male	Progeny				Comment and Conclusion
j 89	B13	W14		L	LH		Confirms W14 no recessive, and suggests its V^1 alleles are the same as the V^1 in B13.
	LH	L		23	24		
		(V^1V^1, cf. h)					

Total for crosses (between marked plants) with unmarked progeny:

	L	0	
Observed	101	20	
Expected (3:1)	90.75	30.25	$0.05 > P > 0.02$

(3) Crosses between other phenotypes and unmarked plants.

Cross	Female	Male	Progeny			Comment and Conclusion
k 53	W24	W30	L			Clear double mark in parent, but marks not classifiable in F_1.
	LH	0	14			
l 131	U13.4i	W30	H	L	0	Poor discrimination between $V^{l}v$ and $V^{h}v$. One mark completely missed.
	LH	0	2	15	1	
m 104	W30	G8.8	L	0		Again, no discrimination between L and H in progeny. Several progeny not identified as marked. Selfing or poor expression.
	0	LH	10	5		
n 52	W30	W29	F	L		Good confirmation of W29 as $V^{l}V^{f}$.
	0	LF	12	7		
o 82	W29	W30	F	L	0	0 presumably by poor expression of mark in $V^{l}v$.
	LF	0	27	11	22	
(reciprocal of n)						
p 113	W30	W86	F	L	0	Good confirmation of W86 as $V^{l}V^{f}$. 0 as above, or by selfing or contamination.
	0	LF	10	19	1	
q 57	A14	W30	F	H		Good confirmation of A14 as $V^{h}V^{f}$.
	HF	0	31	26		
r 85	B14	W30	L	B	0	Fits B14 as $V^{l}V^{b}$ if unmarked progeny are $V^{b}v$ with poor expression.
	LB	0	24	11	3	

General conclusions. Unmarked plants were scored in three crosses where they could not be attributed to selfing (l, o, r), and this probably reflects the fact that the seedlings were scored rather young, and probably not under optimal conditions. The first three crosses (k, l, m; cf. i above) make the point that it is much easier to identify two alleles as different when they are in the same plant and can produce a double mark, than when they are in leaves of different sizes and shapes in different plants, when no confident diagnosis is often possible. This suggests that there may be much more diversity among '$\underline{V}^l$' alleles than can be proved by comparison of different plants with single marks.

(4) Crosses of other phenotypes with simple-marked plants known to be heterozygous for $\underline{v}$.

Cross	Female	Male	Progeny					
s 51	A12	B6	H	LH	B	LB	0	Some V^lV^h probably had very faint L-mark, or had it fused with or overlapped by H-mark. Unmarked plants probably V^bv (cf. r).
	BH	L	40	6	24	25	2	
		(V^lv, cf. e,f)						
t 61	B1	W7	F	L	LH	0		L-mark probably overlapped by F - V^lV^f scored as F. Some V^lV^h probably scored L, some V^hv as L or 0.
	FH	L	15	14	2	1		
		(V^lv, cf. e)						
u 67	D2	A12	L	BL	0			Poor expression of V^b in V^bv and V^bV^l; V^hV^l scored as L ?
	L	HB	17	5	3			
	(V^lv, cf. d)							

(5) Crosses between other phenotypes.

Cross	Female	Male	Progeny	Comment and Conclusion
v 4	A23 LL?	A13 LH	LH LL H 6 3 7	Two kinds of double-mark progeny; three different alleles involved.
w 9	A13 LH (reciprocally)	A7 HY	H HL HY LY 14 22 25 19	Good fit to B V x V^hV^y. Many of Y progeny had no clear yellow tip.
x 16	A6 Ba	A23 LH	H L LBa HBa 27 25 19 16	Good fit to $V^{ba}V$ x V^hV^l.
y 50	B14 BL	A12 BH	B BL BH LH 6 9 7 8	Good fit to V^bV^l x V^bV^h.
z 36	A12 HB	B13 HL	H LH BH BL 20 8 5 13	Possibly some confusion between V^l and V^h marks.
aa 19	A15 HBa	A23 HL	H HL HBa Ba L 12 4 14 1 1	HBa noted as variable while scoring; includes V^lV^{ba}. Ba by selfing? L contaminant.
bb 30	A15 HBa	A27 YL	HY YBa LBa Ba 7 2 3 1	Ba by selfing?
cc 125	W25 LF	W70 LL?	L LH LF HF F 8 8 3 7 2	F are probably V^lV^f.
dd 10	A5 BF	A17 F	F FB 0 35 37 1	Conclude A17 V^fV^f. 0 probably seed contaminant in soil.
ee 15	A6 Ba ($V^{ba}v$, cf. x)	A21 BaF (according to label)	Ba F 0 11 11 3	F progeny presumably include V^fV^{ba}. 0 by selfing?
ff 56	W29 FL (cf. n, o)	A10 YF (according to label - appears LF)	F Y FL YL 29 23 1 5	Some V^fV^l scored F? V^yV^f presumably scored Y or YL.
gg 99A	W28 LF	W29 LF	F LF L 9 5 1	Some V^fV^l scored as F?
hh 7	A15 HBa	A7 HY	H HBa HY HB Ba 2 3 3 2 1	Some V^hV^{by} failed to show yellow tip, scored as HB.

Further conclusions.

(1) Progeny of yellow-tip plants, carrying the $\underline{V}^{by}$ allele, with recognisable chalky white mark tapering towards the midrib, do not always show any yellow tip (crosses w, hh).

(2) $\underline{V}^{f}$ behaves as dominant to $\underline{V}^{ba}$ and sometimes to $\underline{V}^{l}$ by overlap (t, cc, ee, ff, gg).

(3) The presence of unmarked scores where they cannot have arisen by selfing suggests that some L, Ba and B plants can be scored as 0 perhaps especially when young (s, o, r, u, ee), similarly $\underline{V}^{l}\underline{V}^{b}$ as L (cross u).

(4) $\underline{V}^{by}$ seems to show suppressive dominance, and reduce the expression of $\underline{V}^{f}$ to L, and that of $\underline{V}^{l}$ to nothing. Further evidence to support this will be reported below.

Otherwise the results can be explained on the published one-locus basis if a certain amount of selfing, possibly pollen contamination, and contamination by seed known to appear in the compost is allowed for.

(6) Exceptional results: A18 (BBa) transmitting only one allele through pollen.

In cross 11, A18 BBa and A12 BH were crossed reciprocally with each other. The parents are assumed to be $\underline{V}^{b}\underline{V}^{ba}$ and $\underline{V}^{b}\underline{V}^{h}$; the expectation and the results of the two crosses are shown.

	V^hV^{ba}	V^bV^{ba}	V^hV^b	V^bV^b	(V^hV?	V^bV^{ba}?)
	HBa	BBa	HB	B	H	Ba
Expectation	1	1	1	1		
A18 female x A12 male	8	12	8	8	2	1
A12 female x A18 male	10	7	-	-		

This suggests very strongly that when used as male parent, A18 provided only one class of pollen, $\underline{V}^{ba}$.

A18 was also used as male in cross 3, with less clear results.

A6 Ba ($V^{ba}v$, cf. crosses x and ee above) x A18 BBa.

Genotypes	$V^{ba}V^{ba}$, $V^{ba}v$	V^bV^{ba}	V^bv	(V^bv, $V^{ba}v$ or vv)
Phenotypes	Ba	BBa	B	0
Expected	2 :	1 :	1	
Observed	16	1	1	3

These results, taken alone, could also be explained as the result of poor expression of $\underline{V}^b$ in $\underline{V}^b\underline{V}^{ba}$ and $\underline{V}^bv$. In view of the result in cross 11, it appears that they could represent a less extreme case of the same phenomenon; only two plants out of 21 definitely received a $\underline{V}^b$ allele through the pollen, though others, 0 or Ba, may have done so without it being scored. Unfortunately no further crosses were made with this plant, now dead, or its progeny.

2. Expression of Leaf Markings: The Influence of Leaf Size, Age and Environment.

Explanation of the results of the above crosses make it necessary to suppose that some marks were not observed, or identified wrongly, when they were expected to be present. The broken mark for example was often faint where it was seen, and it would not be surprising if it were overlooked in other similar, or even fainter, cases.

Mark development seems to bear an allometric relationship to leaf size. The smaller the leaf absolutely, the smaller relatively and the less conspicuous is the leaf marking. This can be seen most easily on the small leaves which even the largest growing clones produce at early stages of side shoot development, especially under conditions favourable to frequent branching, and also on the small leaf-like structures produced in the inflorescence by plants infected with phyllody virus. There would be a more serious difficulty in mark description when all the leaves were small, or had developed under conditions unfavourable to clear mark expression; bright light and moderately low temperatures seem to enhance the clarity of the marks. Under other conditions the mark on normally marked plants may be reduced to a small roundish spot on each half-leaflet, or only sporadically occurring on an occasional half-leaflet. Under favourable conditions, such extremely reduced marks are less common, and the faintest marks recognised are well extended but very narrow and faint streaks across the leaflet.

The consequences of poor expression of the marks would be the apparent absence of some marks, the lack of detail of others, so that distinctive marks might be classified only as simple marks, and lack of doubleness of double marks - so that, for instance, a $\underline{V}^{l}\underline{V}^{by}$ plant in which the yellow tip was still visible would be scored as By only, $\underline{V}^{h}\underline{V}^{l}$ as L, or $\underline{V}^{l}\underline{V}^{f}$ as F, which amounts to effective dominance.

These considerations qualify the value of scorings under unfavourable conditions - in the field in winter or early spring, or on very old plants grown for a long time in the same soil in pots, or to some extent at all times, in plants with genetically controlled small leaf size. This might lead to an underestimation of the variability in small-leaved populations.

Small leaves are also produced by the seedling, and the development of marks in the seedling may be different for other reasons. The basal mark appears to develop later than the others - not until after the sixth leaf or so - and this may explain its apparent absence in some progenies of the crosses above.

In red clover, which has an elongated flowering stem bearing leaves, P.E. Smith (1950) records that while some plants exhibited clear marks on the first unifoliate leaf, others showed no marks until flowering, when they appeared only on the bract leaves on the flowering stem. In T. repens there are no leaves specially related to the inflorescence, and I have seen no indication that there is any difference in marking between vigorous but entirely vegetative stolons and those bearing flowers, though weakened stolons which would probably bear no flowers might have leaves of reduced size with less clear marks.

Smith described other plants with marks present only at the seedling stage. This may correspond to a phenomenon seen in some of my plants. When the first leaves of near mature size were scored (fifth or sixth leaves) they had marks, and perhaps unusually clear and solidly white ones, making a sharp contrast with the green parts of the leaf. Later scorings showed them as unmarked or very faintly marked. If the observation that these were all unusually clear marks is accurate, it supports the suggestion that the same phenomenon was occurring in all these cases. ~~Five~~ Seven specimens were seen; three from large seed samples from near Copenhagen (N43, N44, N45), two from seed collected on different plots on the 'Park Grass' at Rothamsted (N132, N133), and two in Gumpenstein, Steiermark, Austria (N104).

3. New Phenotypes not previously described.

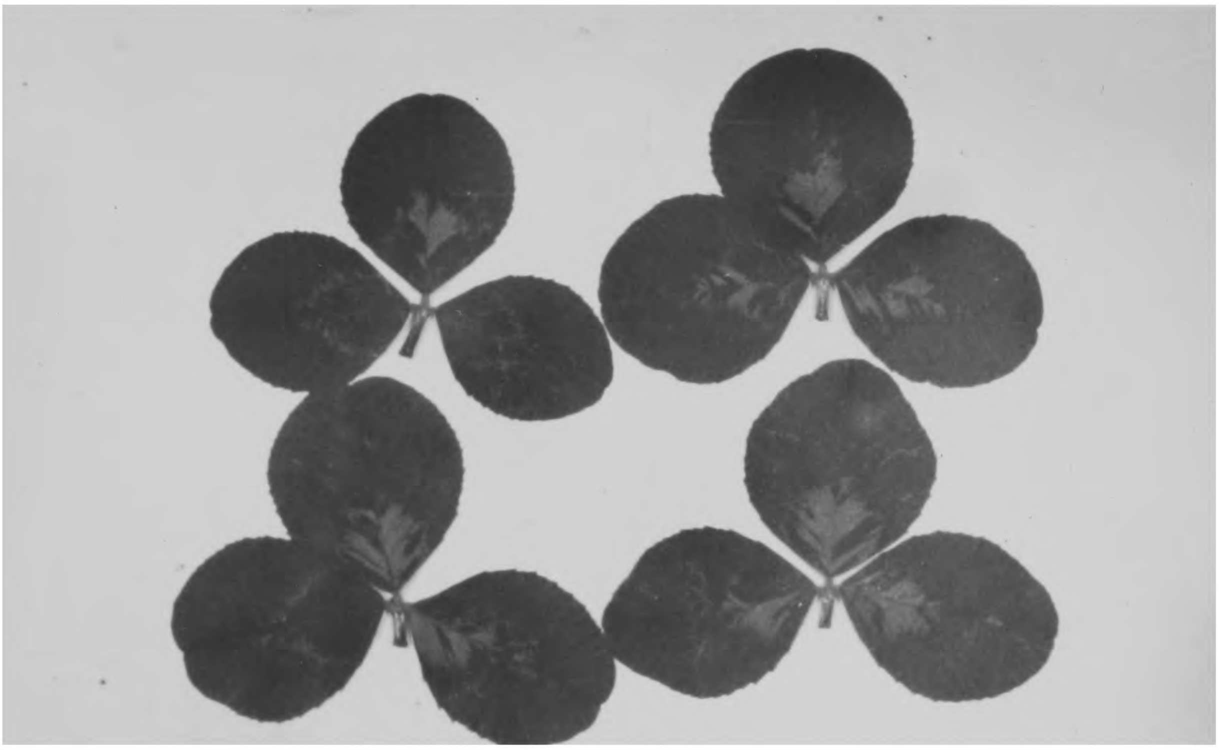
The smeared mark: a new allele with exaggerated expression in the seedling.

In one cross (listed as s above) a $\underline{V}^h\underline{V}^b$ plant (A12) was fertilised by a $\underline{V}^l\underline{v}$ plant (B6, whose heterozygosity was shown in crosses e, f, and i, with other single-marked plants, where unmarked progeny appeared). When first scored, on the appearance of the unifoliate and first trifoliate leaf, it was discovered that a number of seedlings with filled-in marks had been attributed to this cross. Later scoring of about the tenth leaf showed that these seedlings did after all fit the expected phenotypes; no filled marks were present, though then and later a number of the plants which had been filled-in showed some degree of 'smearing' of the mark, with stripes running down from the mark between outlines parallel to the lateral veins, filled with white. A photograph of leaves from an unusually well developed example is shown in Fig. IV(i). Assuming the apparent filled-in phenotype and the smearing were expressions of the same property, the occurrence in the progeny can be shown as follows:

Allele received from plant A12 V^hV^b	V^b		V^h	
Genotype of Progeny	V^bv	V^bV^l	V^hv	V^hV^l
Phenotype	B (or O)	BL	H	(H or) HL
Total	26	25	40	6
Smeared	-	-	32	3
Not smeared	26	25	8	3

Smearing appeared only in those progeny which had received the $\underline{V}^h$ allele from the mother, and smearing must be produced either by that allele, or by other factors which can interact with that allele only. Some degree of smearing occurs in many simple mark phenotypes, and this probably accounts for the scoring of some smeared plants among the $\underline{V}^b$ progeny in two other crosses involving this same plant. In one of them, (with a plant presumed to be $\underline{V}^b\underline{V}^l$), confusion between diagnosis of BL and BH phenotypes could account for what appeared to be $\underline{V}^b\underline{V}^l$ progeny with smearing. Nevertheless both show a significant excess of

Figure IV (i)



(51)53

High simple V-mark with heavy smearing

smearing in the plants receiving the $\underline{V}^h$ allele from A12.

Cross 50		Progeny classes						
		V^b		V^h				
Female	Male	V^bV^b	V^bV^l	V^bV^h	V^lV^h			
B14	A12	B	BL	BH	LH			
V^bV^l	V^bV^h							
		6	9	7	8		V^b	V^h
smeared		-	2	3	7	sm	2	10
not smeared		6	7	4	1	--	13	5

$X^2_{(1)} = 8.90$

$0.01 > P > 0.001$

Cross 36		V^b		V^h				
		V^bV^h	V^bV^l	V^lV^h	V^hV^h			
Female	Male							
A12	B13							
V^bV^h	V^hV^l	5	13	20	8		V^b	V^h
smeared		2	4	15	6	sm	6	21
not smeared		3	9	5	2	--	12	7

$X^2_{(1)} = 7.86$

$0.01 > P > 0.001$

Total: Progeny of three crosses with A12.

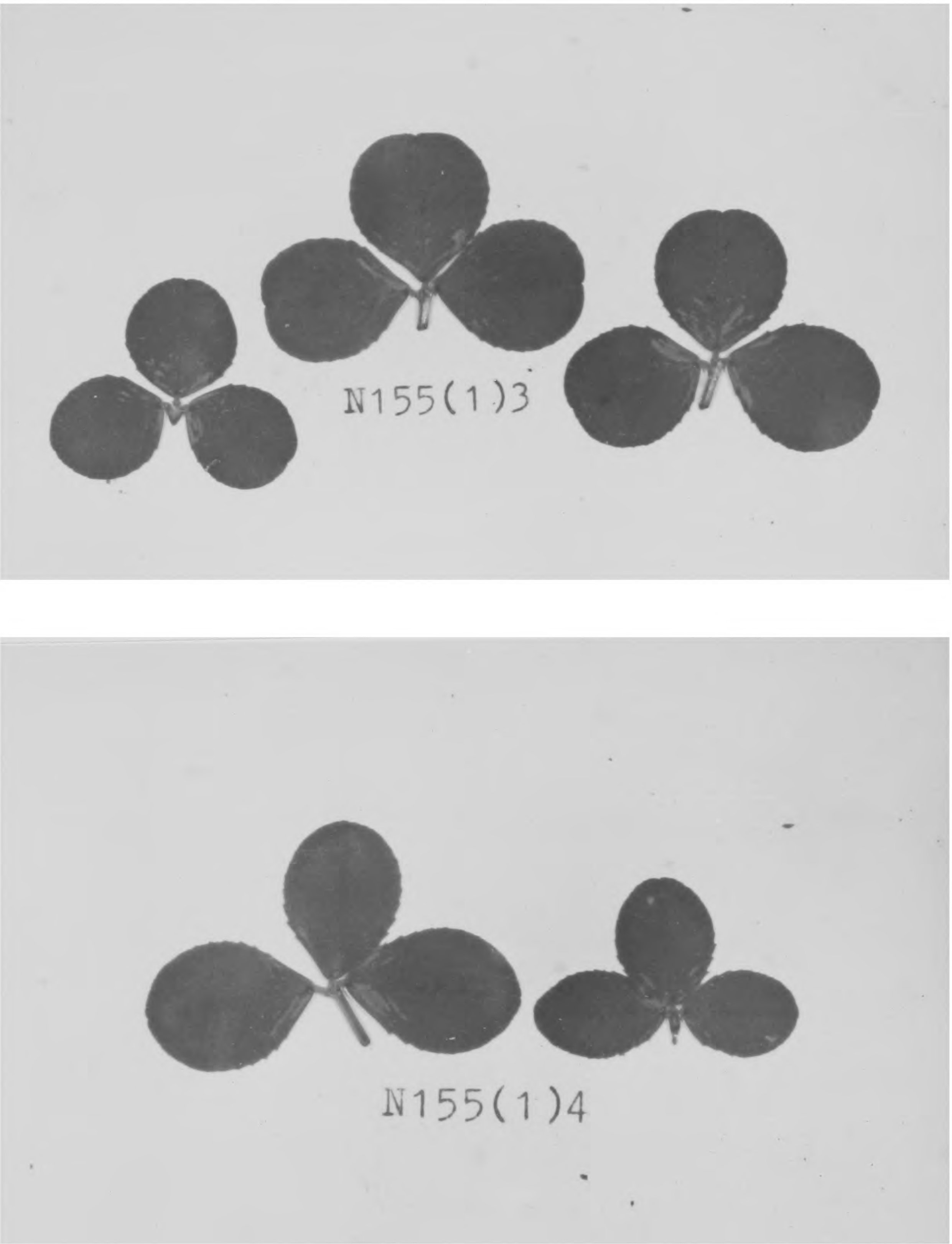
	Allele from A12 carried by progeny		
	V^b	V^h	Total
Plants not smeared	76	23	99
Plants smeared	8	66	74
Total	84	89	

The marginal mark; a new allele or a modification of the broken mark.

One further distinctive leaf-mark phenotype has not apparently been described before. This is the marginal marking, which has the appearance of a widely broken mark, with the bluish-silvery appearance by which the broken mark is usually distinguishable, and is 'smeared' down the edges of the leaflet (smearing in low and high simple marks is normally mainly central). The marginal mark can occur in a double-mark phenotype combined with a simple low or high V-mark. Photographs of leaves from two plants with the simple marginal mark phenotype appear as Fig. IV(ii).

This type of mark has been seen only in two samples of seed sent from areas at 1600 metres altitude near Sondrio in the Italian Alps. One of these samples contained only one marginal marked plant, the other, 33 marginal or double-marks including marginal out of 96. The complete sample consisted of loose florets, which produced in all about 300 seed; it could therefore have been derived from only a few heads, though a specific request had been made that at least ten heads be used in each sample. Other samples sent by the same collector had recognisable heads - respectively 6, 4, 14, and 21. It thus seems likely that the mark was derived from more than one, perhaps several parents, and must occur with a moderately high frequency in the population sampled. The sample was unusual in two other ways. There were very few plants showing simple V's - four

Figure IV (ii)



Leaves from two 'marginal' marked plants showing simple V's alone, one double mark with two simple V's, and 9 showing combinations with broken or marginal marks. Also, there were very many broken marks and derivatives: 49 in all. As this type of mark is similar in position and identical in colour to the marginal mark, it seems likely that there is a connection between this and the high frequency of marginal marks. There might be two alleles, frequent in this population because of similar selective value, or recent origin of one from the other by mutation; or modifying factors might be affecting a single allele - $\underline{V}^b$ - in the 33 scored as M of the 82 plants showing either broken or marginal marks. If this could be shown to be the case, it would be the first clear case of an effect on white leaf-markings in T. repens caused by another part of the genotype.

Shading distal to mark; another new phenotype.

Another phenotype which does not fit into the standard pattern is one in which all the part of the leaf distal to a normal simple V is slightly shaded with white, and thus appears paler than the part proximal to the mark. This phenotype is only visible under some environmental conditions, and was not seen in any of the progeny of crosses carried out with one plant showing it. Genetic determination is however suggested by its absence from many other plants growing under the same conditions as the one showing it.

This has been seen in one plant collected from riverside pasture at the University Farm, Wytham, Oxford, in two plants from Sussex, in one from Ferrybridge, Yorkshire, and in one (wild) from Aberystwyth.

Summary: new phenotypes outside the range previously described.

Four distinct phenotypes are described which have not been reported previously. These are the marks which occur only, or much more conspicuously in the seedling; smearing of (some) high marks under genetical control: marginal mark - probably related in some way to broken mark; and distal shading.

Smeared and marginal marks may represent new alleles related to $\underline{V}^h$ and $\underline{V}^b$ respectively, or may represent the effects of modifiers specific to certain alleles. If so, other genetic loci controlling the white leaf marks beside the V-locus must be postulated.

The marks restricted to the seedling, and the distal shading, only occur in a small minority of plants, and unless they are pathogenic in origin, this suggests that they also are genetically determined.

4. Red Leaf Marking Phenotypes and their Occurrence.

Three classes of red marks were described above, the third class consisting of several phenotypes whose genetics has not been investigated: some may be of purely environmental, perhaps pathogenic, origin.

Two of these newly described phenotypes appear to interact with the white mark in their expression. One, occurring only in a few plants when growing in bright light, is the development of some pink or purplish pigment precisely in the area of the white mark. This 'colour in the white mark' phenotype does not seem to be associated with any distinctive white mark (not with $\underline{V}^{by}$). Its rarity suggests a genetic basis for the ability to express it. The same point was made by W. Williams (1964) in a brief reference to these marks. It has been seen in seven ~~five~~ plants, of the following origins:

Sample no. C44 Greece
C48 Helmburg, Straubing, Bavaria, Germany
N45 Kagsmosen, Vanlöse, Copenhagen, Denmark
N92 Sierosław, Poland, Near Poznań.
Moravska Třebiska, Czechoslovakia; roadside Two plants, one of which LLf phenotype.
On a traffic island at a road junction near Oxford, presumably from a deliberately sown seed mixture.

In the last mentioned plant the red pigment was often brilliant crimson, much more conspicuous than in the other cases mentioned. In these plants, and in those with the separate red V, I have observed that the pigment was confined to the epidermis

It is possible that the description (Allan, 1940) of *T. repens* as having 'white to purple blotches or flecks' may refer in part to these cases. A possible further example of this phenomenon is the plant whose photograph appears below as Fig. IV(iv). The distal component of this apparent double mark was always relatively blue, but in certain conditions (early summer) also appeared to develop some pink to purplish pigmentation. This did not appear in the proximal, yellower, part of the marking, to which belongs the conspicuous reentrant mark tip.

The second phenotype interacting with the white mark is a bronzing of the leaf, associated with a slight stiffening of the leaf into a convex shape, and is restricted to the oldest leaves. Neither the pigment nor the expansion of the tissue occur in the area of the white mark. Leaf bronzing does not always interact with the white mark in this way; it may be a stage in leaf senescence, a response to the physiological conditions, or a result of pathogen attack (e.g. virus infection).

Of the red marks associated with the $\underline{V}^{by}$ allele, the 'red wash' spreading over the distal part of the leaf is quite common, though expressed only under certain conditions, and rarely seen in the greenhouse.

I have seen the red V only in material derived from the original American discoveries, but the blue V and the red speckling in the V-tip have occurred occasionally in the samples studied, usually as one isolated plant in a sample. Blue V tracing inner edge of white.V:

C44	Greece	N7	Kerasovon, Greece (several plants)
C53	Yugoslavia	N41	Damhusengen, Vanløse, Copenhagen
C54	Yugoslavia	N82	Barlinek, Szczecin, Poland
C57	Yugoslavia	N110	La Frétaz, Vaud, Switzerland
C74	Turkey	N155	Sondrio, Italian Alps.

Red speckling in V-tip

N45	Kagsmosen, Vanløse, Copenhagen, Denmark
N83	Cedynia, Szczecin, Poland
N87	Lubiaszów, Radomsko, Łódź, Poland
N90	Kamień Pomorski, Wolin, Szczecin, Poland
N125	Between Motukarara and Springfield, S. Island, New Zealand
SP	Pasture near Bangor, North Wales
	Beside a track along a low cliff overlooking the harbour, Aberystwyth
	Plant collected by Mr. V. Connolly near Clonakilty, C. Cork

Both are detectable only under some conditions favouring the production of large strong leaves.

Of the $\underline{R}$-locus phenotypes, I have never seen $\underline{R}^{l}$ wild, although it is found as a cultivated plant in gardens, particularly a nearly sterile form with a high frequency of multiple leaflets.

The only $\underline{R}^{m}$ phenotypes I have found wild showed only $\underline{R}^{f}$ phenotype when grown in the greenhouse. They were found on riverside pastures at the University Farm, Wytham.

Red flecking, on the other hand, is quite common in wild plants, although because of its uncertain expression no attempt was made to score for it, and no comment can be made on its distribution. As well as varying in the clarity of the flecks, plants clearly vary genetically (as well as phenotypically) in the frequency - which ranges from one on ten or more leaves to ten or more per leaflet. The size of the flecks also varies between individuals somewhat more than within them; one plant with particularly small, very abundant flecks was obtained from Giessen, near Frankfurt-am-Main

It is remarkable that these various types of red marking, though quite different in physical basis, show so many interactions with the white marking system. In genetic control, one class may be determined by factors closely linked to a particular allele at the $\underline{V}$-locus. The position of the mark on the leaf, in the case of the blue V, possibly of the V-tip red speckling, and certainly in the cases of red pigment in the white mark, is related to the position of the white V. Although the distal red wash and the red fleck for instance can be seen superimposed on each other, they seem to respond to similar environmental conditions as each other, and the same as the white marks respond to, to a lesser extent. Both types, except the blue V, appear the same colour. As Carnahan reports, one of the $\underline{R}$-locus phenotypes, $\underline{R}^{m}$, interacts with the $\underline{V}^{by}$ allele in expression, being fainter inside the white V. Two other red pigment patterns, reported above, show pigment either restricted to, or excluded from the area of the white mark. Watkin Williams (1959) suggests that the white marks are 'ghosts' of an earlier pigment pattern which involved simultaneous loss of green pigment and development of red; the two systems would have become dissociated more recently. The interactions between the white marking system and various red markings, listed above, and the existence of a V-shaped red marking supports some connection between the two systems, though the red V does not in fact precisely overlap any white V. The very rare cases of red pigment in the white V mentioned above are the only examples known in this species of red and white markings covering exactly the same area, as postulated by Watkin Williams. Although several other Trifolium species have both red and white V or V-point markings, the only comparable example seems to be in *Parochetus communis*, where a faint pigmentation may develop throughout the white V, though there may be a partly overlapping or completely separate, and more heavily pigmented, red V, and scattered red flecks, more distally ~~higher up on the leaf.~~ The markings in other species will be more fully described below. An illustration (Fig. IV(iii)) shows a plant with the red V, and outside it, only slightly overlapped by it, the chalky-white wings of the By mark (yellow tip very poorly developed if at all) with which it is associated in inheritance.

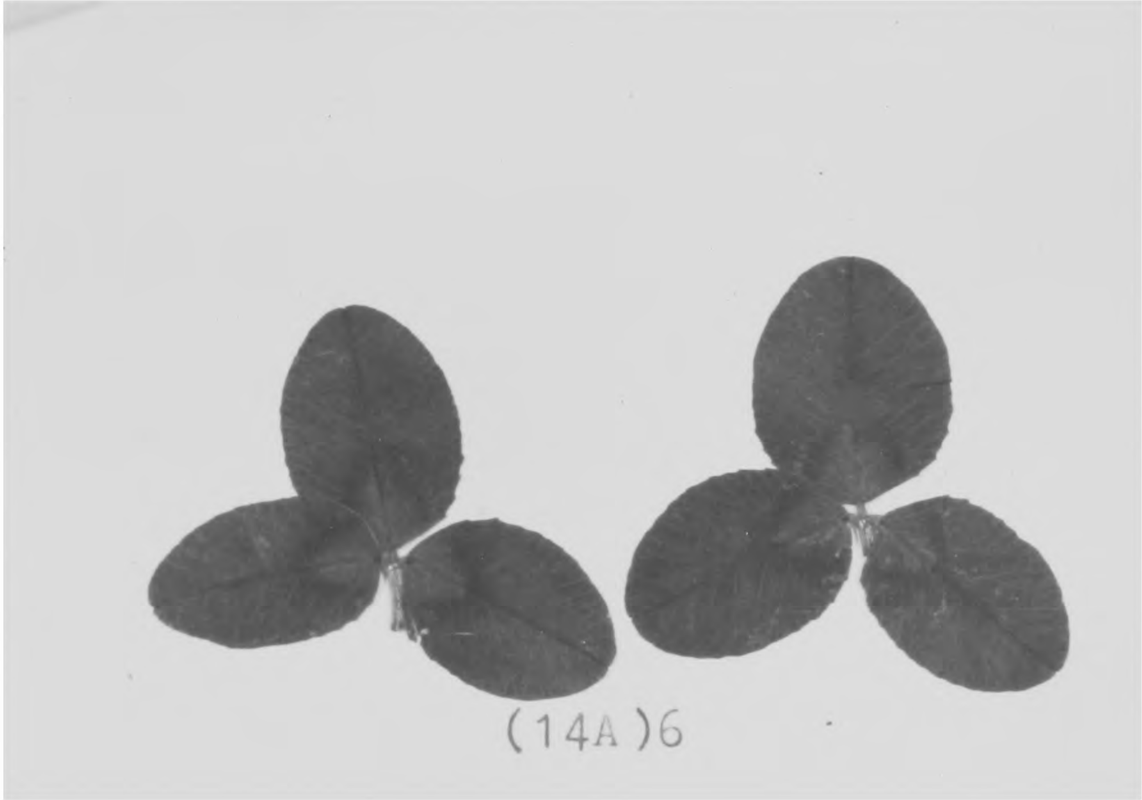
5. General Account of the Variability and Classification of White Markings

The material presented above has consisted of an account of the previous descriptions of leaf marking phenotypes, followed by a number of specific points arising from observations made in the course of this work. It seems valuable to attempt to combine these in a single review, in which the experience gained in this work can set a perspective for a summary of all the available information.

The size of the marked area on a leaflet can be defined in terms of two variables, the extension of the mark across the leaflet, and the width of the marked band measured parallel to the midrib. Because of the variation in leaf size these measurements should be thought of as proportions of the leaflet rather than as absolute sizes; and precise values for them would be impossible.

The width of the mark may vary, or parts of the lateral extent of the leaflet may be unmarked. The 'wings' of most marks taper towards the margin. Few marks reach all the way to the margin, but if the end of the 'wing' of the mark comes close to the margin it tapers there, at least, and is generally curved downwards towards the leaflet base. Where the wings of

Figure IV (III)



Red V-mark on a broken-with-yellow-tip white mark (yellow tip poorly expressed).

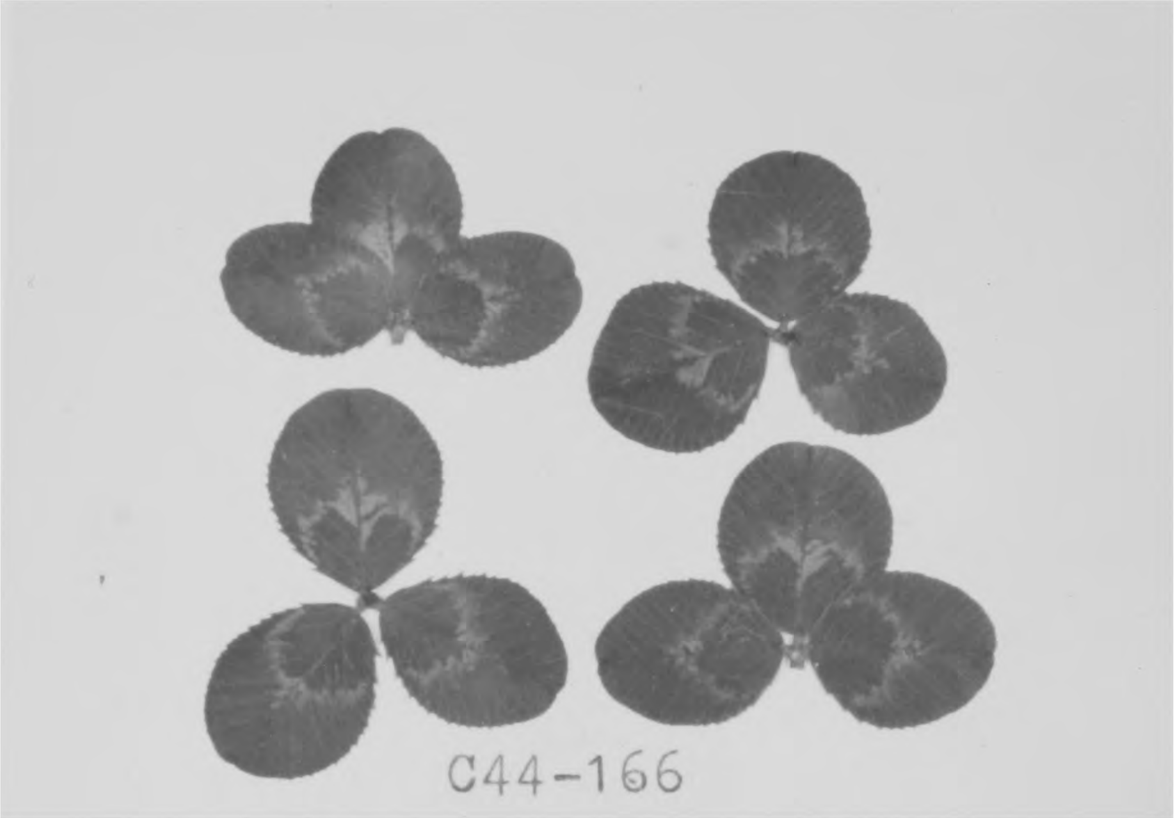
the mark do not run right up to the midrib, they may taper, or may stop abruptly, leaving a green stripe along the midrib (particularly conspicuous in some very acute basal marks), or may leave a more extensive break in the mark. On the other hand the mark may continue across the midrib, or may have a region of greater width, forming a conspicuous thickened point to the V, so that the lower, proximal outline of the mark is more curved or obtusely angled than the distal outline. The tip of the mark may extend up the midrib, producing concavities or reentrant angles on either side. Between any tapering at the ends of the wings, and any tapering, break, or expansion at the midrib, there is often a region of more or less constant width.

Other respects in which different marks may differ are the regularity of the outline of the mark, the abruptness or gradualness of the change from marked to unmarked tissue, and the uniformity of the whiteness of the tissue within the mark. Especially in well-extended but narrow marks, it is obvious that the mark outline is not a smooth line running across the leaflet, but is irregular to some extent. In thinner marks, the mark may actually be discontinuous (for example, the wings of the mark in Figure IV (v) and in Figure IV (vi) below). The width is more or less constant; as though mark development occurs more or less separately in a series of strips of tissue divided by lines parallel to the lateral veins, producing a constant width of mark, but sometimes showing some displacement between adjacent strips. When this displacement is marked, the segments of the mark outline produced by the displacement and running parallel to the lateral veins may be straighter and more sharply marked than the rest. (This may be seen in Fig. IV(i) above, and very strikingly in Fig. IV(iv) and IV(v) which appear together below.)

A case was described above of smearing of the mark appearing to be specially frequent, and possibly under specific genetic control, in one family of plants. A degree of smearing, in which the width of the mark is not constant within each strip, but where there are occasional extensions downwards towards the midrib, is quite common, though not as conspicuous as in the family referred to. It is usually confined to the region close to the midrib, where in an acute mark it produces a thickened triangular mark tip. Smearing of the mark tip in this way may be quite regular in some phenotypes, and when it is accompanied by a similar displacement of the distal margin also near the midrib, the result may be an 'inverted-W' shaped mark with a reentrant tip. In such cases, the straightness and sharpness of the parts of the mark outline running parallel to the lateral veins at right angles to the normal direction is particularly clear. An analogous small straight sided extension of the inner margin of the tip of the red V is also sometimes seen.

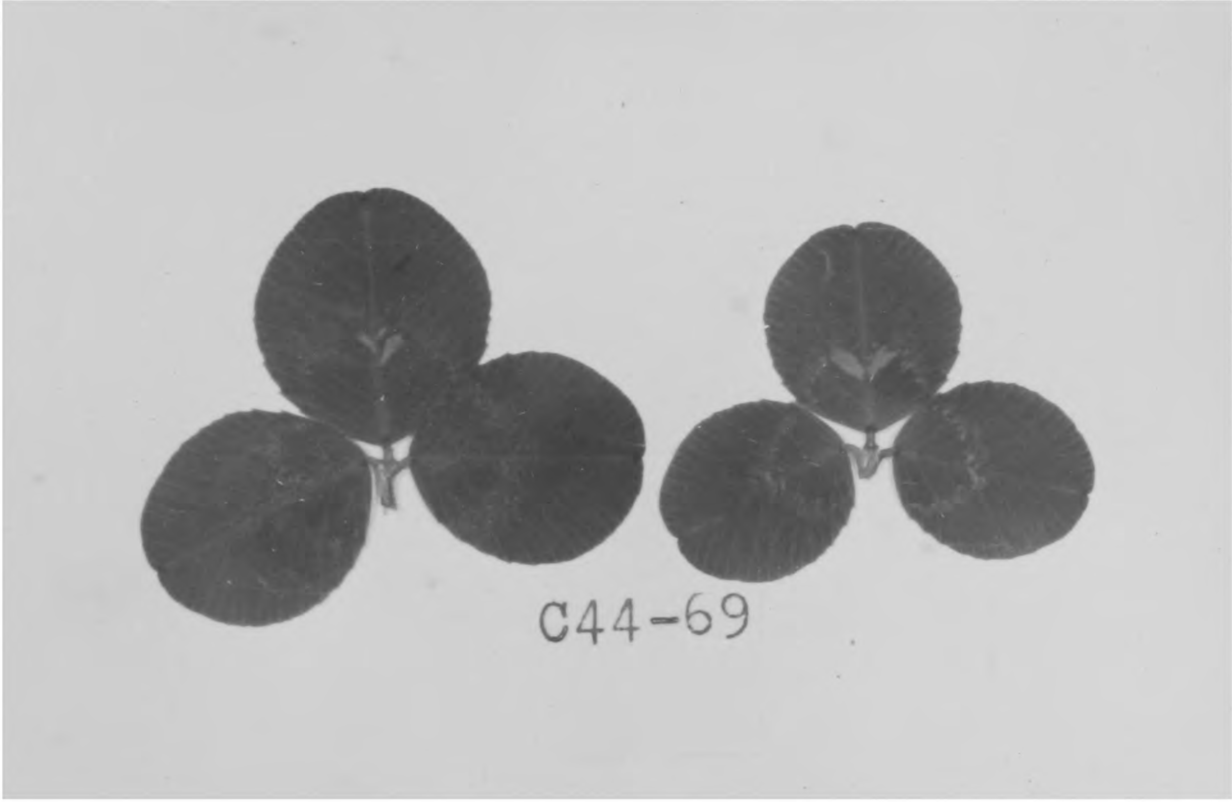
Two very clear examples of such smeared and more or less reentrant tips are shown in Figures IV(iv) and IV(v) which follow. Both are extreme in that they combine such a tip with

Figure IV (iv)



Double mark, outer part bluish, inner yellowish with conspicuously smeared tip.

Figure IV (v)



Mark with irregular discontinuous wings and conspicuous smearing of the tip.

very weakly developed wings. Figure IV(iv) is noteworthy in another respect, since the mark appears to be double. This can be recognised not from separation of two marks by unmarked tissue, but by the difference between the colour of two adjacent marked regions. The proximal part, including all the smeared stripes at the tip is yellowish-green, while the distal part is bluish, and showed an even greater contrast under some conditions when it appeared to develop a faint red pigmentation, as described as a rare phenomenon above. This certainly did not extend to the yellowish part of the mark. If this is correctly interpreted as a double-mark phenotype produced by a heterozygote, the smearing is either a property of only one allele, or at least, only extends the tissue marked in the colour of one allele, and the apparent anthocyanin pigmentation is associated only with the mark produced by the other allele.

The character which has been most important in previous mark classifications is probably the most useful; this is the position of the V on the leaflet, between the base and the tip. The trends of the wings meet at a point on the midrib usually between about three and seven-eighths of the way from the leaflet base to the tip, though the actual mark may lie below this in a reentrant mark, or extend above it in some true V-shaped marks.

Another important variable in the appearance of the mark is the angle which the two 'wings' make with each other. This may be very acute (less than 45°) or quite obtuse (approaching 135°), although some apparently almost flat marks also occur, if the wings do not extend far and there is some development towards a reentrant tip.

The combination, in heterozygotes producing double marks, of marks at different positions and lying at different angles can produce double marks overlapping at the wings but separated in the centre, producing an (uninverted) 'A' shape; or marks joined in the centre but spreading apart away from the midrib. Although mark angle may be affected by leaflet shape, the fact that both these types occur demonstrate that angle is a variable property of the alleles themselves, not completely determined by the height of the mark along the midrib. (cf. Fig. IV(vii), also pp. 44 and 89)

Another property of the mark phenotypes is the development of whitish colouring in parts of the leaflet outside the V-shaped band. A rare 'shaded' phenotype with the distal part of the leaf pale has been described above. The rather variable class of filled-in marks show the alternative condition - modification of the colour of the part of the leaflet below the V.

The published pictures of filled-in marks (Brewbaker, 1955; Carnahan, 1955; W.E. Davies, 1963) seem to be confusing, as they are very extreme types, and most plants showing some degree of filling-in seem to resemble an ordinary simple V much more than these. However, even in Brewbaker's and Davies's illustrations one can make out the V-shape at the edge of the mark as more intensely white than the region within it. A less extreme example is in fact shown by Davies to illustrate the expression of red flecking. It seems possible that the 'spread V' found by Pandey (Brewbaker, 1955), with a spot of chlorophyll at the base of the leaflet, may be an example of a moderately filled-in mark. The intensity of the whiteness in the filled-in area may not be uniform; it may be greater at the leaflet base, or on the other hand it may be concentrated near the V-mark in the centre of each half-leaflet, as though in these cases it was a very faint basal acute mark. In Fig. IV(vi) appears a picture of one of the most heavily filled-in phenotypes seen in the plants of wild origin used in this work. This shows a very irregular and broken V-mark, and also an irregular distribution of the white shading of the 'filling-in', which is concentrated close to the mid-rib.

It seems probable that a complete series of transitional forms between an extreme filled-in and a simple mark could be found, both as inter-plant genetic variation, and also by intra-plant phenotypic variation. Whether the genetic differences reflect a range of different alleles, or the effect of modifying factors is not clear. When parent plants have a fairly intensely filled-in mark it is also seen in their progeny, but this does not discriminate between these alternatives. Further generations of crossing, or crosses between heavily and faintly filled-in plants would provide a better indication.

Another series of transitional forms approaching the filled-in condition runs through the types of smeared marks, although, except in the seedlings, no more than an occasional half-leaflet has been seen with a completely filled-in mark in a plant showing smearing.

One last important character is the colour of the marked area, which may be relatively densely white, or faint and bluish, or yellow or pale green compared to other marks. Figure IV(iv) above shows a plant whose mark is interpreted as double on the basis of difference in colour between yellowish-green and bluish. The difference is fairly clear even in a black-and-white photograph, the fainter, outer half of the stripe of marking being the bluish component. There seems to be no published reference to variation in colour between different marks in _T_. _repens_ (compare the designation of a separate locus controlling white or yellow colour of the mark in _T_. _pratense_ by Wexelsen (1932)). Even the full accounts by Brewbaker (1955) and Carnahan _et_ _al_. (1955) refer to colour only in connection with the By phenotype (cf. also D.R. Davies and Wall, 1959, 1960). Allan (1940) refers to 'white to purplish' marking and may possibly be acknowledging this variation, though he is presumably referring mainly to the two principal classes of white and red marks.

The characters by which the mark on a particular leaf may be described may be listed as follows:

COLOUR and INTENSITY of colour-contrast with green background

POSITION of mark (high or low) between leaflet base and tip

EXTENSION of mark on each half-leaflet towards midrib and margin

WIDTH of the marked band, measured at right angles to extension

VARIATION IN WIDTH - marginal and midrib ends tapered or abrupt, or centre of mark expanded to a triangular tip

REGULARITY OF OUTLINE - smooth or irregular

SHARPNESS OF OUTLINE - abrupt, or gradual and diffuse

SHAPES OF OUTLINES, distal and proximal - projecting tip, angular, curved, reentrant

ANGLE between the straight parts of the two wings

SMEARING of the mark - position and amount

FILLING-IN or SHADING, whitening of leaflet proximally or distally.

Clones or genotypes may be further described in terms of the consistency of mark phenotypes in leaves of the same status (particularly, the sporadic incidence of smearing), and the effect of leaf size, the stage of maturity, the state of vigour or weakness, and the environmental conditions on the phenotype produced.

A 'taxonomy' of mark types would look for tendencies for recurrence of certain combinations of characters, at greater frequencies than related combinations. To take a clear example, broken marks, with a central gap extending half way or more from the midrib, normally extend all the way to the margin, have a bluish or metallic appearance, lie at a fairly obtuse angle fairly high on the leaf, and have a continuous, not very irregular, and fairly sharp outline, with a uniform intensity of marking within it. This combination of characters is fairly well isolated from intermediate combinations, and would seem to qualify as a 'natural group' of marks, since most marks showing several of these characters are found to share the others.

The studies of leaf-mark inheritance carried out by Brewbaker and Carnahan seem to have been carried out with a small group of plants, probably chosen for their mutual distinctness. Acquaintance with the range of natural variation demonstrates a wider and much less discrete range of variation; it is only by arguments such as that in the paragraph above that one can point to marks which can be grouped under one label - hypothetically as produced by a distinct allele.

Some comments may now be made on the value of the reported set of alleles and named mark types in describing the observed variation. A distinction may be drawn between the generally rare marks with distinctive properties - broken, broken with yellow tip, filled-in, basal - and the 'simple' marks, ranging from low to high.

The types of distinctive marks distinguished are fairly acceptable.

Broken mark was quoted above as a good example of a distinct mark type. Its nearest 'relative' is the marginal mark, which shares all its properties except that it spreads downwards on the leaflet from the mark. It may or may not be a distinct allele - it is certainly a distinguishable phenotype.

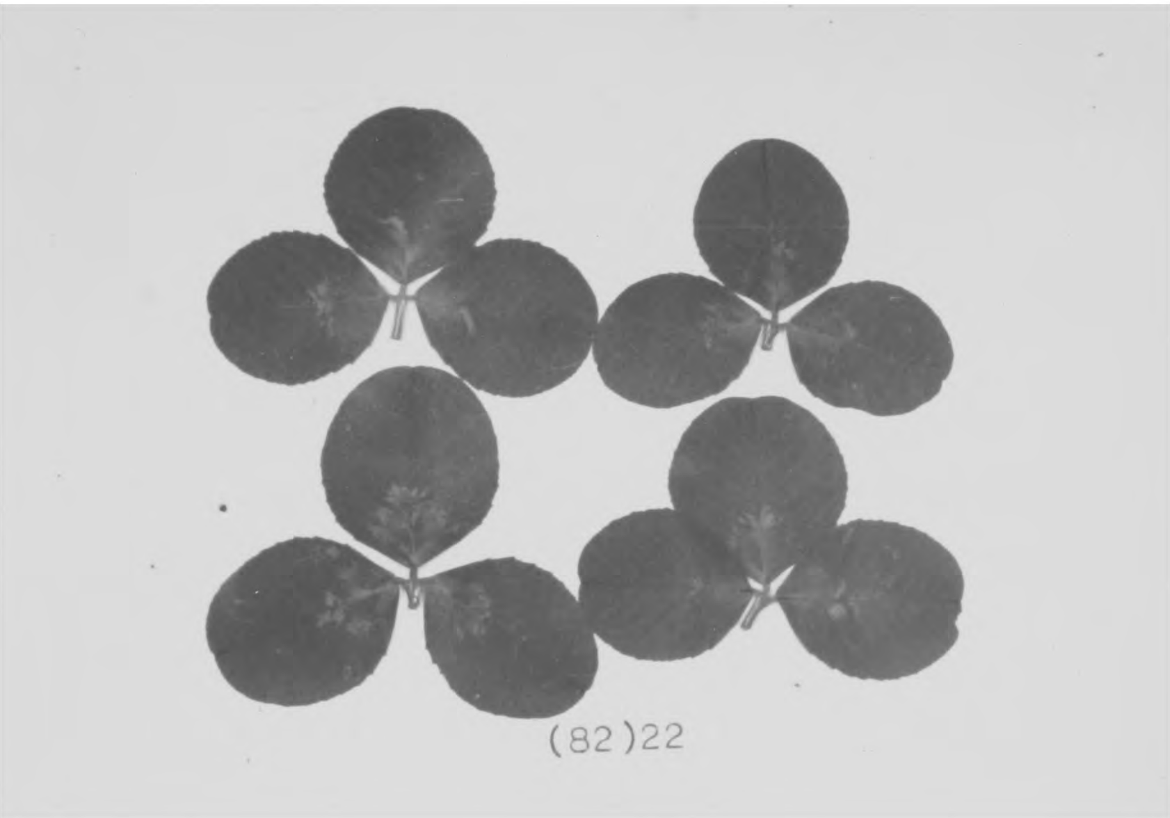
Broken marks with yellow tip is perhaps the most distinctive of all the marks, but includes a wider range of variation than broken mark, and has other peculiarities, such as its double structure and its interactions at various levels with red markings. The yellow tip is not in fact the most useful diagnostic feature, since many leaves and some whole clones fail to show it, or show it only very faintly. (Cf. p. 55 note (1), p. 71, and Fig. IV (iii)) There is however a clear class of marks with a rather high V, somewhat broken (though much less than the simply 'broken' mark), and tapering towards the midrib, and chalky white in colour, producing an unusually sharp contrast with the leaf. Many of these show some amount of yellow tip, and many can develop the 'red wash' on the terminal leaflet. At the other extreme, some leaves (especially perhaps on seedlings?) show very conspicuous yellow tips, and the white mark is reduced to a discontinuous string of dots. The mark is very definitely double in appearance; as well as differing in colour, shape and position, the two components differ in outline - the yellow mark having a diffuse, fairly smooth outline, while the white mark has a rather sharp, sometimes irregular outline.

There are some marks, also double, with a slightly different combination of characters. The tip is yellow-green or pale green and less diffuse in outline, and the wings are bluish-metallic, rather than chalky. This could be interpreted as a double mark produced by a combination of a broken and a simple mark with an enlarged point and little extension. However, these single marks do not appear frequent in the samples where this combination appears, and it may represent a second type of double mark controlled by one allele. These marks were scored as Y (cf. p. 129) This phenotype was seen in the following samples:

Common in	N97-5	Skopje, Yugoslavia
	N97-8	Zagreb, "
Occasional in	N156	Italian Alps
	N159	" "
Possible, not definitely identified	N97-2	Kutina, Yugoslavia
	N82	Barlinek, Szczecin, Poland
	N92	Sierosław, Poznań, Poland
	N96	Krynica, Krakow, Poland.

The filled-in marks are certainly variable, and it seems likely that the existence of a number of filled-in alleles is needed to explain it. As well as varying in the intensity of filling-in, they vary in the width, and the shape (acute-pointed, curved, or reentrant) of the tip. Although a clear distinction between simple marks and filled-in marks can be made - all marks on plants showing any filling in can be called filled-in marks - there seems to be a continuous range, and the V-mark at the edge of the filled-in area varies in much the same way as do the simple marks without filling in. Marks like those used to illustrate filling-in by Brewbaker (1955), Carnahan (1953) and W.E. Davies (1963) are extremely rare in the wild; much commoner are phenotypes with faint filling-in, or a trace only, visible on the best-developed leaves. The mark shown in Fig. IV(vi) shows more intense (though also irregular) filling-in

Figure IV (vi)



Filled-in mark. V-mark irregular in shape, and filling-in stronger near midrib.

than most of the plants of wild origin scored as F (or at first as Lf) in this work. The irregularity and fragmentation of its V-mark, as opposed to the continuous broad mark shown by Davies, and the smooth, diffuse outline of the mark shown for $\underline{V}^f$ by Brewbaker, illustrate the point made above about the variability of the V-mark within this class.

The basal marks have less distinctive properties - a very acute shape, the wings approaching the margin very near the leaflet base, and a yellowish-green rather than whitish colour. However, they do seem to be a distinct group. The lowest of other V's are not acute, and confusion would be most likely with the more acute high V's. The basal class does seem to include some variation. The basal mark in Fig. IV(vii) below falls more or less in the centre of this range. Some marks have tips over half way up the midrib, have a clear green area beneath them, and tapered tips curving inwards towards the midrib at the ends of the wings. At the other extreme are marks like that illustrated for $\underline{V}^{ba}$ by Brewbaker (1955), not reaching half way up the leaf, rather blotchy and irregular in intensity, and without sharp outlines, and either with no green area proximal to the mark, or a green stripe on either side of the midrib which as wide at the mark tip as at the base. This could be regarded as a central break in the mark, as seen in many simple $\underline{V}^1$-marks, as much as an area enclosed by the mark.

The simple marks without the distinctive features of those mentioned above present a much more complicated picture, which the published descriptions seem much more inadequate for. In the population data which will be presented below, they have all been grouped together as 'L' marks, though in some cases a note has been made that some of them should be described as high. Four previously described alleles fall in this class; high, intermediate and low V's, and V-point.

Two kinds of particularly high mark are found. One is relatively flat and obtuse, quite thin, and light green. The second, mainly found in large-leaved plants, is more acute, broad, and yellowish-green. Among the lower V-marks, three relatively distinct rare types may be mentioned first. One is quite broad, a faint silvery colour, and has the tip of its straight V slightly more than half way up the midrib. This, or double marks including it, occurs in over half the seeds from one head, and sporadically in other heads in sample N45, from Kagsmosen, Vanløse, Copenhagen, Denmark. cf p.111. Single plants with possibly comparable marks were seen in

N 42	Damhusengen, Vanløse, Copenhagen, Denmark
N103	Gröbming, Steiermark, Austria
N111	Les Allieres, Fribourg, Switzerland
N122	New Zealand.

One mark lies in a similar position, but is much thinner and sharp-edged, and although quite continuous, it is distinguished by a very definite bluish colour, as distinct as that in a typical broken mark, or more so. It was found as follows

Common	N92	Sierosław, Poznań, Poland
One plant	N82	Barlinek, Szczecin, Poland
	N83	Cedynia, Szczecin, Poland

Possible examples N97-2 Kutina, Yugoslavia
N95 Żegiestow, Krakow, Poland.

A third lies very low on the leaf, and consists of a broad white band, fairly smoothly curved, little more than a quarter of the way up the midrib. A fourth less rare and less distinctive type resembles the last, showing a steadily curved, broadish band, whiter than some other marks, crossing the midrib just below its half-way point.

The remainder - the majority - of the simple marks are harder to divide. Four extreme types may be described, though without definitely claiming that there is any clustering round them or deficiency of intermediate types.

One of these types, perhaps the intermediate V, is a fairly straight sided V, fairly acute, and with its tip over half-way up the leaflet. It resembles the acuter high V, except that it is not quite so broad, so acute, or so high in position. It may be rather less regular in outline and texture at the tip. The second corresponds to V-point, when most fully expressed. The outline of the mark is acute on the distal side, but curved on the proximal side, so that there is a triangular marked tip, and the wings are thin or only slightly extended. The texture within the mark may be uneven - speckled, or showing the triangle is composed of a number of smearing stripes. In small-leaved plants, this, and the two next mentioned, account for all the clearest marks. The next is also seen in larger leaves; it is centrally placed, and the moderately extended wings lie fairly obtusely. The centre of the mark is slightly thinner, and depressed to make a curve of the mark as a whole, or slightly reentrant, with small smeared stripes with sharp outlines parallel to the lateral veins showing near the midrib on the lower side. The last type has a very marked reentrant smeared central region, and the wings are very thin, and run down quite acutely from the ends of the smeared stripes, producing a distinctive W-shape. Some plants show intermediate conditions between these two reentrant marks and the V-point type, the upper part of the point being developed only on some leaflets, and the first mentioned intermediate V and the V-point are linked by forms with well developed wings, an acute distal outline, and various degrees of smearing within the tip at the centre.

In particularly small leaved plants, these simple marks become less and less distinguishable, and a type with a whitish spot either side of the midrib, slightly extended outwards, can correspond to any of these types, or to filled-in types. Basal or broken marks tend to disappear completely, though the distinctively white colouring of the wings of the broken mark with yellow tip makes at least some plants of this form recognisable under conditions when no other type could be identified as anything but 'simple V'.

6. Discussion of the mechanism of expression of the white mark.

Brewbaker (1955) has suggested that the marks represent a concentric set of zones which were at the same stage of development at the time some disturbing factor came into action, for a limited period. The existence of heterozygous types where marks with separated tips converge towards the margin (e.g. the acute high V and the curved mark with the thin centre) show that an increase of acuteness does not follow necessarily from lower position, and the converse arrangement (e.g. the two high V's, or the curved thin-centred V and the V-point lying lower), where wings diverge from an overlapping tip, show the angle changing faster than is conformable with his hypothesis. More positive evidence is the overlapping of marks - the tip of one lying above, and its wings below, the other. This happens with the combination of moderately high-reaching basal marks and the flatter low marks, such as the curved thin-centred (as shown in the phenotype illustrated in Fig. IV(vii)); and with the faint broad silvery V and the curved mark, and in this case the distinctive colour and outline of the silvery mark serve to distinguish the components. Such phenotypes could not be produced by a simple timing control. However, the idea of such a timing control has attraction as an explanation of the phenomenon of smearing, in which the disturbance of the normal process of leaf development continues in a part of the differentiating zone after it should have stopped. The regular smearing which produces reentrant or triangular tips illustrates the sort of interaction

Figure IV (vii)

Double mark of Low and Basal V's, the two marks appearing to cross over one another. (This phenotype is not consistent with Brewbaker's theory of concentric time zones of V-allele activity.)

of the mechanism of timing of mark-allele expression with position between midrib and margin which would also be needed to explain the interchange of position of marks which cross each other. The autonomy of small parts of the differentiating zone such as those which produce smeared stripes is shown by the irregularities seen in the wings of some marks, as though both the beginning and the end of the 'marked' type of development had come a little early or late.

This local autonomy of small regions of tissue producing the mark is supported by the observations of R. Davies and Wall (1959, 1960, 1961). They irradiated two clones of $\underline{V}^{by}\underline{v}$ with gamma rays for a brief period. The first signs of somatic mutation appeared between four and ten weeks later (depending on the season) and 'were recognised as an absence of, or a change in, a small portion of the leaf mark. Later leaflets had larger segments of mutant tissue, then whole leaflets and ultimately whole leaves and tillers were mutant'.

'A considerable number of the mutations observed were small gaps either in the white V or yellow tip' and by the same criteria 'No spontaneous mutation was found in the control plants utilised in all the experiments considered here (ca. 10,000 leaves)'. The small gaps must therefore have been caused by mutation and have been clearly distinguishable from any natural irregularity of the leaf. In other words, a change in the genotype of a small group of cells could prevent them contributing to the mark. Although some response at a higher level to morphogenetic fields in the leaf as a whole must determine which fraction, if any, of the cells in the developing leaflet express the mark, some ability to respond is also required of the cells involved.

The fact that the effect of mutation is to eliminate marked areas supports the idea indicated by the dominance of the positive properties of the alleles that the production of markings is a positive process, interfering with a normal process of development which is provided for completely by quite other factors. Thought of in this way, and if one assumes one dose of an allele is sufficient to produce its full effect, the joint expression in heterozygotes is exactly what would be expected. Independent action of the alleles is all that need be postulated, and Brewbaker's idea of 'mosaic dominance' seems an unnecessary complication.

R. Davies and Wall's work (1959) produces some other results of interest. The latest appearing mutant phenotypes were those affecting the largest amount of tissue, and some, presumably affecting cells in the meristem itself, affected whole tillers. These new self-reproducing phenotypes, as well as completely unmarked forms, included some where only the white mark had been lost, leaving a yellow point; some where only the yellow tip had been lost, leaving a broken white mark which R. Davies and Wall equate with the known broken mark. Indeed the mark they illustrate has neither the sharp contrast nor the tapering by which $\underline{V}^{by}$ marks with faint points were distinguished above from broken marks. These component phenotypes were quite common; considering areas of a leaflet or more, 103 cases of loss of the whole mark occurred, 80 of loss only of the white V, and 18 of loss only of the yellow point. Other stable phenotypes, which no frequency is quoted for, include modified marks, with the white part reduced in intensity, or broken up into spots. These results confirm that such features as the intensity and regularity of the mark are genetically controllable, though they do not indicate whether they are controlled at the V-locus.

Unfortunately, R. Davies and Wall's tillers with mutant phenotypes all behaved in crosses as unchanged $\underline{V}^{by}\underline{v}$. Presumably they were all chimaeras, and the mark development is completely accounted for by a tissue layer which is not involved in gametogenesis. There may have been as many similar mutants in the gametogenic layer - but having no effect on the visible phenotype, these were not selected for closer examination and crossing. Their results also suggest that the $\underline{V}^{by}$ allele contains two functions which can be destroyed or modified separately. This could be so if the $\underline{V}^{by}$ allele involved a reduplication of the chromosome segment containing the $\underline{V}$-locus, and if so, other genetic material involved in the reduplication might control the red markings associated with this and not with other V alleles.

Some complexity in the structure of this locus seems acceptable - in view of the large number of different alleles - 11 published, and if the diversity described above is determined by the V-locus, the number must be considerably larger. Brewbaker (1955) found some BL and some O progeny (only one and two plants respectively, out of 38) in the progeny of a $\underline{V}^{l}\underline{V}^{b}$ x $\underline{vv}$ cross, and suggested that they might result from intra-locus recombination in the heterozygous parent. The O at least could not be derived from selfing.

CHAPTER V

PRINCIPLES OF POPULATION SAMPLING

1. Introduction: Discussion of Problems, and Experimental Studies Related to Them.

The main object of this work has been to obtain data on the distribution of different leaf-mark phenotypes in naturally established populations of white clover, in the hope of finding regularities in the distribution pattern from which it might be possible to deduce the kind of selective pressures which may be acting for or against particular alleles.

Such a pattern might be discernible by comparison of neighbouring populations, or only as a gradual trend over a large area. An attempt has therefore been made to combine study of samples within a limited area (Southern Britain) on which relatively complete information can be obtained, with the study of samples collected from a wide range of sources overseas, particularly in the areas where the species was established before its spread was assisted by deliberate or accidental introduction by man.

Samples used have been both of seed and vegetative material. Vegetative samples describe genotypes which have actually survived for some length of time in the wild, whereas seed samples are relatively unselected. Arguments of convenience favour seed samples, which can be stored for a year or more with negligible loss of viability until it is convenient to grow them, and can be simply and safely sent by post, for which reason most of the samples originating outside Britain are seed samples. Seed samples are grown in more or less uniform favourable conditions, giving good and comparable expression of the phenotypes. It is usually convenient, on the other hand, to score vegetative material in the field, and few of the samples described have been grown up after collection. Studying these phenotypes which have developed under a range of somewhat adverse field conditions must to some extent reduce the accuracy of the descriptions recorded.

The comparability of seed and vegetative samples is a complex problem. Aspects of it are discussed in more detail below, together with experimental studies concerned with some of these aspects.

Experimental Studies bearing on Sampling Procedures.

Before drawing any conclusions from the results of scoring the samples, one should consider the possibility that the techniques of obtaining and handling the sample may have biased the sample in a way that would alter the results. A seedling sample for example must be grown for some time before scoring (modifications of the leaf mark phenotypes on the earliest leaves have been referred to above). Some seedlings die before or after planting out, some seed swells but does not germinate, other viable seed remains hard and unswollen. Any of these causes might apply selectively to some genotypes more than others. The problem of the hard seeds which do not imbibe water is discussed below, and experiments are described to discover the technique best suited to obtaining the maximum yield of seedlings from each sample.

The samples used have been obtained in various ways, and before using them to reveal any correlation with environmental features, one has to consider the possibility that different sampling techniques may introduce different biases, and produce results which are not comparable. Even if perfectly random sampling techniques were used, seed and vegetative samples would still be of different sets of objects; although a biologist may predict a connection between the genetic composition of a population of plants and the seed they carry, this similarity may not be complete.

Within a reproductive cycle, long term stability of adaptation to environment is compatible with a selective equilibrium in which selection favours certain genotypes in the processes leading to seed formation, and others in the processes of development from seed to maturity. Where such a dynamic, cyclical equilibrium of genetic response to selection occurred, vegetative and seed samples would include different proportions of each genotype. The possibility that such discrimination might affect the leaf-mark genotypes being studied should be considered; and if a bias was found, it might in fact identify part of the selection which one would expect to account for the stability of a polymorphism. A comparison is described below between leaf-marking frequencies, in a vegetative sample, and in a sample of the plants bearing inflorescences, which would reveal some of such effects; the results in this case are not significant. A complete study of this kind would involve comparison of several phases of the population; vegetative material, plants producing flowers, genotypes of pollen reaching recessive 'tester' plants, genotypes of mother plants weighted in proportion to the number of seeds they bear, the seed produced in any one season, and the seed lying dormant in the ground. The transitions between each of these phases represent opportunities for selective discrimination for or against particular genotypes.

The possibilities of differences between the genetic composition of the different phases are perhaps even more clear when one considers the inadequacies of the idea of a steady flow through phases of a life cycle. Many of these phases are phases of multiplication, and in a population in equilibrium this must mean later elimination with scope for selection. The idea of a continually flowing cycle may itself be somewhat inappropriate in a species such as this.

Vegetative reproduction can certainly be very effective in a species like this one. A set of observations are described below to illustrate a method which could be used to describe the extent to which single clones can expand. If vegetative reproduction completely took over the functions of dispersal and persistence, as it seems it could over quite long periods, a seed collection would not be the next phase in a cycle, but a sterile side branch; and in view of the difficulties in establishment, this is probably true of a large majority of any seed population in any case. Conversely, from the long term view in which genetic variation, seed dispersal, and dormant survival are necessary, the long life and large size which some clones can attain vegetatively in stable environments, without any selection for sexual fertility, may make their contribution to a vegetative sample deceptive by the inclusion of sub-fertile genotypes with a restricted genetic future.

Another respect in which apparently similar samples may not be strictly comparable concerns not the actual values recorded for different phenotype frequencies, but their accuracy. The number of plants scored in each sample is generally taken as indicating the accuracy of the results, but considerably larger sampling error than thus estimated will be present in samples derived from a set of individuals not chosen independently. A vegetative sample in which plants were chosen from a very restricted area or very close together might well sample the same genotype several times (cf. Harberd, 1958), although the indications are that the area which any one clone occupies to the extent of excluding others is much smaller than the area which its spread can reach. In seed samples, the members of the sample fail to be independent when they consist of seed from the same head, with a common maternal parent, and possibly a quite restricted number of pollen parents. Where the sample is taken from a large batch of seed, this is less important, but when the sample consists of all the seed from as few as five or six heads, a large part of the sampling error depends on the sampling of those five or six parents, not on the forty or fifty progeny. Small samples consisting of loose florets or seed are liable to this problem to an unknown extent - they may represent the whole production of a few heads, or a proportion, selected more or less at random, from many more. Some observations are described below on the seeds grown separately from each head, in a few samples where the heads arrived intact; the divergence of the scores for the progeny of each head give some idea of the effect this easily underestimated sampling error can have. Such a study should also make possible some identification of the maternal genotypes, but the interpretation of the data in this way was more difficult than was expected.

Seed Hardness.

One factor which may bias the scoring of a seed sample is the failure to germinate of hard seed. These are the seed in which the impervious layer developed in the testa is complete, preventing the penetration of water, without which the seed can neither swell to escape its coat, nor start to develop. The proportion of hard seed is lower in commercial samples, partly because of selection in previous generations for prompt germination, and partly because of damage to the impervious layer occurring during mechanical threshing. Wild samples intact in the head or floret, and threshed by hand, are often 90 or even 100% hard when tested on moistened filter paper pads.

To obtain germination of hard seed some process of scarification is necessary to break the impervious layer. Two principal types of scarification are available; mechanical, by abrasion, and chemical, by treatment with a corrosive material such as concentrated sulphuric acid. Abrasion by rubbing with sandpaper can produce germination of every seed in a sample (Chakravarty, 1963a); however it is reputed to depend on the personal touch of the experimenter, and it would be difficult to repeat a successful treatment when the treatment cannot be quantified. This is an important objection, as a slight excess of violence of any scarification treatment beyond the amount needed to break the hardness of a seed can produce damage and death of the embryo. A quantifiable mechanical scarification treatment was devised, consisting of shaking the seed in a glass screw-topped bottle, quarter-filled with silver sand, by means of a Microid Flask Shaker. The results of the longest treatment of this kind, fifty minutes, are compared with various lengths of sulphuric acid treatment in table 5.1 which shows the experimental results obtained with samples of commercial seed, including one which had been previously discovered to show an unusually high frequency of hard seeds.

Table 5.1. Comparison of Scarification Treatments

Material	Treatment	Number of seeds treated	Number remaining hard	Percentage remaining hard
S100	None	299	35	11.7
	Shaking: 50 min.	282	32	11.3
	Acid: 10 min.	148	10	6.8
	20 min.	99	2	2.0
	40 min.	134	1	0.7
S184 (normal)	None	310	42	13.5
	Shaking: 50 min.	290	33	11.4
	Acid: 10 min.	152	3	2.0
	20 min.	138	1	0.7
	40 min.	159	0	0.0
	(Pre-dried) Acid: 10 min.	147	12	8.2
	over 20 min.	158	9	5.7
	$CaCl_2$ 40 min.	148	3	2.0
S184 (hard sample)	None	403	105	26.1
	(Pre-dried) Acid: 10 min.	150	27	18.0
	over 20 min.	148	9	6.1
	$CaCl_2$ 55 min.	148	6	4.1

From these results it appears that the shaking treatment was almost completely ineffective.

The 40 minute acid treatments showed a number of plants damaged by the treatment, with brown radicles which did not grow, although the cotyledons were green, and swelled, and the seedling escaped from the testa. The single treatment by which the maximum proportion of growing seedlings could be produced would seem to be 20 minutes acid treatment, and this was used for most of the seed samples which were handled.

However, if repeated treatments were possible, and if the maximum possible recovery of seedlings was desired, a series of shorter treatments, perhaps 10 minutes, would seem preferable, applied to the hard seeds remaining at each stage following an initial germination without scarification.

3. Examination of Separate Heads within Seed Samples.

A second problem arises in connection with samples provided specifically for this work. These were often quite small, and such small samples could most easily be collected by including all the seed of a small number of heads. Therefore, although the seed number might be quite large, and might represent a similarly large number of pollen parents, they might represent very few seed parents, and exaggerated values of rare genetic factors would appear when a seed parent happened to carry such a factor. A request was made that at least five heads should be used; in the samples which arrived with the heads more or less intact, they could be counted.

A few samples with the heads more or less intact were grown up with each head kept separate, and points from these results are shown below (Figures V(ii), V(iii)).

Variation between progenies of different heads within a sample can have several causes, all of which may be involved in the cases to be described. These are: Variation between the maternal genotypes; Variation between the pollen populations to which they were exposed, resulting from difference in location or flowering time, or from the pollinating insect(s) having visited few, perhaps unrepresentative heads to collect pollen; Sampling variation in the production from diploid parents of particular haploid gametic genotypes involved in the fertilisations which actually produced the seed; and inaccuracies in scoring. A larger number of heads in the sample would reduce the effect of the second cause, and a larger number of seedlings derived from each mother plant would reduce the third.

It was hoped that the progenies would fall into clear classes according to maternal genotype. Using the total progeny as a sample representing the parental population, the frequency of unmarked plants may be taken as an estimate of v^2, where v is the frequency (presumed to be the same in the progeny and the parents) of the recessive allele. $1 - v$ is then the frequency of all other alleles, and the frequency of plants not containing the recessive can be predicted to be $(1 - v)^2$. Only a proportion of these are visibly double marked; let h be the proportion of these $(1 - v)^2$ constituted by the observed doubly marked plants in the progeny.

Considering mother plants lacking the recessive, a proportion v of their progeny will receive it from pollen parents, while a proportion $1 - v$ will lack it, will have two active marking alleles, and may develop double marks. If the progeny of sufficient such mother plants are combined, the mean frequency of double marks should be $h(1 - v)$. However, progenies of individual plants can be expected to have different values, depending on the probability of occurrence in the pollen of alleles which will produce double marks in combination with the alleles carried by each plant. Another plants carrying rare and distinctive marking alleles will produce more double marked progeny, those with common alleles, fewer than the prediction for the whole group.

The frequencies of the differently marked types of progeny in the aggregate progeny of each type of parent may be predicted as set out below.

Table 5.2. Predicted Phenotype Frequencies in Seed carried by Certain Maternal Genotypes.

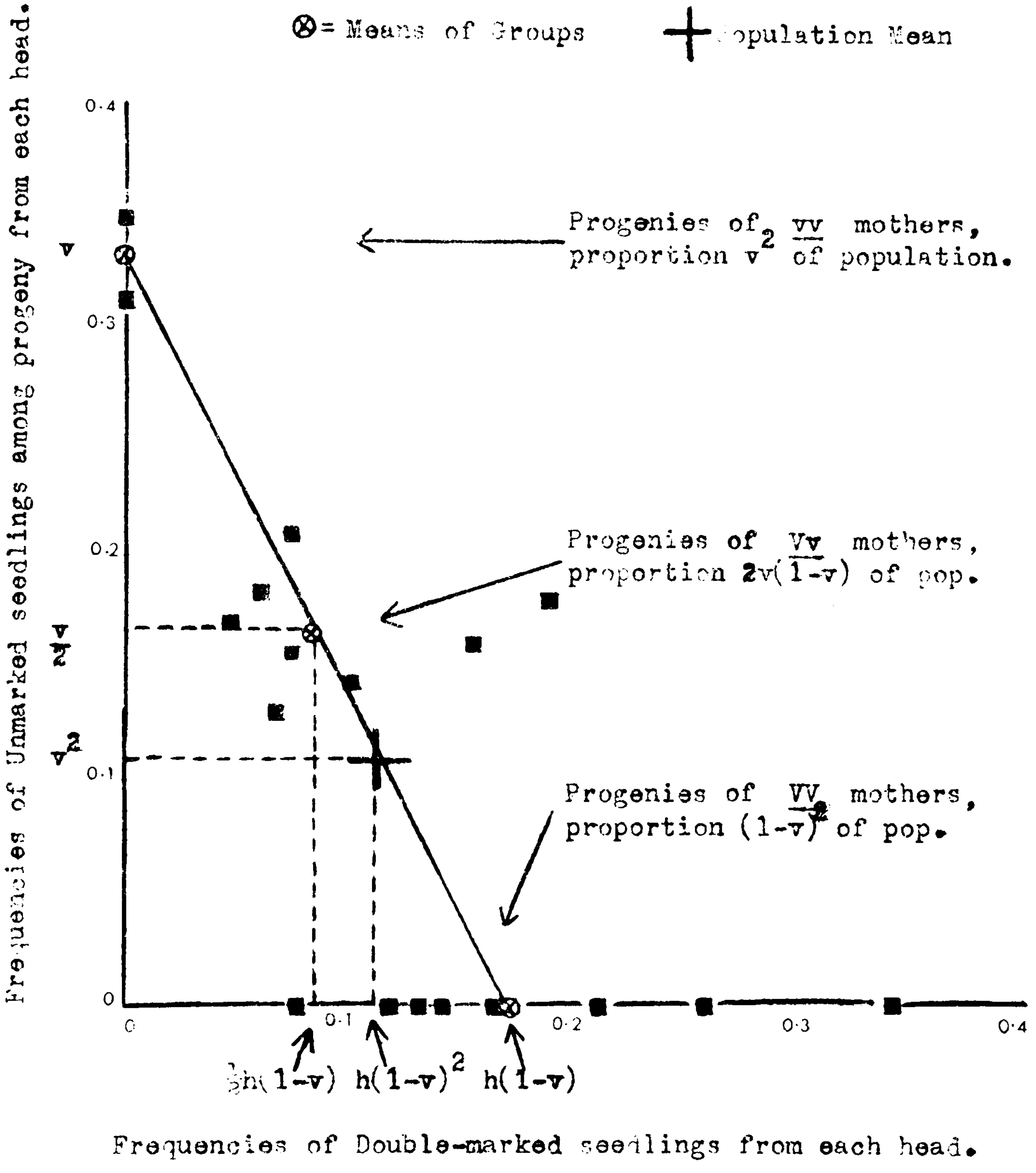
Female parental Genotype	Type of Progeny		
	Unmarked	Simply marked	Doubly marked
vv	v	1 - v	none
Vv	v/2	$\frac{1}{2}(1-v)+\frac{1}{2}v+\frac{1}{2}(1-h)(1-v)$	$\frac{1}{2}h(1-v)$
VV	none	$v + (1-h)(1-v)$	$h(1-v)$

Among the heads in each class, the frequency of unmarked plants should vary only as a result of variation in the frequency of the various alleles in the pollen, and the effect of chance in meiotic segregation; the frequency of double marked plants will also vary from the above values according to the particular alleles carried by the parent, and the predicted values are only means for the whole class of heads. Figure V(i) illustrates the predicted pattern of results.

Figure V (i). Theoretical Model for No-Mark and Double-Mark Frequencies in Progeny of Single Heads.

Arbitrary Values chosen for Model :

$v = 0.33$ $h = 0.25$



Lower frequencies of double marked progeny from mothers with commoner alleles.

Higher frequencies of double marked progeny from mothers with rarer alleles.

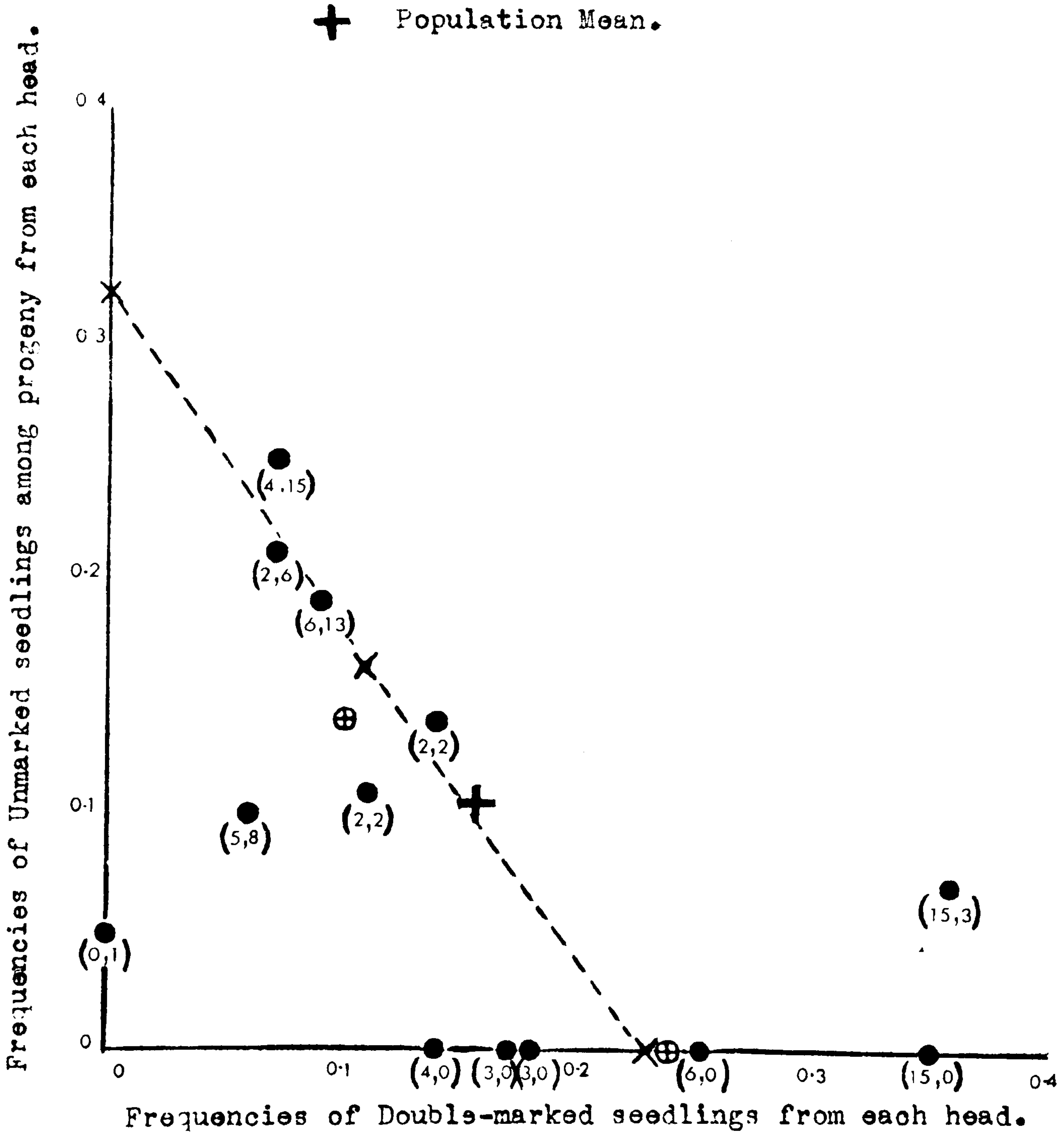
Figure V (ii). No-Mark and Double-Mark
Frequencies in Single Heads.

(a) Sample N 45.

✕ Predicted Group Means.

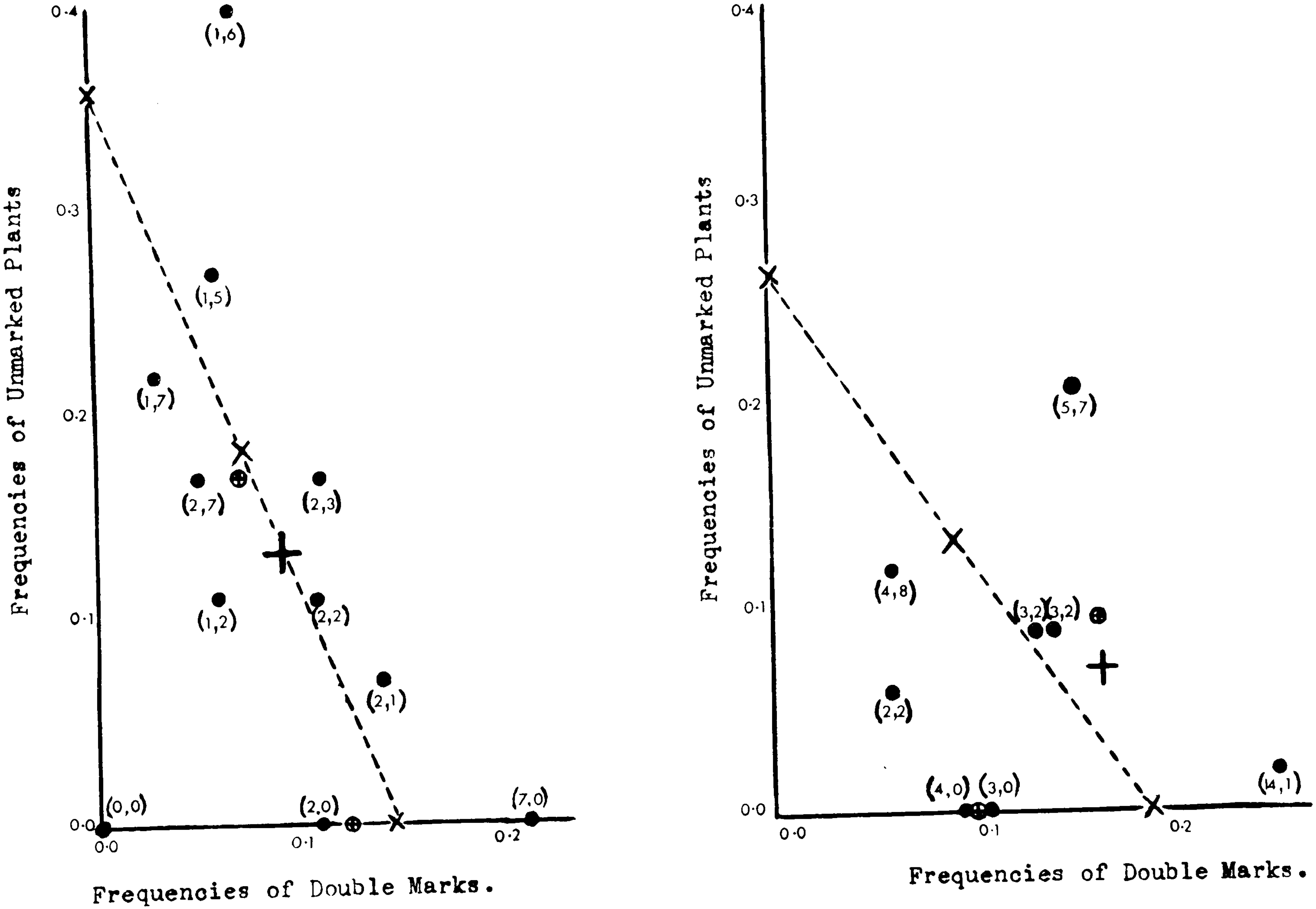
⊕ Means of Observed Groups.

✚ Population Mean.



Beside each point are shown the number of plants of each type (double-marked first) by which the point was plotted, to give an indication of the sampling error.

Figure V (iii). No-Mark and Double-Mark Frequencies in Single Heads


(a) Sample N42

(c) Sample N43

Table 5.3. Data from scoring progenies of single heads, used in preparation of Figures V(ii), V(iii).

Notation

0 (Number of) plants with unmarked phenotype (vv homozygotes)

D (Number of) plants with double marks

N Number of plants in whole sample

$v = \sqrt{O/N}$ Estimated frequency of recessive allele

$h = D/N(1-v)^2$ Proportion, showing double marks, of the calculated number of plants with two active simple alleles

(1) Numbers of Heads and Seedlings

(Loose seed, and heads with less than 12 seedlings scored, counted only in totals)

	Number of heads plotted	Median seedlings scored per head	Mean seedlings scored per head
N42	11	18	24.2
N43	8	32	40.3
N45	13	28	37.0

(2) Total numbers of different types

	N42			N43			N45		
	N	O	D	N	O	D	N	O	D
Progenies with no 0	74	--	9	69	--	7	130	--	31
Progenies with 0	175	33	12	230	22	31	336	50	36
Sum	249	33	21	299	22	38	466	50	67
Other Seedlings	17	3	3	23	1	6	15	--	8
Combined Total	266	36	24	322	23	44	482	50	75

(3) Calculations on whole samples

	N	O	D	$v = \sqrt{\frac{O}{N}}$	$h = \frac{D}{N(1-v)^2}$	h(1-v)
N42	266	36	24	0.368	0.226	0.143
N43	322	23	44	0.267	0.253	0.187
N45	481	50	75	0.322	0.340	0.231

((O,v), $(\frac{1}{2}h(1-v),\frac{1}{2}v)$ and $(h(1-v),0)$ are the predicted class means)

(4) Mean frequencies of Progenies plotted

	Whole pop.		Without 0		With 0		
	D	O	Progenies	D	Progenies	D	O
N42	0.090	0.135	3	0.122	8	0.171	0.069
N43	0.137	0.071	2	0.101	6	0.096	0.135
N45	0.156	0.104	5	0.238	8	0.149	0.107

The results for three samples in which the seeds from each head were grown and scored separately are shown, plotting the frequency of unmarked against the frequency of double-marked plants. The predicted values of these frequencies for the three classes of mother plants are also marked, together with mean frequencies for the whole population, and for the heads appearing to fall into each class.

There is little indication of the progenies containing both doubly marked and unmarked plants being clearly separated from the progenies lacking one type (and plotted on one or other axis), although there is some indication that the progenies without unmarked plants have a higher frequency of double marks than the progenies with both types. None of the progenies observed seems to fit the expected class of progeny of an unmarked plant, with no double marked and a high frequency of unmarked plants.

A clearer indication of the possible effect on a population sample of sampling variation when few female parents are chosen can be seen if rare, distinctive alleles are considered. A generally low frequency would be expected in all heads, derived from a low frequency of the allele in the pollen of the population, but there would also be occasional heads with strikingly different high frequencies, near 50 per cent, among the progeny, when the allele was carried by the mother plant. Table 5.4 shows the occurrence in the heads of sample N45 of phenotypes including the broken, and broken-with-yellow-tip phenotypes, B and Y, attributed to the alleles $\underline{V}^{b}$ and $\underline{V}^{by}$.

Table 5.4. Frequencies of some rare marks in separate heads in the same sample (N45) cf also p 86.

Head	Total Seedlings	Y Seedlings	B Seedlings	Y Frequency	B Frequency
A	24	0	0	-	-
B	19	0	5	-	0.26
C	17	0	0	-	-
E	28	1	0	0.04	-
G	70	0	0	-	-
H	18	0	10	-	0.56
J	83	0	0	-	-
K	59	0	0	-	-
L	42	0	0	-	-
M	43	9	8	0.21	0.19
N	20	0	0	-	-
O	29	2	1	0.07	0.03
Q	14	0	0	-	-
Other seed	15	1	0		
Total	481	13	24	0.027	0.050

The plant bearing head H (possibly also that bearing head B), would seem to be a heterozygote for $\underline{V}^{b}$; it is tempting to suppose that in progeny M also only one allele was involved, although confused in the scoring, the parent being heterozygous for it. The totalled progeny indicates a gene frequency for the two alleles together of approximately 0.04. It is rather surprising that eight of the thirteen heads, including the three largest progenies, show no trace of these alleles. It might be that each pollinator carries pollen from a very small number of heads, in which case rare alleles would either be entirely absent, or present at relatively high frequencies within each head's progeny.

Divergence of Morph Frequencies in Vegetative and Other Samples.

There is a source of confusion in the comparison between seed and vegetative samples which is of greater biological interest than the statistical problem described above. In most cases only a minority of seed was damaged in scarification, remained hard, or died after planting out: the seed sample is thus relatively unselected compared to a sample of genotypes actually established in the field, and may contain new recombinant genotypes which have never been exposed to most of the selective factors affecting this species. Even in so far as selection in the germination phase represented a genetic discrimination, this would only be one, rather distinct, part of the selection experienced in nature. It is possible that some plants could be established in the field and yet function very poorly as seed producers, and any genetic factors predominantly occurring in such plants would be underrepresented in a seed sample. Other genotypes might correspondingly maintain themselves in the population by producing many seed in spite of mediocre vegetative performance, and they would be overrepresented in a seed sample. Any systematic difference in the scores produced by the two methods could affect, for example, the comparison between the British Isles (11620 out of 12232 scores are of vegetative material) with the rest of the world (20530 out of 21041 scores are of seedlings).

Unfortunately no direct comparison of seed samples and vegetative samples from the same population is available. However, one comparison is available which would reveal some of the factors which would be expressed by any difference between seed and vegetative samples. As shown in Table 5.5, this consists of comparisons between a random sample of leaves, and a random sample of inflorescences (that is, of the leaf-marking phenotypes of the runners carrying particular inflorescences).

Table 5.5. Comparison of frequencies of marked and unmarked plants in vegetative and floral samples.

Site	Type of Sample	Number of marked	Number of unmarked	Percentage of unmarked
Blenheim 1	V	18	17	48.6
	F	23	8	25.8
Marston 1	V	45	9	16.7
	F	35	4	10.3
Marston 2	V	32	1	3.0
	F	29	1	3.3
Marston 3	V	23	6	20.7
	F	26	4	13.3
Marston 4	V	60	7	10.4
	F	56	4	6.7
Marston 5	V	44	5	10.2
	F	31	6	16.2
TOTAL	V	222	45	16.85
	F	200	27	11.89

V: Nearest leaf to sample point, or others on same stolon.

F: Nearest inflorescence to sample point followed back to find and score the leaves on the same stolon.

Overall, the difference between the two types of sample in frequency of unmarked plants is not significant $(0.2 > P > 0.1)$, with more unmarked plants in the vegetative sample. If it proved to be significant in a larger sample, it might be because some weak plants flowering little if at all might produce small leaves on which faint marks might not be expressed clearly enough for recognition; such plants, occurring only in the vegetative sample, could be scored as unmarked when they should be scored as marked.

4. The Sample Unit: The consequences of vegetative reproduction on the concept of the individual.

The capacity for vegetative reproduction by stolons which this species has means that each genotype can not only survive over long periods, but parts of it may come to be considerably separated in space, and the clone may at the same time increase considerably in size. At any time a population will contain clones of very different sizes and degrees of dispersion. It would be very difficult to sample in a way that would give each genotype an equal chance of being represented; even if repeated countings of the same genotype could be eliminated, it would be impossible in a sample, as opposed to a complete population study, to eliminate the greater chance for a small clone of being omitted from the sample. Different clones interpenetrate even when intact, the physical connections between parts of the same clone quickly disappear, and parts of the clone might lie separated by an area from which the clone was absent (cf. Harberd, 1963). Genetically identical stolons could only be identified by an elaborate comparison of genetically controlled characters after growth under standard conditions, particularly by identity of self-sterility alleles leading to mutual sterility between parts of the same clone (cf. Harberd, 1958, 1961). Even if such a sample of genotypes was possible, it would not represent a single set of zygotes, but a selected set of zygotes derived in different numbers from many years' production of seed.

The sampling methods which may most easily be devised sample leaves, and a clone's chance of being included in the sample therefore depends on the number of leaves it carried. Increase in size of a clone represents a positive selective adjustment within the population as much as diminution or elimination of another genotype represents a negative effect of selection - the clones represented more than once in a sample are probably those whose large extent reveals their successful adaptation to the environment. Such a sample is therefore one phase of selection further on in the life cycle than the seedlings from which it arose, and this selection represents part of the selection which will determine how much seed will be produced; all other things being equal, more seed will be formed on the larger plants, and in so far as a leaf sample gives different representation to larger and smaller clones, it is providing a prediction of the effect variations in size will have on the next seed population.

It is worth noting however, that Hardy-Weinberg type calculations of allele frequencies have less validity when applied to such a sample than when applied to a set of seed produced at the same time and unselected. Heterozygotic advantage, one of the classic factors producing stable polymorphisms, would have its maximum effect in a sample which had been exposed to selection at all stages from fertilisation to vegetative maturity. Such a sample might include a higher proportion of the heterozygotes than random assortment would predict.

Although a leaf sample may be regarded as adequately representing the population, the study of variation in clone size remains of interest in connection with the comparability of seed and vegetative samples. Knowledge of the extent of variation in clone size would indicate the scale of importance of one of the processes by which an established vegetative population would come to differ from the populations of seedlings from which it was derived. Study of clone size would also provide information about the possible achievements of vegetative reproduction. Where there is continuity in time and space, the functions of persistence and dispersal could be carried out in this species by purely vegetative means. It certainly seems likely that in a dense grassland community, so long as there is little disturbance to produce open bare soil, establishment by newly germinating seedlings may be very rare indeed. In such a situation, which might be semi-permanent, a seed sample would not be a part of a cycle, providing a preview of the genetic material of a future population; and genotypes might become widespread in the population which had very low sexual fertility, and which therefore would make little contribution to a seed sample.

The species is variable enough that many clones can be definitely seen to be different on a very rapid examination, and a population of plants growing in the same environment can be divided into a number of phenotypic groups different from each other, each of which may consist of more than one, or of only one clone. Although the possibility that two similar plants are not the same clone cannot be dismissed until after prolonged growth under standard conditions, and demonstration of identical S-allele genotypes producing mutual sterility, the distribution of such apparent clones provides at least an indication of the results which might be obtained from a more thorough study.

Even if plants of a species mapped are described in only two genotypic classes, knowledge of the size of patches of each type is one step towards estimating a lower limit to mean clone size (though the interpenetration of different clones may confuse the picture). The opposite approach to the problem of clone size and age is provided by studying physical connections between growing systems and deducing past connections from the orientation of living material. The most intensive study of this kind known to me is that of Prime (1955a). Apart from Harberd's work (1961, 1962, 1963), other noteworthy studies on vegetative reproduction and persistence in plant populations are those of Söyrinki (1938, 1939), Lieth (1960) and Tamm (1964).

A brief study on this topic was carried out by the author on a dense patch of clover near a gateway in a field at the Oxford University Farm, Wytham. The scarcity of grass may have been due to trampling and puddling of the soil by cattle passing through the gate during the previous winter, and if so, some or much of the clover may have been recent seedlings (the observations were made in mid-May). Whatever its origin, the density of clover made this a rather unusual area, but also made it suitable for this investigation.

The area, 3.0 x 1.2 metres, was divided into 360 quadrats, 10 cm. square. In all, 19 apparent clones could be distinguished, by examination of leaf mark, leaf colour, and leaf size and shape. The phenotypes occurring in each quadrat were recorded. The results are summarised by Table 5.6, and illustrated by Figures V(iv) and V(v). The results show a very great variation in the size of the apparent clones, and also that the distinct phenotypes interpenetrate quite freely.

Table 5.6. Mapping of Apparent Clones.

Area: 3.0 x 1.2 metres

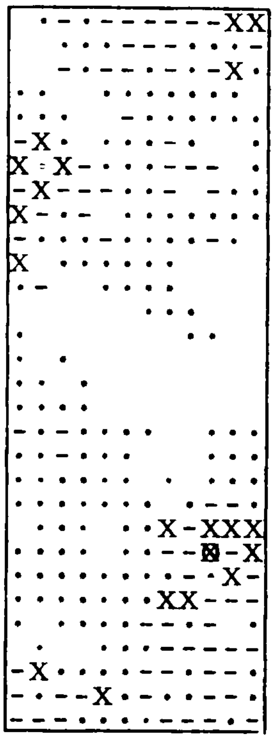
360 x 10 cm. sq. quadrats

Distinct Phenotypes per quadrat	Number of quadrats	Number of records
0	0	0
1	89	89
2	176	352
3	75	225
4	19	76
5	1	5
	360	747

Designation of phenotype	Number of quadrats containing each clone
6	314
8	139
1	110
15	46
4	36
13	19
18	17
5	15
11"	14
14	9
11'	8
12	7
17	4
7	2
10	2
16	2
3	1
9	1
19	1
19 phenotypes	747 records

Figure V (iv).

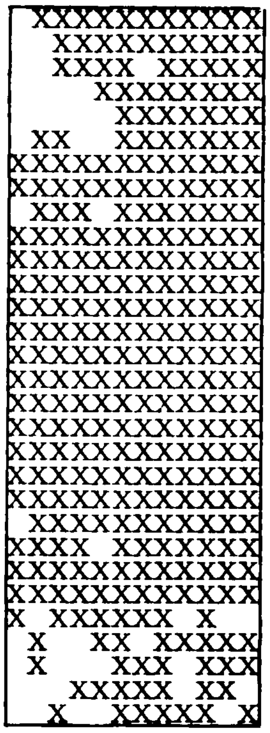
Distribution of Apparent Clones of <u>Trifolium</u> <u>repens</u>.
Area mapped: 1.2 x 3.0 metres, in 10 cm. sq. quadrats.



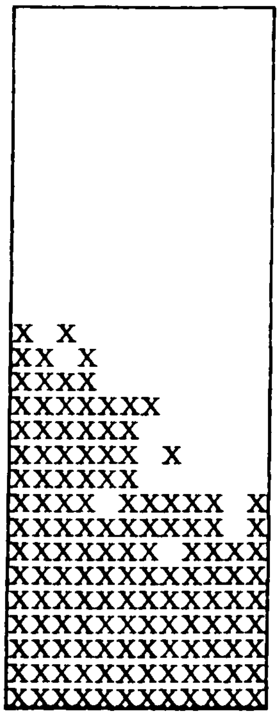
Clones / Quadrat

Key to Map of
Clones / Quadrat

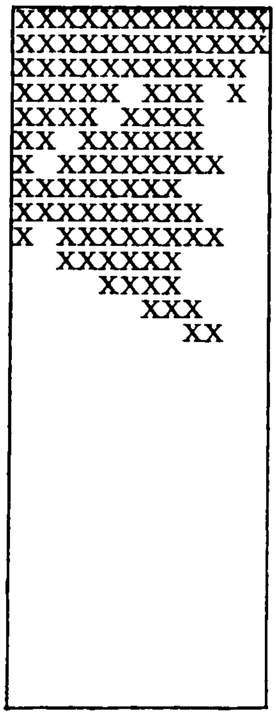
	1 clone
•	2 clones
-	3 clones
x	4 clones
⊠	5 clones



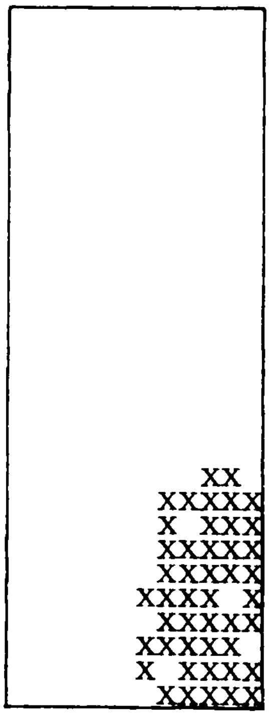
Clone 6



Clone 8



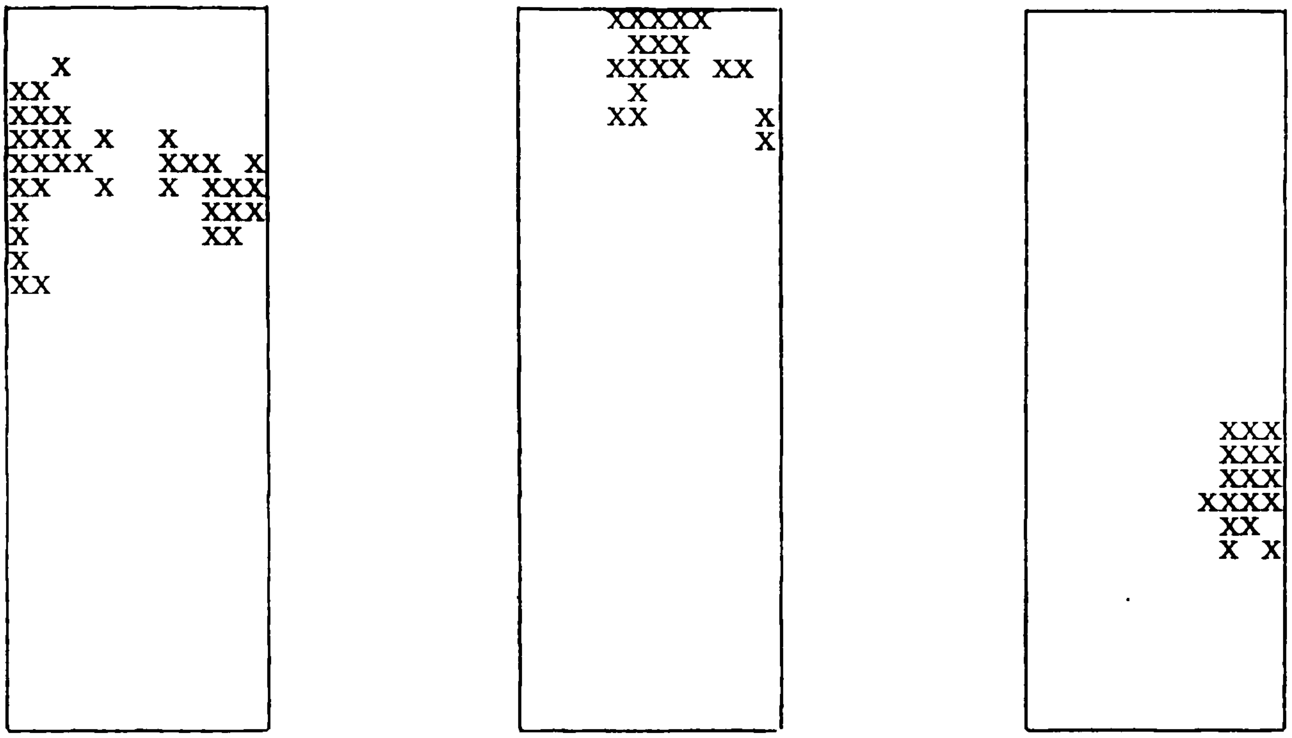
Clone 1



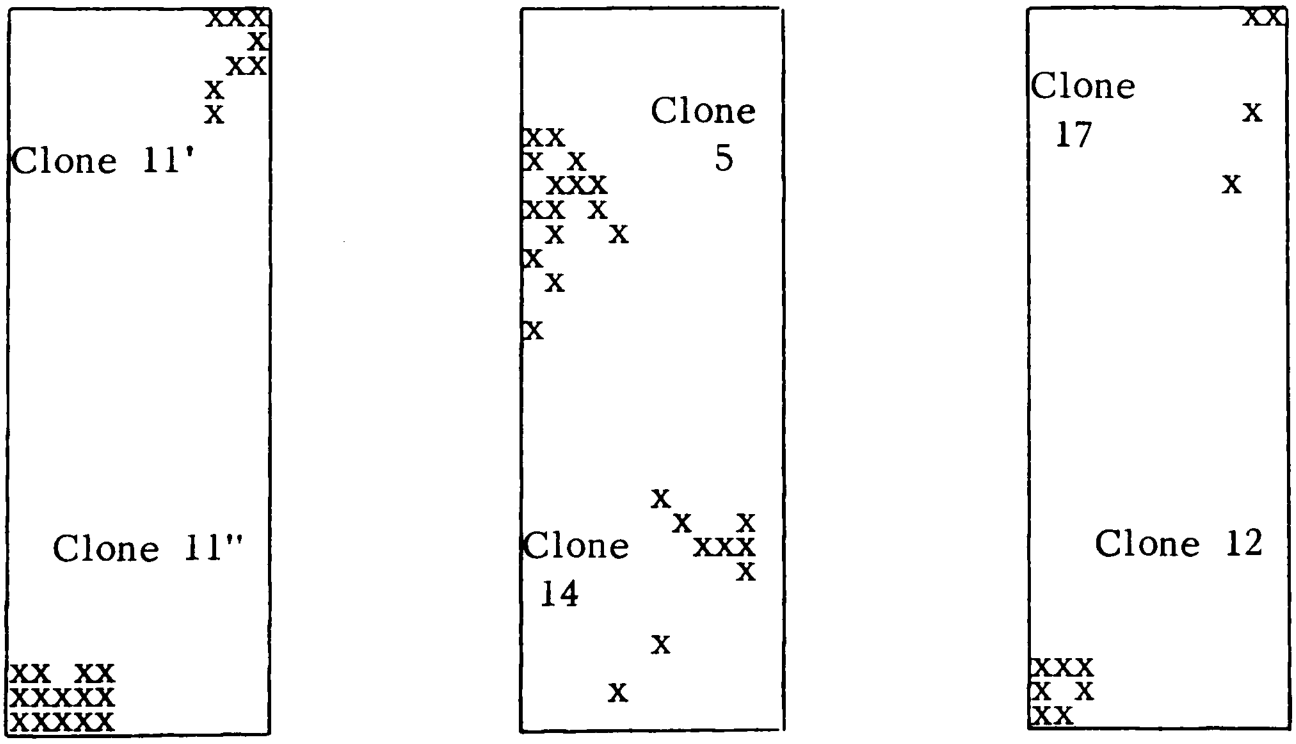
Clone 15

Figure V (v).

Distribution of Apparent Clones of *Trifolium repens*.
Area mapped : 1.2 x 3.0 metres, in 10 cm. sq. quadrats.



Clone 4 Clone 13 Clone 18



PART 3

POPULATION SAMPLING

CHAPTER VI

MATERIALS AND METHODS

1. Sources of Material and Data.

The intention of the work was to combine the study of vegetative samples collected and scored in the field in Britain with the study of the plants grown up from seed samples obtained as widely as possible in the species' range overseas. Requests for seed samples were made to Universities, Agricultural Research Departments and FAO Representatives in a large number of countries both in the species' natural range and in areas of recent introduction associated with man. The requests were made by means of a circular letter of the type described by Daday (1954a), requesting five or more seed-bearing heads of _T_. _repens_ from ecologically differing sites around the area in which the person addressed was working. Samples were to consist of plants which were wild or established as wild, and a request was made for information about the nature of the habitats chosen. The response varied, from close adherence to the terms of the request, to the sending of packets of commercial varieties or of other species, or to no reply at all. Because of limitations of time, materials and space, not all the material received was used. An attempt was made to obtain a balanced coverage of the natural range (especially of areas not covered by samples obtained from other sources described below), and the samples not used were mostly from areas where there were many other samples, or from outside the natural range.

Vegetative samples were collected around Oxford and on field trips to North-East England, Yorkshire, the East Midlands, Sussex, and North Wales. They were taken from agricultural land as well as from more natural and less disturbed habitats, but the samples were mostly taken where it seemed likely from the appearance of the land that ploughing had never occurred or would have been impossible because of the steepness or irregularity of the ground, or where local enquiry obtained information that the land had definitely not been ploughed to the informant's knowledge.

During the course of the work opportunities became available to include data from two other sets of samples. The first were plants grown at the Agricultural Institute, Oak Park, Carlow, Eire. These were many samples of widespread origin, mostly obtained through the United States Department of Agriculture Plant Introduction Service, and intended to provide material as a basis for a selection programme for early growth in spring. I was able to score these plants in person during a week's visit to Carlow, and to obtain information, there and from the U.S.D.A., about the origin of the samples. This always gave the country of origin, but often no more details.

The second set of material was data recorded by the staff of the Welsh Plant Breeding Station on a number of samples grown there over the last ten years, comprising both seed and vegetative material from a wide variety of sources, mostly outside Britain.

Cultivated strains were not included in the seeds used in Oxford, but both the Carlow and W.P.B.S. collections contained some samples which were definitely of cultivated origin, and many more of uncertain origin which may well not have been wild. Many of these were probably local varieties selected only on a bulk basis, and probably representing the effects of the local natural selective forces fairly well. Exclusion of all the cultivated and doubtful varieties would have meant a considerable reduction of the amount of data, and particularly of its geographical coverage (cf. Appendix I). The only samples omitted therefore have been cultivated strains within the British Isles, for which area more samples are available with fuller backgrounds, and cultivated strains which had been grown outside their region of origin, and therefore did not represent an equilibrium with selective forces of any area.

The scoring system used by the Welsh Plant Breeding Station is based, like my own, on the reports and terminology of Brewbaker and Carnahan, and differs mostly in the retention of discrimination between high and low simple V's. The following table describes the results recorded in Oxford by myself and at the W.P.B.S. for three seed lots, obtained from the FAO, of which material was received and grown in both places.

The results show that the two scoring systems do not produce incompatible results. The phenotype labels are my own and represent a 'translation' of those initially used by the W.P.B.S. whose discrimination between L and H is also ignored.

Table 6.1. Comparability of author's and W.P.B.S. Scoring.

Comparison of phenotype distributions in samples from the same batches of seed scored at Oxford, and at Aberystwyth by W.P.B.S. staff.

		Total	O	L+H	LL	Y	LY	FY	F	LF
Belmonte - Guarda Portugal FAO 3584	Oxford N4	99	20	47	1	20	8	1	-	2
	WPBS 2777	49	8	32	-	9	-	-	-	-
Ramada Portugal FAO 3608	Oxford N5	71	-	59	8	-	2	-	2	-
	WPBS 2778	60	-	44	16	-	-	-	-	-
Koutsoufliana Greece FAO 3642	Oxford N6	71	-	50	18	3	-	-	-	-
	WPBS 2779	56	-	49	4	3	-	-	-	-

Table 6.2 summarises briefly the contributions made by the different sources mentioned. A fuller table in Appendix I breaks this down in more detail.

The term 'sample' throughout refers to a group of plants, or the seedlings grown from a group of heads, collected in the same area by the same person on the same occasion. The word 'sample' always refers to a set of plants (in two or three cases only, a collection consisting of a single plant, or from which only one plant survived to be scored).

Table 6.2. Summary of origins of the material providing data.

	Numbers of Samples	Numbers of Plants
Vegetative samples, scored in the field in Britain	192	9497
Vegetative material grown in greenhouse and scored by author	23	377
Seed, mostly foreign, grown and scored in Oxford	72	6504
Vegetative material from S. Ireland, scored at Carlow	19	900
Seed, mostly foreign, scored at Carlow	172	8928
Vegetative material, mostly British, scored by W.P.B.S.	55	1136
Seed samples, mostly foreign, scored by W.P.B.S.	153	6034
Total	685	33273

2. Procedures used in Sampling

i) Collection of Vegetative Samples.

The aim in choosing the plants for these samples was to obtain a fairly random and representative sample with a minimum of time-consuming complication, so that samples could be collected from as many sites as possible.

The quickest method, chosen for most of the samples, was to walk irregularly within an area of twenty or thirty yards across, stopping at regular intervals (every second, third or fourth pace) and studying the leaf nearest to the tip of the forward foot when one halted, if there was one within a two-foot diameter circle in front of and touching that point. If the phenotype was not clear, and particularly if the plant appeared to be unmarked, the stolon bearing the leaf was followed back until other leaves had been found which made a more certain decision possible. A sample of forty or fifty plants was generally aimed at, though smaller numbers occur where the species was hard to find, or shortage of time limited the extent of the search, and larger ones where more time was available and no alternative second site was available. In some cases the path followed was more extensive and regular, especially if there was some indication of heterogeneity in the area of vegetation chosen. Where such heterogeneity was very marked, or where the results of the scoring suggested heterogeneity whether anticipated or not, soil samples were taken separately in the different areas.

ii) Description and Measurement of Environmental Factors.

Soil samples were collected, and pH values obtained, for about two-thirds of the sites sampled. Turves approximately one inch cube were cut out and brought back to the laboratory. Duplicates were collected from the sites near Oxford for which pH values were quoted, and at Aberystwyth; pH values were then obtained separately and averaged. Soil was obtained from the underside of these turves, corresponding therefore to a depth between half an inch and an inch <u>in situ</u>. A record was kept of the colour and texture of the soil.

For estimation of pH, a small volume of soil was placed in a glass tube, and an approximately equal volume of distilled water was added. The mixture was shaken thoroughly and allowed to stand. The liquid was then poured into the sample holder of a battery-operated portable pH Meter (Analytical Measurements Ltd., Richmond, Surrey), and a reading to one tenth of a pH unit taken after thirty to sixty seconds when the needle had stopped moving.

One possible source of error in these values arises from the length of time the samples were kept before scoring; often several days, sometimes up to a fortnight. Those from North Wales, particularly, were mostly very damp when collected, and were kept for about a fortnight in more or less air-tight conditions before scoring. Their low pH values could be partly due to the accumulation under these conditions of carbon dioxide produced by decomposition of organic matter.

The location of the site as a grid reference to the nearest tenth of a kilometre, and the altitude, generally to the nearest ten feet, were recorded for all samples. Notes of a more qualitative kind were also taken of the steepness of slope, aspect, and degree of shelter or exposure of the site, of any indications of water shortage or excess, of the type of vegetation with some details of associated species, of the type of agricultural treatment, and if possible of the kinds of large grazing animals on the site.

(iii) Procedure used in Handling Seed Samples.

When the sample was larger than about 100 seeds, only about that number were used. Where there were distinct heads, an attempt was made to produce a representative rather than a random sample by taking a group of florets from each head.

In accordance with the results of the seed scarification experiments described above, a scarification treatment of 20 minutes was used on the seed samples, and repeated on any hard seed left after the germination treatment. This consisted of exposure to light and warmth on moist filter paper pads in petri dishes covered with a plastic cover with a ventilation hole. The seedlings which germinated were plated out, usually with the cotyledons fully spread and about half an inch of root, into seed boxes at a spacing of approximately 1½ inches. A certain number died because of slug damage, and through drought when watering arrangements failed. In some samples the number of survivors was reduced to less than a quarter.

Final scorings were not carried out until nearly all the plants had produced at least six leaves, by which time many plants had begun to produce stolons.

3. Scoring and Expression of Results.

a) Initial Scoring.

Scoring was carried out and described basically in terms of the phenotypes attributed to the alleles described by Brewbaker and Carnahan, as shown in Table 6.3 (cf. Table 3.1, page 42 above).

Table 6.3. Correspondence of Allelic Symbols and Phenotypic Symbols used in Scoring.

Allele	v	V^{l}	V^{h}	V^{by}	V^{b}	V^{f}	V^{ba}
Phenotype	O	L	H	Y	B	F	Ba

Double phenotypes are described by a combination of two of these symbols: e.g. LY, LBa, YF, presumed to be respectively $\underline{V}^{l}\underline{V}^{by}$, $\underline{V}^{l}\underline{V}^{ba}$, $\underline{V}^{f}\underline{V}^{by}$.

The hardest discrimination was between Y, and B or derived phenotypes. The class Y was used to include all cases, with or without a yellow tip, if the mark was white rather than bluish, and if the inner end of the mark tapered, approached more than half way to the midrib, and appeared to lie on a relatively acute V, especially near the midrib. For some marks with a clear colour difference between a yellow-greenish tip and bluish silvery wings, the choice between Y and BL had to be decided, sometimes with great difficulty, according to whether the central yellower portion had distinct wings or was only a triangular tip. (cf phenotype described on pp 82-83)

Many plants, while showing traces of filling-in - the colour proximal to the mark being paler than that distal to it - seemed much closer to the $\underline{V}^{l}$ phenotype than to the $\underline{V}^{f}$ phenotype as previously described. These were at first scored as Lf, but have all been classed as F below.

It became clear early in the scoring that the clear distinction of a class of high simple marks, suggested by the reported existence of only $\underline{V}^h$ (high) and $\underline{V}^l$ (low) and perhaps $\underline{V}^i$ (intermediate) marks, was impossible to apply consistently, and that if the simple mark phenotype was to be divided at all, many more forms would have to be distinguished. The distance separating the marks in some double-marked forms was less than a quarter of the separation in others, which seemed to be conclusive proof of heterogeneity among what was called the L class. All simple marks appear together as L, and all double marks combining two simple marks as LL.

Notes were made of unusually high marks, though it is not claimed that there was a consistent standard. In the Carlow scorings, a class of L_H marks was described as well as H, In the Oxford seedling scorings, each mark was described in terms of position, shape and intensity. The position scores were in five grades, l, l-m, m, m-h, and h. The H description occurred quite frequently in the W.P.B.S. scoring system, and area by area, a closer fit to it was reached in the other two groups of samples by including the intermediate as well as the extreme class. The H frequencies appearing below are therefore a combination of the three different (and possibly internally inconsistent) standards. Very few scores of high marks were made in the British field samples, and these samples are omitted from the H-frequencies, as these low values represent disinclination to use this rather vague description at least as much as a possible real low frequency.

Corresponding to Brewbaker and Carnahan's $\underline{V}^{p}$ allele (V-point) the W.P.B.S. scoring system distinguished a phenotypic class P, which has been added into the L class in the tabulations below of the data obtained from W.P.B.S. records. The possibility of a clear dividing line seems to be even less than between H and L, and no attempt was made to use this category in the majority of my own scorings.

Other details noticed in scoring or in plants kept as specimens of unusual phenotypes concern very rare phenotypes, and are not presented below, details of the examples of these phenotypes having been given above. These include the marginal phenotype, incorporated with the totals for the broken phenotype in the two Italian Alps samples in which it appears, some relatively distinctive simple marks, included in scores below as L, and a number of anthocyanin-mark phenotypes, including those associated with the $\underline{V}^{by}$ allele.

b) The possibility of Calculating Gene Frequencies from the Data.

The simplest case is that of rare alleles whose expression is assumed always to be recognisable. If they are rare, virtually all plants recorded as showing them will be heterozygotes, and a close estimate of gene frequency can be made by halving the phenotype frequency. A frequency $\underline{p}$ of a dominant allele produces phenotypes showing its presence with a frequency of

$2p(1-p) + p^2 = 2p - p^2$. If p is small, this expression is approximately equal to 2p. The inaccuracy in estimation of gene frequency in this way is an underestimate of the true frequency by a proportion of the true value p/2.

For a recessive allele, the frequency of the gene can be estimated as the square root of the frequency of the recessive phenotype. In this case, the unmarked phenotype has a frequency generally below 30 per cent, and this means that estimated gene frequencies are considerably larger than the frequencies of the recessive phenotype, which may be based on a count of only one or two plants; the transformation to gene frequencies greatly exaggerates the effect of this sampling error.

The frequency of a frequent dominant allele cannot be accurately estimated by a simple halving, as above. It must be calculated by grouping all the other alleles as a single class of recessives, and calculating their frequency as the square root of the frequency of all phenotypes not expressing the allele in question. The estimate of the dominant allele's frequency is the difference between this figure and unity. The same procedure can be used for a group of alleles together, for example the whole class of simple V-alleles. It is possible in principle to treat all the alleles in this system as dominants except the no-mark allele, since the heterozygotes between the other alleles are all supposed to express both alleles they contain. However, there is evidence that this does not happen reliably in practice in respect of the heterozygotes between $\underline{V}^{by}$ and at least the simple $\underline{V}$-alleles, these two classes together accounting for the vast majority of phenotypes other than the unmarked types. Since it is not possible to regard $\underline{V}^{l}$ as dominant in plants carrying $\underline{V}^{by}$, a frequency estimate for $\underline{V}^{l}$ can only be made among the other plants. ($\underline{V}^{by}$ is not so common that this means an important reduction in the accuracy of the estimate.) This provides an estimate of the frequency of $\underline{V}^{l}$ among alleles which are not $\underline{V}^{by}$, and the appropriate correction must be applied to this figure if it is desired to express $\underline{V}^{l}$ frequency in the whole population.

c) Choice of Phenotype Frequencies as Basis for Presentation and Statistical Analysis of Data.

The greatest source of inaccuracy in the conversion of this data to gene frequencies is the exaggeration of sampling error in estimates of the recessive allele's frequency on the basis of a small number of plants. This results from the square root transformation needed to convert phenotype into gene frequency, and the error is at a maximum in the range of frequency of the recessive with which we are concerned.

Because of this and of the complexity of the calculations needed to determine the other allele frequencies, the total of all estimated frequencies often shows considerable divergence from unity. The calculations also involve unproven assumptions about random mating and random sampling, and indeed about the whole genetic system controlling these markings.

It was therefore decided not to use gene frequency calculations where avoidable, and the frequency figures presented below and used in the statistical analysis are basically frequencies of phenotypic classes. Seven morph frequency parameters have been used. The first is the frequency of the unmarked form. The next four are the frequencies of all plants showing respectively the broken-with-yellow-tip, the broken, the basal, and filled-in marks. That is, the 'Y' figure is the summed frequency of Y, YL and YH, YF, etc. The terms Y, B, Ba and F are used inclusively in those cases where double marks are not referred to separately. As was pointed out above, for rare dominant alleles these phenotype frequencies are close to twice the gene frequencies which could be deduced from them, and so any conclusions drawn will be virtually the same, with the saving of an elaborate mathematical step of doubtful soundness. All plants appear in one of the above classes, or in the scores from which the L class below is derived. The marginal and derived phenotypes are counted as contributing to the B class.

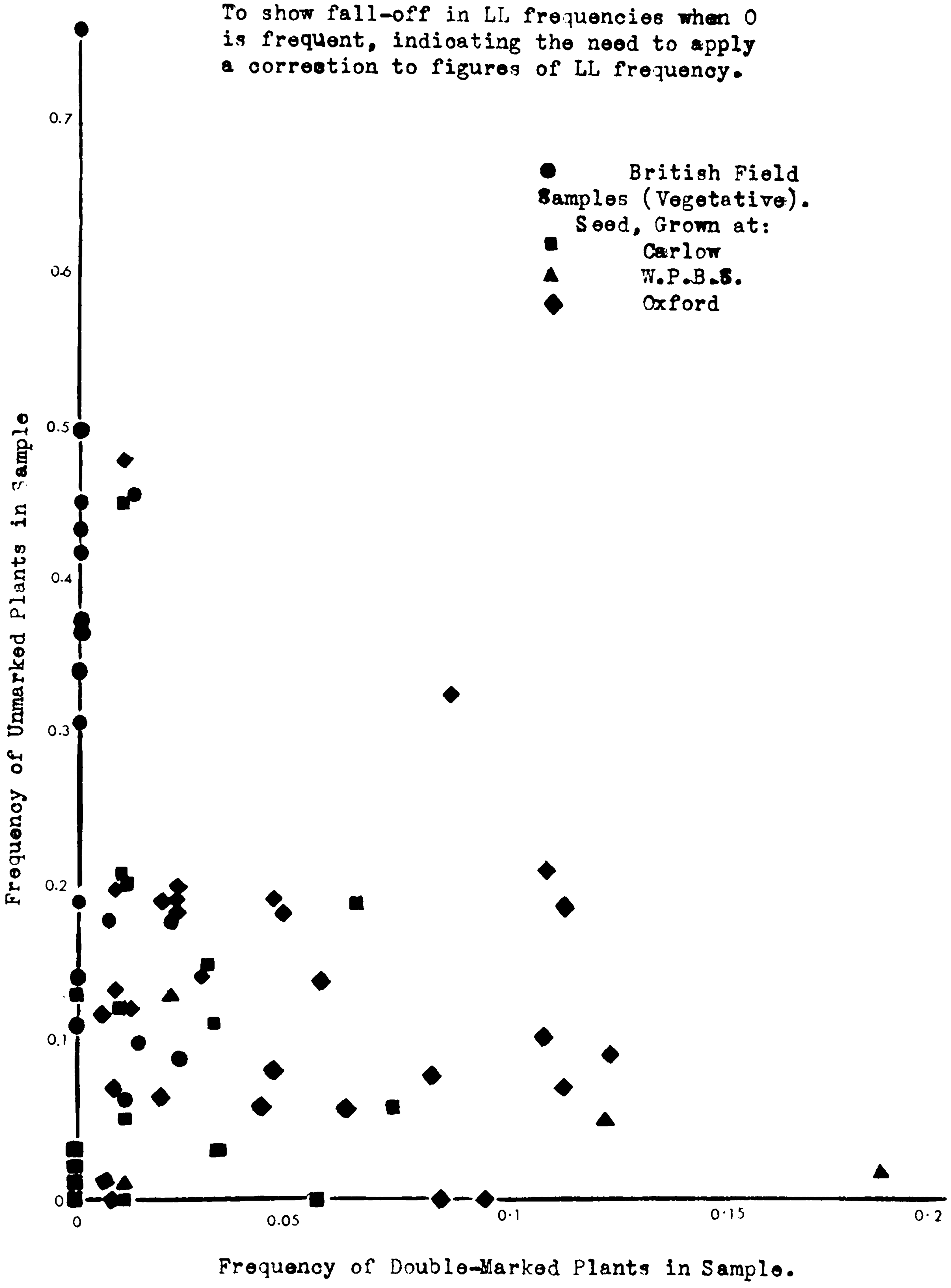
The last two parameters are less simply derived, but are intended to describe the frequency of the simple 'L' marks, and of the LL double marks between members of this class. In both cases allowance has been made for the partial dominance of $\underline{V}^{by}$ over $\underline{V}^{l}$, as a result of which a proportion of $\underline{V}^{by}\underline{V}^{l}$ plants are believed to have been scored as Y rather than YL. (Some evidence for this dominance has been presented above, and more will be presented in the next chapter.) The measure of L frequency stands seventh, and consists of the frequency of plants showing one or more L marks among the plants not showing By marks. Any YL scores therefore do not contribute, and LL plants are only scored once, while other double mark plants, e.g. LBa, LF, are counted twice, once in this and once in another group. This measure of L frequency refers to frequency among the class of marks excluding By, rather than to absolute frequency in the population. It also distinguishes and separates, better than a direct L frequency measure would, the distribution of L from that of O. In about half the samples these (as fractions of N) are each other's complements, and together include all the sample, and therefore map virtually the same distribution pattern, one positively, the other negatively.

Standing sixth is the figure representing the frequency of LL scores. This is conceived to represent the amount of diversity among the alleles in the L class, in a way that would depend on frequencies of distinctive alleles as well as the number of different inter-allelic combinations which could produce recognisable double marks. It is calculated by expressing the observed number of double-L plants scored in a sample as a proportion of the calculated number of plants containing two alleles in the $\underline{V}^1$ class. This was decided after it was noticed that there was a lower proportion of LL in samples with high frequencies of O (the unmarked type). This can be seen from the scatter diagram in Figure VI(i), which shows O and LL frequencies for the largest samples. This negative correlation was attributed to presumed high frequencies of recessive alleles concealed in other phenotypic classes such as L (containing $\underline{V}^{1}\underline{v}$ plants as well as $\underline{V}^{1}\underline{V}^{1}$). However heterogeneous the alleles classified as $\underline{V}^{1}$ were, few or no double marks would be seen if most of them were combined only with the recessive; the test whether two $\underline{V}^{1}$ alleles are different enough to produce a recognisable double mark only arises when they are combined together. The calculated figure is still a very complex derivative, depending on the frequencies and degrees of distinctness of some unknown number of alleles, but by this treatment the contribution to it of the frequency of the unmarked allele at least is allowed for.

Allowance is also made in these calculations for the effect of dominance by $\underline{V}^{by}$. If $\not{Y}$ is the number of plants not showing a By mark, and $\not{L}\not{Y}$ is the number showing neither a By nor an L mark (mostly unmarked), the frequency of the $\underline{V}^{1}$ allele group among the non-By plants (and among the non-$\underline{V}^{by}$ alleles) can be estimated as $1 - \sqrt{\not{L}\not{Y}/\not{Y}}$. The square of this is then a prediction of the frequency, again among non-By plants, of $\underline{V}^{1}\underline{V}^{1}$ plants. Multiplying this by the number of non-By plants, $\not{Y}$, gives a divisor which can be used to derive a frequency of double marks among this number, from the number of double marks actually scored. The final expression for LL frequency is

Figure VI (i)
Frequencies of Unmarked and Double-marked Seedlings in all samples of 90 or more.

To show fall-off in LL frequencies when 0 is frequent, indicating the need to apply a correction to figures of LL frequency.

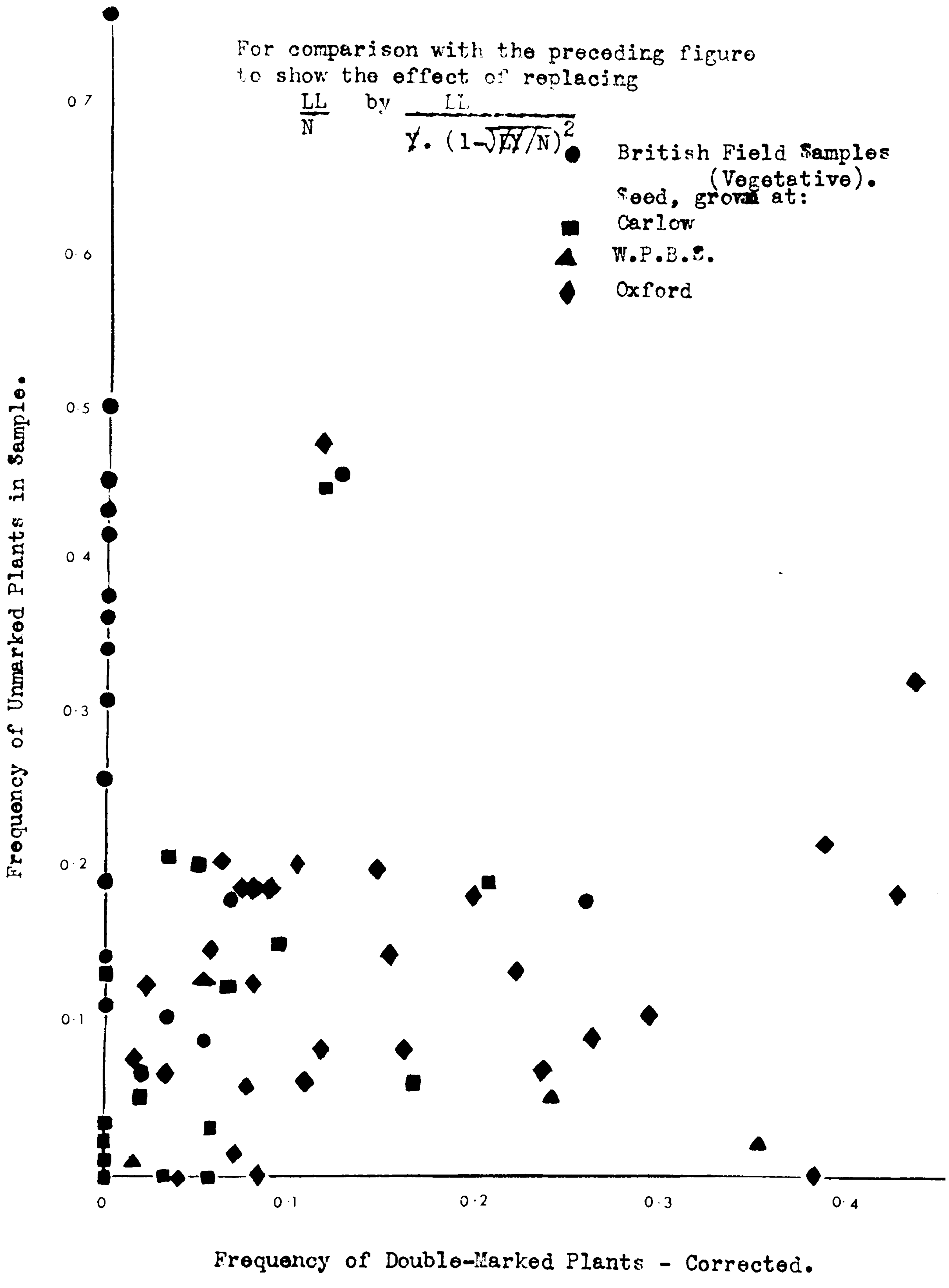


then

$$\frac{LL}{\cancel{Y}.\ (1 - \sqrt{\cancel{L}\cancel{Y}/\cancel{Y}})^2}$$

which is the proportion, among the calculated number of plants carrying two $\underline{V}^1$ alleles, which are scored as double-marked. For an O frequency the same as the overall mean, this would be two or three times the directly expressed frequency of LL plants in the whole sample. Figure VI(ii) shows a plot of the same samples as Fig. VI(i) after correction.

Fig. VI (ii)
Frequencies of Unmarked and Corrected Frequency of Double-Marked Seedlings in all Samples of 90 or more.



For comparison with the preceding figure to show the effect of replacing $\frac{LL}{N}$ by $\frac{LL}{Y.(1-\sqrt{LY/N})^2}$

CHAPTER VII

RESULTS I. INTERNAL COMPARISONS OF SAMPLES

1. Generality of Polymorphism, and Occurence of Unmarked Type.

Some conclusions can be drawn from a study of the sample data before attempting to investigate correlation with environmental factors.

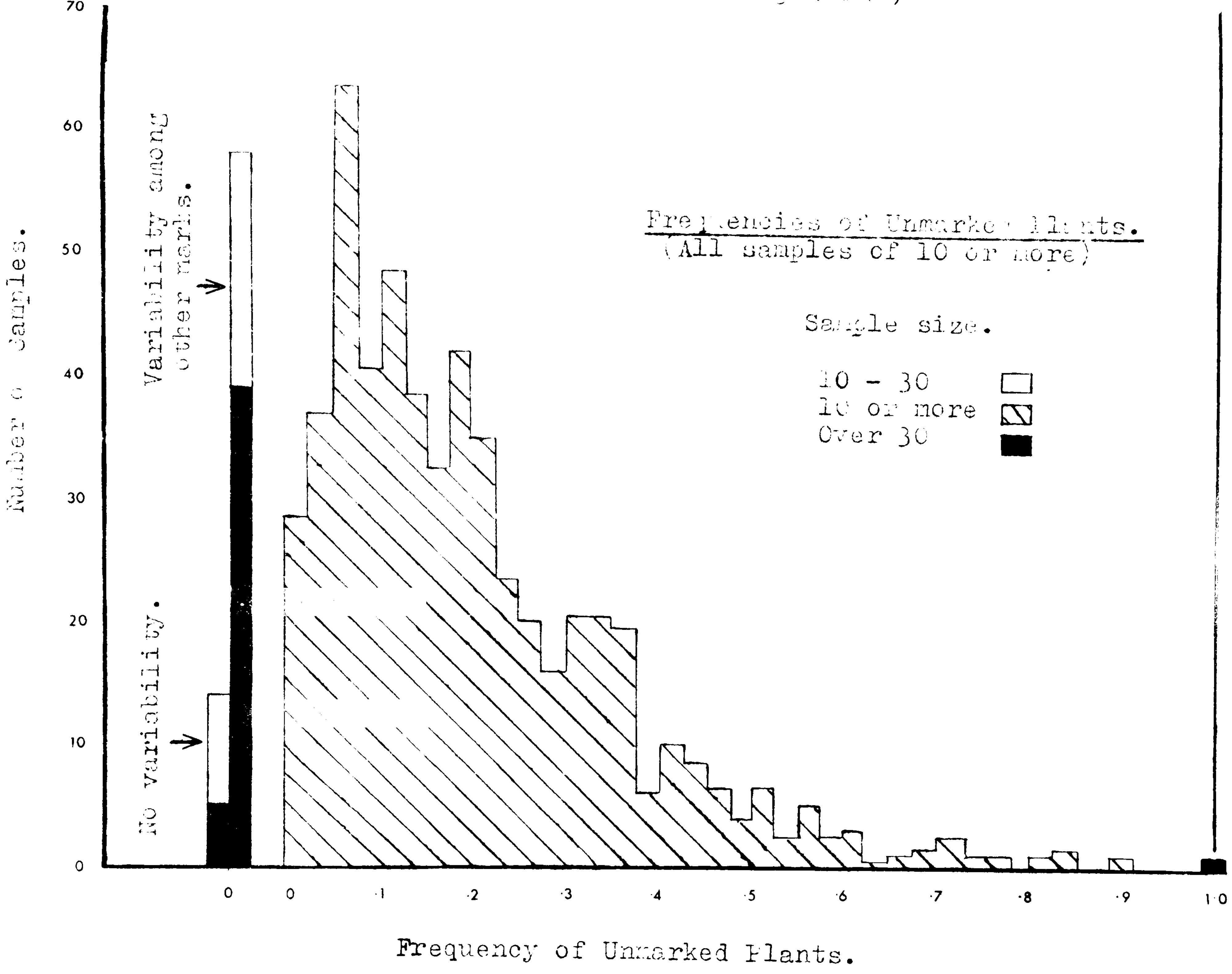
The first notable feature is the generality of the polymorphism, as shown in Table 7.1: only six samples provide serious evidence of uniformity.

Table 7.1. Numbers of Uniform and Polymorphic Samples

		All samples of 10 or more	Samples larger than 30
Uniform			
All unmarked		1	1
All marked:	No variation between marks	14	5
Polymorphic			
All marked:	Several different mark-types	58	39
Unmarked and	marked (one or more types present together)	551	
	Total	624	

Figure VII(i) shows the distribution with regard to the frequency of the unmarked phenotype. It is absent in 72 out of 624 samples, as the table above shows, and all marks are absent in

Fig. VII (i)



Frequencies of Unmarked Plants.
(All samples of 10 or more)

one. The mode of the distribution lies at a frequency between five and ten per cent. The overall frequency among the grand total of all the plants sampled is 17.3 per cent. Slightly more than half the samples fall below 15 per cent frequency, eighty per cent below 30 per cent frequency, and only five per cent exceed 50 per cent frequency of the unmarked form.

2. Evidence for Effective Dominance by the By Allele.

A conspicuous feature of many samples which include plants with the broken-with-yellow-tip mark is the small proportion of these plants which show double-marked phenotypes combining the By mark with another mark. The By mark, when present, rarely exceeds 10 per cent frequency, and over such a frequency range an allele would be expected to occur predominantly in a heterozygous state, and in most samples, half or more of such heterozygotes would be with other mark-producing alleles, and could be expected to show double marks.

To express this deficiency precisely, it is necessary to make calculations of gene frequencies, though bearing in mind the assumptions necessary for such calculations, and the qualified significance of the conclusions, as discussed above.

To simplify the situation, we can describe the population in terms of three alleles; $\underline{V}^{by}$ producing the broken mark with yellow tip, $\underline{v}$, the recessive allele producing no mark, and $\underline{V}^{1}$, the commonest simple V representing all other alleles producing other marks. There will then be four phenotypic classes, produced by the genotypes shown in Table 7.2.

Table 7.2. Summary Classification of Phenotypes and Genotypes in connection with possible Dominance by $\underline{V}^{by}$ over $\underline{V}^{l}$ and other alleles.

Phenotypes	Genotypes
O	vv
L	$V^{l}v$, $V^{l}V^{l}$
Y	$V^{by}v$, $V^{by}V^{by}$; and $V^{by}V^{l}$ (by dominance)
YL	$V^{by}V^{l}$ (when dominance not effective).

The frequency of the recessive allele can be calculated as the square root of the frequency of the O phenotype. For $\underline{V}^{by}$, as top dominant, the calculation is slightly more complicated. If X represents the number of plants not showing the $\underline{V}^{by}$ mark, and N the total number of plants in the sample, $\sqrt{X/N}$ is the frequency of all alleles except $\underline{V}^{by}$, calculated from the frequency of 'homozygotes' between these 'recessives'. The difference between this and 1 is the estimate of $\underline{V}^{by}$ frequency. An estimate of $\underline{V}^{l}$ frequency is then available as the remainder when the sum of (v) and (V^{by}) is subtracted from 1. From these values one can calculate predicted frequencies of $\underline{V}^{by}\underline{V}^{l}$ heterozygotes, expected to show double marks, and of the other $\underline{V}^{by}$ derivatives expected to show the Y phenotype, $\underline{V}^{by}\underline{V}^{by}$ and $\underline{V}^{by}\underline{v}$. Figure VII(ii) shows the proportions of double marked YL

Figure VII(ii)

Predicted and Observed Frequencies of 'YL' Phenotypes among all 'Y' Phenotypes.

This graph plots all samples with 5 or more plants showing a mark of the 'broken-with-yellow-tip' type, whether alone or combined in a double mark.

The purpose of it is to display whether the number of double marked By-derivatives predicted by gene-frequency calculations in fact appears, or whether there is generally a deficiency, which could be attributed to some $\underline{V}^y\underline{V}^1$ heterozygotes being scored simply as Y, as a result of some kind of dominance by $\underline{V}^y$.

Calculation of Predicted Proportion

Data: N = Total number of plants in sample

O = Number of unmarked plants

$\not{Y}$ = Number of plants not showing any By mark.

Estimated Allele Frequencies:

$(v) = \sqrt{O/N}$ Recessive for no-mark

$(V^y) = 1 - \sqrt{\not{Y}/N}$ Dominant for By mark

$(V^1) = 1 - (V^y) - (v)$ All others, mostly $\underline{V}^1$.

Prediction:

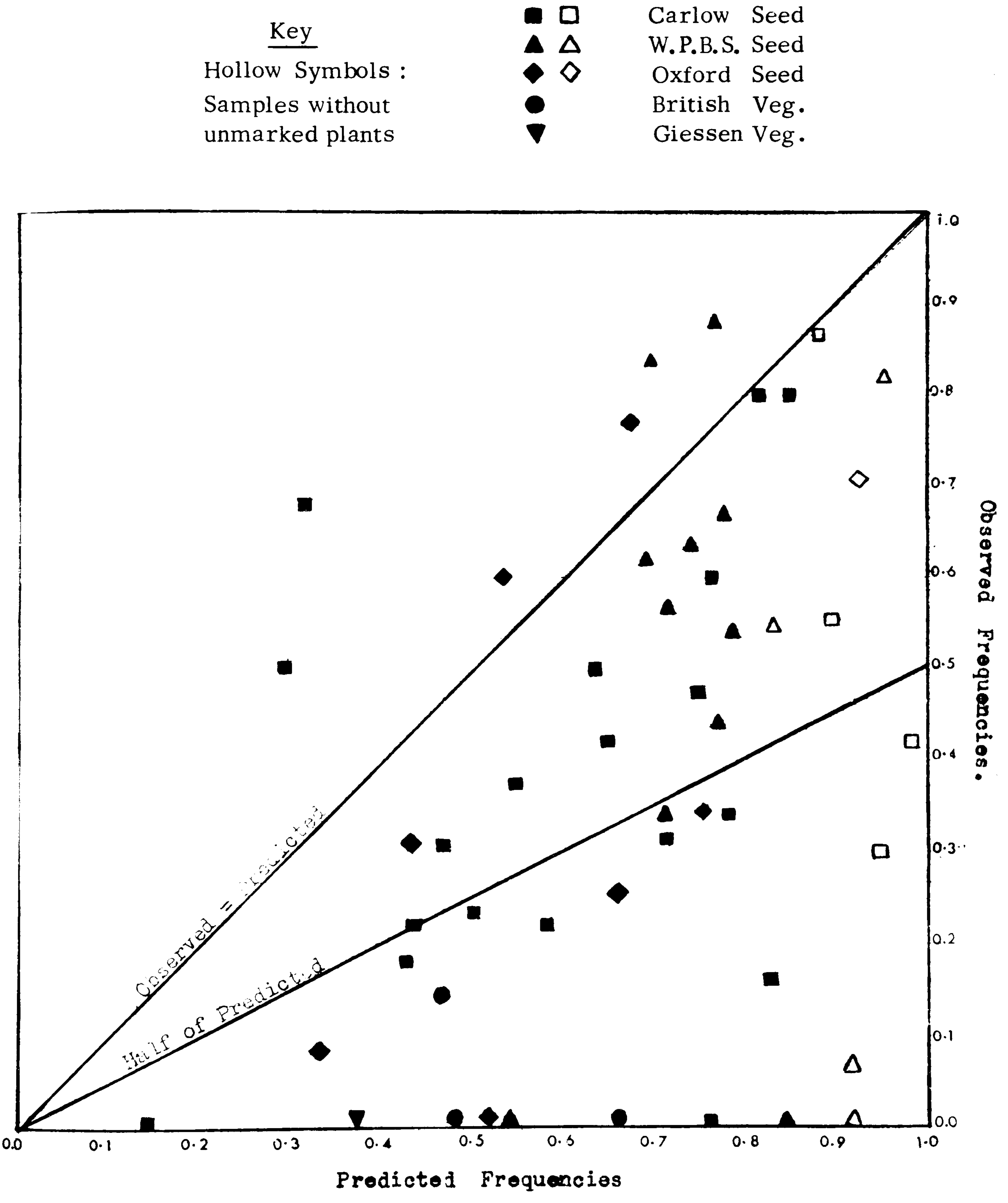
$$\frac{\text{Frequency }(V^yV^1)}{\text{Summed Frequencies }(V^yV^y),\ (V^yv),\ (V^yV^1)} =$$

$$\frac{2(V^y)(V^1)}{(V^y)^2+2(V^y)(v)+2(V^y)(V^1)} = \frac{(V^1)}{\frac{1}{2}(V^y)+(v)+(V^1)} =$$

$$\frac{1-(V^y)-(v)}{1-\frac{1}{2}(V^y)} = \frac{\sqrt{\not{Y}/N} - \sqrt{O/N}}{\frac{1}{2}(1+\sqrt{\not{Y}/N})}$$

Figure VII (ii)

Predicted and Observed Frequencies of 'YL' Phenotypes among all 'Y' Phenotypes



phenotypes, among the total number of plants showing any phenotypes attributed to $\underline{V}^{by}$, (Y + YL), observed values being plotted against predicted ones. To permit reasonably accurate estimates of $\underline{V}^{by}$ frequency, the data is restricted to samples containing 5 or more plants showing the phenotypes Y or YL (including YF, YB etc.).

When the frequency of the recessive is low, its estimation by this method is inaccurate, and it is possible that in most samples where it is not recorded it may be present at a frequency as high as 10 per cent (1 per cent recessive homozygotes would more often than not fail to be found in samples of less than 50). Using an estimated frequency of zero in the calculations would increase the proportion of YL plants predicted, and increase the discrepancy between observation and prediction. Points based on calculations assuming zero frequency of the recessive are therefore distinguished in the plotting.

Table 7.3 summarises the distribution of the points plotted in the figure, and the conclusion that there is effective dominance of $\underline{V}^{by}$ over (many) other mark-producing alleles is inescapable, and is not affected by counting out the samples without unmarked plants. This dominance need not be produced entirely by suppression of the clarity of the second mark, Especially on smaller leaves, two marks may lie so close as to overlap, and a simple V could be taken for part of the white broken V, or as a rather extensively developed yellow tip.

Table 7.3. Deficiency of YL Phenotypes (Double Marks derived from V^{by}.

Samples with 5 or more plants showing V^{by} mark (Y and YL)

	Samples with at least one unmarked plant	All samples
No YL plants at all	8	9
0 - 50% of predicted	12	15
50-100% of predicted	16	21
Less YL than predicted	36	45
More YL than predicted	6	6

This apparently single mark would then be scored as Y because of its double colour and structure. (If any appreciable number of these had been scored as L, the effect shown here would disappear or be reversed.) However, the simple marks lie in a wide range of positions, not all of which would neatly overlap with the By mark. Evidence was also mentioned above (p. 55 note (4)) that the simple mark in recognisable YL plants is often unusually faint, and could often come close to being mistaken for an extended yellow tip with wings running below the white part of the mark. It therefore seems probable that a truly suppressive dominance does exist, and plays some part in the effect shown here.

It would be difficult to explain these results by challenging the assumptions made in calculating gene frequencies, except possibly by a systematic deficiency (in seedling samples as well as field collections exposed to selection) of unmarked homozygotes, leading to underestimation of the frequency of the recessive allele. A failure of random mating would have to be in the direction of assortative mating among By plants, leading to an excess of $\underline{V}^{by}\underline{V}^{by}$ homozygotes, whereas the low frequency of $\underline{V}^{by}$ suggests selection against them. An explanation by selective loss would mean low fitness of most types of heterozygote of $\underline{V}^{by}$, which is hard to reconcile with the presence of this allele, at low frequency, in about 40 per cent of the samples.

3. Interrelations of Different Morph Frequencies.

It was felt that it might be possible to find some kind of pattern among the different morph frequencies, one morph tending to occur with, or to avoid, another. This would imply that some causal sequence was operating and needed explanation, even if no environmental correlations could be found at all.

Morph frequency figures (seven variables, cf. Chapter VI, Section 3(c)) were prepared for all samples of 25 or more, without any combination of samples except between a few samples of closely related origin, all too small to be included alone. This provided 541 sets of frequencies, of which 207 were based on samples from the British Isles. Regression analyses were carried out on this data by computer (cf. Chapter VIII, Section 3 below), and on the data subdivided geographically.

A considerable problem arises at the beginning of this analysis, in the precise articulation of a null hypothesis of independence of the frequencies of two different morphs. Especially for frequent morphs, a negative association would be inherently likely (and between O and L clearly occurs), since a high frequency of one would set a limit to the possible frequency of the other. More specifically, if one was to investigate the regression of, for example, Y frequency on O frequency: should one's null hypothesis be of constant Y frequency, or of a constant proportion of Y among the plants that are not O ? As one assumes a considerable concealed frequency for the recessive gene, a yet more extreme hypothesis would be a constant frequency of $\underline{V}^{by}$ among the complement to the gene frequency of the recessive. This raises again the reservations about gene frequency calculations expressed above.

Calculations were carried out on the basis of the first and second of these hypotheses; for the second, that is, frequencies of Y, B, F and Ba were expressed as proportions of the complement of O frequency. The result can be seen in a shift towards the positive of the regressions of these frequencies on O frequency, compared to the regressions of the simple frequencies themselves. (Correction based on gene frequency calculations would produce a further change in the same direction.)

It is not felt that the problem of choosing the analytical method has been satisfactorily solved, and only the results of the simplest analyses will be reported here, briefly. There were substantial differences between the results of these analyses when carried out on British and on non-British data. This may indicate further scope for investigation, but until such investigations are carried out, it further weakens the conclusions.

Table 7.4. Simple Regressions between Morph Frequencies.

Dependent Variable	Independent Variable	Sign and Significance level of Regression		
		All data (541 samples)	All except British Isles (334 samples)	British Isles (207 samples)
Y	'0'	- 0.001	- 0.05	- N.S.
Y/1-'0'	'0'	- 0.2	- N.S.	+ 0.2
B	'0'	- 0.02	- 0.2	(- 0.3)*
B/1-'0'	'0'	- 0.05	- 0.4	(- 0.4)*
Ba	'0'	- N.S.	+ 0.2	- 0.02
Ba/1-'0'	'0'	+ N.S.	+ 0.1	- 0.05
F	'0'	- 0.01	- 0.1	- 0.1
F/1-'0'	'0'	- 0.02	- 0.3	- 0.1
LL (corr.)	'0'	- 0.05	+ 0.4	- 0.01

* Only two B scores in Br. Is. out of about 10,000 plants.

The clearest of these results shows a negative regression of Y on O, but this is largely removed by the correction, which, as mentioned above, is not as strong as a correction on the basis of gene frequency would be. The negative regressions of B and F on O are much less affected by the correction, and seem to be established as significant.

CHAPTER VIII

RESULTS II: CORRELATION WITH THE ENVIRONMENT

1. Introduction. Previous Reports concerning Morph Frequencies.

Early references to the existence of white leaf markings, and to the fact that they were neither always present nor always absent, were mentioned above. The relative frequencies of different phenotypes are less often mentioned.

Early references refer only to the presence and absence of marks, without discriminating between different types of marks, and appear to include no precise descriptions of frequency. The earliest reference to frequency is probably Gray (1821) 'often spotted with brown or white'. The 'brown' and white spots are presumably respectively the $\underline{R}^f$ red flecking and white V-mark phenotypes, though their difference in position and shape is not mentioned; and 'often' is probably the least committal quantitative term available. A clearer reference on both counts is given by Sir J.E. Smith (1829) 'mostly with a pale transverse stripe'.

Similarly, Bentham and Hooker (1924) 'usually bearing a mark in the centre, which has been compared to a horseshoe', and Clapham, Tutin and Warburg (1962) 'usually with a whitish angled band towards the base' indicate that the presence of the mark is commoner than its absence.

Brewbaker (1955) was the first to give an indication of the relative frequencies of more than two phenotypic classes. He refers to absence of mark, full V, broken V, and V-point as the commonest types of marking, excluding by implication filled V, basal V and broken V with yellow point, and double marked plants, the other phenotypes he describes.

Carnahan *et al*. (1955) were the first to attempt precise description of the frequencies of particular marks. They reported on the frequencies in 480 progenies (meaning presumably 480 seedlings) of unmarked plants in a polycross nursery of 242 clones, and equated these allele frequencies with those in the pollen reaching the 'testers' in this artificial though, for leaf marks, presumably unselected population. From these gene frequencies he predicted phenotype frequencies in a whole population, which he compared with observations on 'more than 2400 plants representing several diverse seed sources'. In spite of the apparent independence of the two sources of material, the results showed substantial agreement, except for a deficiency of double marks. His allele frequencies showed the recessive as the commonest allele (0.31), followed by a large class of simple *V*-alleles, mostly low and intermediate (altogether 0.53), and as rarities broken (0.08) and broken with yellow tip (0.01).

Regional floras and local monographs may be looked to as possible sources of information on frequencies in particular areas, for comparison with the references in the two British

Floras mentioned. However, many fail to mention leaf marking, and those that do may only refer to the existence of marks and their lack of taxonomic value (Hossain, 1961) or to variation in marking within the species, without referring at all to frequency (Foury, 1954; Healy, 1961; Hossain, 1961, p. 413). De Basto Folque (1949: Portugal) and Vicioso (1953: Spain) both refer to leaflets 'frequently' marked with white. Polunin (1959: Arctic) refers to leaflets 'usually with a whitish spot towards the base'. There is certainly no indication of a difference between different areas. Especially as the marks can be seen only with difficulty if at all in herbarium specimens, it may be that some of the reports refer to general knowledge about the species rather than strictly local observations.

Other published data on leaf mark frequency refer exclusively to frequencies in cultivated strains. Carnahan *et al*. (1955) mentions that Louisiana white clover contained no broken or broken with yellow tip marks, and Kent had no plants with double V or a double mark of broken V and simple V.

The clones chosen by Stanford *et al*. (1960) as parents of the new synthetic variety 'Pilgrim' included one unmarked out of 21 clones, but many of the other clones proved to be heterozygous, and the expected frequency of unmarked plants in the first generation, calculated from the parental genotypes deduced from scoring the progeny of each separately, was 13.7 per cent;

frequencies found lay between 9 and 15 per cent. They found no clear pattern of divergence of leaf-mark frequencies in populations multiplied at several different sites (Stanford e̲t̲ a̲l̲., 1962).

W.E. Davies and Young (1963) recorded unmarked and broken yellow frequency for nine strains. These included the S100 No Mark strain, which was deliberately selected for homozygosity for the unmarked recessive so that genetic contamination during increase could be recognised. Unmarked plants are present in all the other strains, at frequencies from 1.7% in Kersey, around 5% in Kent wild white, S100, and NZ Certified Mother Seed, 10% in Ladino, about 15% in S184 and NZ Permanent Pasture, and 34.6% in English Dutch clover. Apart from Kent, with no broken-with-yellow-tip, the broken-yellow frequency values, though lower, were more uniform, varying from 1.6% in S184 to 3.7% in S100.

Daday, ~~in correspondence~~ (1954a), referred to observations during his growing and scoring of world-wide samples for cyanogenesis, and suggests that the clarity, conspicuousness and diversity of the marks and their frequency, as opposed to the unmarked form, increase towards the south in Europe.

2. Information Available about Original Background of Samples.

Quantitative data suitable for statistical treatment are available only for the samples collected by the author in the British Isles, and for these only with regard to geographical location, altitude, and in most cases soil pH. For these factors, an exact analysis is possible by Multiple Regression, leading to the estimation of precise Regression Coefficients for each allele on each environmental variable, and precise estimates of the significance of these coefficients (whether they differ significantly from zero correlation).

Although a fairly accurate geographical location is known for many of the seed samples obtained from overseas, only the country of origin of others is known. This applies particularly to the samples grown at Carlow and the Welsh Plant Breeding Station, not obtained specifically for this work. When data is available for a large number of countries, a large part of any distinct general distribution pattern would be shown by mapping, even if only a single value in plotted for each country, calculated from the sum of all samples (cf. Appendices III and III). In fact, for the rarest alleles, it is necessary to go beyond this, and sum the data for adjacent groups of countries to produce large enough numbers of the rare phenotypes in each group for quantitative expressions of their frequency to have any degree of accuracy. These groupings of countries have been arranged, so far as possible, to combine countries with relatively similar overall frequencies of markings.

Data can therefore be presented more or less quantitatively for consideration of possible correlations as shown in Table 8.1.

Table 8.1. Methods of Analysis and Sets of Data.

Data	Environmental Factor	Morph Frequencies	Method of Investigation
British Veget., field-scored.	Grid Reference Altitude	O,Y,Ba, F,LL,L.	Multiple Regression (and Scatter Diagrams)
" (not all)	Soil pH	"	
All British Is.	Location	O,Y,LL	Mapping
Western Europe	Location	O,Y	Mapping
All Nat. Range	Country	O,Y,B,Ba, F,LL,H	Mapping
WPBS Collections Spain and Portugal	Altitude	O,Y,B,Ba, F,LL,L	Simple Regression
	Wet/dry Agric. Use	O,Y,F	Contingency Tables
WPBS Collections South Wales	% Rye-Grass	O,Y,Ba LL,L	Simple Regression
" "	Agric. Use	O	Contingency Tables
Polish Samples	Agric. Use Wet/Dry	O,Y,F	Contingency Tables
Swiss Samples	Agric. Use	O,Ba,F	Contingency Tables
Other Foreign Samples	All factors	O	Direct Comparisons within groups of samples.
British Samples, field scored	All factors	O	

3. Multiple Regression Analysis of British Field-Scored Data.

a) Data and Treatment.

Data was tabulated for 148 samples of 25 or more plants collected in Britain and scored, mostly in the field, by the author.

The data for each sample consist of Grid Reference to the nearest 0.1 km., and Altitude, expressed in feet, generally to the nearest 10 feet, though in some cases (especially high altitude values) only to the nearest 100 feet.

Information from smaller samples was incorporated where possible, either by combining small samples or by adding a small sample to a nearby larger one. Such combinations were of course made only between samples closely similar in location and altitude.

All the morph frequency parameters are expressed to three places of decimals as absolute frequencies. No figure for the frequency of the broken mark appears since no such marks were scored in these samples. For the complex expressions representing LL and L frequency, calculations where samples were combined were made on each sample separately, and the numerators and denominators of the final fraction added before division. The four remaining frequencies refer to O, Y, Ba, and F marks (cf. above, Chapter VI, Section 3(c)).

Soil pH data expressed to one tenth of a pH unit were available for over half of these samples. The data were tabulated for a second treatment as 90 samples with soil pH data as well as the other figures referred to above. In a few cases more than one soil pH value was available within the sample, and the plants could be allocated to two areas corresponding to these soil samples. Where this was so, and the pH values differed, the sample was split, provided the parts each contained 25 or more plants. Six samples visited twice, but only tested for pH once, appear each as two sets of figures in the first tabulation, but only combined as one in the data including pH.

No attempt has been made to weight the values according to sample size and the corresponding accuracy of the frequency measurements except by the exclusion of samples smaller than 25.

A large amount of material is available from a restricted range of habitats in a restricted area near Oxford (riverside pastures at the University Farm, Wytham). The possibility of bias resulting from this is partly allowed for by condensing the 28 samples of 25 plants or more to 14 in the first set of data; only 8 of these appear in the data with pH values, as the sites of the others had been ploughed up when revisited to collect soil.

It was mentioned above that the soil pH values for material from North Wales may have been biassed by the treatment of the samples before scoring. The data with pH values was therefore presented again for full analysis without these seventeen samples, to see whether this would affect the regression coefficients on pH and their significance. Removal of this block of data also greatly reduces the amount of variation of Grid Reference in the East-West direction, as all but four of the remaining samples lie within a belt about 200 km. wide running North to South. (It is also true to a lesser extent of the fuller sets of data, that they represent a considerably greater North-South range than East-West. This accounts for the greater significance of regression coefficients, of the same size, on northing as opposed to easting.)

To investigate the possibility of any effect of the still large group of samples from Wytham, a repeated treatment of each of the three sets of data indicated so far was carried out with the Wytham samples excluded.

A seventh set of data was prepared to indicate to what degree differences between the conclusions drawn from the two main sets of data were due, on the one hand to the exclusion of the particular samples which had no pH values (and the modifications in other sample), and to what extent it was due to the incorporation of pH data in the analysis. For this purpose the data tabulated with pH values were taken, but the pH values were ignored.

Table 8.2. British Field Samples: Sets of Data used in Statistical Treatments.

	Number of Independent Variables	Number of Samples
1. All data	3	148
2. All data except Wytham	3	134
3. Data with pH values	3	90
4. Data with pH values	4	90
5. Data with pH values except Wytham	4	82
6. Data with pH values except N.Wales	4	73
7. Data with pH values except N. Wales and Wytham	4	65

Independent Variables: 3: Grid Reference (East and North), Altitude.

4: Grid Reference, Altitude, Soil pH.

Dependent Variables : 6: O, Y, Ba, F, LL, L frequencies.

Multiple Regressions were calculated for each of the morph frequency variables in each of the sets of data described above on the set of independent variables indicated in Table 8.2. This was done by means of the KDF 9 Computer at the Oxford Computing Laboratory, using a library tape (Stat. 15) for General Multiple Regression Analysis prepared by the laboratory staff.

b) Results of Multiple Regression Analyses: Presentation.

The first of the Tables which follows (Table 8.3) shows all the pairs of variables which reach a significance value of 0.2 in any of the seven analyses listed above (Table 8.2). The next three Tables (Tables 8.4, 5, 6) show the full set of means and standard errors of the variables, and all the regression coefficients for three of the analyses. These refer to the full set of 148 samples, and to the set of 90 samples which have soil pH values, the latter analysed both with and without inclusion of the soil pH data.

When reading these tables, it should be remembered that smaller regression coefficients for the same level of significance will be found when the variation of the corresponding dependent variable is small. The standard error of the F frequency figure is less than a tenth of those of the O and L figures; the corresponding regression coefficients would be expected to stand in the same proportion if they reach similar levels of significance.

It should be remembered that the L and LL figures shown are not simple frequencies, but are respectively $(L\chi/\chi)$ and $(LL\ /\ \chi.(1-\sqrt{L\chi/\chi})^2)$. The correction applied to LL allows principally for the negative relation between O and uncorrected LL frequency (cf. Chapter VI, Section 3(c) and Figures VI.1 and 2). If its use was distorting the data appreciably there would tend to be high values of corrected LL frequency where O was high. In fact, the regression coefficients for O and LL on two of the three main variables, Northing and Altitude, are large and opposite in sign, an effect opposite to any that could have been produced by the correction. On the other hand, LL shows a clear regression on Easting opposing that of L, which suggests that the correction has not been inadequate.

The presentation of results of the seven analyses side by side in Table 8.3 permits comparisons between the different analyses to reveal the effects of the differences between the different sets of data. The opportunities for these comparisons may be summarised as follows:

1. Inclusion in the Analysis of Soil pH as fourth Indep. Variable:
   Compare Columns 3 and 4; more detail in Tables 8.5 and 6.
2. Exclusion of Samples from Sites without pH Values:
   Compare Columns 1 and 3 (more details in Tables 8.4 and 5); or compare Columns 2 and 5.
3. Removal of Wytham Samples:
   Compare Columns 1 with 2, 4 with 5, or 6 with 7.
4. Removal of North Wales Samples:
   Compare Column 4 with 6, or 5 with 7.

When the reader's concern is not with these comparisons, Columns 1 and 4 (and Tables 8.4 and 6) will be of most use, but it should be remembered that comparison between them involves both of the first two effects listed above.

Table 8.3. The Most Significant Correlations between Morph Frequencies and Environmental Factors.

This Table shows the <u>Regression Coefficients</u>, with <u>Estimates of their Significance Levels</u>, derived by Multiple Regression Analysis, for all pairs of variables whose Significance Level passes $P = 0.2$ in any of these sets of Data.

<u>Notes for Tables 8.3-6</u>

1) Variables Used in Multiple Regression Analysis.

Independent: Environmental Factors.

(refer to number quoted above each column of Table)

3: Grid Ref., Easting and Northing: Altitude.
4: Grid Ref., Easting and Northing; Altitude; Soil pH.

Dependent: Morph Frequencies

O	Frequency of Unmarked Phenotype
Y	Frequency of Phenotypes showing Broken-with-Yellow-Tip.
Ba	Frequency of Phenotypes showing Basal marks.
F	Frequency of Phenotypes showing Filled-in marks.
LL	$(= LL/\not{Y}.(1-\sqrt{L\not{Y}/\not{Y}})^2$ representing the Frequency of Double marks consisting of two 'L' Simple V-Marks.
L	$(= L\not{Y}/\not{Y})$ representing Frequency of Simple V-Marks ('L').

2) Regression Coefficients expressed as

Change, in parts per thousand, of Morph Frequency per	Approximate Total Range among Samples. ↘
(100 km. Eastward	200 km.
(100 km. Northward	550 km.
(1000 ft. Upward	1900 ft.

3) Significance and	***	**	*	N.S.
Probability Levels	$P < 0.001$	$P < 0.01$	$P < 0.05$	$P > 0.4$

Figures appearing below the Regression Coefficient in each cell of the Table are the greatest Significance value (lowest Probability value) passed by the calculated value of <u>t</u>.

Table 8.3 (continued)

Data Set: (cf. Table 8.2)		1 All	2 All exc. W	3 With pH	4 With pH	5 With pH exc. W	6 With pH exc. NW	7 With pH -W, -NW
Number of Indep. Vars.		3	3	3	4	4	4	4
Morph	Env. Factor							
O	N	+21.70 0.01 **	+26.11 0.001 ***	+22.16 0.05 *	+22.24 0.05 *	+23.87 0.02 *	+22.24 0.05 *	+23.88 0.02 *
L	N	-21.58 0.01 **	-24.91 0.001 ***	-18.60 0.1	-18.77 0.1	-19.81 0.1	-18.37 0.1	-19.43 0.1
LL	N	- 6.25 0.02 *	- 6.92 0.01 **	- 6.48 0.2	- 6.52 0.2	- 8.32 0.1	-10.56 0.05 *	-12.75 0.02 *
Ba	N	- 0.97 0.4	- 1.21 0.3	- 2.83 0.2	- 2.87 0.2	- 3.42 0.1	- 3.42 0.2	- 4.01 0.1
F	N	- 0.61 0.3	- 0.74 0.2	- 0.65 N.S.	- 0.58 N.S.	- 0.80 N.S.	- 0.55 N.S.	- 0.77 N.S.
O	E	-19.23 0.3	-17.91 0.3	-27.90 0.1	-27.41 0.2	-22.43 0.2	-20.89 0.4	-16.31 N.S.
L	E	+16.72 0.4	+15.71 0.3	+28.20 0.1	+27.10 0.2	+23.28 0.2	+20.27 0.4	+17.11 N.S.
LL	E	- 8.96 0.2	- 9.14 0.2	-15.43 0.05 *	-15.66 0.05 *	-18.98 0.02 *	-40.17 0.001 ***	-45.40 0.001 ***
Y	E	+ 4.21 0.1	+ 4.10 0.2	+ 4.26 0.2	+ 4.89 0.1	+ 4.79 0.2	+ 7.51 0.1	+ 7.26 0.2
O	Alt	+81.32 0.05 *	+92.72 0.01 **	+18.80 N.S.	+18.17 N.S.	+16.27 N.S.	+22.48 N.S.	+23.65 N.S.
L	Alt	-79.12 0.05 *	-87.78 0.01 **	-24.68 N.S.	-23.29 N.S.	-20.78 N.S.	-35.63 N.S.	-36.30 N.S.
LL	Alt	-18.35 0.2	-19.99 0.2	-44.49 0.05 *	-44.19 0.05 *	-46.27 0.05 *	+ 8.62 N.S.	+ 6.81 N.S.

Table 8.4. Multiple Regression on Grid Reference and Altitude.

Regression Coefficients and Significance Levels for all British Field-Scored Data (148 Samples).

Coefficients represent change of frequency (parts per thousand) per		Grid Reference Easting	 Northing	Altitude
		100 km. Eastward	100 km. Northward	1000 ft. Upward
Mean		4238.2 km.	3450.3 km.	387.6 feet
Standard Error		± 75.92 km.	±1695 km.	331.7 feet
Dependent Variable	Mean freq. and Standard Error			
O	0.2723 ±0.1467	-19.23 0.3	+21.70 0.01 **	+81.32 0.05 *
Y	0.0149 ±0.0207	+ 4.21 0.1	- 0.86 N.S.	- 5.24 0.4
Ba	0.0070 ±0.0182	+ 1.66 N.S.	- 0.97 0.4	- 1.83 N.S.
F	0.0021 ±0.0086	+ 1.28 0.3	- 0.61 0.3	+ 1.48 N.S.
LL	0.0206 ±0.0469	- 8.96 0.2	- 6.25 0.02 *	-18.35 0.2
L	0.7200 ±0.1440	+16.72 0.4	-21.58 0.01 **	-79.12 0.05 *

Table 8.5. Multiple Regression on Grid Reference and Altitude.

Regression Coefficients and Significance Levels for all data which also have soil pH values (90 samples, as expressed for Regr. on 4 Variables).

Coefficients represent change of frequency (parts per thousand)		Grid Reference Easting	Grid Reference Northing	Altitude
	per	100 km. Eastward	100 km. Northward	1000 feet Upward
Mean		4340.2 km.	2846.2 km.	335.4 feet
Standard Error		±867.3 km.	±1297 km.	± 274.8 feet
Dependent Variable	Mean freq. and Standard error			
O	0.2260 ±0.1182	-27.90 0.1	+22.16 0.05 *	+18.80 N.S.
Y	0.0117 ±0.0189	+ 4.26 0.2	+ 1.02 N.S.	- 4.77 N.S.
Ba	0.0083 ±0.0204	+ 0.10 N.S.	- 2.83 0.2	- 1.66 N.S.
F	0.0032 ±0.0107	+ 1.68 0.3	- 0.58 N.S.	+ 3.96 N.S.
LL	0.0247 ±0.0513	-15.43 0.05 *	- 6.48 0.2	-44.49 0.05 *
L	0.7689 ±0.1195	+28.20 0.1	-18.60 0.1	-24.68 N.S.

Table 8.6. Multiple Regression of Grid Reference, Altitude and Soil pH.

Regression Coefficients and Significance Levels for all Data with Soil pH values (90 samples).

Coefficients represent change of frequency (parts per thousand per		Grid Reference		Altitude	Soil pH
		Easting	Northing		
		100 km. Eastward	100 km. Northward	1000 feet Upward	1 pH Unit more Alkaline
Mean		4340 km.	2846 km.	335.4 feet	pH 6.18
Standard Error		± 867 km.	±1297 km.	±274.8 feet	±pH 1.06
Dependent Variable	Mean freq. and Standard Error				
O	0.2260 ±0.1182	-27.41 0.2	+22.24 0.05 *	+18.17 N.S.	- 8.95 N.S.
Y	0.0117 ±0.0189	+ 4.89 0.1	- 0.92 N.S.	- 5.56 N.S.	-11.32 N.S.
Ba	0.0083 ±0.0204	+ 0.75 N.S.	- 2.87 0.2	+ 1.94 N.S.	+ 4.04 N.S.
F	0.0032 ±0.0107	+ 1.57 0.4	- 0.60 N.S.	+ 4.09 N.S.	+ 1.86 0.4
LL	0.0247 ±0.0513	-15.66 0.05 *	- 6.52 0.2	-44.19 0.05 *	+ 4.15 N.S.
L	0.7689 ±0.1195	+27.10 0.2	-18.77 0.1	-23.29 N.S.	+19.75 N.S.

c) Results of Multiple Regression Analyses: Interpretation.

These results give a clear indication that Soil pH (as measured) can explain very little if any of the observed variation. None of the coefficients of regression on pH reach even a significance level of P = 0.4 in any of the analyses. Another expression of the apparent irrelevance of pH is seen when Columns 3 and 4 of Table 8.3 are compared; the inclusion of pH as a fourth independent variable in the analysis has had practically no effect on the coefficients of regression on the other variables or their significance.

On the other hand, the two principal analyses (Columns 1 and 4) differ considerably, and comparison of Columns 1 and 3 shows that this is due almost entirely to the change in the set of samples used. The main feature of this change was the exclusion of nearly all the samples from the North-East of England. This greatly reduced the amount of variation in Northing, and also in altitude, as most of the high altitude samples were in this group. This would be expected to make it harder to establish the significance of a regression on either of these variables even if equally well followed by the morph frequencies. Thus there is decline in significance without appreciable change in regression coefficien for the regression of O on N, of LL on N, of F on N, and very clearly for O on Altitude. The principal exception is the regression of LL on Altitude (cf. also Ba on N). The effect here is a considerable increase in both the significance and the coefficient, which could be explained in two ways. On the one hand there may be heterogeneity in the application of this correlation, the block of samples which were removed being exceptions from it, so that while they remained they concealed it. Alternatively, the effect is unreal, and in the smaller sample the more restricted range of habitats studied produced an unrepresentative picture of variation in the species. The variation in Easting remains substantially unchanged, however, and a greater part of the variation in morph frequencies is attributed to it; there is an increase in coefficient and in significance for O, L and LL, regressions on Easting.

The effect of removing the Wytham samples (already somewhat reduced in numbers to limit their contribution) is to increase the coefficient and especially the significance of all the regressions on N, and also to some extent on O and L on Altitude. This is what would be expected if a group of samples near the centre of the range of northing were removed, having had rather untypical morph frequencies for that part of the range. It will be seen from the maps below that the Wytham group and from Appendix II that the 'South Midlands' group (dominated by Wytham), resemble the northern samples rather than their neighbours. It appears that these samples do have distinctive properties, and that they would be capable of producing bias if over-represented.

The removal of the North Wales samples was intended to eliminate any bias caused by possible inaccuracy in their pH data. No appreciable pH effects are present, either before or after removal of these samples. Any effect of removing a bias might therefore not be recognised, and none was. As mentioned above, removal of these samples greatly restricts the amount of variation in Easting and also (this group containing the bulk of the high altitude samples with pH values) in altitude, and thus reduces the possibility of establishing regressions on these factors. The regressions of O and L lose considerably in significance, and that of Y increases its coefficient without appreciable change in significance. On the other hand high levels of significance are revealed for the regression of LL on E. (Although reacting to Easting in the same direction as O, the LL regression reaches its highest significance here while the O regression becomes fainter). The same choice of interpretations applies as above to LL on Altitude. This removal of the North Wales samples has an even more dramatic effect on regression of LL on Altitude; not only does it remove its significance, previously at $P = 0.05$, but it removes the regression completely, replacing it by a small one of opposite sign. This suggests that the previously significant regression depended entirely on (some of) the North Wales samples being extreme in altitude and LL frequency.

d) Comparison of Simple and Multiple Regression Analysis.

By simple regression analysis of a morph frequency with regard to one environmental factor alone, the attempt is made to explain all the variation in morph frequency in terms of that independent variable. Simple regression coefficients are therefore usually larger and appear to be more significant than multiple regression coefficients. It can be argued that the amount by which the simple exceeds the multiple regression coefficient is a spurious contribution, which can be eliminated by allowing for the effect of the other environmental variables measured. Therefore, the multiple regression coefficients have been presented here as the best available estimates of the extent of the correlation. (It is also true that they would probably be reduced still further if other environmental variables were allowed for, and that the multiple regression coefficients presented/may remain overestimates.)

However, these multiple regression coefficients only allow for two or three of very many possible environmental variables. The simple regression coefficient for each environmental factor, though overestimated, is not biased by allowance for particular environmental factors chosen merely because measurements of them were available. It is also a better basis for comparison with other data where only one environmental factor is measured (cf. below, Section 5, part (a)).

Simple regression coefficients and their significance levels are presented in Table 8.7 for comparison with the corresponding multiple regression coefficients, all being derived from calculations involving Grid Reference and Altitude for the full set of 148 British field-scored samples (set 1 in Table 8.3 above). The principal differences are significance at or near 5% for association of high Y frequency with southern position and low

Table 8.7. Comparison of Simple and Multiple Regression Coefficients.

Simple R.C.'s above, Multiple R.C.'s below.

	Easting	Northing	Altitude
	423.8 km. ± 75.9 km.	345.0 km. ±169.5 km.	387.6 ft. ±331.7 ft.
Mean, S.E.			
O 0.2723 ± 0.1467	-50.27 ±15.44 0.01 **	+30.93 ± 6.69 0.001 ***	+135.79 ±34.82 0.001 ***
	-19.23 ±16.37 0.3	+21.70 ±7.44 0.01 **	+81.32 ±36.79 0.05 *
Y 0.0149 ± 0.0207	+5.73 ±2.21 0.05 *	-1.99 ±1.00 0.05 *	-9.96 ±5.11 0.1
	+4.21 ±2.47 0.1	-0.86 ±1.12 N.S.	-5.24 ±5.55 0.4
Ba 0.0070 ± 0.0182	+2.79 ±1.97 0.2	-1.40 ±0.88 0.2	-4.85 ±4.53 0.3
	+1.66 ±2.21 N.S.	-0.97 ±1.00 0.4	-1.83 ±4.97 N.S.
F 0.0021 ± 0.0086	+1.61 ±0.93 0.1	-0.73 ±0.42 0.1	-0.59 ±2.15 N.S.
	+1.28 ±1.04 0.3	-0.61 ±0.47 0.3	+1.48 ±2.34 N.S.
LL 0.0206 ± 0.0469	-0.74 ±5.11 N.S.	-5.94 ±2.23 0.01 **	-23.27 ±11.53 0.05 *
	-8.96 ±5.54 0.2	-6.25 ±2.52 0.02 *	-18.35 ±12.45 0.2
L 0.7200 ± 0.1440	+47.33 ±15.20 0.01 **	-30.19 ±6.57 0.001 ***	-131.48 ±34.23 0.001 ***
	+16.72 ±16.11 0.4	-21.58 ±7.32 0.01 **	-79.12 ±36.21 0.05 *

Units: Morph Frequencies: Absolute Frequencies.
Coefficients: change in morph frequency in parts per thousand per 100 km. or 1000 ft.

Data: 148 Field-scored British samples (set 1 in Table 8.3).

altitude, and the absence among the simple regressions of any indication of dependence of LL on distance east.

e) Results of Multiple Regression Analyses: Conclusions.

A clear positive regression of O (unmarked frequency) on northing is consistently present throughout these analyses, of the order of 0.02 or 0.025 O-frequency change per 100 km. Its significance is considerably clearer when the full range of variation in northing is included. This is more markedly true for the regression of O on Altitude, which is of the order of 0.085 increase in O frequency for 1000 feet increase in altitude.

Regression coefficients and significance values for L closely parallel those of O, with change of sign, but are generally slightly smaller. Because of this, and because, as has been said above, the L figure certainly combines a number of different phenotypes, no further reference will be made to the L regressions; they can be assumed to be the opposite of the O regressions.

The regression of LL on Northing is also clear; the correction used to produce the LL figure almost certainly weakened it.

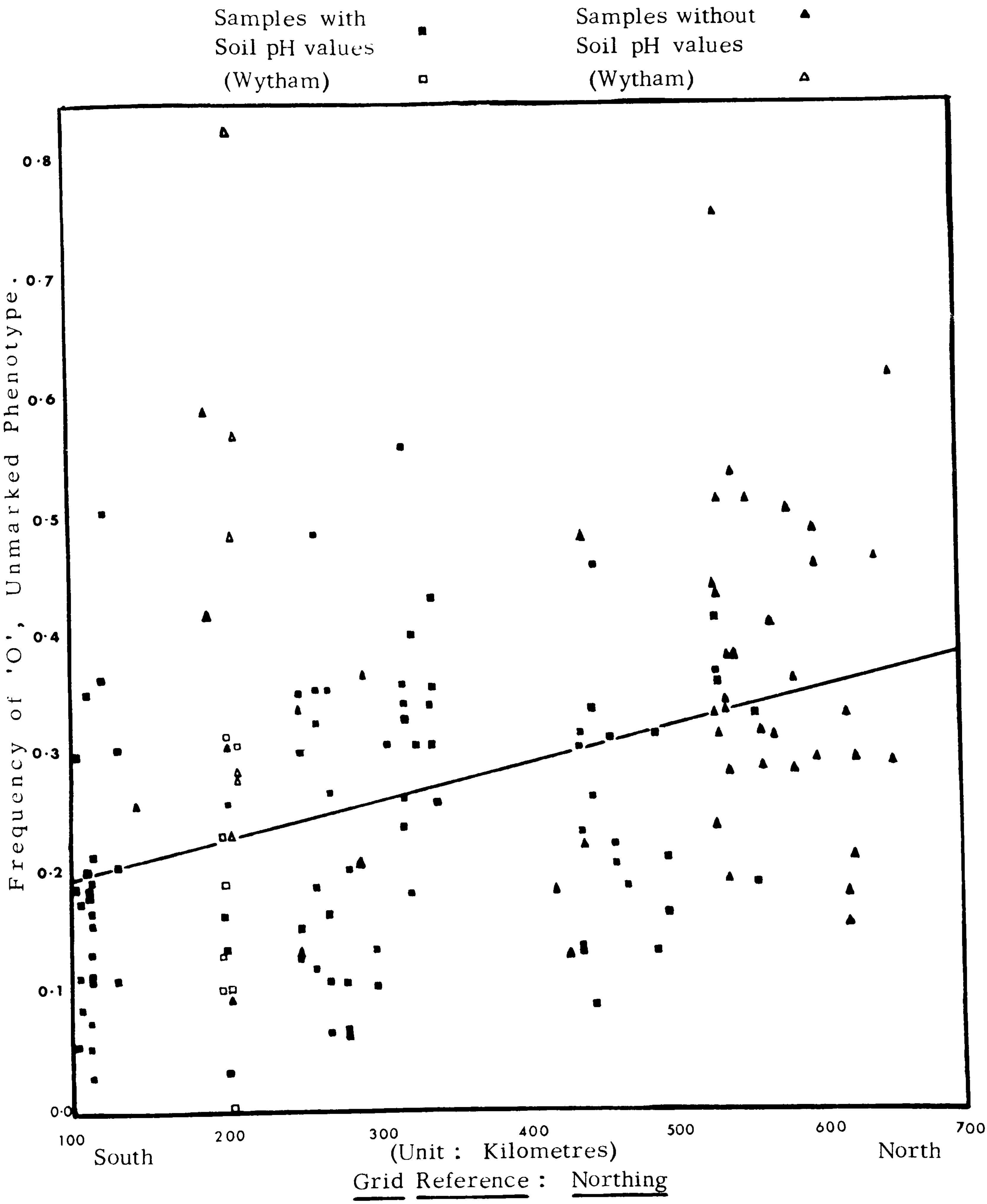
With so many variables and so many comparisons, perhaps one significance level of 5 per cent might be reached by chance. The three clear cases above, with significance levels well past 5 per cent, therefore together make a strong case for denying that leaf-marking frequencies are unresponsive to environmental differences, for denying therefore that they could be of no selective importance.

It is less clear how to interpret the cases where significance is reached only after removal of some samples for reasons unconnected with the factors concerned. The principal examples are the regression of LL on Easting and on Altitude. However, the coefficients in the complete set of data, though not significant, are large enough to suggest that these regressions may be real. More data would be needed to establish significance for regressions of this size, and this is true also of the other weaker regressions listed in Table 8.3.

Scatter diagrams (Figures VIII(i) to (ix)) follow, to illustrate the actual distribution of the samples with respect to the nine most clearly indicated regressions, listed in Table 8.3. The samples with no pH values (whose removal produces the differences between analyses 1 and 3) and those from Wytham are plotted with different symbols. On each Scatter Diagram is shown the simple regression line, morph frequency on environmental factor, for the two variables concerned. The simple regression equation is shown with the diagram, using the units shown on the diagram (absolute morph frequencies, kilometers (Grid Ref.) and feet (altitude)), in the form:

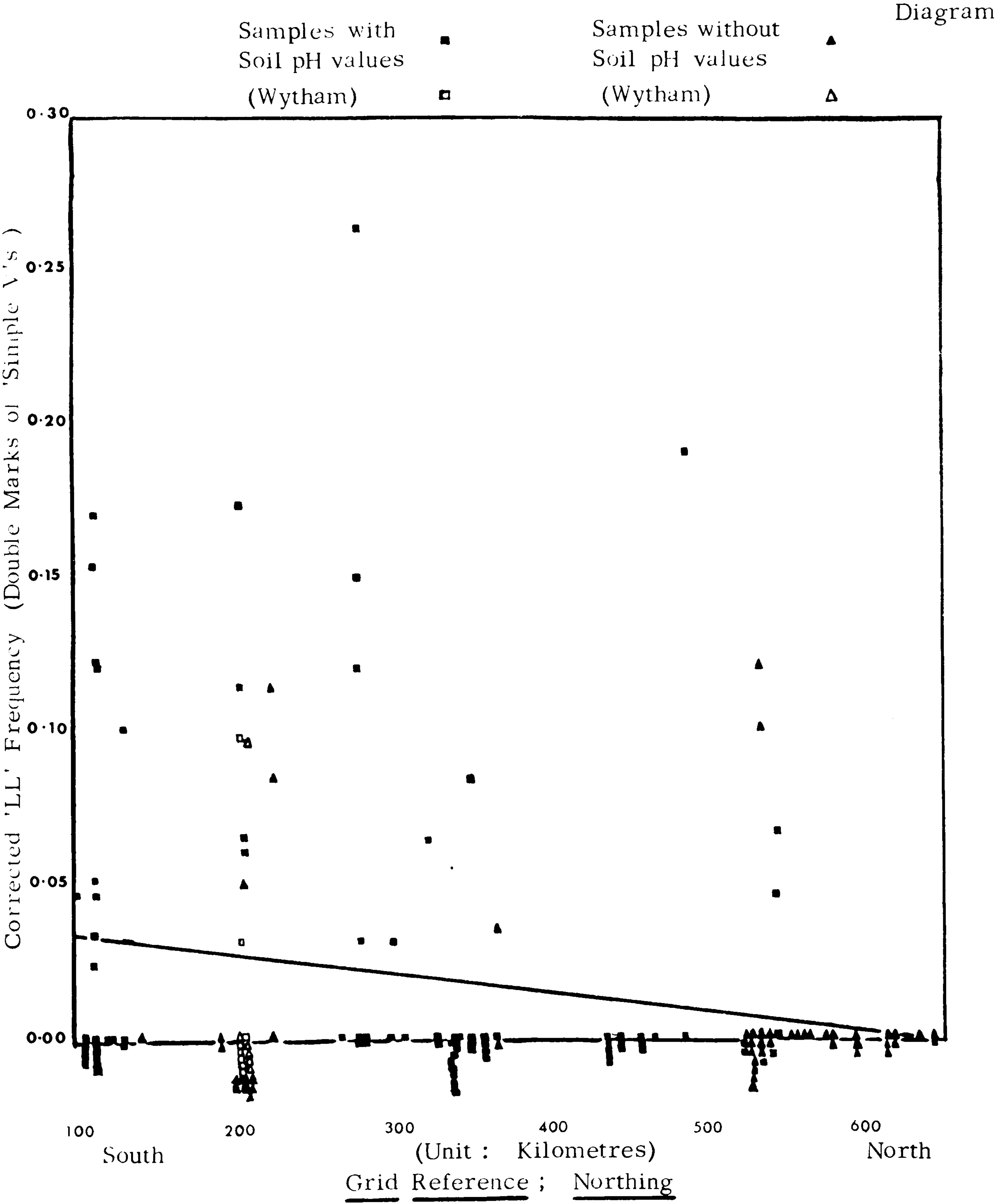
(Morph Frequency (Predicted)) = (Mean Morph Frequency, ± S.E.)
+ (Simple Regression Coefficient, ± S.E.) x ((Environmental Factor (Measured)) - (Mean for Environmental Factor, ± S.E.)).

Figure VIII (i) 'O' Frequency - Northing Scatter Diagram



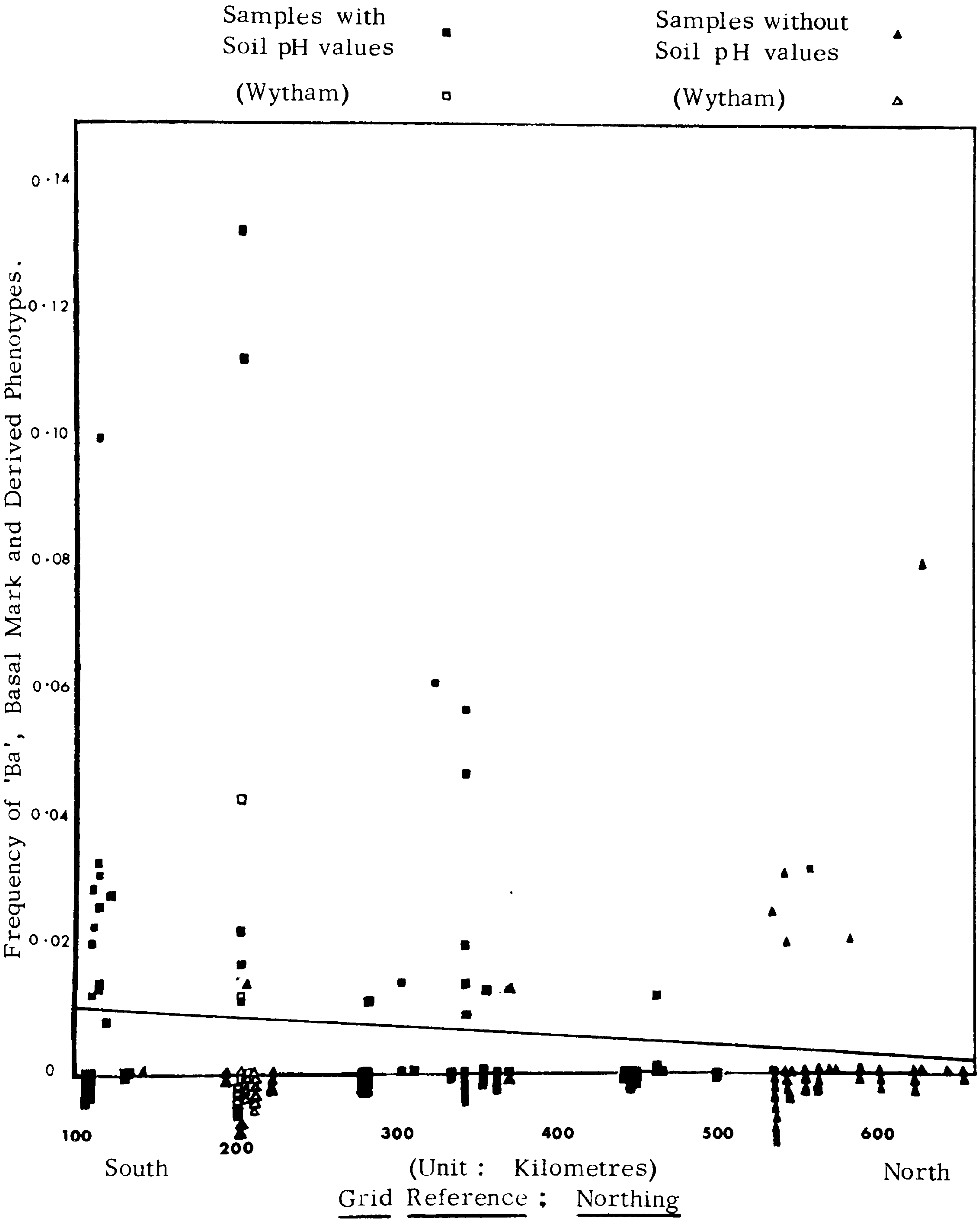
Simple Reg. Eq. : (O) = 0.2723(±0.1467) + 0.000309(±0.000067) (GR(N)km - 345(±170))

Figure VIII (ii) Corrected 'LL' Frequency - Northing Scatter Diagram



Simple Reg. Eq. : (LL) = 0.0206(±0.0469) - 0.000059(±0.000022) (GR(N)km - 345(±170))

Figure VIII (iii) Basal Mark Frequency - Northing Scatter Diagram



Simple Reg. Eq. : (Ba) = 0.0070(±0.0182) - 0.000014(±0.000009) (GR(N)km - 345(±170))

Figure VIII (iv) Filled-in Mark Frequency - Northing Scatter Diagram

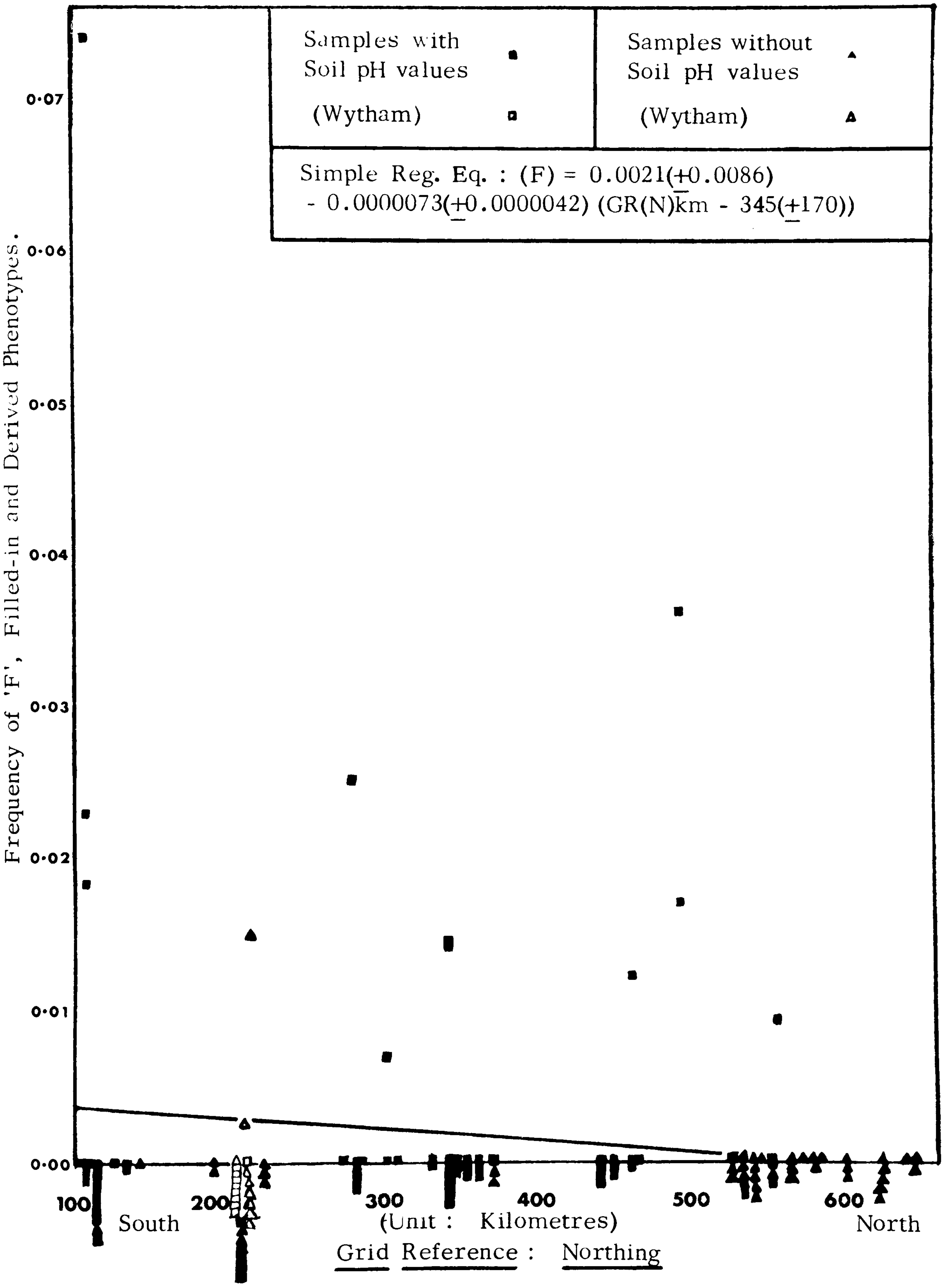
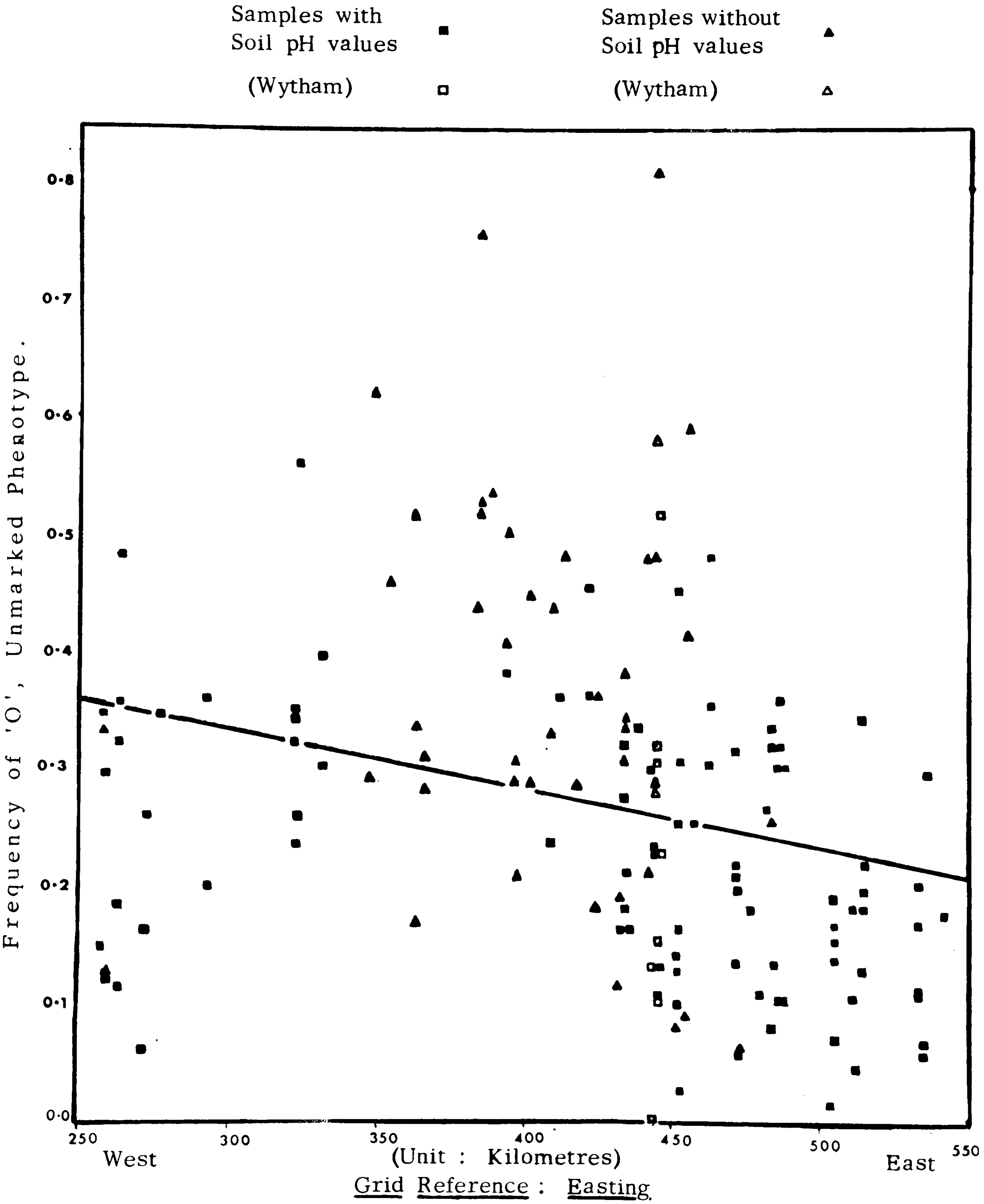
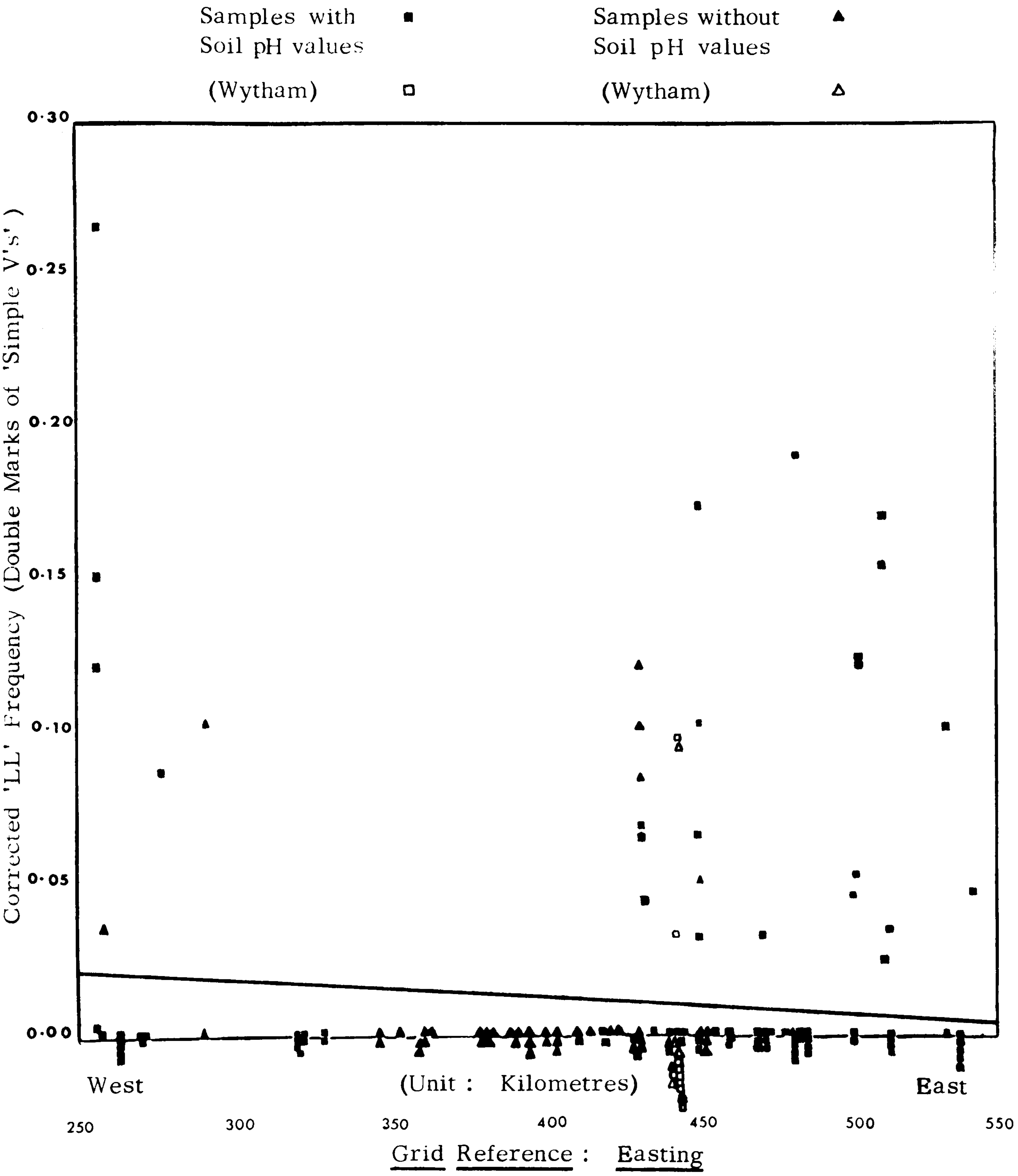


Figure VIII (v) 'O' Frequency - Easting Scatter Diagram



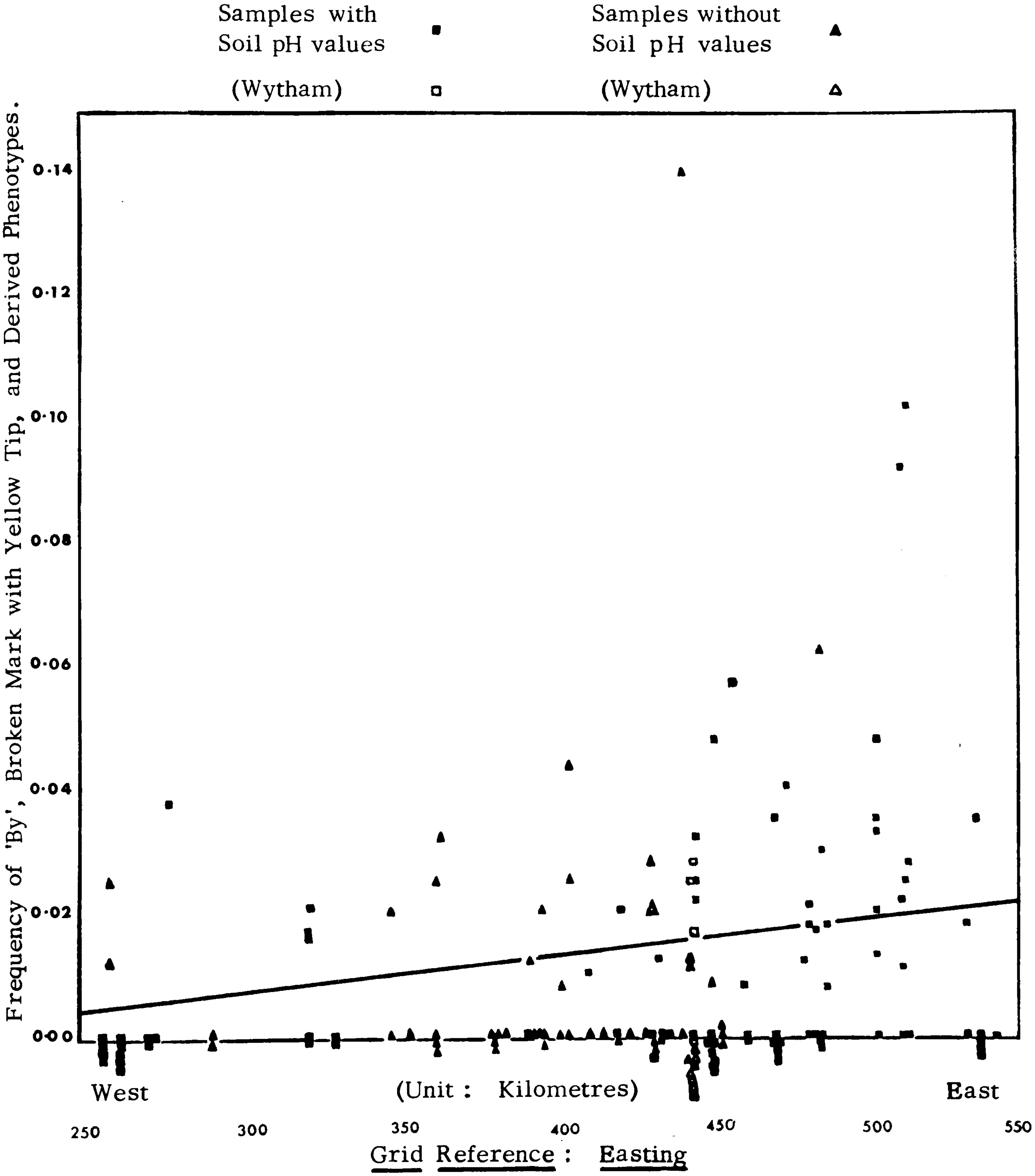
Simple Reg. Eq. : (O) = 0.2723($\pm$0.1467) - 0.000503($\pm$0.000154) (GR(E)km - 424($\pm$76))

Figure VIII (vi) Corrected 'LL' Frequency - Easting Scatter Diagram



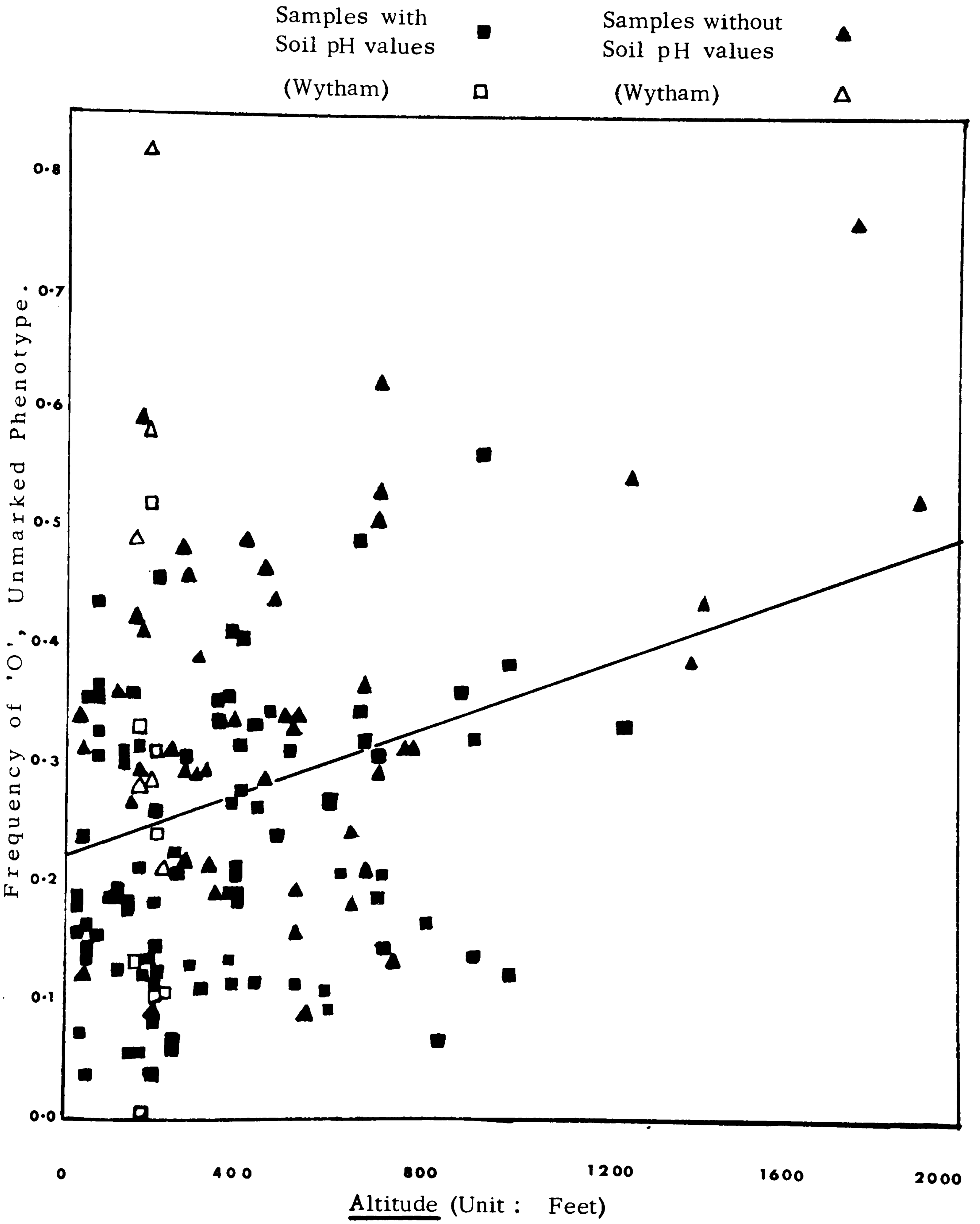
Simple Reg. Eq. : (LL) = 0.0206(±0.0469 - 0.000007(±0.000051) (GR(E)km - 424(±76))

Figure VIII (vii) 'Broken-Yellow' Mark Frequency - Easting Scatter Diagram



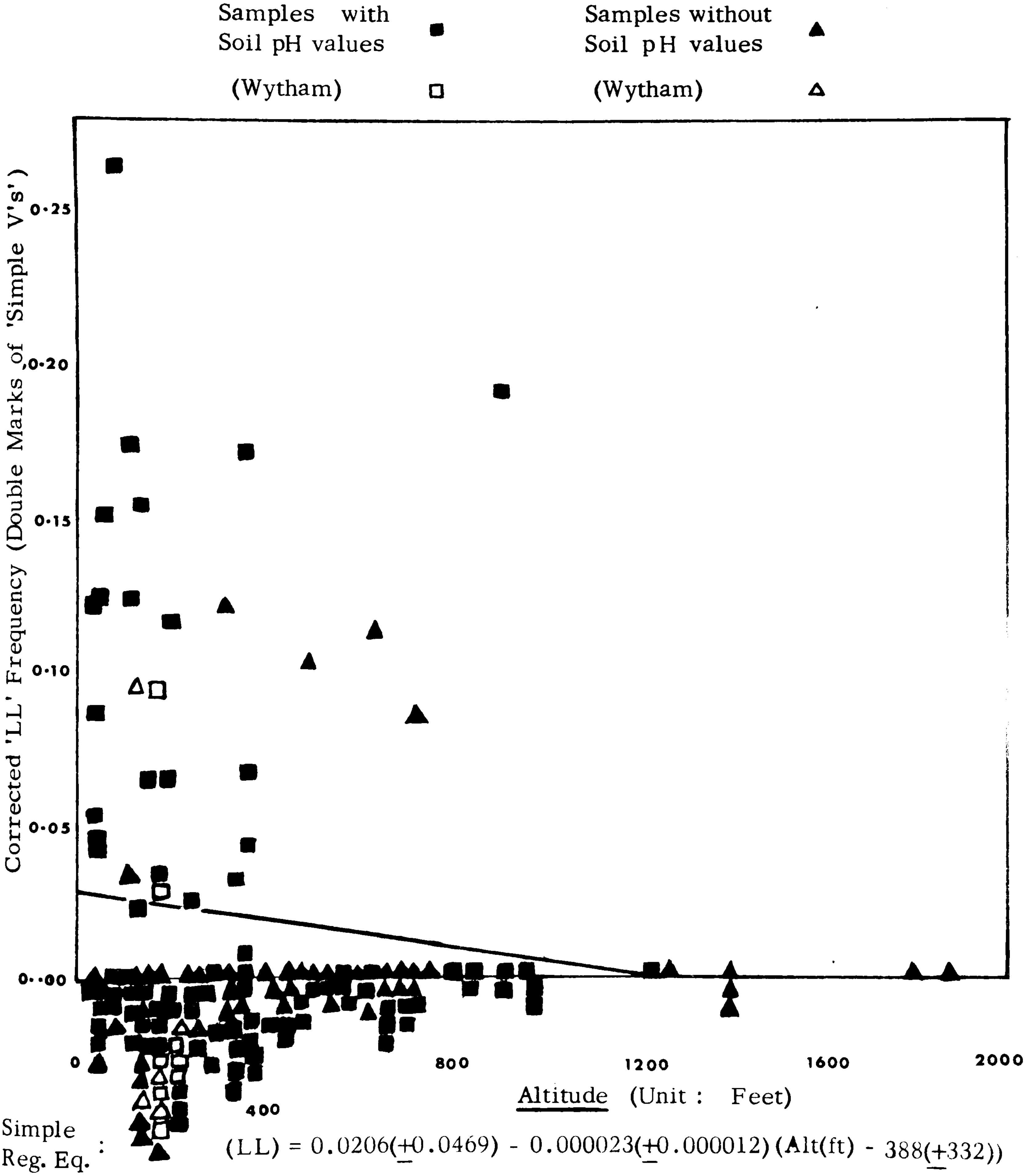
Simple Reg. Eq. : $(Y) = 0.0149(\pm 0.0207) + 0.000057(\pm 0.000022)\ (GR(E)km - 424(\pm 76))$

Figure VIII (viii) 'O' Frequency - Altitude Scatter Diagram



Simple Reg. Eq. : (O) = 0.2723(±0.1467) + 0.000136(±0.000035) (Alt(ft) - 388(±332))

Figure VIII (ix) Corrected 'LL' Frequency - Altitude Scatter Diagram



Simple Reg. Eq. : $(LL) = 0.0206(\pm 0.0469) - 0.000023(\pm 0.000012)(Alt(ft) - 388(\pm 332))$

4. Mapping.

A set of maps follows below to illustrate the geographical distribution of frequencies of the various phenotypes.

a) British Isles. (Cf. Appendix III (a)).

Figures VIII(x) to (xii) show frequencies of the unmarked, broken-with-yellow-tip, and corrected frequencies of LL double-marks for the British Isles. They include other data as well as that from field scored samples analysed in the section above.

Each circle represents a sample or more often a group of samples added together. As distinct from the field-scored samples, those circles which represent principally material grown and scored in the greenhouse are marked with a broken outline.

These maps illustrate clearly the North-South cline in the frequency of the unmarked form, the higher frequency of the broken-yellow marks in the South-East, and the higher frequency of the LL type toward the west (which is supported by the inclusion of samples from Ireland).

b) Western Europe. (Cf. Appendix III (b)).

Figures VIII(xiii) and (xiv) show for Western Europe all the samples (except very small ones) which can be precisely located, those very close together being grouped. Samples or groups of samples based (mainly),on vegetative material are indicated by broken outlines.

A north-south cline in O frequency is not so clear, but there are definitely lower values around the Mediterranean (with the exception of the unmarked sample from Sicily). Frequencies of By seem to move in the opposite direction and reach high values around the Mediterranean.

c) Whole of Natural Range. (Cf. Appendices III(c), III(d)).

Figures VIII(xv) and (xvi) show all the samples available for most countries. However, in some countries (France, Poland, Spain, Portugal) where all or nearly all the samples are precisely located, it is possible to represent several groups of samples, and in this case unlocated samples are ignored. Two samples, indicated by broken outlines, are included, from Ethiopia and the Azores, which are probably outside the natural range. It should be borne in mind that compared to these and other outlying samples on the whole-range maps, the central circles representing many combined samples are very much larger: extreme values in the small isolated peripheral samples are less important than they may appear on the maps.

Because of the apparent difference (cf. part (a)) between British field scores and others, the British data in these two large-scale maps is based only on greenhouse-grown and scored material. Almost all of the Irish material was vegetative, and was grown transplanted, but in the open, and is included here.

The group of smaller scale maps (Figures VIII(xvii-xxii)) show frequencies for all the rare phenotypes, including the high V-marks (H), which have not been mentioned before in this chapter. For them the only British data used has been that not scored in the field. Inconsistencies in the scoring of these marks were discussed above (Chapter VI, Section 3(a)) and it was pointed out that the virtual absence of H scores in the British field scored data was probably due to reluctance to make the distinction between H and L, rather than to a genuine extremely low frequency. This presumption is supported by the appearance of a moderately high frequency of H scores in the other British data. It should be remembered that any plants scored as HL will have contributed both to the H frequency map and the LL frequency map.

d) Conclusions.

In Section 3 an estimate of 2 to 2½ per cent per 100 km. was obtained for the slope of the North-south O frequency cline. This predicts a change of frequency of about 30 per cent between the North Mediterranean, and North Britain and South Scandinavia. Even in Figure VIII(xv) where the British O frequencies have been considerably reduced by the exclusion of field scored material, the presence of a cline at least half as steep as the British one is supported by the map, although in detail it looks like an abrupt change as the Mediterranean coast is approached, rather than a smooth cline. Inclusion of the field-scored data would make the cline appear considerably steeper (cf. Figure VIII(xiii)).

Among the other maps, only those of F and Ba show simple clines; F frequency increasing towards the south-east, and Ba frequency towards the north-west. The LL map suggests high frequency in the centre but especially towards the south-west and to some extent towards the north-east, and low values both in the north-west and the south-east. The B and M, By, and H maps all suggest regions of high frequency in the centre of the range, for B and M in the Alps and the Balkans, for By in the Pyrenees and in Greece, and for H in France. The By distribution seems particularly complex, and could be interpreted as a low frequency periphery, a (semi-) circular belt of high frequency, and a central region of low frequency which coincides with the region of high B frequency.

Fig VIII(x)

Frequency of Unmarked Phenotype O in British Isles

Full circle = 100% Frequency

Sample Size: ○ 25-99

Sample Size: ◯ 100 or more

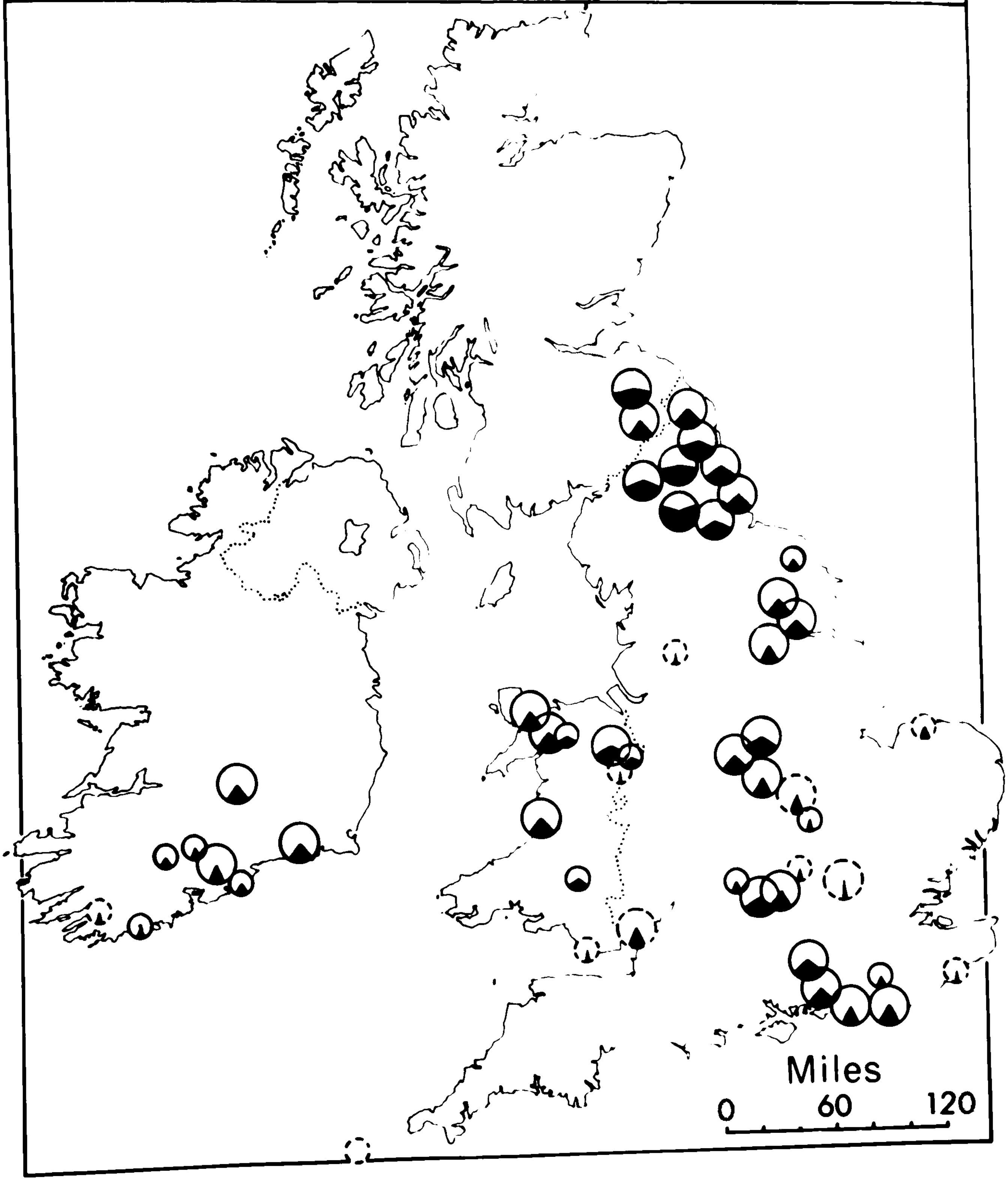


Fig VIII(xi)

Frequency of By and derived Phenotypes in British Isles

Full circle = 5% Frequency

Sample Size ○ 25-99 ◯ 100 or more

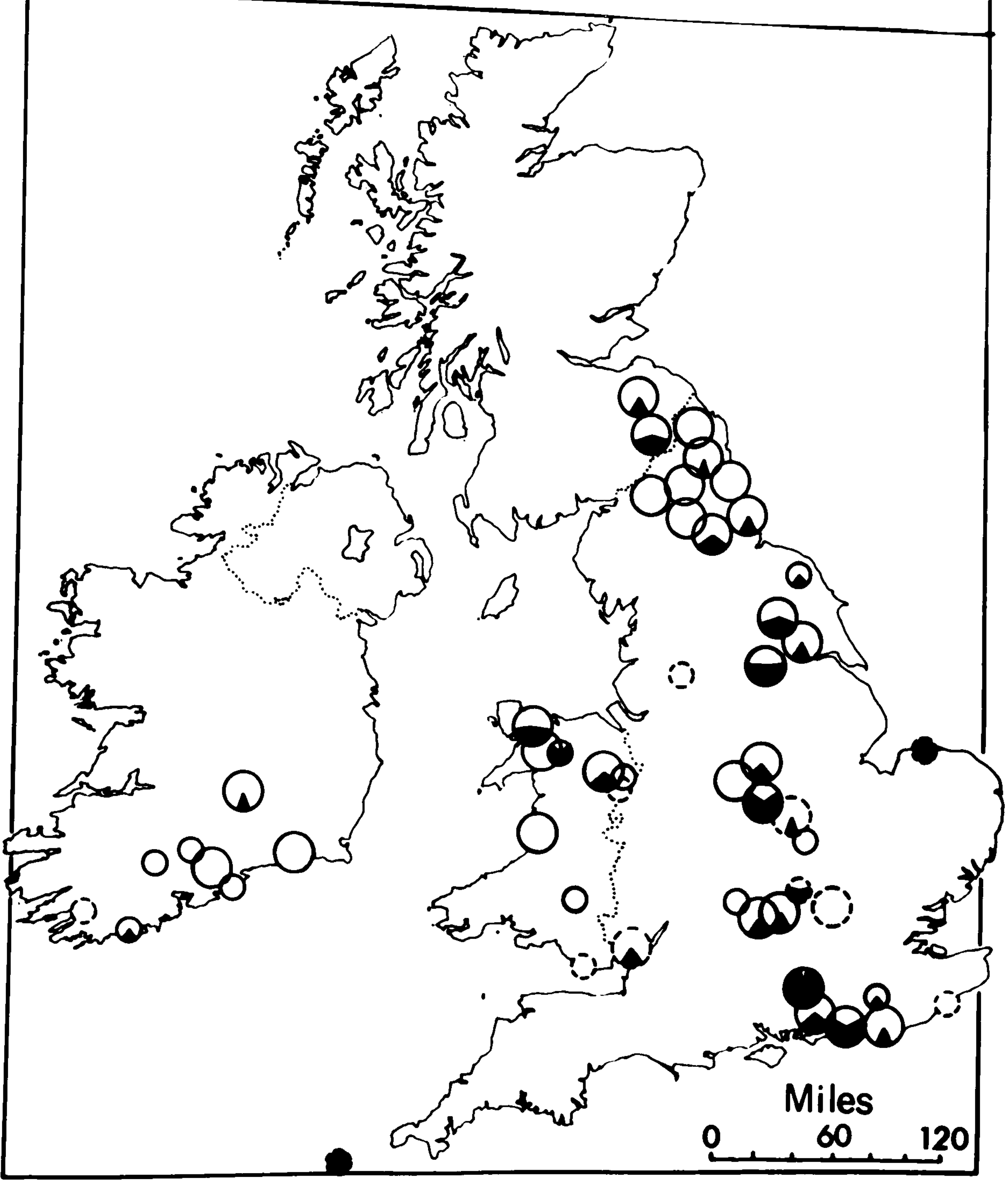
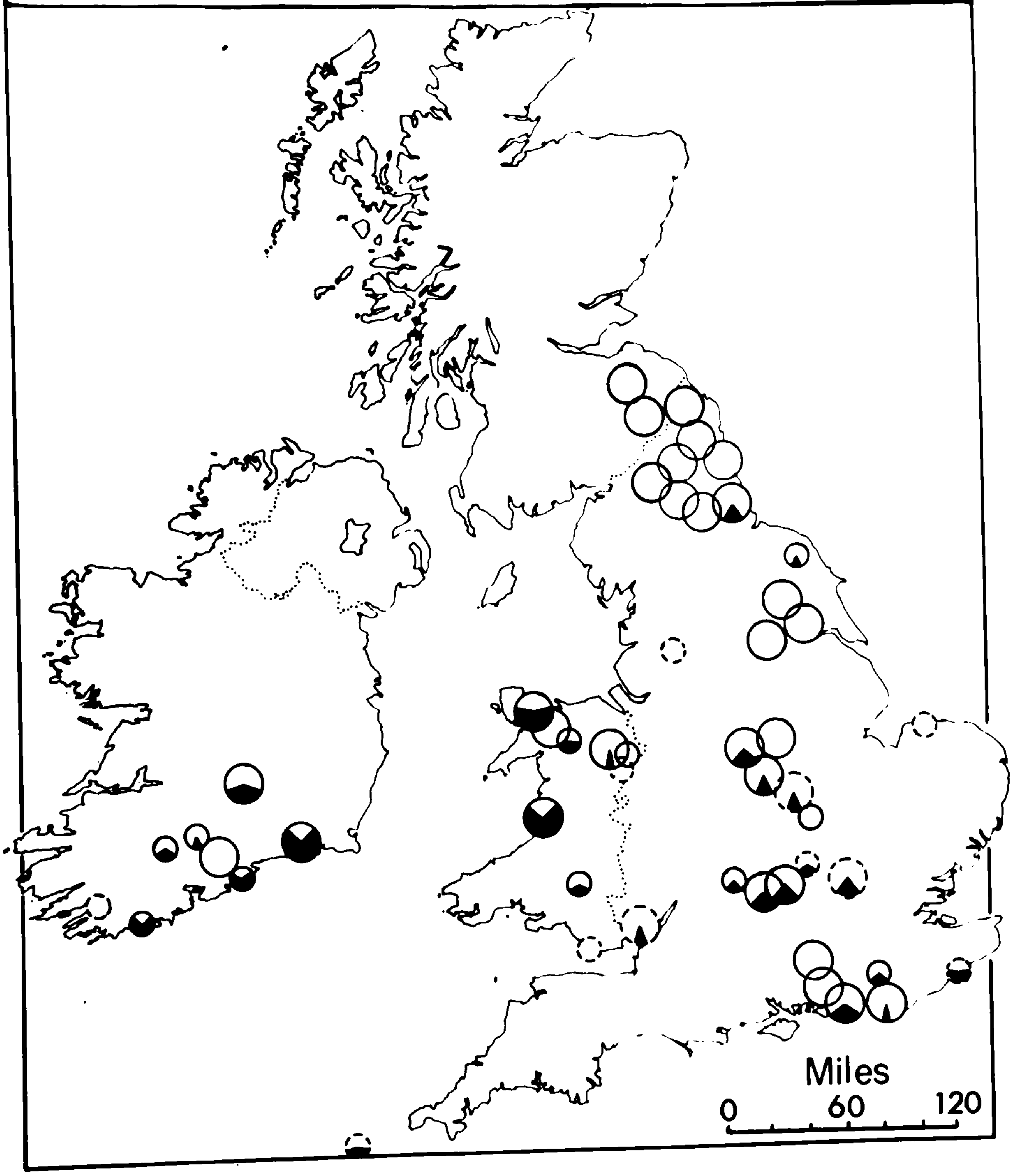


Fig VIII (Xii) Corrected Frequencies of LL Double-marked Phenotypes in British Isles

Full circle = 20% Frequency

Sample Size ○ 25-99 ◯ 100 or more



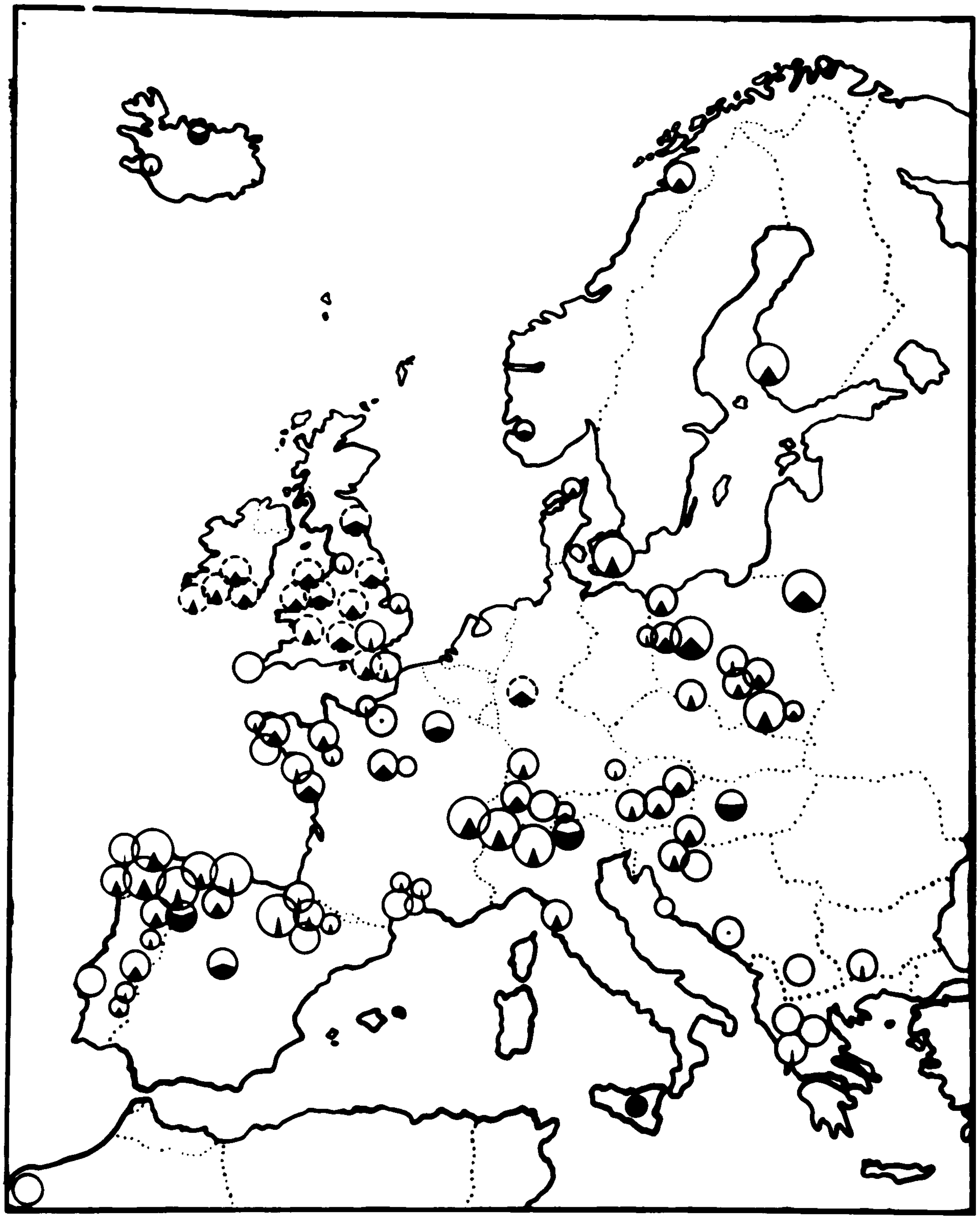


Fig. VIII xiii Frequency of Unmarked Phenotype O in Western Europe, Known Locations

Full circle = 100 % Frequency

Sample Size
○ 14 - 39
○ 40-199
◯ 200 or more

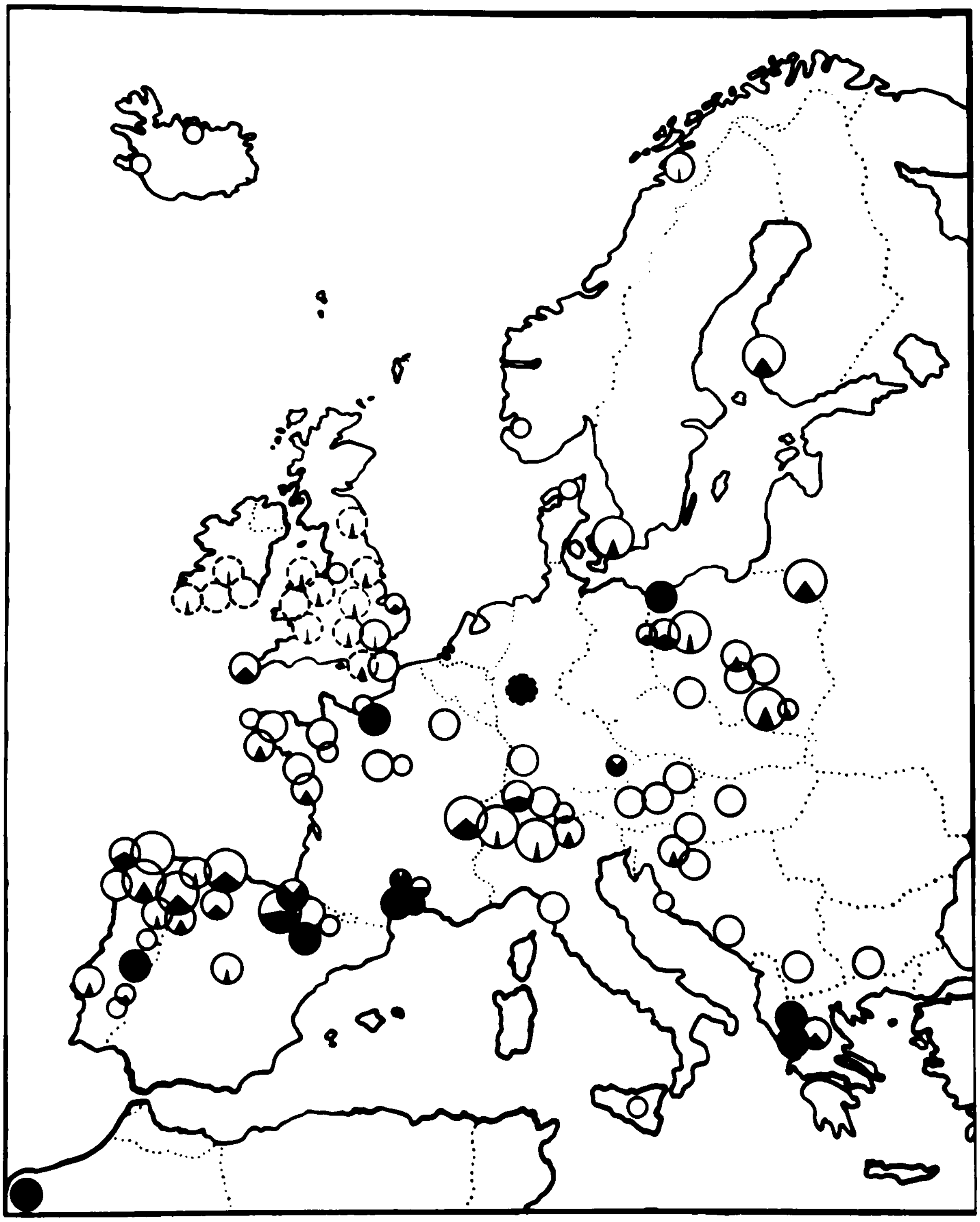


Fig.VIII xiv Frequency of By and derived Phenotypes in Western Europe, Known Locations

Full Circle = 20% Frequency

Sample Size
○ 14-39
○ 40-199
○ 200 or more

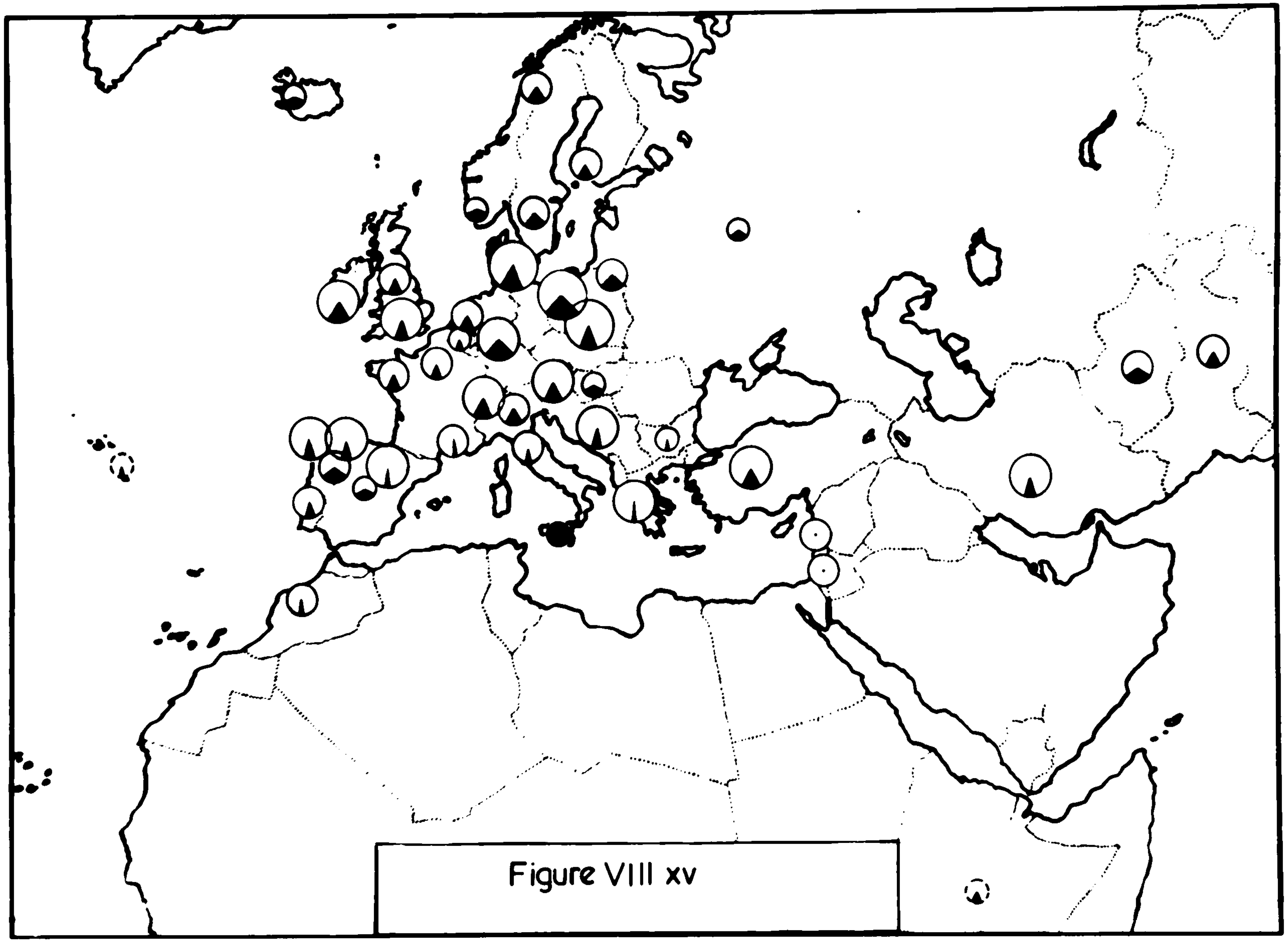


Figure VIII xv

Frequency of unmarked Phenotype O throughout Natural Range

Full circle = 100 % Frequency

Sample Size ○ 30 – 124
○ 125 – 499
○ 500 or more

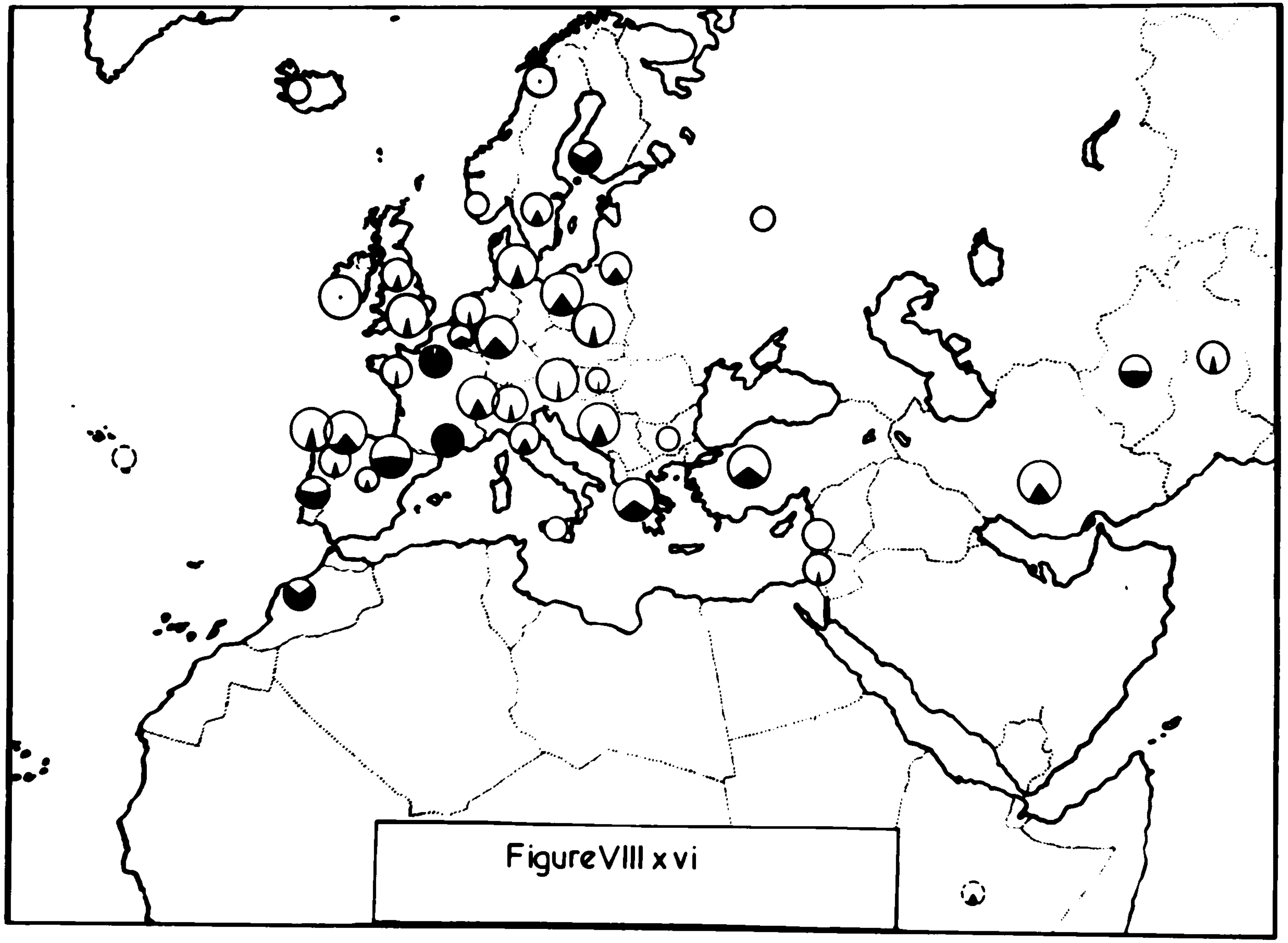


Figure VIII xvi

Frequency of By and derived Phenotypes throughout Natural Range

Full circle =	Sample	○	30-124
20% Frequency	Size	○	125-499
		○	500 or more

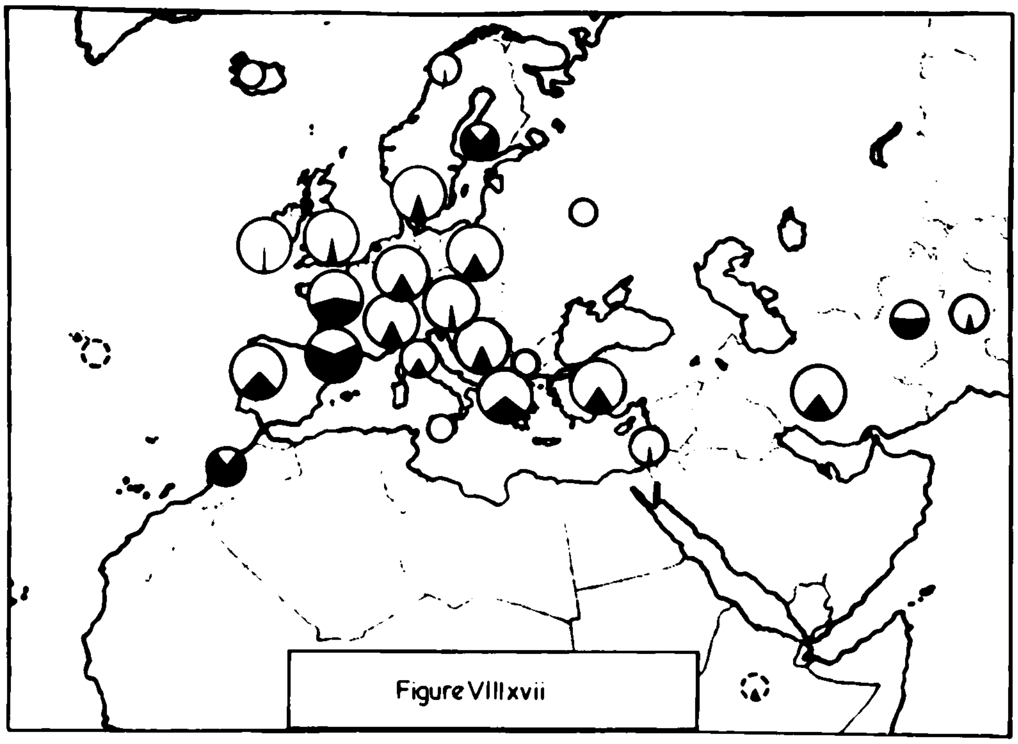


Figure VIII xvii

Broken Yellow Tip By Full Circle = 20%

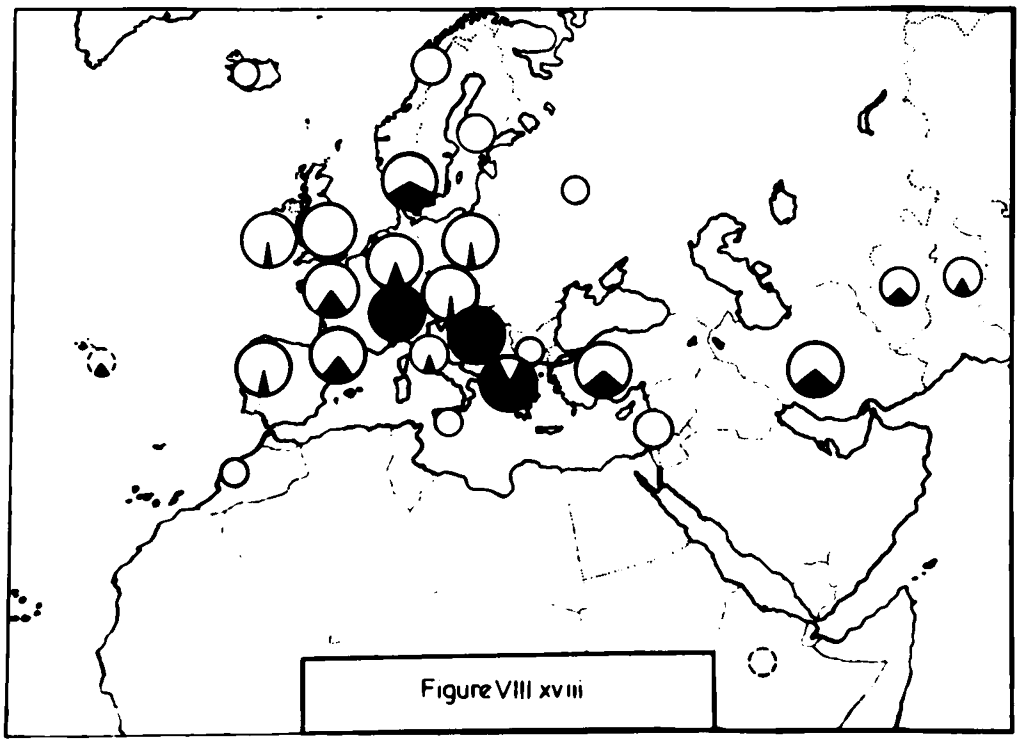


Figure VIII xviii

Broken mark B Full Circle = 5%

Sample Size ○ up to 200 ○ 200 to 700 ○ over 700

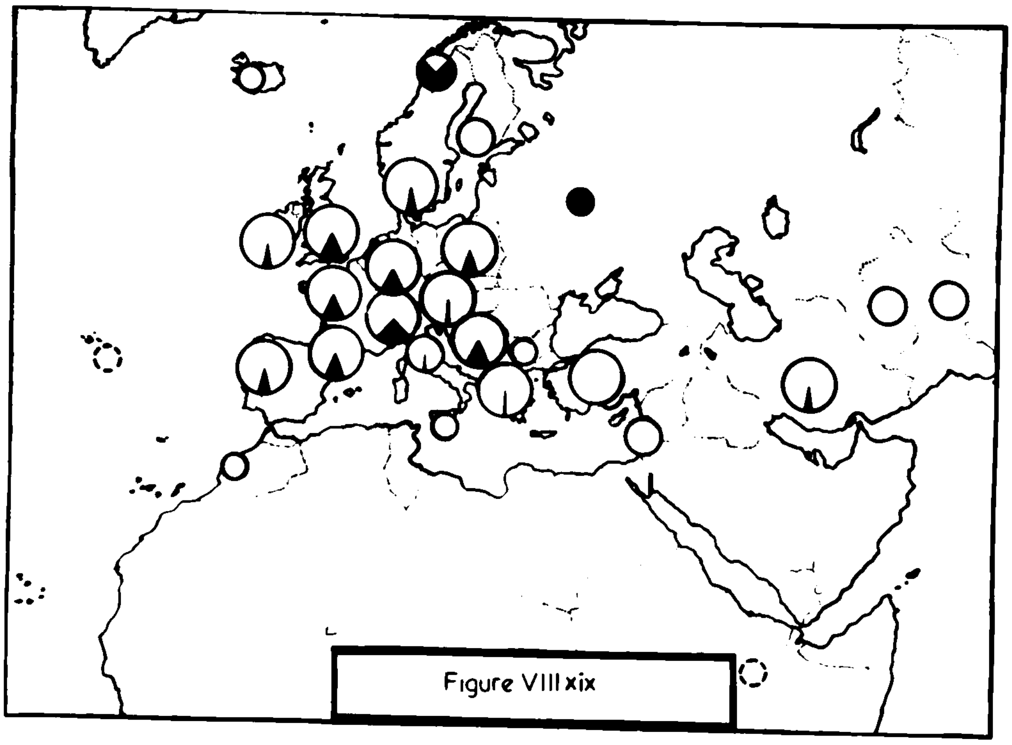


Figure VIII xix

Basal mark Ba Full Circle = 5 %

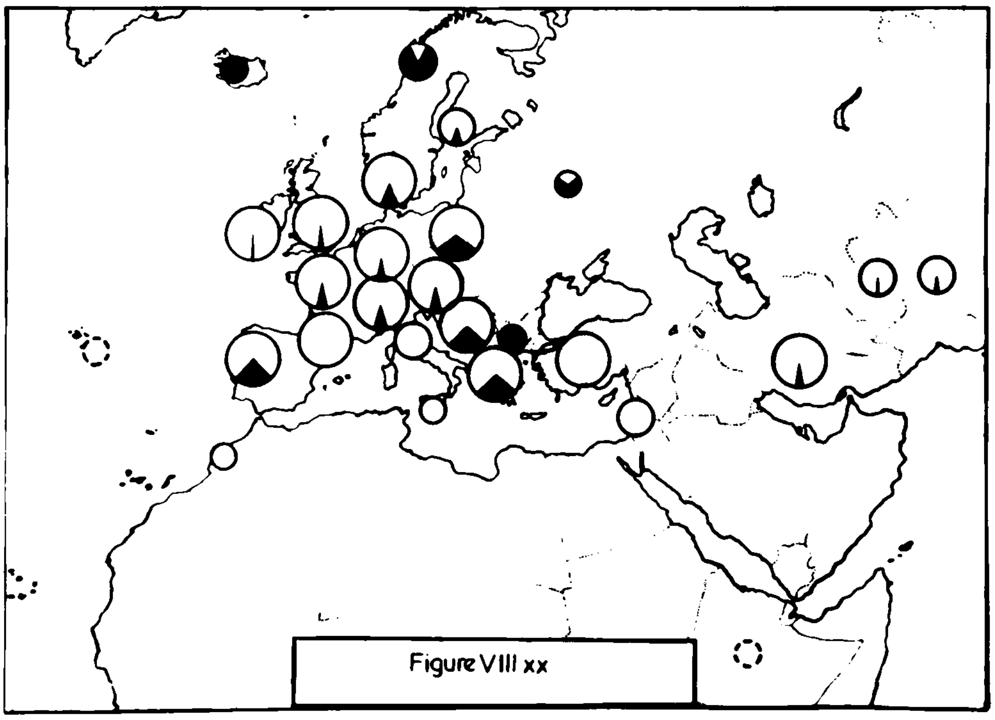


Figure VIII xx

Filled-in mark F Full Circle = 10 %

Sample Size ○ up to 200 ○ 200 to 700 ○ over 700

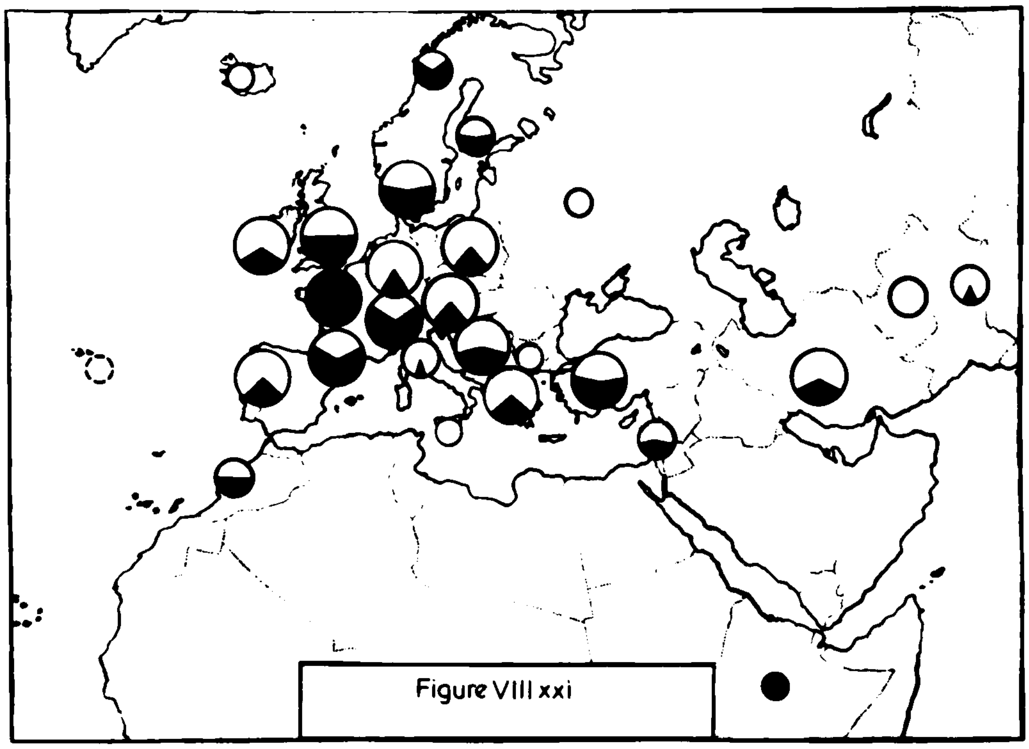


Figure VIII xxi

High simple V-mark H Full Circle = 5 %

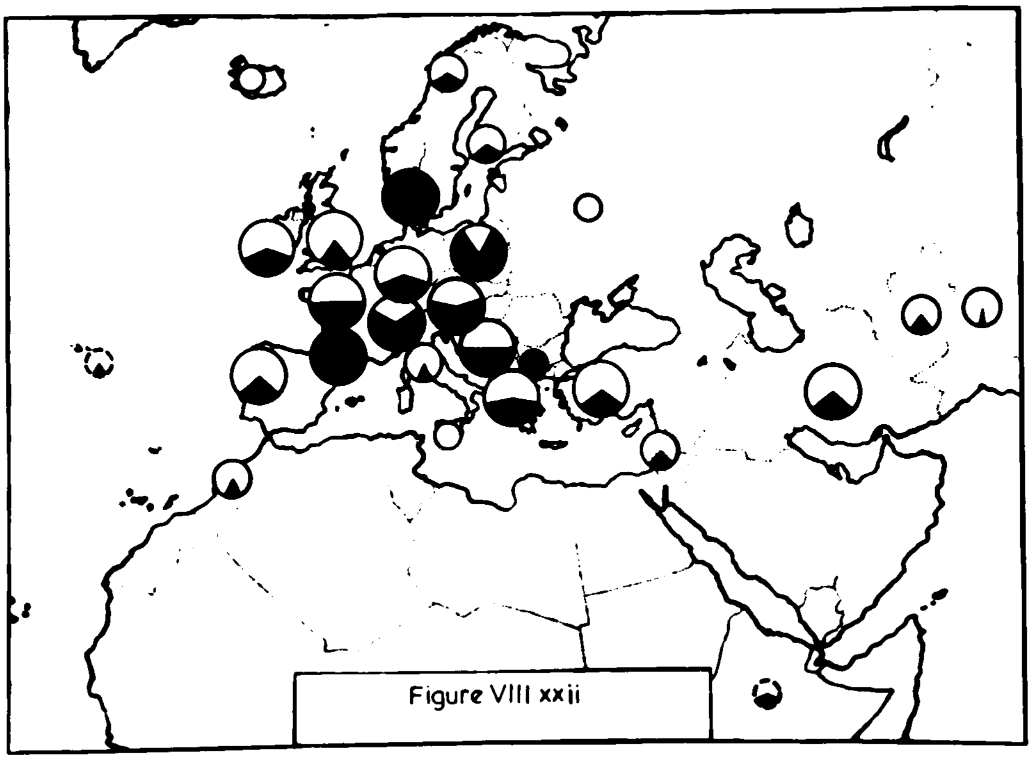


Figure VIII xxii

Double mark (simple V's) LL (corrected) Full Circle = 20 %

Sample Size ○ up to 200 ○ 200 to 700 ○ Over 700

5. Other Sets of Data sufficient for Quantitative Comparisons.

a) W.P.B.S. Collections in Spain and North Portugal.

The data acquired from the Welsh Plant Breeding Station included a large number of samples from the north-west of the Iberian Peninsula with detailed descriptions of their original sites. 46 samples from which 25 plants or more had been scored were provided with figures of the altitude of the original site. Simple regressions for each morph frequency were calculated on altitude. The results are shown in Table 8.8, and the simple regression coefficients for the British data (cf. Table 8.7) are included for comparison. It will be seen that there is fairly close agreement between the two sets of figures, though one is based on seed samples from Spain, and the other on vegetative material scored in the field in Britain. The main conclusion is that there is a clear increase of O frequency with altitude. A scatter diagram of the Spanish data is shown in Figure VIII(xxiii).

Although the background descriptions are detailed, it is difficult to use much of the information. One simple procedure is to arrange the samples in order of morph frequency and observe which environmental features appear to cluster at one or other end of the series.

Table 8.8 Spanish Collections: Regression on Altitude.

Simple Regression Coefficients for each Morph Frequency on Altitude. Figures for British field-scored data in brackets for comparison.

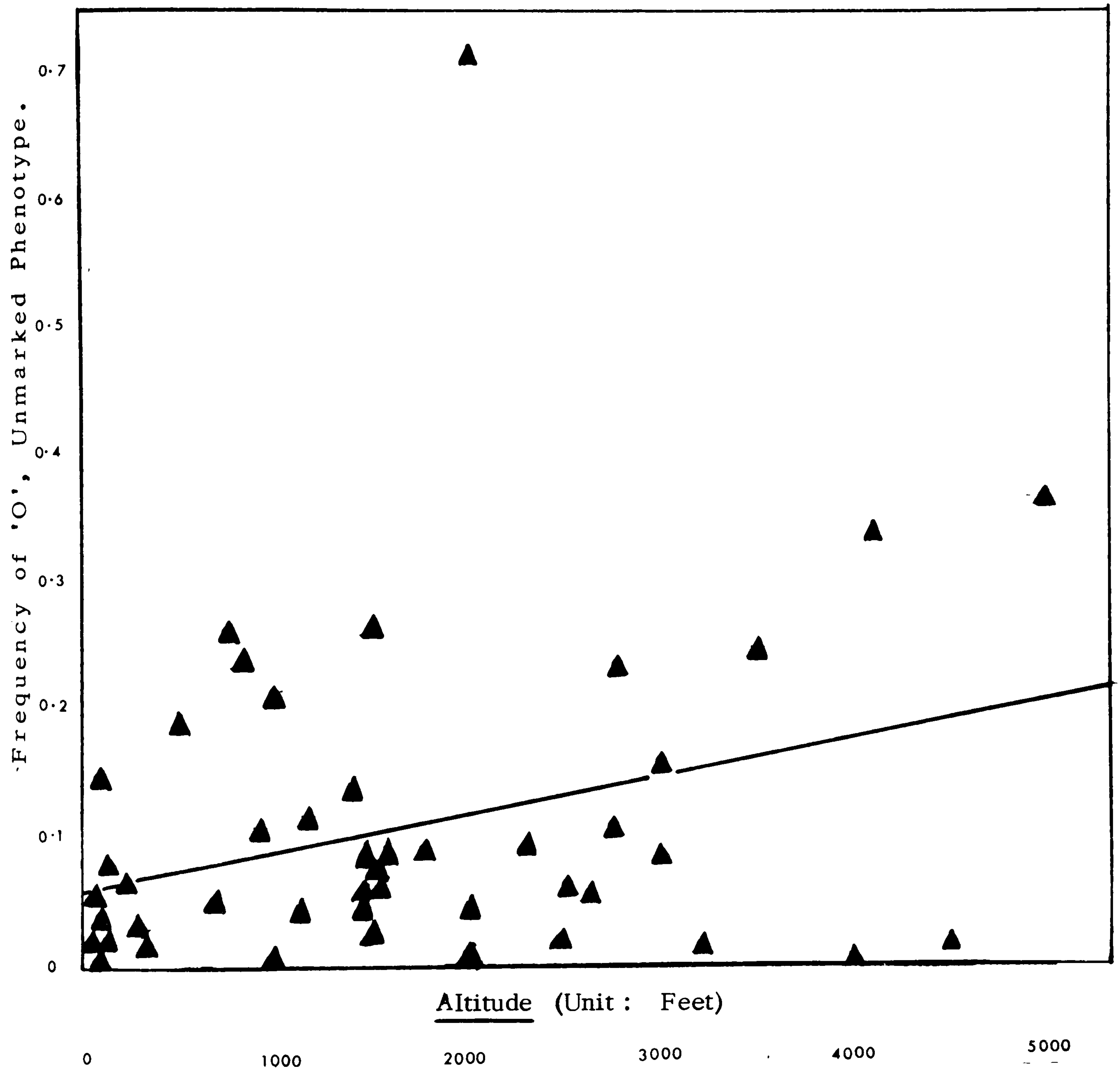
Units: Mean and Standard Errors of Frequencies in absolute proportions; Coefficients: Changes in frequency in parts per thousand per 1000 ft.

	Morph Frequency		Regression Coefficient	Standard Error	Significance (P-level)
	Mean	S.E.			
O	0.1089	±0.1287	+30.02	±14.45	* 0.05
			(+135.79	±34.82	*** 0.001)
Y	0.0412	±0.0652	-8.91	±7.55	0.3
			(-9.78	±5.14	0.1)
B	0.0017	±0.0103	-0.17	±1.21	N.S.
			(No B scored in Britain)		
Ba	0.0036	±0.0100	-2.02	±1.14	0.1
			(-4.85	±4.53	0.3)
F	0.0177	±0.0490	+2.05	±5.92	N.S.
			(-0.59	±2.15	N.S.)
LI	0.1382	±0.1535	-18.73	±17.84	0.3
			(-23.27	±11.53	* 0.05)
L	0.8659	±0.1344	-32.69	±15.03	* 0.05
			(-131.48	±34.23	*** 0.001)

Mean Altitude 1658.0 ±1287.0

Figure VIII (xxiii)

Spanish Samples : 'O' Frequency - Altitude Scatter Diagram



Simple Reg. Eq. : (O) = 0.1089($\pm$0.1287) + 0.000030($\pm$0.000014) (Alt(ft) - 1658($\pm$1281))

By this method the following species and genera appeared to some extent to be associated with high O frequency: *Holcus* spp., *Festuca rubra*, and *Cynosurus*; with low O frequencies, *Trifolium pratense*, *T. fragiferum*, *Festuca arundinacea* and *Lolium* spp. The procedure for listing the species was, it is understood, not very systematic, and too much weight should not be attached to these lists.

There appeared to be associations of high O frequency with wetness, and with pastures as opposed to waste places and roadsides. The second classification appeared to have some relation to F frequency; and figures for Y frequency and dryness are also presented for comparison with those in Part (c) below. The numbers of plants are summed for all samples with similarly described sites.

Table 8.9. Morph Frequencies and Agricultural Use of Site.

	N	O	O/N	F	F/N	O (Unmarked)
Meadow	832	96	0.115	10	0.0120	$X^2_{(5)} = 46.64$
Combined Meadow and Pasture	221	32	0.145	-	0.0	$P \ll 0.001$
Pasture	910	143	0.157	36	0.0396	***
Waste	535	50	0.093	13	0.0243	F (Filled-in)
Roadside	259	7	0.027	16	0.0618	$X^2_{(5)} = 42.83$
Undescribed	240	15	0.063	-	0.0	$P \ll 0.001$ ***
Total	2997	343	0.114	75	0.0250	

Table 8.10. Morph Frequencies and Water Regime in Spanish Samples.

	N	0	0/N	Y	Y/N	0 (Unmarked)
Wet or Moist	916	98	0.107	34	0.037	$X^2_{(5)} = 204.85$
Irrigated	346	18	0.052	17	0.049	
Near Water	267	98	0.367	3	0.011	$P \ll 0.001$ ***
Dry but near Water	304	44	0.145	8	0.026	Y (Broken-Yellow)
Dry	477	28	0.020	25	0.052	$X^2_{(5)} = 10.51$
Undescribed	687	58	0.084	30	0.044	$0.1 > P > 0.05$
Total	2997	344	0.115	117	0.039	

It will be seen from these figures that there is clear heterogeneity between the habitats in the first three comparisons. In the first case, 0 seems to be frequent in pastures and infrequent on roadsides; and F frequent in both these habitats but rare where there was any mowing. In the second case, it is difficult to summarise the patterns; the conditions favouring high 0 could perhaps be adequate water with good drainage.

b) Old Rye-Grass Pastures in South Wales.

A second set of data of some size, also from the Welsh Plant Breeding Station, consisted of seed samples from old rye-grass pastures in coastal Monmouth and Glamorgan. 19 samples in all with more than 15 plants were analysed, by

Simple and Multiple Regression. Although Grid Reference and Altitude were known, they were all within very narrow ranges, and gave no approach to significance except in the case of LL frequency increasing northward (away from the sea). One other quantified factor with a greater range of variation was the estimated percentage of rye-grass in the field (Range: 0-60%). Results of simple regression analysis for the morphs present appear in Table 8.11.

Table 8.11. Simple Regression Coefficients on Percentage of Rye-Grass in Original fields of Monmouth and Glamorgan Samples.

Unit: Increase in frequency parts per thousand per 10% increase in estimated rye-grass content.

Rye-Grass Estimated Percentage: Mean 25.26 ± S.E. 18.32%

	Mean	S.E.	Coefficient	Significance (P Level)
O	0.1207	±0.0854	+12.45	0.3
Y	0.0082	±0.0250	+6.31	* 0.05
Ba	0.0058	±0.0184	+1.64	N.S.
LL	0.0256	±0.0566	-0.10	N.S.
L	0.8765	±0.0852	-11.77	0.3

The frequency of Y seems to follow the percentage of rye-grass, as perhaps also does that of O.

Some differentiation of O frequencies also seems to exist according to the type of management; this is shown in Table 8.12.

Table 8.12. South Wales Samples: 0 Frequency and Management.

Mown only	48	8	0.167
Mown and grazed	151	11	0.073
Grazed only	279	45	0.139
Undescribed	109	13	0.119
Total	632	77	0.122
(Mown ± Grazing)	199	19	0.095)
Saltings	44	2	0.045

$X^2_{(3)} = 5.15$

$0.2 > P > 0.1$

There are apparently higher 0 values in grazed than mown fields, though the small number of fields only mown and not grazed are out of line, as are the sea-side 'salting' pastures.

c) Polish Seed Samples.

A set of 17 seed samples from various parts of Poland grown and scored at Oxford provide descriptions of the original habitats as wet or dry and mown, grazed or waste, and permit comparisons of morph frequencies among the different classes.

Table 8.13. Polish Samples: 0 Frequency and Mown, Grazed or Waste.

	N	0	0/N	F	F/N
Pasture	374	95	0.254	3	0.008
Meadow	292	45	0.154	3	0.010
Waste	302	27	0.089	35	0.116
Total	968	167	0.173	41	0.042

	0	F
$X^2_{(2)} =$	32.70	58.49
P	< 0.001 ***	< 0.001 ***

Table 8.14. Polish Samples: O and Y Frequency and Wetness.

	N	O	O/N	Y	Y/N
Dry	276	76	0.275	1	0.004
Intermediate or Undescribed	467	70	0.150	9	0.019
Wet	225	21	0.093	23	0.102
Total	968	167	0.173	33	0.034

	O	Y
$X^2_{(2)} =$	32.02	42.63
$P <$	0.001	$<$ 0.001
	***	***

The figures for F and agricultural use of the land are included for comparison with those above (Table 8.9); all the 35 F or derived scores from waste land are in one sample ('wayside place' at Wilczowice Stare, near Łódż) without which there would be no indication of any effect of this factor on F frequency.

d) Swiss Samples.

This type of analysis, summing samples sharing a certain feature to isolate its effects, depends on the features concerned being distributed independently; a genuine causal relationship with one factor could cause an apparent effect of another factor if the two environmental factors were correlated. Such associations are almost inevitable when there are very few separate samples. The last candidate for this type of analysis is a set of seven Swiss samples.

Table 8.15. Swiss Samples: Morph Frequencies and Mown or Grazed.

	N	O	O/N	Ba	Ba/N	F	F/N	Basal
Only Pasture	443	61	0.138	4	0.009	1	0.002	$X^2_{(1)} = 9.57$
Frequently Mown	403	54	0.134	17	0.042	7	0.017	$P < 0.01$ **
								Filled-in
Total	846	115	0.136	21	0.025	8	0.009	By exact test: chance of this or more uneven distribution = 0.032 *

e) Vegetative Samples from Giessen, near Frankfurt, Germany.

	N	O	O/N		O/N	O / Altitude
Heath	22	6	0.273)	all over	0.189	$X^2_{(1)} = 2.55$
Pasture	15	1	0.067)	1000 ft.		
Meadow	62	16	0.258)	all below	0.258	$0.2 > P > 0.1$
Sports Field	33	15	0.454)	1000 ft.		

Eight sets of vegetative samples were received, and were grown up in the greenhouse before scoring. The higher O frequencies appear to be associated either with low altitude (if so, in opposition to much other data), or with the different type of habitat.

6. Smaller Groups of Foreign Seed Samples.

These samples can only be presented individually, and correlations can at most be suggested, and put on record for comparison with other sets of samples.

i) Vanløse, near Copenhagen, Denmark (cf. Chapter V, Table 5.3 above)

	N	O	O/N	
N41	21	3	0.143	Meadow on filled-in lake.
N42	266	36	0.135	Rather dry
N43	322	23	0.071	Meadow (gone out of cultivation).
N44	108	52	0.481	Dry, sandy, uncultivated.
N45	481	50	0.104	Boggy, very wet, little cultivated.

High O frequency perhaps associated with dryness.

ii) Sondrio, Italian Alps.

	No. of Heads	N	O	O/N	L	L/N	B+M	B+M/N	
N155	?	96	12	0.125	16	0.167	82	0.854	meadow.
N156	(6)	71	3	0.042	55	0.775	20	0.282	pasture.
N157	(4)	42	24	0.571	18	0.428	--	0.0	dry stream bed.
N159	(14)	89	12	0.135	67	0.752	11	0.123	pasture.

High O in dry stream bed, low L in meadow.

iii) Austria: Gumpenstein and Gröbming, Steiermark.

	O	N	O/N			Exposure
N103(24)	134	11	0.082	meadow	2600 ft.	Flat
N104(13)	124	18	0.145	pasture	2400 ft.	North
N105(13)	131	26	0.198	pasture	2400 ft.	South

High O in pasture.

iv) New Zealand (naturalised wild-growing populations)

A set of 12 samples was obtained from points at regular intervals along a steadily rising transect from Motukarara at about 30 feet above sea level to Springfield at 1100 feet, 45 miles to the west. Unfortunately, in spite of the favourable arrangement of these samples, no clear trend could be recognised.

Sample No.	120	121	122	123	124	125	126	127	128	129	130	131
Scored	10	56	20	48	12	36	28	54	37	41	17	41
Unmarked	2	20	1	11	5	4	4	20	9	9	3	7
O/N	.20	.38	.05	.23	.42	.11	.14	.37	.24	.22	.18	.17

7. Small Groups of British Field-Scored Samples.

The association between morph frequencies and quantitatively measured environmental factors has been dealt with above in Section 3. For the remaining features of variation, the only method of presentation possible seems to be comparison of samples of closely similar origins but divergent morph frequencies, in the hope of discovering associations between the morph frequency differences and the remaining differences in background. For each comparison, an explanation can only be suggested; a stronger indication would only be provided if a similar pattern appeared in several such comparisons.

Because of their low frequencies, it is not possible to make any useful comparisons of frequencies of any other morphs except the unmarked type.

i) Wytham, Oxford. Field 3A. Grid Ref. SP 471 101.

	N	O	O/N	
01	25	21	0.84	Centre of field, water standing after rain.
02	13	1	0.08	Sloping bank within 2-3 ft. of river.

(Transect 200 yards long across field, from South to North)

	N	O	O/N	
1	48	15	0.31	30 ft. from stream.
10	39	20	0.51	Close to patch of Caltha.
2	43	16	0.37	Some Juncus; water standing after rain.
3	59	9	0.15	Drier than (2).
4	89	22	0.25	Near dense Juncus.
11	40	18	0.45	Near dense Juncus.
5	41	15	0.37	Middle of dense Juncus.
7	33	14	0.42	Behind raised river bank.
6	27	6	0.41	Bank beside River Thames, 1-2 ft. higher.

The presence of Juncus and Caltha can probably be taken as an indication of persistent waterlogging and bad drainage; drainage would be best beside the stream and the river, which in summer would be 2 or 3 feet below the field level.

There would seem to be an association between high O frequency and bad drainage.

These samples were collected in areas only about 20 feet across; it seems likely that different parts of the same clones may have been sampled several times, and that the sampling error is therefore greater than would appear at first sight from the above figures.

ii) Wytham, Oxford. Field 3B. Grid Ref. SP 468 101.

	N	O	O/N	
1	54	22	0.40	Near stream bank.
2	37	9	0.24	Higher drier part (molehills).
3	17	3	0.18	Behind raised river bank.
4a	61	13	0.21	Middle of field, some Juncus.
4b	40	33	0.83	Centre of field, away from drainage.
5	34	11	0.32	Very damp, abundant Juncus.

These results are consistent with the association of high O frequency with bad drainage reported in (i), though they are not so clear.

iii) Wytham, Oxford. Field 13. Grid Ref. SP 472 098.

1	40	11	0.28)	Higher part of field, 3-4 ft. above other.
2	15	2	0.13)	
3	76	8	0.11)	
4	115	50	0.44)	Riverside lower part of field.
5	45	31	0.69)	
6	45	25	0.56)	
7	4	3	(0.75))	

There would appear to be a divergence of O frequency between the two halves of the field, which can probably be attributed to the change in height affecting the drainage in the same way as above.

iv) Blenheim Park, Woodstock, Oxon. Grid Ref. SP 440 163.

1	35	17	0.49	Short worn turf beside drive, dry, exposed.
2	50	11	0.22	Clearing beside lake, longer grass, damper, sheltered.

High O in exposed dry conditions, short turf.

v) Marston Ferry, near Oxford. Grid Ref. SP 522 090 (One field).

1	91	13	0.14	Mixture of short turf and longer grass.
2	60	2	0.03	Close-grazed turf near footpath.
3	59	10	0.17	Close-grazed, exposed, dryish.
4	123	11	0.09	Longer grass, damper, sheltered by trees.
5	84	11	0.13	10 ft. lower than others on river bank.
6	90	41	0.46	Longer grass close to hedge, sheltered.

No clear pattern.

vi) Sherburn, Co. Durham. Grid Ref. NZ 332 414.

1	50	19	0.38	Waste ground beside road, long grass; clover mostly in short turf on trodden paths.
2	75	21	0.28	Hard grazed pasture, with thistles.
4	50	17	0.34	Hill top, floor of disused quarry. Clover only on paths, acid-soil vegetation elsewhere.
5	49	17	0.35	On and beside more or less bare paths among dense bushes.
6	31	6	0.19	Open hill top, long grass.

High O in sheltered places and on trodden compacted soil.

vii) Wolsingham, Co. Durham. Grid Refs. NZ 075 367, 073 378.

Hill	79	19	0.24	Rich pasture, N-facing slope, 600 ft.
Town 1	54	24	0.44	Damp ground, narrow strip beside river.
Town 2	45	15	0.33	Same field, 10 ft. higher (about 450 ft.); dryish pasture field.

High O in damp place, low O in more fertile field.

viii) Stoneygate, Co. Durham. Grid Refs. NZ 350 508, 342 508.

1	159	44	0.28	Coarse grass, thistles.
2	175	32	0.18	Finer grass, better pasture, closer grazed.

High O in denser vegetation.

ix) Thropton, Northumberland. Grid Ref. NU 029 026.

1	130	59	0.45	River bank, silt left by flood; rough grass.
2	85	25	0.29	Slightly higher, firm ground, drier, closer grazed.

High O with dampness, less intensive use, longer grass.

x) Oxton, Berwickshire. Grid Ref. NT 497 536. (Adjacent fields).

1	50	31	0.62	Rich fertile close grazed pasture.
2	31	9	0.29	Coarser grass, few patches close grazed.

High O with greater fertility, more intensive use.

xi) Transect up North side of Chanctonbury Hill, Sussex, from Buncton Manor Farm (low, N. end of transect, sandy soil) and Bushovel Farm to high ground at South end on chalk hills. Grid Refs. TQ 147 136 - TQ 140 122.

						Alt.(feet)	Aspect
B.M.Fm.		134	23	0.17	Close fine turf	130	flat
Bush	1	59	3	0.05	Coarser grass, thistles	180	(S)
	2	53	12	0.23	Longer grass, less clover	150	flat
	3	132	17	0.13	Rough grass	180	(N)
Cha.	2	83	22	0.27	Fine turf on slope, mole-hills, little clover.	380	N
	3	30	6	0.20	Coarse turf.	700	N

No clear pattern.

xii) Between Fulmer ane Kingston, Sussex. Grid Ref. TQ 385 093 - TQ 384 079. Transect up northern side of hills.

					Altitude	Aspect
1	70	21	0.30	Pasture, long grass	100	S
3	57	3	0.05	Waste, long grass	140	NW
4	27	2	0.07	Waste, long grass	200	NW
5	57	10	0.18	Hillside fairly long grass	340	E
6	19	3	0.16	Valley on hillside, mixed short turf and tussocks.	400	SE
7	41	4	0.10	Steep slope, hillside, do.	430	NNE

High O in pasture, low on waste.

xiii) Barton Hill, Yorkshire. Grid Ref. SE 720 655.

					Alt.	pH
1	85	19	0.22	Hilltop, rough pasture on sand	260	6.0
2	52	11	0.21	West facing, damper, finer grass	150	6.9
3	66	21	0.32	Damp, flat, almost on level of plain to W., finer grass	110	(6.0 (6.6

Third sample appeared to have more O in one part; more acid soil sample collected there.

Higher O with dampness and perhaps with acidity (especially if overall trend of increase with altitude is allowed for).

xiv) Saltergate, Yorkshire. Grid Ref. SE 852 939.
100 yd. Strip of roadside verge above steep west-facing slope.

N	28	9	0.32	Brown soil, pH 7.2
C	48	8	0.17	Dark brown, pH 6.4
S	11	-	0.0	Black soil, pH 5.5

No other visible difference except that clover was less abundant on the more acid soil.

Strong suggestion of higher O on less acid soil.

xv) Warter, Yorkshire. Grid Ref. SE 844 505.
All in one field, grazed by cattle (and rabbits).

2	48	4	0.08	Top of field, slight slope, brown loam.
3	53	18	0.34	Slightly lower, steep SE slope, on sandy soil round rabbit holes.
4	41	11	0.27	Flat damper bottom of field, 100 ft. lower, dark loam.

xvi) Market Harborough, Leics. Grid Ref. SP 743 873.

1	90	6	0.07	Waste ground, herb layer not closed.
2g	66	4	0.06	(Same field,) Areas of very close grazed turf.
21	25	5	0.20	(horse pasture)Areas of longer grass.

(significance of difference between the last two, $0.1 > P > 0.05$).

High O perhaps where clover part of dense vegetation.

xvii) Llangollen, Denbighshire. Grid Refs. SJ 228-243 402-421.

1	145	50	0.35	Fine dense turf. Exposed	660	5.7	flat
2	80	26	0.33	Rough pasture (gorse); clover only on short-grazed patches	1250	5.2	flat
3	34	20	0.59	Fine dense turf. Clover mostly near and on track	930	4.3	S
4p	11	8	0.7	Path, close trodden turf	900		NW
4g	8	2	0.25	Close to above, little clover			
5	50	12	0.24	Dense turf, dryish pasture	460	5.3	NW
6	60	16	0.27	Rich damper pasture	370	6.3	flat
7	64	23	0.36	Rough pasture between main road and river, with tussocks	320	4.9	(N)

High O on trodden paths, lower with high pH.

xviii) Ty'n-y-Maes, near Bethesda, Caerns. Grid Ref. SH 636 636.

1	37	7	0.19	Dry S-facing hillside; clover in depressions.
2	27	13	0.48	Flat marsh land level with river.
3	40	13	0.33	On earth bank below road; damp, above marsh.

High O in dampest conditions.

xix) Northpark, Sussex. Grid Ref. TQ 051 157. Transect across wet pasture field, almost completely surrounded by woodland.

1	74	2	0.03	Dry corner, 5 ft. higher.
2	50	7	0.14	Damp ground beyond grid of drainage channels, *Juncus* abundant.
3	69	11	0.16	Banks of drainage channel.
4	86	6	0.07	Firm ground near drainage channel, little *Juncus*.
5	59	8	0.14	Lowest part of field, abundant *Juncus*.
6	10	4	0.4	Less *Juncus*, not quite so low as (5).

Higher O frequency with worse drainage.

xx) Bangor, Caerns. Combined results of several areas near harbour; Penrhyn Port and Penrhyn Estate, quayside, carttrack in woodland, pasture, and waste ground.

Closed, ungrazed veg.	56	5	0.09	Higher O frequency in:
Pasture	21	5	0.24	
Open vegetation	39	13	0.33	Open vegetation
Shaded	32	3	0.09	
Exposed	84	20	0.24	Exposed conditions
Short, close-grazed	36	5	0.14	
Not close-grazed	80	18	0.23	Longer herbage.

xxi) Definog, Brecknockshire. Grid Ref. SN 925 279.

On flat strip of ground between stream (3-4 ft. below field level) and W-facing slope in higher part of field.

1	29	6	0.21	10 feet from stream.
2	38	14	0.37	50 feet from stream, at foot of slope.

Higher O with worse drainage.

Features which these comparisons suggest may be associated with high O frequency are dampness, trodden paths and perhaps denser as opposed to close-grazed herbage. The dampness factor appears not to extend to well-drained positions such as river banks. It would seem plausible to combine the first two features, suggesting that compacted, badly aerated and waterlogged soil produces a higher frequency of unmarked plants. It may be remembered that the opposite association between O frequency and wetness was suggested above (Section 6, Part (c) and Part (f,i)). Wetness was also considered in Part (b) (Spanish samples), with results which were less clear, though higher O values were found in places described as near water than those described as wet.

8. Conclusions.

a) Unmarked Phenotype.

The most abundant clearly definable class is that of unmarked plants ('O'), and it is about their frequency patterns that the most indications of reaction to the environment are available. An association of O frequency with greater distance north is the most definite of the results of the multiple regression analysis of the British field-scored data (Figure VIII(i)) and is supported by the mapping of data from the rest of the natural range (Figs. VIII(x), (xiii), (xv)). When attempting to link up the two sets of data, there is a strong suggestion that the frequencies of O are higher in the field-scored samples (cf. Figure VIII(x)).

A second factor affecting O frequency appears to be altitude, on the basis of the British data (Figure VIII(viii)) and also the Spanish data (Figure VIII(xxiii)). There is also a suggestion in the British data of higher O frequency further west.

The type of agricultural exploitation, or its absence, appears to affect O frequencies. Low O frequencies appear on waste ground (Spanish and Polish data) and higher frequencies under grazing than mowing (Polish, Spanish, S. Wales and Austrian data).

There are several indications of effects of water regime, but the pattern is not simple. In the Polish samples, dry sites showed higher O frequencies; and this may be supported by data from Denmark and the Italian Alps. The Spanish samples offer a more confused picture, with higher O frequency on intermediate sites. In the British field data on the other hand, high O frequencies in wet places are indicated by several comparisons (particularly the transects in fields at Wytham and Northpark). Again there is a suggestion that good water supply is not the factor alone, so much as excessive water supply, leading perhaps to exclusion of air by waterlogging.

This can be linked with the finding of high O frequency on trodden paths as opposed to longer nearby vegetation in some British samples. On the other hand, denser vegetation in contrast to short grazed (rather than short-worn) seems to be associated with increased O frequencies (particularly the horse-grazed pasture at Market Harborough).

b) Simple V-alleles.

The frequencies of L are the complement of those of O, and reflect the same environmental patterns.

The measure of heterogeneity among the L marks, presented as the corrected LL frequency, shows an increase with southern and western position and low altitude in the British Isles (Figures VIII(ii), (vi), (ix), (xii)). This fits the indication of the map (Figure VIII(xxii)) of low frequencies in the extreme northwest (and southeast) of the range rising to a ridge of high frequencies between them.

H frequency is presented only by means of one map

(Figure VIII(xxi)) which suggests a high frequency around France, and somewhat lower frequencies elsewhere. If the contribution of H (plants scored as LH) were removed from the LL map, defining the class of L-marks more narrowly, it would be converted to two regions of high frequency in northeast and southwest Europe, with a trough, or at least a saddle, of low frequency between them.

c) Frequencies of the Broken-yellow mark may increase towards the east in the British Isles (Figures VIII(vii), (xi)), which would fit the decline towards the edge of the range shown by the maps (Figures VIII(xiv), (xvi), (xvii)). Both British and Spanish data suggest a slight decrease towards higher altitudes. The Polish data suggest an association with wetness, but this may only reflect a general tendency to move in the opposite direction to O frequency.

d) The Broken mark is apparently absent from Britain and rare elsewhere. Its high frequency in Switzerland, Yugoslavia, and Greece lies in the same area as the isolated occurrence of the marginal type, and supports the idea of a connection between them. This has been assumed, and M frequencies added into the figure presented in the map (Figure VIII(xviii)).

e) The Basal mark is generally rare, and within Britain only shows a slight association with low altitude (also in the Spanish data) and with southern position (Figures VIII(iii)), which opposes the interpretation of the map pattern

(Figure VIII(xix)) as a cline rising to the northwest, and suggests it should rather be a falling off in all directions from a centre of high frequency in France.

f) The Filled-in mark frequency is also slightly associated with southern position in Britain (Figure VIII(iv)), which fits the map pattern of an increase towards the south and east (though this does not extend beyond Europe: Figure VIII(xx)). There are also suggestions in the Spanish and Polish data that it may be more frequent on waste ground and less frequent in meadows.

PART 4

COMPARATIVE STUDIES

CHAPTER IX

LEAF MARKING IN SPECIES RELATED TO T. REPENS

1. Significance and Possibility of Comparisons.

There is a polymorphism in T. repens for the presence (and variation) or absence of whitish V-shaped leaf markings. One may presume that the effective expression of this character depends on the interaction of many genetically determined processes, but the observed variation in phenotype can probably all be accounted for by a set of alleles at one locus.

Even in an outbreeding species, drift or selection would tend to eliminate genetic variation. There is a strong presumption that examples of variation actually found are rarely those which have recently arisen and may soon disappear, but very often those which have persisted because of some balancing mechanism opposing any tendency towards fixation. Evidence of a long history of the presence of such variation would provide a very strong argument for the existence of such a mechanism.

The presence of markings of a similar kind exclusively in a restricted group of related species would be a strong indication of inheritance of the basic mechanism producing the markings from a common ancestor. The distribution, within such a group, of uniformly marked, variably marked, and uniformly unmarked species might give some further information on the ability to survive under selection, both of the mechanism by which marks can be produced, and of any mechanism favouring the persistence of variation in this respect.

Markings very similar in appearance have been described in other species of Trifolium. Their appearance in related species suggests they may be truly homologous and have common ancestry. Similar markings have also been mentioned and illustrated by Healy (1953) in Parochetus communis, also in the Trifolieae (Willis, 1955). Unfortunately most of the rather few reports of marking in Trifolium are much less detailed.

Red markings of various types resembling those in T. repens have also been described in other related species. Details of these are also of interest here bcause there are reasons such as the occurrence of V-shaped markings both red and white, which suggest some connection between the two. Some references also confuse the two colours.

In Medicago arabica (also in the Trifolieae) the leaflets are 'usually blotched' (Clapham et al., 1962). This 'purplish-brown blotch ... is sometimes absent' (Healy, 1953; cf. Taubert, 1894). I have seen specimens of Medicago with a red blotch which was thumbnail- or crescent-shaped, and more directly comparable with V-markings in Trifolium. A blotch also occurs in M. intertexta (Stahl, 1896). I do not know of any other report of comparable markings in the Leguminosae.

It seems plausible to assume that the mechanism by which white V-markings are produced in these species arose only once, in a population ancestral probably at least to all the genus *Trifolium* and *Parochetus*. The coincidence of shape and the genetical and physiological associations between red and white markings suggest that one may have arisen in connection with the other (cf. p. 69), and that the ancestor from which at least the red V-marks are derived is also ancestral to *Medicago*.

Direct investigation of many other species would have been beyond the possible scope of this work. Some observations made in other species of *Trifolium* and on *Parochetus communis* will however be described below.

An attempt has also been made to collect information on the occurrence of markings in other species. The published references are few and scattered - the only published reference principally concerned with leaf markings which refers to them in more than two species is Brewbaker (1955), who lists seven other species in which marks like those of *T. repens* occur. The greatest amount of information compiled specifically about leaf markings is unpublished.

Markings presumably often seem, to taxonomists concerned to give a general description of species, to be of trivial importance, and because of their variability of even less use in species identification. Perhaps also for reasons of space, many authors of floras and monographs make no mention of them at all. Others do mention them in some species - most commonly in T. repens and T. pratense - but in some cases omit any reference under these species while referring to markings in other cases. For example, there is no reference under T. repens in Grenier and Godron (1848), though four other species are referred to as having markings; and none under T. pratense in Bentham and Hooker (1924) though marks are referred to in three other species. A most remarkable case is that of Lowe (1868) who actually refers to the absence of markings in four species, and to its presence in four others, but makes no reference to them under T. pratense. Most authors only refer to marks when they are present, and lack of reference suggests therefore that markings are absent, though the examples above show that this is not reliable. No author seems to have gone so far as to say that since markings occur in some species, their presence or absence should be referred to systematically in all; and among those references that are made there are often differences of phraseology without difference of meaning, suggesting no standardisation.

The questions one would like to ask about each species begin with whether white V-shaped markings occur at all. Many references refer to markings without any indication of their shape or position, and often not of their colour. Descriptions like 'leaflets spotted', or 'leaflets often marked' probably refer to white marks, but are ambiguous. Further confusion between the white and red marks is produced by some remarks about frequency: for example, Gibelli and Belli (1891) on *T. fragiferum*: 'usually with whitish or dark marks'. Similarly, Allan (1940) in *T. repens* 'often variously marked with white to purple blotches or flecks', and Ascherson and Graebner (1906-1910) on *T. pratense* 'often with dark or light half-moon-shaped or irregular spots'. This latter presumably refers to light half-moon-shaped V-marks and to dark irregular spots: the former are usually present, and the latter probably very scarce. Further questions one would ask about each species are whether there is variation between different types of marking, and if so, what their relative frequencies are.

Many references do not answer these questions clearly. Because of the scattered, incomplete and sometimes ambiguous nature of the published information on this topic, because quite a large amount of unpublished information is available, and because all the sources available were insufficient to answer any of the questions above for more than a fraction of the genus, it seemed worth while to tabulate together all the information that could be found before proceeding to draw any conclusions from it about correlations of marking with phylogeny.

Out of 290 species in the genus *Trifolium* (Clapham, Tutin and Warburg, 1962), some indication of the presence of white markings has been found and is presented below for about ~~twenty-five~~ thirty species, and statements of absence of white marking for thirty more. Because most authors have only or usually referred to marks when present it seems likely that many at least of the better-known species not mentioned below also lack marks, and do not appear because no author consulted felt a need to say so positively. The twenty-five marked species thus probably represent a sample of rather more than the fifty-five species reported on below.

For many of the species on the list the information, particularly about the occurrence of variation and relative frequency of different forms is far from adequate. In other cases confirmations would be valuable since in several of the cases where there are a number of reports they are not identical or even compatible. For other species there is too much detail relevant to present in the table, and this information has been added after the table in the form of notes.

Before presenting the table general accounts will be given for some species for which there is particularly abundant information. New observations made on these species in the context of the present work will also be described.

2. T. pratense

a) New Observations.

Many of the T. repens habitats investigated contained T. pratense, and some acquaintance with its phenotypes was acquired, though no systematic scoring was done. The phenotypes previously reported (see below) have all been seen - unmarked, central, basal, and apical V, the central-position V particularly being variable from very broad to thin, and sometimes being reduced to a triangular V-point.

Two plants were seen in a pasture at the Oxford University Farm, Wytham, with moderately broad central marks, but with on one half-leaflet of some leaves a mark extending to the base of the leaflet. This did not seem to affect less than half a leaflet at a time, but otherwise rather resembles the 'smeared' variation of some V-marks described above in T. repens. The description of what may be the same phenotype by Smith (1950) is referred to below.

Only one plant of T. pratense with any kind of red leaf marking has been seen during this work. This plant, with an intense red flecking, was found growing as a weed in the garden of the Oxford Botany School. The flecks in their colour, sharp outlines, and some elongation parallel to the lateral veins of the leaflet resembled the R^f flecked phenotype of T. repens: they differed in being larger and more frequent, covering about a quarter of the upper surface of the leaflet, and by being no less frequent near the leaflet margin than near the midrib. Ascherson and Graebner's rather unclear reference, mentioned above, could well be to a similar type.

b) Genetic Control of Whitish Leaf Marking in T. pratense.

This is the only species for which genetical information comparable to that on T. repens has been published; genetical investigations were in fact carried out on this species many years before T. repens, and the same is true of work on the anatomical basis of the marking (Kajanus, 1912).

The presence of the marks was mentioned by Sowerby (1794) and Smith (1829), though without mentioning their variation. Variation was mentioned by Grenier and Godron (1848).

Kajanus (1912) first reported the presence of mark as genetically dominant to absence in this species. He also reported variation among the marked types and a genetical basis for it, central leaf spot being dominant to basal. Gmelin (1914, 1916) published similar observations. Wexelsen (1932) made many observations on leaf markings in the segregating families produced by successive generations of sib-crossing in a programme designed to produce inbred lines of T. pratense. He did not apparently do any systematic crossing to investigate the interactions of the various segregating systems he found affecting leaf markings.

He confirms the dominance of the presence of leaf mark, and records that heterozygotes for the factors for presence and absence of mark were no less strongly marked than the marked homozygotes. He reports one plant whose progeny segregated as though the parent was heterozygous for marking, although scorings before it died recorded it as unmarked. This, and some unusual segregations, are taken to indicate mark-suppressor genes. In inbred lines breeding true for leaf marking he distinguishes three marked phenotypes, with marks small, large, and extended (towards the leaflet apex), as well as unmarked forms. The difference between large and small is unifactorial, large being dominant, while segregations for large and extended show no clear pattern. He reports a phenotype with a weak mark; strong and weak marks segregated sometimes unifactorially and sometimes apparently bifactorially with strong mark dominant (3:1 and 15:1 ratios). He reports unifactorial determination of leaf-mark colour, yellow mark being dominant to white. He also reports oligogenic variation in leaf colour. Other abnormal phenotypes in his inbred families include albino and yellow seedlings, seedlings with a mosaic of white spots or entirely white, and a form whose cotyledons have a large yellow spot in the centre.

P.E. Smith (1950) presenting, in a thesis, work apparently not further published, reports crosses carried out specifically to investigate leaf mark inheritance. He confirms the recessivity of the allele m for no mark, and its epistasis to the genes modifying the mark.

He reports that Mb, the allele determining central as opposed to basal mark (mb), is very closely linked to the M/m locus. The difference between this and a one-locus-three allele system more closely resembling the *T. repens* V-locus is that a chromosome carrying the recessive epistatic factor *m*, giving an unmarked homozygote, can also carry the dominant factor *Mb*, and thus, combined with a chromosome *M mb* from a line pure breeding for basal mark, can produce in the F_1, a central mark, different from both parental phenotypes.

He reports a pair of complementary factors, *A/a* and *T/t*, epistatic to *Mb/mb*, giving an apical mark in any *M A T* genotype. However, he also records some plants as having both apical and central marks, though there is no place for this phenotype in his table of genotype-phenotype relationships.

He reports some central marks having white markings also below the white mark - and that this occurs sometimes only on some leaves of the plant, while others have normal central marks. The occurrence of what may be this phenotype is referred to as a new observation above.

Smith also reports that while the markings are plainly visible on the first unifoliate leaf in some plants, in others no marks were shown until the bracts on the flowering stem. Some plants behaving as *Mm* in crosses were never recorded as marked at all. This resembles the case quoted by Wexelsen. In other plants, the mark was seen only in the seedling. The possibility, and some evidence of similar variation with time in *T. repens* has been discussed above.

Thus, in *T. pratense*, four separate loci at least have been described affecting the mark phenotype, and each has been described as possessing only two alleles. Altogether, as complete as possible a contrast with the one-locus-many allele system found in T. repens.

This suggests that although the underlying mechanism for the production of marks may be homologous in these species, and can be traced back to a common ancestor, the genetic mechanism producing contemporary variation in leaf-mark phenotypes are largely if not completely unrelated; the V/v and M/m do at least resemble one another in dominance, which leaves the Mb, A, and T loci without known variable homologues in T. repens.

c) Red Leaf-Markings in T. pratense.

The observation of a single, isolated example of a T. pratense plant with red fleckings on the leaves has been referred to above. Ascherson and Graebner (1906-1910) describe T. pratense as 'often with dark or light half-moon shaped or irregular spots'. 'Lightish half-moon shaped' clearly enough refers to the whitish V-marks which my experience and other reports (Kajanus, 1912; Healy, 1953; Foury, 1954) indicate as usually present. 'Dark' and 'irregular' together may then refer to a fleck marking like that described, though there is no other evidence that this occurs often.

R.D. Williams (1937) describes complete linkage between M, Mb, and yellow-mark colour loci, and an 'anthocyanin factor'. W. Williams (1964, p. 15, cf. 74), quoting this source, says 'the presence or absence of pigment in the mark behaves as an additional allele of the leaf mark series', but I understand (personal communication) that, although this remark immediately follows reference to red markings, he is referring in this sentence to yellow or white colour, not the anthocyanin factor.

Evidence for rare, irregular flecked, red marks has been mentioned above. It is surprising also to find references, in American floras (from outside the species' natural range), to T. pratense leaflets 'often with a purplish spot near the middle' (Rydberg, 1922, cf. Abrams, 1944).

3. T. nigrescens; Homology with T. repens at V-locus.

One of the few hybridisations achieved in this genus is between T. repens (2n = 32) and T. nigrescens (2n = 16). When colchicine-treated autotetraploid parents are used, a fertile allohexaploid hybrid is produced (Brewbaker and Keim, 1953). Segregation in F_2 and backcrosses suggest tetrasomic inheritance for the V-locus and the S incompatibility locus. From this it is concluded that the T. nigrescens genome is closely similar to one genome of the presumed autotetraploid bivalent-forming T. repens, and specifically that the allohexaploid possesses four chromosomes bearing the V-locus, all sufficiently similar to pair easily. In a later paper Brewbaker (1962) says that further unpublished work confirms the homology. This suggests that T. repens inherited its V-locus from a diploid parent. closely related to T. nigrescens, which itself shows polymorphism for leaf-marking. T. nigrescens occurs in the Mediterranean area (Taubert, 1894; Hossain, 1961), not extending to the

British Isles; in most taxonomic treatments it is placed fairly near T. repens; an important difference is that it does not root at stolon nodes and is annual.

4. T. fragiferum: New Observations, and Previous Conflicting Reports.

This species is referred to by Smith (1829), Lowe (1868), and de Basto Folque (1949) as unmarked, and many authors who do refer to marks in other species where they report their occurrence make no mention of them in this species, suggesting that they are taken to be absent. Gibelli and Belli (1889) however say that it usually has whitish or dark marking on the upper surface of the leaflet. Evans (see below) also refers to the presence of marks.

In the course of this work this species has been observed and collected and grown in the greenhouse (sometimes being mistaken for unmarked T. repens). It was observed that although an unmarked form was common and perhaps predominant, some plants showed a faint thin yellowish-green V about half-way up the leaflet. Most of these plants, and some with no detectable pale V, also had a thin red V, lying parallel to the pale V when present and a millimetre or so closer to the tip of the leaflet. These observations of marks all refer to plants collected in riverside fields at Wytham, near Oxford; marks are perhaps less frequent or even absent in other populations. This red V may correspond to the dark marks referred to by Gibelli and Belli.

5. Other Species of *Trifolium*.

a) British Species not described as marked in the Flora.

In the Flora of the British Isles (Clapham, Tutin and Warburg, 1962), the reference to the frequent presence of white markings in *T. repens*, *T. pratense*, and *T. medium* leaves at least a suggestion that the other species described are unmarked. In fact, for *T. ochroleucon* part of the description by which it is keyed out is 'leaflets never spotted' (thus distinguished from *T. pratense* and *T. medium*). There are three species with full descriptions not referring to markings in the Flora, and not positively described for presence or absence of markings elsewhere; *T. ornithopodioides*, *T. strictum*, and *T. micranthum* (though this may be what Brewbaker means by '*T. filiforme* (? *dubium*)'). It seems likely that they are unmarked, though they have not been included in the tabulations.

On the other hand, there are references elsewhere to markings in species which are described in the Flora without reference to markings. References in the Key to Varieties of Subterranean Clover, and by Brewbaker to markings in *T. incarnatum*, seem specific enough that there is no occasion to question them. The verbal references to markings in *T. glomeratum* are both over 100 years old, but very specific, e.g. Sowerby, (1794) 'Leaflets often marked with a transverse white or yellowish spot': a V-mark is illustrated by Clapham et al (1957). For *T. dubium*, there is only a passing reference to marking (Brewbaker, 1955) not repeated by this author in his later list;

this is opposed by Evans' report of it as unmarked. *T. scabrum* is described by Lowe (1868) as 'pale-spotted' and by Lojacono (1891) as 'white-marked'. In both cases information from direct observation would be particularly interesting.

b) New Observations.

T. dubium has been seen frequently around Oxford, never with any kind of marking.

A visit was made to a locality for *T. scabrum*, Pen Dinas Hill, Aberystwyth, and material was collected of *T. scabrum*, *T. dubium*, and *T. campestre*. *T. scabrum* was scarce, two small patches being found, about fifty plants in all. None showed any form of marking. No white marks were seen in the other species either, but in both, plants with occasional red flecks, irregular in shape and elongated along the leaflet lateral veins, were seen; one plant out of twenty in *T. campestre*, and two out of a larger number in *T. dubium*.

Brewbaker, and Lowe and Lojacono, may indeed have seen white-marked plants of *T. dubium* and *T. scabrum*, respectively, but it does seem fair to be very reluctant to accept the report of marking in *T. dubium*.

Plants of *T. hybridum* have been examined, sown in fields in Britain and among seedlings growing from seed sent from Bulgaria as *T. repens*. As reported by various authors, they are never marked.

Plants of *Trifolium medium* were examined growing wild in Tatranska Lomnica, Czechoslovakia. Some groups of ten or twenty plants all lacked markings, in others a minority showed very faint central white spots, often shapeless, while a few of these, and one plant with a distinctly much clearer mark, approached the broad lowish flattish V-shape illustrated in Clapham, Tutin and Warburg, (1957).

6. Parochetus communis: White and Red V-markings outside Trifolium.

Healy (1953) illustrates a V-shaped mark in Parochetus communis, and also describes it as usually having dark flecks on the leaflets. As the only member of the Trifolieae (Willis, 1955) and outside the genus Trifolium to show white V-marks this is of some interest. (Red spots in the centre of the leaflet, sometimes approaching a crescent shape, are known in Medicago.)

The species is native at high altitudes in the East Indies (Oliver, 1871), but is found also in tropical Africa (Willis, 1955). The Botanic Garden of the University of Marburg an der Lahn, Germany, lists this species in their exchange list, and from them a seed sample was requested and obtained.

On examination of twenty plants five weeks old, mostly with a dozen or so leaves and one or two runners an inch long, they all showed some sign of marking. Younger plants in a less brightly lit position showed much less conspicuous marking, but (consistently with Healy's description) no clear variation could be seen between plants under the same conditions. As in Healy's illustration, the white mark is lower on the leaflet than is ever seen in *T. repens*. The mark is very obtuse, almost flat, and each wing of the mark is slightly convex distally, so that at the midrib there is a slightly reentrant configuration. All the plants showed some sign of red markings, consisting of irregular scattered flecks, but also of a red V, which was at this stage almost exactly superimposed on the white V when both were well developed. The red V was slightly more distally placed than the white V, and several weeks later they were often completely separated. The clarity of both markings varied greatly from leaf to leaf - the majority of leaves at five weeks old showed no trace of either V-mark, only scattered red flecks; all the plants however showed some trace of both V-markings. The clarity of the two types of marking was fairly closely correlated; sometimes both were present on one side of a leaflet and absent on the other, but at other times one might appear with full clarity without the other. Healy does not refer to this great degree of intra-plant variation, nor to the red V.

When both marks were well developed, but separated, as in the older plants, a fainter development of red pigment was often seen in the area of the white V. There seem thus to be parallels here to the separate red V and to the development of red pigment precisely confined to the white V, both described above in *T. repens*. As in the $\underline{R}^t$ red flecks, the separate red V, and the red pigment in the white V in *T. repens*, the pigment of the red flecks and the red V in *P. communis* is confined to the upper epidermis. In the area of the white mark, the leaf is reduced in thickness by about a quarter, and the cells of the upper mesophyll are little if at all elongated perpendicular to the surface of the leaf.

7. Table of Information on Occurrence and Variation of Leaf-Marking Phenotypes in species of *Trifolium*.

a) Sources of Information.

Leaf-mark phenotypes of a few species are mentioned in many floras and monographs. The largest number of species with marks referred to is probably in Gibelli and Belli's series of papers (Gibelli and Belli, 1887, 1889a, b, 1891, 1892, 1893; Belli, 1894).

The most important unpublished source is a list received indirectly from Professor J.L. Brewbaker. It was prepared by him in about 1960, representing the extent of his knowledge to date concerning twenty-five species he had worked with, and others in Dr. E.A. Hollowell's collection, making thirty-seven in all. The table was prepared to combine information about breeding system, chromosome number and leaf marking occurrence and variation.

Dr. A.M. Evans has provided a similar list with details of twenty-seven species, on the basis of observations made during her thesis work.

Other information has been acquired in the course of correspondence with some of the authors arising from the papers quoted (Gillett, 1952; Hossain, 1961); the anonymous 'Varietal Key to Subterranean Clover', prepared at the Waite Agricultural Research Institute, Australia, was lent by Prof. J. Black after a request for further information after reading a paper by him (Black, 1960).

Some information was obtained from the cyclostyled list of 'Seeds Available and Descriptive Notes' from Regional Plant Introduction, U.S. Dept. of Agriculture, Geneva, New York, prepared by Dr. D. Dolan.

Information was also obtained in correspondence with Mr. A.V. Bogdan, of the National Agricultural Research Station, Kitale, Kenya, and arising out of this, with a former worker at Kitale, Dr. D.C. Edwards.

In the table below, these unpublished sources are referred to by name only; other dated references appear in the main reference section. Under each species, information is given in order of publication, one line for each source. Authors' names have been added to some specific names where they were not given in the source, but where there is any confusion or complication, these names are given exactly as they appear in each source. Some references to T. repens and T. pratense have been omitted; for the other species this is a complete list of the references discovered.

b) Notation used in Table IX.1 to represent descriptions.

White Marks

O	Unmarked.
+	Marks occur (whitish).
V	" " " ; some reference to shape and position.
++ or VV	Marks occur, and are of more than one kind.
+,O or V,O	Marks occur, but some plants are unmarked.

(Letters as used by Brewbaker, 1955; one letter alone indicates no variation, more than one refer to a range of marks in a variable species.)

O	Unmarked.
A	Simple mark.
B	Broken mark.
C	V-point.

Forms clearly indicated as more common stand first, followed by semi-colon.

Red Marks

R	Red or dark marks, shape and position not clearly indicated.
RV	Red V-shaped marks.
RT	Red tip associated with a whitish V.
RF	Scattered red flecks (cf. R^f in T. repens).
RL	Whole leaf red (cf. R^l in T. repens).
+ (after red-mark symbol)	Common.
±	Variable (relative frequencies with and without not indicated).
-	Rare, usually not present.
All marks *	Mark illustrated as well as described.
All marks **	Information based only on illustrations, no reference or description in text.

Table IX.1. Information on the Leaf-Mark Phenotypes of *Trifolium* species.

SPECIES	WHITE MARKS	RED MARKS	SOURCE
agrarium L. (aureum Poll.)	0		Evans
agrarium (aureum)	0		Brewbaker
alexandrinum L.	0		Evans
	0		Brewbaker
ambiguum M.Bieb. 2x=16	0; A		Evans
6x=48	C,A,B,&c (Note 1)		Evans
	* +		Bobrov, 1947
	+		Brewbaker, 1955
	A, ?0		Brewbaker
arvense L.	0		Evans
baccarinii Chiov.		RV	Bogdan
berytheum Boiss.	0		Evans
bocconii Savi	0		Brewbaker
campestre Schreb. (procumbens L)	0		Evans
campestre	0		Brewbaker
	0	RF -	(this thesis)
carmeli Boiss.	0		Evans
cernuum Brot.	0		Brewbaker
ciliolatum Benth.	+,0		Abrams, 1944
dalmaticum Vis.		* RV	de Visiani (1847-52)
davisii Hoss.		R +	Hossain, 1961
	* +	* RV	Hossain
diffusum Ehrh. (as pratense ssp diffusum)	V		Gibelli+B 1889
as diffusum Ehrh,	+		Bobrov 1949
dubium Sibth. (minus Sm.)	0		Evans
dubium	+		Brewbaker 1955
	0	RF -	(this thesis)

SPECIES	WHITE MARKS	RED MARKS	SOURCE
echinatum M.Bieb.	0		Brewbaker
elizabethae Grossh.	** +		Bobrov, 1947
filiforme (? dubium) (filiforme L ? p.p.)	0		Brewbaker
fragiferum L.	0		J.E. Smith 1829
	0		Lowe 1868
	+,0	R ±	Gibelli+B. 1891
	0		de Basto F. 1949
	+		Evans
	0; faint A	RV ±	(this thesis)
glomeratum L.	V,0		Sowerby 1794
	0; V		J.E. Smith 1829
	0		Lowe 1868
	** +		Clapham, T&W 1957
	0		Evans
hirtum All.	+ (Note 2)	RV ±	Gibelli+B. 1889
	0		Brewbaker
	0; +	(? R)	Hossain 1961
		RV	Hossain
hybridum L.	0; V		Bentham & H, 1924
	C		Bobrov, 1949
	0		Evans
	0		Brewbaker
incarnatum L.	0		Evans
	0,A,C		Brewbaker
isthmocarpum Brot.	0,A		Foury 1954
	0,A	RT ±	Brewbaker
johnstonii Oliv. (Note 3)	0		Evans
johnstonii	0		Brewbaker
lappaceum L.	0		Brewbaker
lupinaster L.	0		Evans
maritimum Huds. (squamosum L)	0		Evans
mattirolianum Chiov.	+	R	Bogdan

SPECIES	WHITE MARKS	RED MARKS	SOURCE
medium L.	+		Evans
	+		Brewbaker 1955
	O,A,C		Brewbaker
	V		Dolan
	* O,A		Clapham, T&W, 1957, 1962
	O; V faint; V clear		(this thesis)
mexicanum Hemsl.	O		Brewbaker
michelianum Savi	+		Brewbaker 1955
	O,A	RT ±	Brewbaker
nigrescens Viv.	+,O		Lojacono 1891
	often spotted		Asch.&G. 1906-1910
		RF ±	Borg 1927
	O		de Basto F. 1949
	+		Evans
	+		Brewbaker 1955
	O,A,B	RT ±	Brewbaker
occidentale Coombe	O; ?V(Note 4)	RF -	Coombe 1961
	O		Evans
	O		Géhu 1963
ochroleucon Huds.	O		Clapham, T&W 1962
pallidum	O		Brewbaker
physodes Stev.	+; O		Gibelli+B. 1891
	O; +		de Basto F. 1949
		V-shaped spots	Hossain 1961
		RV	Hossain
polymorphum Poir.	O		Brewbaker
pratense L.	V		Sowerby 1794
	V		J.E. Smith 1829
	sometimes spotted		Grenier+G. 1848
	V,O (Note 5)		Gibelli+B. 1889
	V; O		Kajanus 1912
	O,VV		Gmelin 1914, 1916
	O,V?	RF+ ±	Ascherson+G 1906-10

SPECIES	WHITE MARKS	RED MARKS	SOURCE
pratense L.(contd.)	+,0		Borg 1927
	further genetical variation		Wexelsen 1932
		'often dark spot near the middle'.	Abrams 1944
	+,0		de Basto,F. 1949
	further genetical variation		P.E. Smith 1950
	V; 0		Healy 1953
	V; 0		Foury 1954
	+		Brewbaker 1955
	* V,0		Clapham, T&W, 1957, 1962
	0,A,C	RF	Brewbaker (this thesis)
cf. diffusum (pratense ssp. diffusum)			
procumbens L.	0		Lowe 1868
procumbens (agrarium) (procumbens L. p.p. = agrarium Huds.)	0		Brewbaker
purpureum Loisl.	0		Brewbaker
radiosum (radiosum Whlbg. = globosum L.)	A		Brewbaker
reflexum L.	A,B(RT)	RT in white B	Brewbaker
repens L.	** V		Sowerby 1794
	+	R ±	Gray 1821
	V; 0	R(?F) -	J.E. Smith 1829
	+; 0	R(?F)	Lowe 1868
	+		Lojacono 1891
	** +,0		Reichenbach 1903
	V; 0		Bentham+H. 1924
	+,0		Borg 1927
	0,many V's		Atwood 1937-44
	+,0	RF ±	Allan 1940
	V-locus		Brown 1947
	V-locus; Double-V marks		Brewbaker+A. 1952
	0,A,B,C; other V's		Brewbaker 1955
	A,0; other V's		Carnahan et al. 1955
		R-locus; 4 alleles	do.
		Red V (assoc. V^{by})	Hovin + G. 1961
	**V; 0		Clapham et al. 1957, 1962

repens L. (contd.)	V-locus	R-locus	Davies 1963
	Other red (assoc V^{by})		do.
	(New Phenotypes, see above)		(this thesis)
resupinatum L.	+		Lowe 1868
	+,0		Gibelli+B. 1891
	+,0		Borg 1927
	+,0		Allan 1940
	** V	RV	Foury 1954
	0		Evans
	0		Brewbaker
ruepellianum Fres. (cf. subrotundum)	0		Bogdan
scabrum L.	+		Lowe 1868
	+		Lojacono 1891
	0		(this thesis)
scutatum Boiss.	0		Brewbaker
semipilosum Fres.	0,A,white midrib		Evans
	0		Brewbaker
	white midrib; V		Bogdan
perhaps two taxa (i) (ii) (Note 6)	(white midrib only) (0,A)		Edwards
spumosum L.	often spotted		Grenier+G. 1848
	+		Lojacono 1891
	+,0		Gibelli+B. 1892
	0		de Basto F. 1949
	** C		Foury 1954
	0,C		Brewbaker
squarrosum L.	0		Evans
	0		Brewbaker
stellatum L.	0	RT ±	Brewbaker
striatum,L.	0,C		Brewbaker
subrotundum Steud.	0,A,C	RT,RV, RV-point	Brewbaker
(Gillett 1952 places the greater part of this species in T. ruepellianum, q.v.)			
subterraneum L.	sometimes spotted		Grenier+G. 1848
	V		Lowe 1868
(Note 7)	+,0		Gibelli+B. 1893
	0,+		de Basto F. 1949

subterraneum L.	O,+	RF ±	Healy 1953
	V,O		Foury 1954
	+		Brewbaker 1955
		Red lf. (1 dom.gene)	Brockwell 1956
		Red leaf only expressed if nitrogen shortage	Frankel+B. 1958
	+		Evans
	++		Black 1960
	O,A,B	Red lflt, base ± Red V distal to white	Anon (cf. Black 1960)
suffocatum L.	O		Lowe 1868
tembense Fres.		Red base or RV-point	Brewbaker
tomentosum L.	O		Evans
	O		Brewbaker
uniflorum L.	O		Evans
variegatum Nutt.	A,B,C	RV	Brewbaker
vavilovi Eig.	O		Evans
vesiculosum Savi	+,O		Grenier+G.1848
	V,O		Gibelli+B. 1892
	+,O		Asch.+G. 1906-10
	V		Bobrov 1949
	+,O		Vicioso 1953
	+		Evans
sspp. **multistratum Koch**	V,O		Gibelli+B. 1892
mutabile Portenschlag	O; faint mark		Gibelli+B. 1892
var. 'Amclo'	V;O		Beaty, et al. 1965
Parochetus communis Buch. Ham. *	V	RF	Healy 1963
	V ? always	RV + RF ? always	(this thesis)

Note 1.

In hexaploid T. ambiguum, Evans refers to some phenotypes as resembling the low, high, broken, and broken-with-yellow-tip phenotypes of T. repens, as well as to unmarked plants, and also to two new phenotypes. These contain a V with a reentrant apex made up by straight segments running out from the midrib at a slight slope towards the leaflet tip; more than half way to the leaflet margin these turn at a sharp angle and thin extensions run towards the leaflet base. In one of these types, the reentrant angle is filled by a yellow blotch. The other, simpler mark sounds rather like a more extreme version of the 'W' mark, with 'smearing' confined to the midrib region, described above in T. repens.

Note 2.

According to Gibelli and Belli (1889), a form T. hirtum All. var. pictum Roth has been described, with reddish coloration on the leaflets. They grew plants of this form from seed received from Botanic Gardens at Lyon and Madrid, and observed that the red mark was irregularly arrow-shaped, or less often without a definite shape. This mark was very distinct in some forms, but quite absent in others. Below this mark when present, (it is not clear whether this means on the same part of the leaf and covered by the mark, or lower down the leaflet towards the base), there was almost always an irregular whitish zone. These authors do not feel that the other morphological differences from the species supposed to distinguish this form are clear enough to give it taxonomic recognition.

Note 3.

According to Gillett (in litt.) the true _T_. _johnstonii_ Oliv. is now classified as a subspecies - _T_. _burchellianum_ Ser. ssp _johnstonii_ (Oliv) Cufod.; but the name _T_. _johnstonii_ Oliv has been commonly misapplied by writers on agriculture in Kenya to a plant which is in fact _T_. _semipilosum_ Fres. var. _glabrescens_ Gillett (cf. Gillet, 1952), though the two may be related. It is therefore possible that material described under this name may belong to either of these two taxa.

Note 4.

_T_. _occidentale_ shows little variation. Among the ten clones originally described by Coombe, most were identical and unmarked. One has 'dark markings along the lateral veins' (perhaps equivalent to $\underline{R}^f$ in _T_. _repens_). Another has 'obscure yellowish-white markings on the leaflets'; it is not clear that these should be regarded as comparable to the V-markings in other species.

Note 5.

Gibelli and Belli (1889) describe the main species _T_. _pratense_ as often marked with a whitish crescent- or arrow-shaped mark. As varieties they describe var. _sativum_ Rchbch. and describe for the first time var. _collinum_ (Gib. et B.). One character in which the two descriptions differ is leaf-marking, though the authors do not lay any special stress on it. Var. sativum is described as having a large, arrow-shaped white mark, while var. collinum is described as lacking arrow- or crescent-shaped marking.

Note 6.

According to Edwards the form of T. semipilosum with the white band along the midrib is morphologically rather distinct, and consistently breeds true for the leaf mark phenotype. It may be genetically isolated and taxonomically distinct from the other T. semipilosum, which include both V-marked and some unmarked plants.

Note 7.

The Waite Varietal Key (Anon.) uses mark characters to identify varieties of T. subterraneum and Foury (1949) refers to a V-mark being present in some varieties and not in others. Since the species is self-fertile, the cultivated varieties are probably strictly inbred and genetically uniform, and the uniformity of each one and the consistent differences between them in this respect do not suggest any particular action of selection on leaf-marks as a similar situation might in an outbreeding species.

8. Infra-generic Arrangement of Marked and Unmarked Species.

Having gathered this information, one would like to compare it with a phylogenetic arrangement of the species concerned. Gillett (in litt.) quotes an account by Seringe in De Candolle's Prodromus (1825) as the most recent survey of all the species in the genus. There have been many treatments of species in particular areas since that time, some with more or less ambitious attempts to set up a system of infra-generic classification. Unfortunately these arrangements all differ, and even the authors least concerned with phylogenetic speculation in effect produce a classification of their own if they attempt infra-generic classification at all, by combining selected features from a number of their predecessors.

None of them mentions all the species for which data is listed above, and so one is forced to using references in one or more other classifications to place several of the species in relation to whichever is accepted as the principal authority. It is difficult or impossible to know whether they are then placed in a way that the principal authority would accept, since it can be clearly seen to disagree on other points with any secondary authorities consulted.

The most recent account, dealing with 90 species in one of the main regions of diversity in the genus, is that of Hossain (1961), from whom is taken the classification used below into eight subgenera, and of subgenus Amoria into four sections.

This much as a basis for the classification is substantially compatible with other authorities; these groups seem more or less accepted, though there is disagreement about ways of combining them. Hossain differs from most other authors, who use the term section for his subgenera, and subgenus for an initial division of the genus into two parts, Subgenera Lagopus (Hossain's subgenera Trifolium and Calycomorphum) and Trifoliastrum (the remainder). Hossain says "The genus Trifolium consists of several major and distinct natural groups" (his eight subgenera) "which, however, are not very closely related. There is no obvious link between these different groups and no sign of hybridisation. Combining these two groups under two subgenera (mainly on the basis of the presence or absence of the floral bracts) leads to an artificial grouping rather than to a natural one."

Hossain's subgenus Trifolium (some authors' section Lagopus) and section Amoria contain particularly large numbers of species for which leaf mark data is available. Section Amoria has been subdivided into subsections roughly according to the arrangement used by Taubert (1894). Subgenus Trifolium has been split into very small groups, without classifying these at a higher level into sections in any of the differing ways used by, for example, Lojacono (1883), Gibelli and Belli (1889b), Taubert (1894) or Bobrov (1949).

Table IX.2. Taxonomic Arrangement of Trifolium species, with their Leaf-Markings.

Notation:

O	Only unmarked plants reported.
V	Marks reported, no report of unmarked forms.
VVV	All marked, more than one kind of marked phenotype.
O,V	Both unmarked and marked individuals occur.
O,VVV	Unmarked and several kinds of marked individuals occur.

Symbols for red marks as above; any type known is recorded, even if rare.

In most cases, the reports for each species have been simplified to a single note; only the more irreconcilable contradictions are shown as such, with a colon separating the two versions.

Classification	Species	White Marks	Red Marks
Subgenus 1 Trifolium			
	medium	O,VVV	
	pratense	O,VVV	RF, ?RV
	diffusum	V	
	pallidum	O	
	hirtum	O, V	RV
	lappaceum	o	
	arvense	O	
	bocconei	O	
	scabrum	O : V	
	dalmaticum		RV
	striatum	O, V	
	stellatum	O	RT
	incarnatum	O,VVV	
	scutatum	O	
	purpureum	O	

Classification	Species	White Marks	Red Marks
	alexandrinum	O	
	vavilovi	O	
	berytheum	O	
	carmeli	O	
	echinatum	O	
	maritimum (squamosum)	O	
	squarrosum	O	
	ochroleucum	O	
	davisii	V	RV
Subgenus 2 Calycomorphum	subterraneum	O,VVV	RV,RF,RL &c.
	radiosum (globosum)	V	
Subgenus 3 Paramesus			
Subgenus 4 Involucrarium	variegatum	VVV	RV
Subgenus 5 Galearia	tomentosum	O	
	resupinatum	O : V	RV
	physodes	O, V	RV
	fragiferum	O : O,V	RV
Subgenus 6 Mistylus	spumosum	O, V	
	vesiculosum	O, V	
	ssp. multistriatum	O, V	
	ssp. mutabile	O, V	
Subgenus 7 Amoria Section 1 Lupinaster	lupinaster	O	
Section 2 Cryptosciadium	uniflorum	O	
Section 3 Amoria Subsection 1 Thalia	hybridum	O : O,V	
	repens	O,VVV	RF,RL,RV, &c
	occidentale	O, ?	RF
	semipilosum	(O, V (white midrib stripe)	

Classification	Species	White Marks	Red Marks
	ambiguum	0,VVV	
	elizabethae	V	
	johnstonii	0	
Subsection 2 Neoamoria	reflexum	VVV	RT
	ciliolatum	0,V	
	mexicanum	0	
	polymorphum	0	
Subsection 3 Isthmocarpa	subrotundum	0,VVV	RT,RV
	mattirolianum	V	R
	baccarinii		RV
	tembense		RV &c.
	nigrescens	0,VVV	RT
	isthmocarpum	0, V	RT
Subsection 4 Fistulosa	michelianum	0, V	RT
Section 4 Micrantheum	cernuum	0	
	glomeratum	0 : 0,V	
	suffocatum	0	
Subgenus 8 Chronosemium	agrarium (aureum)	0	
	procumbens (agrarium)	0	
	campestre (procumbens)	0	RF
	dubium (minus)	0 : V	RF
	filiforme (dubium)	0	
Parochetus communis		V	RV + RF

9. Conclusions.

The first conclusion that stands out from the above data is that the leaf mark character is very often, perhaps always, variable. Of the eight species noted as 'V' in the second table above (including one of two conflicting accounts for T. resupinatum and T. dubium), seven amount only to statements of the occurrence of marks without specifically excluding the occurrence of unmarked plants. The exception, T. radiosum, is reported as 'A' (cf. Table IX.1) by Brewbaker in his unpublished list where variation is explicitly described in many other species.

Secondly, this variation almost always involves the occurrence of an unmarked form. Beyond the eight cases referred to, in only two further species are marks (variable) reported without unmarked forms. Of all the twenty-five or so species where marks are reported, only for these two, T. variegatum and T. reflexum, and for T. radiosum, is there an indication of any strength of the absence of unmarked forms; and two of these three are among the ten species for which there is reported variation among the marked forms, leaving only T. radiosum as marked and not variable.

The first impression produced by the taxonomic arrangement of the leaf-mark descriptions of the species is that the markings occur in all the subdivisions of the genus. On closer examination, two possible exceptions appear. The only report of marking in subgenus Chronosemium is that by Brewbaker (1955) for T. dubium, and it is opposed by Evans' report of the absence of mark, implicitly by the absence of any other reference to the absence of marks (e.g. in the Flora of the British Isles) in this quite common species, and by my own observations.

Secondly, a part of the subgenus Trifolium seems to lack marks; species related to T. purpureum, T. alexandrinum, T. maritimum, and T. squarrosum. This corresponds to a greater part of Gibelli and Belli's section Stenostoma, with the exclusion of T. ochroleucon and T. davisii (newly described by Hossain, as a close relative of T. canescens). Alternatively, the unmarked group comprises Bobrov's section Stenostoma, with the same exception, and of part of his section Hiantia, which includes another unmarked species, T. arvense, and another marked one, T. hirtum.

One further aspect of importance that arises in the earlier sections of this chapter is the remarkable difference between the systems of genetical control of the various markings in T. repens and T. pratense, the only species where genetical work on leaf markings has been published in any detail. Homology between the two species could not be argued for more than the principal epistatic M-locus in T. pratense, whose recessive allele for no mark could correspond to the recessive allele at the V-locus in T. repens.

The virtual absence of uniformly marked species suggests that there is an intrinsic property of the mark-producing system, perhaps expressed as an inferiority of the marked dominant homozygote, which very effectively opposes the fixation of marked types. Evidence pointing in the same general direction exists within _T. repens_, where wild populations are very nearly always variable; and, in spite of a generally low frequency of unmarked plants, they rarely lack the unmarked type. Gene frequencies of ten per cent or more could easily have been undetected in the populations sampled as all marked, few of which were larger than 100 plants (cf. Figure VII (i)).

Such an effect would also be produced if a selective process acting on the end products of the gene activity - in this case perhaps the leaf mark itself - acted so as to favour diversity as such. This would resemble the situation in certain prey animals. (cf. Ford (1964), p. 160 and p. 274). De Ruiter (1952) has shown that some predators may learn to recognise each morph as food separately. A morph with a high frequency would be liable to predation by a correspondingly large number of predator individuals which might not predate the other morphs. A role for such a mechanism in maintaining polymorphism has been suggested by Cain and Sheppard (1954) with regard to banding in the snail, _Cepaea nemoralis_, and by Kettlewell and Berry (1961) in a polymorphism for (non-industrial) melanism in the moth, _Amathes glareosa_.

Whatever this hypothetical force opposing the fixation of marking might be, it would appear to act consistently throughout the genus, and would have to be inseparable from the most fundamental properties of the genes which produce the marking.

If one accepts that all the marks have a common origin, then there is a strong case for believing that all marked forms had a continuous sequence of marked ancestors, tracing back to a common ancestral population, and the argument above suggests that these would all have been members of poly-morphic populations. It would then follow, unless the taxonomists' judgments are a very poor guide to phylogeny, that all, or many, of the now unmarked species are derived from ancestral populations that were marked or probably polymorphic for marking. This implies many occasions on which populations became fixed in the unmarked condition; suggesting that the forces preventing fixation of marking act specifically against fixation only of a marked type.

A modification of this conclusion would follow if suppressor genes were postulated; then a continuous sequence of marked ancestors of all marked populations would not be assumed. For example, in the case of T. pratense, fixation of mm would conceal all the effects of the other loci. The concept of a suppressor implies that such a mechanism would conceal rather than destroy or replace the detailed and perhaps quite elaborate genetic resources controlling the physiological production of marking. (The presumed complexity of this mechanism is one reason for supposing that the capacity to produce marks only arose once, the other being their visible resemblance in different species.) Taking the m gene in T. pratense as an example of a potential suppressor of the kind suggested requires the further assumption that the bulk of this elaborate mechanism would still be present in mm plants, either elsewhere on the genome, or at an M-locus whose inactivity was due to a small reversible change rather than complete loss.

If there were such suppressors, marked or polymorphic populations could be derived by a simple mutational change from unmarked ancestors. If some of the present unmarked species had suppressed the marking system as opposed to losing it, such species should be liable to occasional mutation revealing the marked character. One could test this using artificial mutagenic treatments.

If the conclusion of a continuously acting force preventing fixation of marking is not accepted, the widespread occurrence of polymorphism for marking must be traced to more than one case of occurrence and establishment of new genetic variation, perhaps by the mutation of a suppressor. The genetic mechanisms controlling the variation might then not be homologous, though the underlying mechanism producing marking would still be so. If variation had been established several times, this would provide grounds for thinking that the apparent advantage, suggested by its prevalence, which polymorphism for leaf marking confers on a population, is a property of variability in the phenotype we recognise, leaf marking, and not of some hidden property of the locus controlling the polymorphism, which in this case would differ from species to species. This would mean, not only that the variation observed at the V-locus would be of selective significance, but that the variation in the actual character we observe is selectively important.

CHAPTER X

LEAF-MARKINGS IN OTHER FAMILIES

1. Introduction.

It may be of some value to consider cases in other genera where leaf-markings are found sharing some of the properties of those of *Trifolium repens*.

First one may list the significant properties of the leaf marks of *T. repens*.

White marks.	V shape
	Produced by modification of palisade structure and perhaps of chloroplasts
	Genetically variable.
Red marks.	Flecks $\underline{R}^{f}$
	Central or extensive patches $\underline{R}^{m}$, $\underline{R}^{l}$, Red on tip (assoc. By).
	V-shapes Red V, Blue V (assoc. By); fainter red in other white V's.
	Genetically and environmentally variable.
Interrelations.	Coexistence in same species of white and red marks
	Coexistence of systems of genetic variability
	Coexistence of V-shaped marks in both colours
	Interaction in expression (red-in-V mark, partial suppression of $\underline{R}^{m}$ in $\underline{V}^{by}$, approximation of Blue V mark to inner side of white mark and other mark if heterozygous)
	Association in genetic control ($\underline{V}^{by}$-controlled red marks).

2. *Ranunculus* species

The genus which seems to have the most points of resemblance to *Trifolium*, including occurrence in similar habitats such as short, grazed pasture, is *Ranunculus*. Three species that I have noticed have both red and pale marks.

In *Ranunculus ficaria* the white marks are sharp edged, small flecks and may be more or less arranged in an arc, none being very close to the margin or to the base of the leaf. The red mark is a sharp edged irregular streak up the midrib from the base. Stahl (1896) reports that they develop more clearly in moist shady places, and may almost vanish in a cold greenhouse. All or nearly all plants can show pale marks, but many show no red marks According to Turrill (1948), variation in leaf blotch caused by red pigment in the upper mesophyll is genetically controlled in *R. ficaria* & *R. acris*

In *Ranunculus omatophyllus* there is a similar red mark, centrally placed, and extended along the three main ribs. There are pale patches, located below the marginal sinuses between the main veins. (In this species the margin consists of a series of convex segments linking sinuses alternately associated with main veins and with pale patches.) These patches however are not sharp edged.

A similar situation can be seen in *Ranunculus repens*, with pale diffuse-edged patches associated with the major sinuses. Here however the distribution of 'red', effectively blackish, colour is different; it does not occur in sharp edged uniform patches, but as a speckling which only becomes continuous in the centre of a marked area, or on very extensively pigmented leaves, and is distributed more sparsely and fades out at the margin. In this case there does seem to be an association with the pale patches, or both may be associated with a third factor; the pale patches occur below, proximally to some sinuses, and the densest part of the pigmented patch, when this is moderately developed, is beside the base of the sinus - the pigmented patches occurring in pairs beside each of the sinuses that has a pale patch associated.

3. Genetically Dominant Pattern Variegations.

The white V-markings of Trifolium are an example in which a set of cells of the same genetic constitution differentiate so as to produce a variegated pattern. The majority of such cases are produced by often rare recessive factors. However, Tilney-Bassett and Kirk (1966?, in press) list some examples of dominant pattern genes (Hiorth, 1931; Correns, 1931; Parker, 1933).

The most interesting of these for comparison with the V-locus of T. repens is that of Collinsia bicolor (Hiorth, 1931). Five dominant and one recessive allele are described at one locus, producing various combinations of white spots or white veins on the leaves and cotyledons. When an allele producing white veins, and an allele producing white spots, in the cotyledons, are combined, the heterozygotes have both white veins and white spots on the cotyledons. Here, as in T. repens, is applicable the interpretation that dominance is the result of each allele being active to disturb (in places) a process provided for by other genetic material (cf. p. 92). However the analogy breaks down at a structural level - these white marks are produced in the epidermis. Another analogy exists however, since in this species there are also red markings which (1) affect the principal leaf veins (one of the white-marks alleles produces white areas round all the veins) and (2) are closely linked with the white-mark locus.

Extensive variation in leaf appearance involving red marking as well as white is also known in *Coleus* (and here the white marking is an absence of chlorophyll, not a light reflecting blister). Correns (1931) reports the dominance of a variegation expressed as a central leaf blotch; on crossing green and variegated plants nearly all the progeny were variegated. The expression varied in the selfed progeny of variegated plants, ranging from a large blotch to a mid-vein stripe, or even to scattered spots.

Parker (1933) describes a condition with scattered yellow spots on the leaves of *Phaseolus vulgaris* this is only partially dominant, as the spots were larger and more nearly fused together in the homozygote. With this example we return to the *Leguminosae*, and perhaps find a closer analogy in *T. repens* with the dominant (complementary) factors producing Atwood and Kreitlow's (1946) mottling rather than with V-marks.

In the tetraploid Cyclamen persicum there are a number of differing leaf marks produced by epidermal blisters. Seyffert (1955) after work on experimentally produced polyhaploids (2x) refers to variation affecting leaf marking at two loci, and quotes a case of 9:3:4 segregation. However, after further work (Seyffert, 1957 and in litt.) he lists four alleles at only one locus. These are a basal recessive for no mark, and a series of three dominant alleles, dominant over each other in sequence. The top dominant produces a peripheral mark round the edge of the leaf, the next a circular medial band, and the last a central blotch. The medial phenotype is the most common in wild (tetraploid) populations. The phenotype produced by the top dominant, peripheral, is (in the greenhouse) less vigorous and produces smaller flowers. Other photographs of Cyclamen were obtained in correspondence to illustrate these marks (cf. Wellensiek, 1961). These show some variation in the intensity of the medial mark, and illustrate the central mark with a separate fainter band round it in the medial position, a compound phenotype not described by Seyffert.

4. Arum maculatum.

In Arum maculatum, some plants have a number of red patches on the leaves - and in some cases, the larger of these areas are distorted and bulge out above or below the leaf.

Pethybridge (1903) records that in the flat marked areas, the leaf is thinner, mostly due to a shortening of the palisade cells, in which the pigment is located. The intercellular spaces also seem to be larger in the marked areas, and in the distorted spots, the pigment may also, or only occur in the spongy mesophyll. As in Ranunculus spp., it does not occur in the epidermis, as does the pigment, in some at least (R-alleles, Carnahan et al., 1955 Also the red V, and the red pigment in the white V, according to my own observations) of the Trifolium red markings. These marks therefore combine features both of the white and of the red markings in Trifolium; that is, a modification of leaf structure (specifically a less organised and differentiated palisade), associated with the presence of red pigment.

These marks do not seem to be subject to environmental variation. Each plant consistently produces either marked or unmarked leaves. The data of Colgan (1911) suggest that it is under oligogenic control: eleven seeds gathered from a marked plant transplanted into his garden and growing with two other marked plants as its only near neighbours, grew up to include six entirely unmarked plants.

Prime (1955b) publishes a map of the frequency of the unmarked and marked forms; in Lancashire and Yorkshire and further south, the marked forms comprise 10 to 20 or rarely 30 per cent of the population. North of this they are rare or absent, except north of the line which is thought to mark the limits of the species' natural distribution in Scotland; it is thought that these are introduced plants, which may have been chosen with discrimination in favour of markings. In Germany, on the other hand, the spotted form is commoner in the north.

5. Analogies with *Trifolium repens* markings.

Among the examples above there are analogies particularly in *Collinsia bicolor* and *Cyclamen persicum* to the dominant expression controlled by a multiple allele series seen in *Trifolium repens*. Analogies to *T. repens* in a second respect are shown by the association of red and white markings in *Collinsia bicolor* and *Ranunculus* spp.

A further point of resemblance is in the shape and appearance of the whole marks. Red V or crescent shapes on the leaf are known also in *Polygonum persicaria* and a very regular V of almost uniform width was seen on a plant labelled as *Polygonum capitatum*, now in the Oxford Botanic Garden.

However, the mark on the whole leaf, as opposed to the leaflet, is in *Trifolium* roughly circular or polygonal, and comparison of shape is then possible between *T. repens* V-marks and the white marks of *Cyclamen*, with the red 'zone' of *Pelargonium zonale* and *P. hederifolium*, and perhaps with the spots in *Arum maculatum*: though not forming a continuous band, the larger spots all lie along an oval line parallel to the leaf margin and about half way from it to the attachment of the petiole. One could extend the analogy in the same way to cover the ring of white flecks in *Ranunculus ficaria*.

6. Discussion.

Very many other plants show leaf markings of many kinds, both 'white' and 'red'. They are perhaps more abundant in flora exotic to Britain, as can be seen in tropical glasshouses; one of the most conspicuously variable groups is *Begonia*.

Little comparative work seems ever to have been done on leaf markings or on their significance. One of the few discussions is that of Stahl (1896). He suggests, firstly for red coloration, three functions: to discourage animals (and reports experimental evidence supporting this); to accelerate warming of the leaf (and showed differences of the order of a degree soon after exposure to light); and possibly to convert light of some wavelengths to others which could be absorbed by chlorophyll (the absorption spectra of chlorophyll and 'erythrophyll' are as he points out closely complementary). For white leaf patches, he mentions only effects on temperature, reporting in *Anthurium crystallinum* a temperature difference of a third of a degree immediately after exposure to light, and in *Begonia argyrostigma* and *Pteris cretica albolineata* that the white flecks cool more slowly than the green tissue.

PART 5

GENERAL DISCUSSION

CHAPTER XI

GENERAL DISCUSSION

1) The Conclusions and their Validity.

It was stated in the introduction that no obvious explanation of the significance of leaf markings was available to the intuition. On this basis it would be possible to suggest that leaf markings were of no selective importance, and that at least in a variable outbreeding species their variation might occur in spite of rather than because of the action of selection. The principal conclusion reached by this work is that the unmarked form is significantly more frequent further north and further uphill. These two points, and especially the second, seem to eliminate the possibility that the differences between the marking alleles are too trivial to be of selective importance.

One might attempt to explain away a simple overall cline as a side-effect of some other phenomenon, or as a result of gene flow between centres of population which had diverged in this respect by drift during a period of isolation. However, the rise in unmarked frequency with altitude cannot be discounted in this way when it occurs in separated areas, for example, both in Britain and in Spain.

Mark expression is dependent to an extent on environment, and plants growing in unfavourable conditions show their marks less clearly and may sometimes effectively lose them. The increase of unmarked frequency in the field-scored British samples could be supposed (as more definitely could the higher unmarked frequency in field- than greenhouse-scored material) to be a consequence of distance north and higher altitude providing lower temperatures and less light, and making mark expression and recognition difficult. The results, it would be argued, represent a change in phenotype of purely environmental origin, and no change in genotype. The author's assessment of the extent to which environmental influences on expression could account for the results suggests that the results are substantially real. This assessment is admittedly subjective, and it may be felt that prejudice in favour of finding a positive result could have biased it. However, the parallel altitude cline in the Spanish material cannot be attacked in the same way, as these plants were all scored at Aberystwyth under standard (or at least not systematically different) conditions. The latitude cline across Europe can be defended in the same way; if these clines exist elsewhere, they are probably true, as they appear to be, in British material.

Other studies of polymorphism were discussed in the introduction, and it was suggested that the next step after discovering a pattern was to explain it as that of some particular selective factor. With regard to cyanogenesis, the common feature deduced by Daday (1954a, b, 1958) from altitude and latitude clines was an effect of temperature, and subsequent experiment substantiated this conclusion (Daday, 1962, 1965).

The most definite factors associated with high unmarked frequency are distance north, higher altitude (one is tempted to follow Daday and deduce low temperature as the common factor); (in Britain, at least) wet (waterlogged), or compacted soil, and dense vegetation. The distribution pattern is not necessarily explicable in terms of one principal factor. However, if there is one, it might be something related to increased wetness and poor soil aeration. On the basis of the available data it is not possible to go beyond these two suggestions of temperature and water regime as the selective factors.

2) The Significance of Leaf Markings.

A second question remains unanswered: is it leaf markings and their variation that matters, or is leaf marking a trivial by-product of some other activity of the V-locus, about which nothing has been directly observed? Wetness or low temperature may be the important selective factors, but it is hard to see any connection with markings on leaves. It would seem reasonable to suggest that the markings are side effects of alleles which achieve something, quite different, related to one of these selective factors.

We perceive the markings as a result of the reflection of light from the leaf. Interaction with light seems almost certain to be part of the true significance, if any, of leaf markings as opposed to the system which produces them. The suggestion by Stahl (1896) that leaf markings alter the temperature regime for the marked tissue is the sort of explanation wanted, but his experimental results (mostly on red marks, and in other species) indicate only small temperature differences, confined to occasions of rapid light intensity change. One could perhaps approach by means of the pattern of the clines demonstrated and suggest that in northern latitudes and at high altitudes there is more frequent cloud and less light, and therefore markings, reducing photosynthetic capacity, are disfavoured. This could mean that markings are favoured elsewhere (and even in these places, since they are present in a majority in samples from all areas) because light supply is above optimal: this is hard to believe. Otherwise, even if the cline is explained, the positive significance of markings still needs an explanation.

The interactions between red and white markings have been referred to in several places in the text. Developing Watkin Williams' suggestion (1959, p. 15), one might suppose that following the evolution of red marks, modification of the underlying (or nearby) tissue was also favoured, that the system evolved to achieve this was distinct, became separated, and developed its own selective advantages: in Trifolium repens to-day most plants show white markings but no red ones.

The complete linkage between $\underline{V}^{by}$ and red $\underline{V}$ in Trifolium repens (Hovin and Gibson, 1961; W.E. Davies, personal comm.) may therefore have evolved because variation at both loci affected the same character. This would accord with the prediction of Fisher (1930, p. 117) that selection would favour close linkage of loci affecting the same phenotypic character. The double character (in expression, and in mutability, cf. R. Davies and Wall (1959)) of the $\underline{V}^{by}$ allele could perhaps indicate, as the mechanism by which this linkage was brought about, a reconstruction of part of the chromosome, involving reduplication of the V-locus, including a locus affecting red leaf pigmentation. This whole region might fail to recombine with normal chromosomes, explaining the complete linkage of the red V only to $\underline{V}^{by}$ among the V-alleles.

Linkage (complete) is also reported in T. pratense between the loci controlling mark presence and position, white or yellow mark colour, and an 'anthocyanin factor' (R.D. Williams, 1937). One may also compare the genetic linkage in Collinsia bicolor between loci for red and white markings along the leaf veins (Hiorth, 1931).

If the close linkage in this case has evolved because it has been selected for, it is implied that there are powerful selective forces acting on the leaf markings that we see, since the physiological and morphological bases of the two markings are very different, and they seem to be related only by their shape and position on the leaf. More circumstantial evidence supporting the possibility of selective significance of red and white leaf markings in other plants has been referred to above, for example in Ranunculus spp.

What, we may still ask, is the significance of what should perhaps be regarded as, at least in origin, a combined red and white marking system? Stahl's three suggestions remain: the improvement of temperature regime, or of light utilisation (though the observed clines suggest lower frequencies of markings where stress in these respects would be greater); or, some effect on animals.

A number of cases of markings of similar shape, mostly approximating to a circular shape, were referred to in Chapter X. It seems possible that such a shape would, like a flower, stand out against a less ordered background of vegetation, attract an animal's attention, and in some way modify its behaviour to the plant's advantage. Markings could perhaps serve as secondary foci of visual attraction to pollinators (the inflorescence in this species is perhaps visually less conspicuous from a distance than single flowers of simpler and more striking geometrical shapes). On the other hand, they might serve as warning signs to predators: the marks in Trifolium repens are generally commoner in the areas where cyanogenesis is commoner (cf. Daday 1954a, 1958) and it has been shown experimentally that cyanogenic forms are recognised, and are avoided, by some predators (Jones, 1962).

3) Points arising for further investigation

The previously published accounts of the genetics of leaf marking in this species are not challenged by this work, though somewhat modified (for example in respect of the frequent effective dominance of $\underline{V}^{by}$ over $\underline{V}^{l}$). It appears however that there is much more variation than they recognise. The question whether this variation can be attributed entirely to the V-locus was raised above in connection with the marginal mark phenotype. Genetical investigation of this and several of the other phenotypes described might supply an answer and would break new ground.

The studies described in Chapter V suggest a whole field of investigation of the dynamics of population structure. This is a different, wider, and more difficult but perhaps more significant topic than the matter of leaf markings which concerns us here. Even in so far as population structure affects sampling procedures in <u>Trifolium</u> <u>repens</u>, the studies described go only a short way towards discounting possible causes of incorrect conclusions in studies on leaf markings. There is great scope for quantitative investigation of the rate of establishment and expansion of new genotypes, and of displacement of others, in different kinds of vegetation, and for quantitative study of variation between different genotypes in effectiveness of the various component processes of vegetative and sexual reproduction. It would probably be found that sexual fertility and vegetative competitive ability differed between seed and vegetative samples, because of the different selective processes to which they had most recently been exposed. Differences might also be found in respect of the frequencies of more easily described polymorphic characters such as leaf markings. The studies reported, on the genetic composition of single heads, and on mapping of clone size, illustrate how genetic variation, particularly of vegetatively expressed polymorphisms such as leaf marking and cyanogenesis, could be used as a tool in investigation of population dynamics.

Only a degree of progress can be claimed with regard to the main problem of defining the environmental variables whose variation the variation in morph frequencies reflects. One way to improve this would be a repeated study of the same kind with improved methods. It can be suggested that these should include the use only of vegetative samples, of larger size, perhaps sampled in a more precisely standardised way, and all grown up after collection in standard conditions before scoring. A more systematic coverage of different habitats and different parts of the species' range would be desirable, as would fuller and more quantitative information on the sample sites, measured and presented in the same way for each site. Such a study would involve a great deal of labour, both in preparation and execution, and indefinite expansion along these lines could be expected to lead to diminishing returns.

The clines already appearing in this study provide a starting point for another more radical approach by experimental studies. Direct study of relative growth of marked and unmarked types as affected by the possible selective factors (suggested above to be aspects of temperature and water regime) are clearly indicated as part of the next step in attacking the main problem. Genetic variation, both that between different marked types and also that abundantly present in the rest of the genotype, would need to be allowed for by careful design of such experiments. This could be done partly by extensive replication, and partly by comparisons between marked and unmarked progeny of the same cross, or within inbred lines.

With regard to the other species, future work would clearly have to replace the rather bibliographical treatment above by a great deal of direct observation on material of as many species as possible, and from as many sources as possible. Morph frequency surveys in the field, particularly on _T_. _pratense_, could be combined with those on _T_. _repens_.

The systems of inheritance of leaf markings described in *T. repens* and *T. pratense* are very different. It would be interesting to study the genetics of differing leaf markings in other species of *Trifolium*, especially the genetics of differences between marked types, as well as between them and the unmarked type. Further information of this kind would provide a much better foundation for discussion of the relation of leaf markings and their polymorphisms to phylogeny.

REFERENCES

Abrams, L. (1944)
Illustrated Flora of the Pacific States.
Vol. II. Stanford.

Ahlgren, G.H. and Sprague, H.B. (1940)
Bull. N.J. Agric. Expt. Sta., 676.
A Study of Variability in White Clover (Trifolium repens L.) and its Relation to Pasture Improvement.

Allan, H.H. (1940)
A Handbook of the Naturalised Flora of New Zealand.
D.S.I.R. Bulletin No. 83. Wellington.

Allison, A.C. (1954)
Trans. Roy. Soc. Trop. Med. and Hyg., 48, 312-318.
The Distribution of the Sickle Cell trait in East Africa and elsewhere, and its Apparent Relationship to the Incidence of Subtertian Malaria.

Anonymous (University of Adelaide, Waite Agricultural Research Institute)
Key for Distinguishing the Commoner Strains of Subterranean Clover (Trifolium subterraneum).

Apirion, D. and Zohary, D. (1961)
Genetics, 46, 393-399.
Chlorophyll lethal in natural populations of the orchard grass (Dactylis glomerata L. ssp. judaica). A case of balanced polymorphism in plants.

Ascherson, P. and Graebner, P. (1906-1910)
Synopsis der Mitteleuropäischen Flora. Band VI, Teil 3.
(Engelmann, Leipzig).

Atwood, S.S. (1937-1944)
Ann. Repts. U.S. Regional Pasture Res. Lab., State College, Pennsylvania.

Atwood, S.S. (1938)
2nd Ann. Rept., U.S. Reg. Pasture Res. Lab., pp. 24-28.
Self-pollination in Legumes.

Atwood, S.S. (1941)
Genetics, 26, 137.
Cytogenetic basis of incompatibility in Trifolium repens.

Atwood, S.S. (1942a)
J. Agric. Res., *64*, 699-709.
Genetics of pseudo-self-compatibility and its relation to cross-incompatibility in *Trifolium repens*.

Atwood, S.S. (1942b)
J. Amer. Soc. Agron., *34*, 353-364.
Genetics of Self-Compatibility in *Trifolium repens*.

Atwood, S.S. (1942c)
Genetics, *27*, 129-130 (Abs.) and 333-338.
Oppositional alleles causing cross incompatibility in *Trifolium repens*.

Atwood, S.S. (1944)
Genetics, *29*, 428-435.
Oppositional Alleles in Natural Populations of *Trifolium repens*.

Atwood, S.S. (1945)
J. Amer. Soc. Agron., *37*, 991-1004.
Behaviour of the Self-Compatibility Factor and Its Relation to Breeding Methods in *Trifolium repens*.

Atwood, S.S. and Hill, Helen D. (1940)
Amer. J. Bot., *27*, 730-735.
Regularity of meiosis in microsporocytes of *Trifolium repens*.

Atwood, S.S. and Kreitlow, K.W. (1946)
Amer. J. Bot., *33*, 91-100.
Studies of a genetic disease of *Trifolium repens* simulating a virosis,

Atwood, S.S. and Sullivan, J.T. (1943)
J. Hered., *34*, 311-320.
Inheritance of Cyanogenetic glucoside and its hydrolyzing enzyme in *Trifolium repens*.

Barber, H.N. (1955)
Evolution, *9*, 1-14.
Adaptive gene substitutions in Tasmanian Eucalypts.
1. Gene controlling the development of glaucousness.

Bateman, A.J. (1947)
Nature, *160*, 337.
Number of S-alleles in a population.

Beaty, E.R., Powell, J.D., and Young, W.C. (1965)
Crop Science, 5, 284
Amclo Arrowleaf Clover.

Beatty, D.W. and Gardner, F.P. (1961)
Crop Science, 1, 323-326
Effect of Photoperiod and Temperature on Flowering of White Clover (Trifolium repens L.)

Beinhart, G., Gibson, P.B., Halpin, J.E. and Hollowell, E.A.
Crop Science, 3, 89-92.
Selection and Evaluation of White Clover Clones. III. Clonal Differences in Branching in Relation to Leaf Area Production and Persistence.

Belli, S. (1894)
Memorie delle Reale Accademia delli Scienze di Torino, Ser II, XLIV, 233-292.
Rivista critica delle specie di 'Trifolium' italiane, comparate con quelle straniere, delle Sezione 'Lupinaster' (Buxbaum).

Bentham, G. and Hooker, Sir J.D. (1924)
Handbook of the British Flora.

Black, J.N. (1960)
Australian Jour. Agric. Res., 11, 277-291.
The significance of petiole length, leaf area, and light interception in competition between strains of subterranean clover (Trifolium subterraneum L.) grown in swards.

Bleier, H. (1925)
Jb. wiss. Bot., 64, 604-636
Chromosomenstudien bei der Gattung Trifolium.

Bobrov, E.G. (1947)
Acta Inst. Bot. Acad. Sc. URSS Ser I(6), 164-336.
(Revision of clovers of USSR).

Bogdan, A.V. (1963)
Heredity, 18, 365-368
Chloris gayana without anthocyanin coloration.

Borg, J. (1927)
Malta Flora. (Malta).

Bosemark, N.O. (1957)
Hereditas, 43, 211-235
On Accessory Chromosomes in Festuca pratensis.
V. Influence of Accessory Chromosomes on Fertility and Vegetative development.

Bradshaw, A.D. and Snaydon, R.W. (1959)
Nature, 183, 129-130
Population Differentiation within Plant Species in Response to Soil Factors.

Brewbaker, J.L. (1955)
J. Hered., 46, 115-123
V-Leaf Markings of White Clover.

Brewbaker, J.L. (1962)
J. Hered., 53, 163-167
Cyanidin-Red White Clover.
A Duplicate Recessive Mutant in *Trifolium repens* L.

Brewbaker, J.L. and Anderson, W.P. (1952)
Genetics, 37, 568
Multiple alleles conditioning leaf markings in *Trifolium repens*.

Brewbaker, J.L. and Carnahan, H.L. (1956)
J. Hered., 47, 103
Leaf marking alleles in White Clover - Uniform Nomenclature.

Brewbaker, J.L. and Keim, W.F. (1953)
Amer. Nat., 87, 323-326
A Fertile Interspecific Hybrid in *Trifolium*: 4n *T. repens* x 4n *T. nigrescens*.

Brigham, R.D. and Wilsie, C.P. (1955)
Agronomy J., 47, 125-127
Seed setting and vegetative vigour of Ladino clover (*Trifolium repens* Leyss) clones and their diallel crosses.

Britten, E.J. (1960)
Science, 131, 100-101.
Genetical and Environmental Control of Flowering in *Trifolium repens* in the Tropics.

Britten, E.J. (1961)
Agronomy J., 53, 11-14
The Influence of Genotype and Temperature on Flowering in *Trifolium repens*.

Brockwell, J. (1956)
J. Austral. Inst. agric. Sci., 22, 260-265
The use of the red leaf strain of subterranean clover for rapid evaluation of strain effectiveness in *Rhizobium trifolii*.

Brown, K.G. (1947)
Abstracts, 1947 Meeting, American Society of Agronomists.
Inheritance of patterned leaf-markings in white clover.

Bula, R.J., May, R.G., Garrison, C.S., Rincker, C.M. and McAllister, D.R. (1964)
Crop Science, 4, 295-297
Growth Responses of White Clover, *Trifolium repens* L., progenies from Five Diverse Geographic Locations.

Cain, A.J. and Sheppard, P.M. (1954)
Genetics, 39, 89-116
Natural Selection in *Cepaea*.

Carnahan, H.L. (1960)
Agronomy J., 52, 302
A comparison of 1st and 2nd synthetic generation forage yields of Ladino and white clover in association with Bromegrass.

Carnahan, H.L., Hill, Helen D., Hanson, A.A. and Brown, K.G. (1955)
J. Hered., 46, 109-114
Inheritance and frequencies of leaf markings in white clover.

Carrier, L. and Bort, K.S. (1916)
J. Amer. Soc. Agron., 8, 256-266
History of Kentucky bluegrass and white clover in the United States.

Chakravarty, A.K. (1963a)
Indian J. Genet. and Pl. Breeding, 23, 185-196
Inheritance of Cyanogenesis in White Clover.

Chakravarty, A.K. (1963b)
Indian J. Genet. and Pl. Breeding, 23, 197-204
Linkage Relationships between genes controlling Cyanogenesis in White Clover and the genes for V-shaped Leaf marking, self-incompatibility and growth.

Clapham, A.R., Tutin, T.G. and Warburg, E.F. (1957)
Flora of the British Isles. Illustrations (Part 1)
Drawings by Sybil J. Roles.

Clapham, A.R., Tutin, T.G. and Warburg, E.G. (1962)
Flora of the British Isles (Second Edition).

Colgan, N. (1911)
Irish Naturalist, XX, 210-217
On the Inheritance of Pitted Leaf-Blotchings in *Arum maculatum* and on FloralVariations in this species.

Coombe, D.E. (1961)
Watsonia, 5, 68-87
Trifolium occidentale, a New Species related to *T. repens*.

Corkill, L. (1952)
NZ J. Sci. Tech., *34*, A, 1-16.
Cyanogenesis in white clover (*Trifolium repens* L.).
VI. Experiments with high glucoside, and glucoside-free strains.

Correns, C. (1931)
S.B. Preuss. Akad. Wiss., 220-231
Vererbungsversuche mit buntblattrigen Sippen.
XI *Coleus hybridus* forma (?) *albopicta*.

Crowder, L.V. (1960)
Agronomy J., *52*, 608-609
The response of white clover varieties grown at high elevations in Colombia.

Crowder, L.C. and Craigmiles, J.P. (1960)
Agronomy J., *52*, 382-385
Effect of soil temperature, soil moisture, and flowering on the persistence and forage production of white clover stands.

Curran, P.L. (1963)
Nature, *197*, 105-106

Balanced Polymorphism in *Dactylis glomerata* ssp *woronowii*.

Daday, H. (1954a)
Heredity, *8*, 61-78
Gene Frequencies in wild populations of *Trifolium repens*
I. Distribution by latitude.

Daday, H. (1954b)
Heredity, *8*, 377-384
Gene Frequencies in wild populations of *Trifolium repens*.
II. Distribution by Altitude.

Daday, H. (1955)
J. Brit. Grassl. Soc., *10*, 266-274
Cyanogenesis in strains of white clover.

Daday, H. (1958)
Heredity, *12*, 169-184
Gene Frequencies in wild populations of *Trifolium repens*.
III. World Distribution.

Daday, H. (1962)
Ann. Rept., Div. of Plant Industry, C.S.I.R.O., Canberra 1962.
(a) p. xiii
Natural Selection in Cyanogenetic Strains of White Clover.

(b) p. 14
Mechanism of natural selection.

Daday, H. (1965)
Heredity, *20*, 355-365
Gene Frequencies in *Trifolium repens*.
IV. Mechanism of Natural Selection.

Darlington, C.D. and Janaki-Ammal, E.K. (1945)
Ann. Bot., *IX* (N.S.), 267-281
Adaptive isochromosomes in *Nicandra*.

Darlington, C.D. and Shaw, G.W. (1959)
Heredity, *13*, 89-121
Parallel Polymorphism in the heterochromatin of *Trillium* spp.

Darwin, C. (1891)
The Effects of Cross and Self-Fertilisation in the
Vegetable Kingdom (Third Edition).

Davies, D.R. and Wall, E.T. (1959)
Nature, *184*, 1957
Separation by gamma-irradiation of two elements at the V^{by}
locus in *Trifolium repens*.

Davies, D.R. and Wall, E.T. (1960)
Heredity, *15*, 1-15
Induced mutations at the V^{by} locus of *Trifolium repens*.
I. Effects of Acute, Chronic and Fractionated doses of
gamma-radiation on induction of somatic mutations.

Davies, D.R. and Wall, E.T. (1961)
Genetics, *46*, 787-798
Induced mutations at the V^{by} locus of *Trifolium repens*.
II. Reduction below the additive base line by fractionated
doses of gamma-radiation.

Davies, W.E. (1963)
Leaf Markings in *Trifolium repens*.
in Teaching Genetics, ed. C.D. Darlington and A.D.
Bradshaw. (Oliver and Boyd. Edinburgh and London).

Davies, W.E. (1965)
Demonstration at Genetical Society Summer Meeting,
Aberystwyth.

Davies, W.E. and Young, N.R. (1963)
Ann Rept. (1962), Welsh Pl. Breed. Sta., 33-35.
The use of genetic markers for the identification of
white clover cultivars.

De Basto Folque, N.A.P. (1949)
Melhoramento, *1*, 11-120
Subsídios para o estudo do género *Trifolium* L. em Portugal.

De Reuter, L. (1952)
Behaviour, 4, 222-232
Some experiments on the camouflage of stick caterpillars.

Dessureaux, L. (1949)
Agriculture (Montreal), 6, 127-131
Variabilité des populations non selectionnées de trèfle blanc Ladino.

De Visiani, R. (1847-1852)
Flora Dalmatica. Lipsiae. Vols. II, III.

De Vries, H. (1907)
Plant Breeding. Comments on the Experiments of Nilsson and Burbank. London.

Dobzhansky, T. (1951)
Genetics and the Origin of Species. Columbia Press, New York.

Dolan, D.D. (1962)
N.Y. State Agr. Exp. Sta., Reg. Plant Introd., Geneva, N.Y.
Seeds Available and Descriptive Notes, 1960 and 1961 Scorings. Forage Legumes and Grass. (cyclostyled).

Dunn, G.M., Calder, F.W. and Kilpatrick, R.A. (1962)
Agr. Exp. Sta., Univ. of New Hampshire, Sta. Tech. Bull. 104.
Variation in white clover in relation to improvement. pp.1-22.

Dyer, A.F. (1963)
Chromosoma, 13, 545-576
Allocyclic segments of chromosomes and the structural heterozygosity that they reveal.

Erith, A.G. (1924)
White Clover (Trifolium repens L.). A Monograph. London.

Erith, A.G. (1928)
J. Genetics, 19, 351-355
Some hybrids of varieties of white clover (Trifolium repens L.)

Evans, A.M. (1962)
Euphytica, 11, 164-176
Species hybridization in Trifolium.
I. Methods of overcoming species incompatibility.

Fenner, F. (1959)
B. Med. Bull., 15, 240-245
Myxomatosis.

Fisher, R.A. (1930)
The Genetical Theory of Natural Selection.
(Cf. Second Edition. Dover, New York, 1958).

Ford, E.B. (1940)
Polymorphism and Taxonomy.
in The New Systematics, ed. J. Huxley, Clarendon Press, Oxford.

Ford, E.B. (1964)
Ecological Genetics. Methuen, London.

Foury, A. (1954)
Les légumineuses fourragères au Maroc.
Rabat. Service de la recherche agronomique.

Frankel, O.H. and Williams, J.D. (1958)
J. Aust. Inst. Agric. Sci., 24, 162-163
A record of natural crossing in subterranean clover.

Fröst, S. (1958)
Hereditas, 44, 75-111
The Geographical Distribution of Accessory Chromosomes in *Centaurea scabiosa*.

Gamble, J.S. (1935)
Flora of the Presidency of Madras. Vol. I. London.

Géhu, J-M. (1963)
Bull. Soc. Bot. Nord. France, XVI, 203-209
Répartition de *Trifolium occidentale* D.E. Coombe dans l'Ouest de la France et observations écologiques.

Gibelli, G. and Belli, S. (1887)
Atti Accad. Sci. Torino, XXII, 628-672
Intorno alla morfologia differenziale esterna ed alla nomenclatura delle specie di Trifolium della sezione *Amoria* (Presl.) crescenti spontanae in Italia.

Gibelli, G. and Belli, S. (1889a)
Malpighia, III, 193-233, contd. 305-319.
Rivista critica della specie di '*Trifolium*' italiane della Sezione '*Chronosemium* Ser. in DC Prodr'.

Gibelli, G. and Belli, S. (1889b)
Mem. R. Accad. sci. Torino, Ser 2, XXXIX, 245-426
Rivista critica e descrittiva della specie di *Trifolium* italiane e affini comprese nelle Sezione *Lagopus* Koch.

Gibson, P.B., and Trautner, J.L. (1965)
Crop Science, *5*, 477-479
Growth of White Clover with and without Primary Roots.

Gibelli, G. and Belli, S. (1891)
Mem. R. Accad. sci. Torino, Ser. 2, *XLI*, 149-224
Rivista critica delle specie di *Trifolium* italiane, comparate con quelle del reste d'Europa e delle regione circummediterranee delle sezioni *Galearia* Presl. - *Paramesus* Presl. - *Micrantheum* Presl.

Gibelli, G. and Belli, S. (1892)
Mem. R. Accad. sci. Torino, Ser. 2, *XLII*, 179-222
Rivista critica delle specie di *Trifolium* italiane, comparate con quelle del reste d'Europa e delle regione circummediterranee delle sezione *Trigantheum* Nobis (*Mistyllus* Presl. p.p.).

Gibelli, G. and Belli, S. (1893)
Mem. R. Accad. sci. Torino, Ser. 2, *XLIII*, 169-228
Rivista critica delle specie di *Trifolium* italiane, comparate con quelle del reste d'Europa e delle regione circummediterranee delle sezioni *Calycomorphum* Presl. - *Cryptosciadium* Celak.

Gibson, P.B. (1957)
Agronomy J., *49*, 213-215
Effect of flowering on the persistence of white clover.

Gillett, J.B. (1952)
Kew Bull., *3*, 367-404
The genus *Trifolium* in Southern Arabia and in Africa south of the Sahara.

Gmelin, H.M. (1914)
Mededeelingen van de Rijks Hoogere Land-, Tuin- en Boschbouwschool, Del 7, Afl. 5.
(First series of investigations with reference to the improvement of red clover.)

Gmelin, H.M. (1916)
Cultura, *28*, 414-430
Proefnemingen met Roode Klaver. Tweed Reeks Van Onderzoekingen met Betrekking Tot De Roode Klaververedeling.

Gray, S.F. (1821)
Anatomical arrangement of British Plants. Vol. II.

Grenier, M. and Godron, M. (1848)
Flore de France. Vol. I.

Haggar, R.J. (1961)
Nature, *191*, 1120
Flower Initiation in Kent Wild White Clover (*Trifolium repens* L.) under Controlled Environmental Conditions.

Haller, A. von (1768)
Historia Stirpium indiginarum Helvetiae. Vol. I. Bern.

Harberd, D.J. (1958)
Nature, *181*, 138
A Spurious Significance in Genecological Trials.

Harberd, D.J. (1961)
New Phytol., *60*, 184-206
Population Structure in *Festuca rubra* L.

Harberd, D.J. (1962)
New Phytol., *61*, 85-100
Some observations on natural clones in *Festuca ovina*.

Harberd, D.J. (1963)
New Phytol., *62*, 198-204
Observations on Natural Clones of *Trifolium repens* L.

Harland, S.C. (1947)
Heredity, *1*, 121-125
An alteration of gene frequency in *Ricinus communis* due to climatic conditions.

Healy, A.J. (1961)
N.Z. Weed Control Proceedings, 22-38
The Identification of Clovers in New Zealand.

Hector, J.M. (1936)
Introduction to the Botany of Field Crops. Vol. II.
Central News Agency, Johannesburg.

Hiorth, G. (1931)
Z. indukt. Abst. Vererbungslehre, *59*, 236-269
Genetische Versuche mit *Collinsia* II. Die Blatt- ind Kotyledonenzeichnungen von *Collinsia bicolor*.

Holding, A.J. and King, J. (1963)
Plant and Soil, *18*, 191-198
The effectiveness of indigenous populations of *Rhizobium trifolii* in relation to soil factors.

Hollowell, E.A. (1952)
Ladino and other White Clovers.
in Forages. Hughes, H.D., Heath, M.E. and Metcalfe, D.S.
Iowa State College Press. pp. 180-189.

Hooker, Sir J.D. (1879)
Flora of British India. London.

Hossain, M. (1961)
Notes, Roy. Bot. Gdn., (Edinburgh), XXIII, 387.
A revision of Trifolium in the Nearer East.

Hovin, A.W. and Gibson, P.B. (1961)
J. Hered., 52, 295-296.
A Red Leaf Marking in White Clover.

Huxley, J. (1955)
Heredity, 9, 1-52
Morphism and Evolution.

Jones, D.A. (1962)
Nature, 193, 1109
Selective Eating of the Acyanogenic Form of the Plant Lotus corniculatus L. by various animals.

Kajanus, B. (1912)
Botanisker Notiser, Lund, 39-44
Über die Blattzeichnung des Rotklees.

Kalm, P. (1819)
In John Pinkerton's Voyages and Travels. London

Karpechenko, G.D. (1925)
Bull. Appl. Bot. and Pl. Breeding, 14, 271-279
Karyologische Studien über die Gattung Trifolium L.

Kerner von Marilaun, A. and Oliver, F.W. (1895)
The Natural History of Plants. Blackie, London.

Kettlewell, H,D.B. and Berry, R.J. (1961)
Heredity, 16, 403-414
The study of a cline.

Kimura, M. and Kayano, H. (1961)
Genetics, 46, 1699-1712
The Maintenance of Supernumerary chromosomes in wild populations of Lilium callosum by preferential segregation.

King, J. (1963)
Plant and Soil, 18, 221-224
Ecotypic differentiation in Trifolium repens.

Kirk, T. (1899)
The Students' Flora of New Zealand. Wellington.

Komarov, V.L.
Flora URSS Editio Academiae Scientiarum U.R.S.S., Moscow.

Lange, J. (1887)
Meddelelser om Grønland, 3, 233-308
Conspectus Flora Groenlandicae.
1). Tillaeg til Fanerogamerne og Karsporeplanterne.

Laude, H.M., Stanford, E.H. and Enloe, A.J. (1958)
Agronomy J., 50, 223-225
Photoperiod, temperature, and competitive ability as factors affecting the seed production of selected clones of Ladino clover.

Lieth, H. (1960)
Patterns of Change within Grassland Communities, pp. 27-39. in The Biology of Weeds, ed. J.L. Harper. Blackwells, Oxford.

Lojacono Pojero, M. (1883)
Nuovo giorn. botan. ital., 225 ff.
Clavis specierum Trifoliorum.

Lojacono Pojero, M. (1891)
Flora Sicula. Vol. I, Part 2. Palermo.

Lowe, R.T. (1868)
Manual Flora of Madeira. London.

McCloud, D.E. and Cole, W.A. (1951)
Proc. 48th Annual Conv. of Assoc. of Southern Agricultural Workers, pp. 137 ff.
The Effect of Photoperiod on the Flowering of Ladino and Louisiana White Clovers.

Mirande, M. (1912)
C.R. Acad. Sci. Paris, 155, 651-653
Sur la présence de l'acide cyanhydrique dans le trèfle rampant (Trifolium repens L.).

Oliver, D. (1871)
Flora of Tropical Africa. London.

Pandey, K.K. (1957)
J. Hered., XLVIII, 278-281
A self-compatible hybrid from a cross between two self-incompatible species in Trifolium.

Parker, M.C. (1933)
J. Hered., 24, 481-486
The inheritance of a yellow spot character in the bean.

Pethybridge, G.H. (1903)
Irish Naturalist, 12, 145-151
The leaf-spots of Arum maculatum.

Pickering, C. (1879)
Chronological History of Plants. Boston.

Polunin, N. (1959)
Circum-Polar Arctic Flora. Oxford University Press.

Prime, C.T. (1955a)
Watsonia, 3, 175-178
Vegetative Reproduction in Arum maculatum.

Prime, C.T. (1955b)
Watsonia, 3, 181-185
Variation in Arum maculatum

Reichenbach, L. and Reichenbach, H.G. (1903)
Icones Florae Germaniae et Helveticae. Vol. XXII.

Roberts, R.H. and Struckmeyer, B.E. (1938)
J. Agr. Res., 56, 633-677
The effects of temperature and other environmental factors upon the photoperiodic responses of some of the higher plants.

Rydberg, P.A. (1922)
Flora of Rocky Mountains and Adjacent Plains. (2nd Ed.).
New York.

Saunders, E.R. (1918)
J. Genetics, 7, 215-228
On the occurrence, behaviour, and origin of a smooth-stemmed form of the common foxglove (Digitalis purpurea).

Scheibe, A. (1955)
Züchter, 25, 97-103
Die Wirkung der natürlichen Auslese bei Pisum arvense. Formen mit und ohne Wachsschicht.

Seringe, (1825)
Trifolium.
in Prodromus Systematis Naturalis Regni Vegetabilis, Vol. II.
A.P. de Candolle, Paris.

Seyffert, W. (1955)
XIV Int. Hort. Congress, Netherlands, 929-937.
Der Genetik der Cyclamenfarben.

Seyffert, W. (1957)
Gartenwelt, 57 (4), 57-58.

Smith, Sir J.E. (1829)
English Flora, Vol. III. London.

Smith, P.E. (1950)
The Inheritance of Leaf Markings in Red Clover (Trifolium pratense L.)
Ph.D. Thesis, Pennsylvania State College.
Doctoral Dissertation Series. Publication No. 2243.
(Microfilm copy in Library of Welsh Plant Breeding Station, Aberystwyth).

Smyth, A.H. (1887)
Life and Writings of Benjamin Franklin, 2, 383.

Snaydon, R. and Bradshaw, A.D. (1962)
J. Brit. Grassl. Soc., 17. 113
The performance and survival of contrasting natural populations of white clover when planted into an upland Festuca/Agrostis sward.

Sowerby, J. (1794)
English Botany. Vol. III.

Söyrinki, N. (1938)
Ann. Bot. Soc. Zool.-Bot. Fenn. Vanamo, 11, 1-323
Studien über die generative und vegetative Vermehrung der Samenpflanzen in der alpinen Vegetation Petsamo-Lapplands.

Söyrinki, N. (1939)
Mem. Soc. Fauna et Flora Fennica, 15, 201-209
Generative und Vegetative Vermehrung in der alpinen Vegetation Petsamo-Lapplands.

Stahl, E. (1896)
Annales du Jardin Botanique de Buitenzorg, XIII, 137-215.
Ueber bunte Laubblatter.

Stanford, E.H., Laude, H.M. and Enloe, J.A. (1960)
Agronomy J., 52, 149-152
Effect of harvest dates and location on the genetic composition of the Syn_1 generation of Pilgrim Ladino clover.

Stanford, E.H., Laude, H.M. and Booysen, P. de V. (1962)
Crop Sc., 2, 497-500
Effect of Advance in Generation Under Different Harvesting Regimes on the Genetic Composition of Pilgrim Ladino Clover.

Stapledon, R.G. (1924)
Jour. Min. Agr. (G.B.), 30, 33-38
White Clover.

Starzycki, S. (1959)
Hodowla Roślin Aklimatyz. Nasiennictwo, *3*, 277-319
Zmienność mieszańców koniczyny białej (*Trifolium repens* L.)
z koniczyną czerwoną (*Trifolium pratense* L.).
(Variability of hybrids between white clover and red clover.)

Starzycki, S. (1961)
Hodowla Roślin Aklimatyz. Nasiennictwo, *5*, 285-330
Badania nad mieszygatunkowymi koniczyn *Trifolium repens* L.
x *Trifolium pratense* L.
(Studies on interspecific clover hybrids.)

Strickland, W. (1801)
Observations on the Agriculture of the United States.
Rept. British Bd. Agric.

Suckling, F.E.T. (1952)
N.Z. J. Sci. Tech., *33*, *A*, 64-77.
Dissemination of white clover (*Trifolium repens*) by sheep.

Tamm, C.O. (1964)
Proc. X Int. Bot. Cong., Edinburgh. Abst. No. 629.
Survival of individual perennial herbs, with some remarks
on the role of plant persistence in vegetation dynamics.

Taubert, P. (1894)
Leguminosae. *In* Engler and Prantl, Die Natürliche
Pflanzenfamilien. Leipzig. Teil III. Abth. 3.
pp. 70-388 (*Trifolium*, 249-254).

Thomas, R.G. (1961)
Aust. J. Agric. Res., *12*, 227-238
The influence of environment on Seed Production Capacity
in White Clover (*Trifolium repens* L.).
1. Controlled Environment Studies.

Tilney-Bassett, R.A.E. and Kirk, J.T.O. (1966?: In press).
The Plastids: their structure, biochemistry and genetics.
L.H. Freeman, & Co., London.

Trimble, J.P. and Hovin, A.W. (1960)
Agronomy,J., *52*, 485
Interspecific hybridization of certain *Trifolium* species.

Turrill E.B. (1948)
British Plant Life. New Naturalist.

Ved Brat, S. (1965)
Chromosoma, *16*, 486-499.
Genetic Systems in *Allium*. I. Chromosome variation.

Vicioso, C. (1953)
Tréboles Españoles. Revisión del género *Trifolium*.
Cons. Sup. de Invest. Cient., Instituto Botanico A. Cavanilles,
Madrid.

Ware, W.M. (1925)
J. agric. Sci., *15*, 47-67
Experiments and observations on forms and strains of *Trifolium repens* L.

Wellensiek, S.J. (1961)
Euphytica, *10*, 259-268
The Breeding of Diploid Cultivars of *Cyclamen persicum*.

Westbrooks, F.E. and Tesar, M.B. (1955)
Agronomy J., *47*, 403-410.
Tap Root Survival of Ladino Clover.

Wexelsen, H. (1928)
Univ. Calif. Publ. Agric. Sci., *2*, 355-376
Chromosome numbers and morphology in *Trifolium*.

Wexelsen, H. (1932)
Hereditas, *16*, 219-240.
Segregations in red clover (*Trifolium pratense* L.).

Whyte, R.O. (1960)
Crop Production and Environment. (2nd. Ed.). London.

Williams, R.D. (1931a)
Bull. Welsh Pl. Breed. Sta., *H12*, 209-216.
Self- and Cross-fertility and flowering habits of certain herbage grasses and legumes.

Williams, R.D. (1931b)
Imp. Bur. Plant Genetics, Herbage Plants, Bull., *3*, 46-76.
Methods and Technique of Breeding Red Clover, White Clover, and Lucerne.

Williams, R.D. (1937)
Rept. IVth. Int. Grass. Congr., Aberystwyth, 238-250.
Genetics of Red Clover and its bearing on Practical Breeding.

Williams, R.D. (1939)
J. Genet., *37*, 441-458, 459-482
Genetics of Chlorophyll Deficiencies in Red Clover - *Trifolium pratense* L.
I. Albinos.
II. Yellow seedling factors.

Williams, W. (1945)
Bull. Welsh Pl. Breed. Sta., *H16*, 19-26
Varieties and Strains of Red and White Clover - British and Foreign.
Section B. The Varieties of White Clover used in British Agriculture.

Williams, W. (1964)
Genetical Principles of Plant Breeding.
Blackwells, Oxford.

Williams, W. and Brown, A.G. (1956)
Heredity, 10, 237-245.
Genetic Response to Selection in Cultivated Plants:
Gene Frequencies in *Prunus Avium*.

Willis, J.C. (1955)
A Dictionary of the Flowering Plants and Ferns.
Cambridge University Press.

Wolter, J. (1958)
Ann. Univ. M. Curie-Skłodowska, Sect. E., 13, 297-325.
Badania nad krajowymi populacjami konicxyny białej
(*Trifolium repens* L.).
(Investigations on native populations of white clover.)

Zohary, D. and Imber, D. (1963)
Heredity, 18, 223-231
Genetic dimorphism in fruit types in *Aegilops speltoides*.

Zwingli, W. (1956)
Thesis (D. Tech. Wiss.), Eidg. Tech. Hochsch., Zürich.
Untersuchungen über die Fertilitätsverhältnisse in
schweizerischen Weissklee-Populationen mit ergänzenden
zytologischen Studien.

Published in full: Z.Pflanzenz., 36, 237-288

Appendix I

Table of Numbers of Samples and Plants and Their Countries of Origin.

The table is presented so that it is possible to see how many samples and plants are based on seed and how many on vegetative material; seed samples are underlined in the figures for the British Isles, the majority being vegetative, and vice versa the minority of vegetative samples is underlined elsewhere. The table also shows how many of the samples from each country were grown and scored by the author in Oxford (and a few in Bangor), how many were grown at Carlow and scored there by the author, and how many were grown and scored by the staff of the Welsh Plant Breeding Station, and within each grouping by country of origin and place of scoring, indicates which samples were known to be of established, spontaneous growing wild origin, which are unrepresentative of their population or represent bred strains, and for which there is no information to indicate which of these is the case.

In each cell of the table, the first figure represents the number of samples, and the second the total number of plants contained in them.

Summary of Totals

Place Scored	TOTAL	Field	Oxford		Oak Park		Carlow	Welsh P.B.S.		
Wild or Selected		Wild	Wild	?	Wild	?	Seltd.	Wild	?	Seltd.
Vegetative	289 11910	192 9497	23 377	- -	19 900	- -	- -	55 1136	- -	- -
Seed	396 21363	- -	69 6180	3 324	13 533	123 6385	35 1907	83 3966	68 2010	2 58
Combined Total	685 33273	192 9497	92 6557	3 324	32 1433	123 6385	35 1907	138 5102	68 2010	2 58

Part 1. Natural Range outside British Isles (Seed unless underlined).

Place Grown and Scored	TOTAL	Field	Oxford		Carlow			Welsh P.B.S.		
Wild or Selected		Wild	Wild	?	Wild	?	Seltd	Wild	?	Seltd
Iceland	2 30		2 30							
Norway	5 361		2 213	1 99					2 49	
Sweden	6 294					3 147	3 147			
Finland	2 282		1 275						1 7	
Russia	1 86					1 86				
Poland	24 1462		18 1197			2 96			4 169	
Denmark	9 1414		5 1198			2 110	1 58		1 48	
Germany	21 638		8 132			3 116	7 330		3 60	
Netherlands	11 451					7 327	3 116		1 8	
Belgium	2 59						1 49		1 10	
France	26 1067		1 82		3 148	3 200	1 50	(14 468) (1 29)	3 90	
Switzld & Lcht	19 1342		7 846		1 28	5 316	1 40		5 112	
Italy	17 776		5 300		1 10	7 345			3 67	1 54
Spain	57 3222					2 87	1 150	49 2879	5 106	
Portugal	18 678		2 170			2 88	1 30	(10 377) (3 13)		
Morocco	7 161					2 89		4 16	1 56	
Austria	8 613		3 389			3 158			2 66	
Hungary	3 93					2 89			1 4	
Yugoslavia	27 1545		4 375	1 105		18 914			4 151	
Greece	15 1031		2 199			9 687	1 40	1 56	2 49	
Bulgaria	1 59		1 59							
Turkey	10 713				1 50	5 299	2 274		2 90	
Lebanon	2 130					1 90			1 40	
Israel	7 405			1 120		1 57	2 150		3 78	
Iran	16 723				2 67	12 587	1 49		1 20	
Afghanistan	12 428					9 313	3 115			
West Pakistan	6 249					5 221		1 28		
Totals (Pt. 1)	334 18312		61 5465	3 324	8 303	104 5422	28 1598	83 3866	46 1280	1 54

Part 2. British Isles (Vegetative unless underlined)

	TOTAL		Field		Oxford		Oak Park				Carlow		Welsh P.B.S.					
			Wild		Wild	?	Wild		?		Seltd		Wild		?		Seltd	
NE Eng. SE Scot.	46	2471	45	2462									1	9				
Lancs & Yorks	18	732	15	701									3	31				
Wales & M (N)	39	1150	23	960	14	152							1+1	9+29				
N.Midlands	23	1127	14	982									9	145				
Wales & M (S)	31	886	6	227									2+23	27+632				
S. Midlands	65	2847	60	2482	2+1	238+93							2	34				
South-East	33	1762	29	1683									4	79				
Cornwall, Sc.Is.	7	178											2+5	50+128				
Unlocated	1	58							1	58								
Ireland	23	1021					19	900	1	50			2	63	1	8		
Total (Pt. 2)	286	12232	192	9497	17	483	19	900	2	108			55	1236	1	8		
Part 3.	Outside Natural Range (All are Seed Samples)																	
Azores	1	106			1	106												
Canada	5	249			1	103									4	146		
U.S.A.	18	719					5	230	6	278	4	152			2	55	1	4
Jamaica	1	58							1	58								
Costa Rica	1	46							1	46								
Peru	1	40													1	40		
Brazil	1	19													1	19		
Uruguay	1	50							1	50								
Argentina	3	176							2	139					1	37		
New Zealand	15	518			12	400					1	38			2	80		
Australia	10	461							3	197	2	119			5	145		
Japan	3	120													3	120		
India	3	88							2	48								
Ethiopia	1	39							1	39								
South Africa	1	40													1	40		
Total (Pt. 3)	65	2729			14	609	5	230	17	855	7	309			21	722	1	4

Appendix II

Numbers and Frequencies of main Marking Types, by Countries of Origin.

These figures are the totals of all plants showing each mark; that is, double marks (except LL) are counted twice, and the sum of the other figures on each line may therefore exceed that of the 'total' figure. LY scores are added into the L total, and the L figures are therefore not quite equivalent to those used below in the statistical treatment, where allowance is made for the dominance of $\underline{V}^{by}$ by counting L only among plants not showing Y.

The figures for two countries (France and Italy) are divided, geographically defined groups with distinctive marking frequencies being separated from the (larger) remainder, in which remain the samples not located more precisely than by the country of origin.

The table is presented in three parts, the British Isles and the samples from outside the species' natural range being removed into separate sections.

NB. A decimal point should be read before the frequency figures. Except for those for the totals, they are presented as parts per thousand.

Area	Total	O	L	Y	B+M	Ba	F	O	L	Y	B+M	Ba	F
Part 1. Natural Range of Species (excluding British Isles)													
Iceland	30	11	15	-	-	-	5	367	500	---	---	---	167
Norway	361	82	261	1	-	12	27	227	723	003	---	033	075
Sweden	294	78	212	7	-	-	2	265	721	024	---	---	007
Finland	282	48	225	10	-	-	3	170	798	135	---	---	011
Russia	86	26	49	-	-	9	6	302	570	---	---	105	070
Poland	1462	308	1068	47	3	8	43	211	731	032	002	005	029
Denmark	1414	211	1151	30	29	4	19	149	814	021	021	003	013
Germany	638	152	454	29	6	5	7	238	712	045	009	008	011
Netherlands	451	71	380	5	-	2	-	157	843	021	---	004	---
Belgium	59	5	51	4	-	1	-	085	864	068	---	017	---
France (exc. SE)	905	104	750	76	9	6	7	115	829	084	010	007	008
France, South-east	162	5	138	39	2	-	-	031	852	241	012	---	---
Spain	3222	346	2711	158	6	18	75	107	841	049	002	006	023
Portugal	678	69	572	47	-	-	5	102	844	069	---	---	007
Morocco	161	6	136	22	-	-	-	037	845	137	---	---	---
Switzld & Lcht	1342	197	1108	40	1	20	13	147	826	030	001	015	010
Austria	613	88	519	3	1	-	4	144	847	005	002	---	007
Italian Alps	300	51	158	4	113	-	4	170	527	013	377	---	013
Italy (exc. A, S)	438	29	399	14	2	1	-	066	911	032	005	002	---
Sicily	38	38	-	-	-	-	-	1000	---	---	---	---	---
Hungary	93	32	59	1	-	1	1	344	634	011	---	011	011
Yugoslavia	1545	113	1348	38	80	12	41	073	872	025	052	008	027
Greece	1031	19	967	59	44	1	28	018	938	057	043	001	027
Bulgaria	59	2	53	-	-	-	6	034	898	---	---	---	102
Turkey	713	115	561	42	10	-	-	161	788	059	014	---	---
Lebanon	130	1	129	-	-	-	-	008	992	---	---	---	---
Israel	405	1	402	3	-	-	-	002	993	007	---	---	---
Iran	723	71	628	27	10	2	5	098	869	037	014	003	007
Afghanistan	428	137	253	42	5	-	1	320	592	098	012	---	002
West Pakistan	249	37	209	3	2	-	1	149	839	012	008	---	004
Total, Pt. 1.	18312	2453	14966	751	323	102	303	1340	8173	0410	0176	0056	0165

Area	Total	O	L	Y	B+M	Ba	F	O	L	Y	B+M	Ba	F
			Part 2.	British Isles									
NE Eng. + SE Scot.	2471	900	1545	18	-	10	1	364	625	007	---	004	0004
Lancs Yorks	732	164	557	11	-	1	3	224	761	015	---	001	004
Wales + Marches N	1150	326	803	12	-	7	7	283	698	010	---	006	006
North Midlands	1127	218	891	15	-	13	1	193	791	013	---	012	001
Wales + Marches S	886	136	744	5	-	4	1	153	840	006	---	005	001
South Midlands	2847	734	2085	22	-	17	3	258	732	008	---	006	001
South-East	1762	303	1405	41	-	20	10	172	797	023	---	011	006
Cornwall, Sc. Is.	178	11	165	2	-	4	1	062	927	011	---	122	006
'Eng' unlocated	58	5	51	1	-	-	1	086	879	017	---	---	017
Ireland	1021	177	841	2	2	3	2	173	824	002	002	003	002
Total, Pt. 2.	12232	2974	9087	129	2	79	30	2431	7429	0105	0002	0065	0025
			Part 3.	Outside Natural Range									
Azores	106	7	99	-	1	-	-	066	934	---	009	---	---
Canada	249	33	205	7	-	1	5	133	823	028	---	004	020
United States	719	82	579	63	38	2	17	114	805	088	053	003	024
Trop. America	163	17	139	9	1	1	-	104	853	055	006	006	---
Temperate S. America	226	33	180	20	3	-	2	146	796	088	013	---	009
New Zealand	518	107	408	12	-	4	4	207	788	023	---	008	008
Australia	461	37	374	67	7	1	2	080	811	145	015	002	004
Japan	120	20	100	-	1	-	-	167	833	---	008	---	---
India (Nilgiri Hs)	88	8	80	-	-	-	-	091	909	---	---	---	---
Ethiopia	39	6	32	1	-	-	-	154	821	026	---	---	---
South Africa	40	-	40	-	-	-	-	---	1000	---	---	---	---
Total, Pt. 3.	2729	350	2236	179	51	9	30	1283	8193	0656	0187	0033	0110
Combined Total	33273	5777	26289	1059	376	190	363	1736	7901	0318	0113	0057	0109

Appendix III(a)

Frequency Data Used in British Isles Maps (Figures VIII (x-xii).

	Grid Ref.	N	O	O/N	Y	Y/N	LL	LL Freq.‡ (Corr.)
Oxton; Blainslie	NT5054	122	59	484	1	008	00	---
Jedburgh	NT6521	160	36	225	3	019	--	---
Wooler; *Berwick	NT9930	131	33	252	-	---	--	---
Thropton; Longframlington	NU0502	271	111	410	1	004	--	---
Morpeth; Longhirst; Seaton Sluice	NZ2585	155	49	316	-	---	--	---
Bellingham; Chollerford	NY9478	156	73	468	-	---	--	---
Catton; Thirlwall; Gilsland	NY7062	167	65	389	-	---	--	---
Dirt Pot; Moss Moor	NY8542	249	153	614	-	---	--	---
Brancepeth; Sunnyside; Wolsingham; Stanhope; Rookhope	NZ0838	464	164	353	7	015	--	---
Sherburn; Stoneygate	NZ3546	594	157	264	5	008	5	035
Saltergate	SE8594	87	17	195	1	011	1	031
Malton; Barton Hill	SE7468	253	60	237	5	020	--	---
Warter; Mkt. Weighton	SE8646	248	66	266	2	008	--	---
Ferrybridge	SE4823	113	19	168	3	027	--	---
*Great House (Helmshore)	SD7821	31	2	065	-	---	--	---
Bangor(*); *Newborough; *Port Dinorwic	SH5772	146	29	200	3	021	5	115
Ty 'n-y-Maes; Nant Ffrancon; Llyn Ogwen; Llugwy Valley; *Pen-y-Pass; Capel Curig	SH6960	374	87	233	-	---	--	---
Betws-y-Coed	SH7957	80	28	350	3	038	1	087
Llangollen	SJ2441	452	157	347	6	013	1	014
Gobowen	SJ3035	69	24	348	-	---	--	---
*Oswestry	SJ2930	29	1	034	-	---	--	---
Burton Joyce; Lambley; Dorket	SK6346	258	87	337	3	012	--	---
Morley; *Dovedale; Swarkeston	SK3740	162	39	245	-	---	3	049

(Britain: * : Sample or Group scored in greenhouse; if greater part of group, plotted with broken outline on map).

‡ Corrected LL Frequency calculated as sum of all LL scores divided by sum for each sample of $Y.(1-\sqrt{YL / Y})^2$.

Appendix III(a) continued

		Grid Ref.	N	O	O/N	Y	Y/N	LL	LL Freq. (Corr.)
Groby; Markfield		SK4910	200	32	160	7	035	2	027
*Medbourne; Skeffington; *Ch.Langton; *Tur Langton; Mkt.Harborough; Rothwell		SP7488	411	47	114	2	005	5	026
Isham		SP8975	34	4	118	-	---	--	---
*Holkham		TF8944	62	9	145	3	048	--	---
Aberystwyth; *Clarach		SN5881	170	38	224	-	---	7	149
Definog		SN9228	67	20	299	-	---	1	073
*Glamorgan		SS9268	57	5	088	-	---	--	---
*Monmouth		ST3784	575	72	125	5	009	4	019
Burford; Ch. Norton		SP2920	122	19	156	-	---	2	044
Marston; Woodstock; Shotover; *Oxford		SP5210	786	176	198	8	009	12	049
Wytham; Cothill; Wittenham		SP4809	1664	548	329	13	008	16	044
*Creslow		SP8122	34	3	088	1	029	1	062
*Rothamsted		TL1311	238	8	034	-	---	7	043
*Romney Marsh		TR0430	79	5	063	-	---	5	113
Selsfield		TQ3534	98	16	163	1	010	2	057
Fulmer-Kingston; Newhaven		TQ3908	340	54	175	2	006	1	009
Chanctonbury; Wiggonholt; Northpark		TQ1014	881	129	146	29	033	20	059
Midhurst; Hemley (Sx)		SU8923	236	60	252	3	013	--	---
Nutcombe; Mar.'s Bdge		SU8738	128	37	289	6	047	--	---
*Scilly Isles		SV9211	40	0	---	2	050	8	083
	Lat.	Long.							
Thurles	52.42N	7.50W	268	55	205	1	004	7	079
Waterford	52.15N	7.08W	208	45	216	-	---	9	151
Ardmore	51.57N	7.42W	64	11	172	-	---	3	137
Clonakilty	51.38N	8.53W	87	11	126	1	011	5	146
Fermoy	52.08N	8.17W	72	10	139	-	---	1	034
Mallow	52.08N	8.39W	94	18	191	-	---	2	064
Tallow	52.06N	7.59W	107	14	131	-	---	--	---
*Bantry	51.41N	9.27W	54	5	093	-	---	--	---

(Ireland : * : Seed Sample, plotted with broken outline. All others transplanted, scored at Carlow.)

Appendix III(b)

Frequency Data Used for Western Europe Maps (Figures VIII (xiii, xiv)

	Lat.	Long.	Total	O	O/N	Y	Y/N
Ulvila (Finland)	61.26N	21.56E	275	47	171	10	036
Rogaland (Norway)	59 N	6 E	40	16	400	--	---
Vågønes (Norway)	67.17N	14.24E	312	60	192	1	003
Vanløse (Denmark)	55.30N	12.30E	1198	164	137	27	023
Jutland (Denmark)	57 N	9.30E	48	9	188	--	---
Vogar, Thing (Iceland)	63.59N	22.24W	14	1	071	--	---
Botn Eyjafjardar (Iceland)	66 N	19 W	16	10	625	--	---
Kamien Pomorski (Poland)	53.58N	14.49E	77	12	156	17	221
Gredziec; Barlinek (Poland)	53.02N	15.08E	44	9	205	3	068
Cedynia (Poland)	53 N	14.20E	18	1	056	1	056
Poznań; Kobylepole; Plewiska; Sieroslaw; Zakrzewo	52.25N	16.53E	474	115	243	5	011
Bierutowice, Karkonosze (Poland)	50.30N	16.30E	40	4	100	-	---
Wilczowice Stare (Poland)	52.05N	19.10E	44	2	045	1	023
Jasień (Poland)	51.30N	19.40E	58	10	172	-	---
Lubiaszow (Poland)	51.10N	19.20E	61	8	131	-	---
Krynica (Poland)	49.25N	20.56E	47	10	213	1	021
Żegiestow (Poland)	49.39N	20.40E	250	31	124	6	024
Suwałki (Poland)	54.05N	22.55E	229	61	266	9	039
Straubing, Bavaria (Germany)	48.53N	12.53E	20	1	050	3	150
Hohenheim (Germany)	48.43N	9.15E	40	4	100	-	---
Giessen (Germany) ((Veget.))	50.36N	8.42E	132	38	288	14	212
Rheims (France)	49.30N	4.00E	40	16	400	--	---
Lochrist, Le Conquet (France)	48.10N	4.45W	20	1	050	--	---
Crozon; Pentrez Plage (France)	48.12N	4.20W	122	22	180	--	---
Rouen; Isneauville (France)	49.32N	1.02E	128	1	008	49	383
Yvetot (France)	49.37N	0.45E	30	1	033	--	---
Chamffleuri (France)	48.50N	2.40E	20	1	050	--	---
Houdan (France)	48.48N	1.36E	40	10	250	--	---
Bernay (France)	48.05N	0.04W	20	1	050	--	---
Fougères (France)	48.21N	1.12W	119	18	151	--	---
Tréogat (France)	47.55N	4.15W	39	0	---	1	026
Cordemais (France)	47.18N	1.53W	40	2	050	--	---

Appendix III(b) continued

	Lat.	Long.	Total	0	0/N	Y	Y/N
Chantonnay (France)	46.41N	1.03W	29	8	276	1	034
S.André de Lancize (France)	44.15N	3.45E	33	2	061	6	182
(NW of) Montpellier (France)	43.35N	3.40E	50	-	---	10	200
Aigues Mortes (France)	49.32N	4.05E	39	-	---	20	513
(NW of) Cavaillon (France)	43.52N	4.59E	30	1	033	3	100
Belmonte - Guarda (Portugal)	40.20N	7.20W	148	28	189	38	257
Ramada (Portugal)	38.50N	9.20W	194	--	---	4	021
Estremoz - Redondo (Portugal)	38.44N	7.43W	19	3	158	-	---
Castello de Vide (Portugal)	39.26N	7.26W	28	1	036	1	036
Pocinho (Portugal)	41.08N	7.07W	18	1	056	-	---
Outerio; Milhao; Grandais; Vinhaes; Vinhaes - Chaves; Vinhaes - Vilar d'Ossos (Portugal)	41.49N	7.02W	153	23	150	2	013
La Coruña; Arteijo; Carballo (Spain)	43.16N	8.29W	164	2	012	11	067
Guitiriz; Baamonde (Spain)	43.10N	7.49W	377	47	125	--	---
Baralla; Vega de Valcarce; Villafranca del Bierzo; Toreno; Paramo del Sil; Folgoso de la Ribeira; Astorga (Spain)	42.40N	6.30W	463	34	073	22	048
Leon - Oviedo (Spain)	42.56N	5.41W	218	23	106	1	005
Ribadesella; Cangas de Onis; Colunga (Spain)	43.26N	5.08W	200	6	030	11	055
Oseja de Sajambre; Cangas de Onis - Riano (Spain)	43.11N	5.03W	159	26	164	8	050
Mediano (Spain)	42.21N	0.10W	38	2	053	-	---
Vega del Rio Gallego; Aula Dei, Zaragoza (Spain)	41.45N	0.50W	56	-	---	12	214
Pamplona; Larrasoana; Aoiz (Spain)	42.51N	1.32W	282	13	046	26	092
Erro; Roncesgalles (Spain)	42.57N	1.26W	76	2	026	12	158
Bailo (Spain)	42.31N	0.48W	86	7	081	--	---
Sierra Guadarrama (Spain)	41 N	4 W	74	27	365	1	014
Muelas del Pan - Fonfria; Fonfria - Alsanices (Spain)	41.37N	6.08W	108	66	611	3	028

Appendix III(b) continued

	Lat.	Long.	Total	O	O/N	Y	Y/N
Marin; Cangas; Meano; Armentera; Viascon (Spain)	42.25N	8.42W	284	27	095	1	004
Pontevedra - Lalin; Lalin; Silleda; Monte Pedroso, Santiago; Enfesta (Spain)	42.46N	8.17W	338	46	136	12	036
(South of) Marrakesh (Morocco)	31.13N	8.00W	72	--	---	20	278
Le Pont; Changins; La Frétaz; Les Allieres (Switzerland)	46.40N	6.40E	550	76	138	31	056
Orsieres; Bluche; : Alpage Frid (Switzerland)	46.06N	7.25E	346	46	133	3	009
Zürich; Stein-am-Rhein (Switz.)	47.23N	8.33E	66	14	212	5	076
Schaanwald, Liechtenstein	47.11N	9.31E	40	--	---	-	---
Ardez (Switzerland)	46.47N	10.13E	28	9	321	-	---
Gruppo Bernino, Valmalenco (Italy)	46.20N	9.50E	42	24	571	1	024
Sondrio (Italy)	46.11N	9.52E	256	27	105	3	012
Pisa (Italy)	43.43N	10.24E	50	6	120	-	---
Sicily	37 N	15 E	38	38	1000	-	---
Gröbming (Austria)	47.28N	13.55E	134	11	082	-	---
Gumpenstein (Austria)	47.29N	14.56E	255	44	173	-	---
Michelbach; Rupprechtshafen (Austria)	48.10N	15.50E	66	13	197	-	---
Fajtacyujtmeny, Martonvasor (Hungary)	47.15N	18.45E	40	21	525	-	---
Kutina (Yugoslavia)	45.28N	16.47E	119	--	---	-	---
Zagreb; Maksimir (Yugoslavia)	45.49N	16.00E	133	14	105	3	023
Beltinci (Yugoslavia)	46.30N	16.15E	48	7	146	-	---
Bokanjac Lac, Zadar (Yugoslavia)	44.07N	15.13E	31	-	---	-	---
Mostar (Yugoslavia)	43.20N	17.49E	116	1	009	-	---
Skopje (Yugoslavia)	42.00N	21.28E	109	-	---	-	---
Kerasovon (Greece)	40.20N	20.45E	128	-	---	-	---
Koutsoufliana (Greece)	39.45N	21.10E	127	-	---	38	297
Yannina (Greece)	39.40N	20.51E	40	1	025	6	047
Er Kjupria, Rhodopus Mts. (Bulgaria)	42 N	24 E	59	2	034	-	---

Appendix III(b) continued

	Total	O	O/N	Y	Y/N
*NE England, SE Scotland ()	2302	835	363	18	008
*Yorkshire	701	162	231	11	016
Great House, Rossendale, Lancs.	31	2	065	--	---
*North Wales (West) ()	600	144	240	6	010
*Llangollen etc. ()	550	182	331	6	011
Aberystwyth; Clarach	170	38	224	-	---
South Wales (+)	699	97	139	5	007
*North Midlands ()	1065	209	196	12	011
Holkham, Norfolk	62	9	145	3	048
*Oxon. and Berks ()	2575	723	281	21	008
Rothamsted; Creslow, Bucks.	272	11	040	1	004
*Sussex and Surrey	1683	298	177	41	024
Romney Marsh	79	5	063	--	---
St. Mary's, Scilly Isles	40	-	---	2	050
Thurles	268	55	205	1	004
Waterford	208	45	216	-	---
Tallow; Ardmore; Mallow; Fermoy	337	53	157	-	---
Clonakilty; Bantry	141	16	113	1	007

(British Isles : * : Majority of samples scored at site
(+) Some of samples scored at site
() Some of samples not scored at site.

Appendix III(c)

Frequency Data used for Larger Scale Maps of Whole Range (Figures VIII (xv, xvi))

Data as for Countries, see Appendix II, with the following exceptions:

	Total	0	O/N	Y	Y/N
N. Norway (Vågønes)	312	60	192	1	003
S. Norway (Rogaland)	40	16	400	-	---
(Norway: omitted)	(9)	(6)		(-)	---
N.E. Poland (Suwałki)	229	61	266	9	039
N.W. Poland	731	182	249	30	041
S. Poland	502	65	129	8	016
N. France	258	28	109	49	190
N.W. France	389	52	134	2	005
S.E. France	162	5	031	39	241
(France: omitted)	(258)	(24)		(25)	
N.E. Spain	538	24	045	50	093
Central Spain (S. Guaderrama)	74	27	365	1	014
N.W. Spain	992	88	089	42	042
Extreme N.W. Spain	1211	123	102	24	020
(Spain: omitted)	(417)	(31)		(38)	---
N. Portugal, Central N.W. Spain	279	90	323	5	018
S. Portugal	389	32	082	45	116
N. Britain (greenhouse-scored only)	240	34	142	3	013
S. Britain (greenhouse-scored only)	1278	141	110	12	009

Appendix III(d)

Frequency Data used in Smaller Scale Maps of Whole Range: Figures VIII (xvii-xxii)

	Total	Y	B+M	Ba	F	H	LL	Y	B+M	Ba	F	H	LL (corr.)	Est. No. of $\underline{V}^1\underline{V}^1$
Britain	11211	127	-	76	28		115	011	---	008	003		040	2908.83
(scoring for 'H')	1641					41						025		
Ireland	1021	2	2	3	2	16	28	002	002	003	002	016	078	357.64
N. France	895	76	9	6	7	48	34	085	010	007	008	054	099	344.93
S. Fr., NE. Sp.	710	89	7	4	-	23	77	125	010	006	---	032	229	335.78
NW. Sp., Portugal	3362	155	1	14	80	43	207	046	0003	004	024	013	148	1401.86
Morocco	161	22	-	--	--	4	2	137	---	---	---	025	030	66.14
Sicily	38	--	-	--	--	-	-	---	---	----	----	---	---	---
Italy (ex. A., S.)	438	14	2	1	--	2	5	032	005	002	---	005	026	195.86
Switz., It. Alps	1642	44	114	20	17	56	74	027	069	012	010	034	135	547.47
Neth., Belg., Germ.	1148	38	6	8	7	10	20	033	005	007	006	009	059	336.49
Den., Swed., S.Nor.	1748	37	29	4	21	47	139	021	017	002	012	027	239	580.46
Finland	282	10	--	-	3	8	6	135	---	---	011	028	063	94.86
N. Norway	321	1	--	12	27	11	6	003	---	037	034	034	070	85.67
Iceland	30	-	--	--	5	--	-	---	---	---	167	---	---	2.57
Russia	86	-	--	9	6	--	-	---	---	105	070	---	---	10.14
Poland	1462	47	3	8	43	16	62	032	002	005	029	021	163	380.29
Austria, Hungary	706	4	1	1	6	8	29	006	0014	0014	008	011	115	252.60
Yugoslavia	1545	38	80	12	41	32	76	025	052	008	027	021	101	755.35
Greece	1031	59	44	1	28	14	60	057	043	001	027	014	091	659.45
Bulgaria	59	--	--	-	6	--	14	---	---	---	102	---	512	27.37
Turkey	713	42	10	2	5	13	20	037	014	003	007	018	060	331.60
Israel, Lebanon	535	3	--	-	-	12	22	006	---	---	---	022	046	474.33
Iran	723	27	10	2	5	13	20	037	014	003	007	018	060	331.60
Afghanistan	428	42	5	-	1	--	3	098	012	---	002	---	041	72.78
West Pakistan	249	3	2	-	1	2	1	012	008	---	004	008	011	94.59

Regions on fringe of range: Mapped as broken circle. _Trifolium_ _repens_ probably present only by introduction.

	Total	Y	B+M	Ba	F	H	LL	Y	B+M	Ba	F	H	LL (corr.)	Est. No. of $\underline{V}^1\underline{V}^1$
Azores	106	-	1	-	-	-	2	---	009	---	---	---	034	58.53
Ethiopia	39	1	-	-	-	2	1	026	---	---	---	051	077	12.95

The last column shows the divisor for the (corrected) LL frequency figures, that is, the number of plants calculated to possess two alleles in the $\underline{V}^1\underline{V}^h$ group, calculated as the sum of $X(1-\sqrt{LL/X})^2$ for each sample.