THE VARIATION OF SOME LEAF PHENOLICS AND ISOZYMES IN POPULATIONS OF BRITISH SPECIES OF *LIMONIUM*, AND ITS RELATION TO THE BREEDING SYSTEM.

BY

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NOTE ON AUTHORITIES

Authorities for British species are as given in Clapham et al. (1962) and are not quoted. Other species referred to from other research are given the names which the authors used in that work. In the few cases where no authorities were given for species, no attempt has been made to name them.

ABSTRACT

The three species studied were L. vulgare Mill., L. humile Mill. and L. binervosum (G.E.Sm.) C.E. Salmon. They are self-incompatible, self-compatible and apomictic respectively. Using leaves sampled directly from natural populations of all three species, alcoholic extracts of leaf phenolics (mainly flavonoids) were studied by thin-layer chromatography. Population samples of L. binervosum were also chromatographed after cultivation. No attempt was made at chemical identification of the phenolics. The leaf enzymes esterase, leucine aminopeptidase and 6-phosphogluconate dehydrogenase of L. vulgare and L. humile only were studied by horizontal starch gel electrophoresis. Leaf enzymes of L. binervosum could not be detected, despite attempts using a variety of extraction techniques. Leaf morphology measurements were also taken from some population samples, particularly cultivated L. binervosum.

During a detailed consideration of the anticipated relationships between variation and the breeding system, a hypothesis particularly attributable to H.G. Baker was described. Stated simply, this was that variation would be found within populations of an outbreeding species, and that this variation would reduce distinctions between such populations. Conversely, for an inbreeding species there would be little variation within populations, but variation between these populations would be high. An apomictic species would show a similar pattern of variation to that of an inbreeding species.

An attempt was made to relate the observed biochemical variation within and between populations of the three species to this general hypothesis. It was found that the variation detected in some ways corresponded, and in other ways conflicted, with expectations. In the case of the chromatographic results, variation within populations conformed to the expected pattern, there being generally most variation in L.vulgare and least in L.binervosum populations, although some populations showed exceptions, and there was some

overlap between species. However, chromatographic variation between populations was mainly the reverse of expectation, there being more variation between L. vulgare populations than L. humile, and the L. binervosum samples showing similar levels of interpopulation variation to L. vulgare.

These departures from expectations could be related to environmental influences on the production of leaf phenolics in wild material, and this was supported by the results from L. binervosum material cultivated in a uniform environment. Further, these cultivated samples showed a strong relationship between the breeding system and chromatographic variation within and between populations, and in addition this variation was related to the geographical origin of the populations and to leaf morphology.

The results from electrophoresis showed isozyme variation within L. vulgare populations, but little significant variation between them, but for L. humile the results were not as anticipated, there being a total lack of isozyme variation both within and between populations. The most likely reason for this was considered to be the action of the founder effect on populations established by self-fertilisation and long-distance dispersal. If this was the case then there should also have been an absence of chromatographic variation between L. humile populations, but some variation existed, although probably caused by environmental factors.

It was also concluded that only under uniform growth conditions could chromatography of leaf phenolics be used as a reliable indicator of any genetic variation that may be present, but as has been shown by many other workers, electrophoresis of isozymes is a valuable tool for studying population and evolutionary genetics.

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CHAPTER 1.

THE INFLUENCE OF THE BREEDING SYSTEM ON POPULATION STRUCTURE AND EVOLUTION.

1. 1. INTRODUCTION

The synthesis of Mendelian principles and Darwinian thought, combined with deductions from the Hardy-Weinberg law, brought about a rapid development of the study of theoretical population genetics in the 1920's and 1930's. One of the main aims of this study was to identify the factors that influence the amount of genetic variation found in populations. This problem is still the subject of much discussion, e.g. Lewontin (1967). A recent attempt to classify these factors was made by Mayr (1970) pp.130-1. The significance of understanding them lies not only in the interpretation of the existing genetic structure of a population, but also in assessing its evolutionary potential.

It became clear from calculations that an important force moulding population structure was the breeding system. This term has been defined by Darlington & Mather (1949) as:

> "The habit of mating within the group" (p.237) and "In particular populations or mating groups of plants and animals there will be an average degree of hybridity. This will follow from the average relationship of the parents. And this in turn will depend on a variety of conditions of which the most obvious and most important is the relative frequency of self- and cross-fertilisation. This habit is the breeding system of the population, or group, or species" (pp.239-240).

The breeding system is seen as just one interacting component of the whole "genetic system" of a species (Darlington 1939,1971).

A distinction needs to be made between the two pairs of terms 'inbreeding and outbreeding' and 'self-fertilising and cross-fertilising'. The term inbreeding is applied when

> "the individuals which mate are more closely related to each other than are random members of an infinitely large population" (Allard, Jain and Workman 1968).

The most extreme form of inbreeding, principally available to plant species,

can be achieved by self-fertilisation, although this ability does not guarantee inbreeding as it does not always preclude cross-fertilisation. Degrees of inbreeding can also be classified formally by terms such as 'sib' or 'cousin' mating, but matings in natural populations do not usually follow regular patterns. Outbreeding requires that the individuals of a population mate at random, one of the conditions under which the Hardy-Weinberg law operates, but cross-fertilisation does not necessarily create outbreeding if the individuals are genetically related. The range of breeding systems possible in plant species was summarised by Baker (1959a); for animals see Mayr (1970) pp. 238-242.

There has also been much discussion about the often-used but rarely defined word 'population'. One definition useful here because it does not presuppose a given breeding system is:

> "The community of potentially interbreeding individuals at a given locality" (Mayr 1970 p.82).

1. 2. BREEDING SYSTEMS AND POPULATION STRUCTURE

In the Nineteenth Century it had been observed that inbreeding had harmful effects on individuals, sometimes expressed as the 'Darwin-Knight Law' and 'Inbreeding Depression'. An early demonstration of the uniformity of a self-fertilising species was made by Johannsen who in 1909 produced experimental results which showed that selection in pure lines of the inbreeding Phaseolus vulgaris was ineffective (Briggs and Walters 1969 pp.61-63). Theoretical work, predicting the genetic consequences to whole populations when they adopted different degrees of inbreeding, was described by Jennings (1916) and Wright (1921). From the latter came the conclusion that continued inbreeding without selection in a population yielded an array of pure homozygous lines. The most rapid approach to this state would be through the most extreme form of inbreeding, self-fertilisation. In contrast, in a large population with random breeding, many of its members would remain genetically heterozygous, as the Hardy-Weinberg law had already shown. More complex intermediates between extreme inbreeding and outbreeding, and involving selection, were also

investigated, later reviewed by Wright (1951).

The various predictions from mathematical models were restated and then taken a step further by, for example, Wright (1931) and Haldane (1932) in significant works where the evolutionary possibilities of different breeding systems were described. However, there still remained scope for more considerations of population structure and breeding system on an empirical basis. These were made by Baker (1953a, 1959a). In the form of a table (see Table 1.1.) he described the anticipated structure of a natural population colonising a new environment under different breeding systems and selection pressures. The essential features of this table were that within obligately outbreeding populations there would be considerable variation, while in habitually inbreeding populations there would be relatively little. Certain shortcomings were indicated, such as the assumption that no further mutation took place, and that the characters were polygenically controlled.

Baker also described the nature of the differences expected to occur between populations:

> "It is to be expected that the relatively continuous morphological variation within a population of outbreeders will tend to obscure the clear-cut distinction between two populations. This will be the case particularly when some of the morphological characters are not directly connected with ecologically significant variation by linkage or pleiotropy.... At the other extreme, where the variation is rendered discontinuous by nearly complete inbreeding, or by apomixis or vegetative reproduction, a stable pair of populations, if they are not identical, will tend to be relatively clearly distinguishable." (Baker 1953a).

He also considered how the various types of breeding system might interact differently with ecological or climatic factors over a large area. In an outbreeder distributed along a climatic or ecological gradient, its continuous variation would create a cline, making the recognition of ecological races difficult. On the other hand, the discontinuity generated by inbreeding would aid the circumscription of discrete ecotypes. Other factors were mentioned

TABLE 1. 1.

THE PREDICTED APPEARANCE OF COLONISING POPULATIONS UNDER DIFFERENT BREEDING SYSTEMS AND SELECTION PRESSURES. TABLE FROM BAKER (1953a).

		Obligate outbreeding	Facultative apomixis (Outbreeding when sexual)	Habitual inbreeding	Obligate apomixis or Vegetative reproduction
Very little selection pressure in new environment	Biotypes	Many	Moderate number	Few	Very few or one
	Potential biotypes	Very many	Many	Moderate number	-
	Appearance of population	A vast number of character combinations (some more common because of linkage)	A considerable mixture of rather definite character combinations	A moderate mixture of definite character combinations	Probably a single character combination
Moderate selection pressure in new environment	Biotypes	Moderate number	Few	Very few	One or none
	Potential biotypes	Many	Moderate number	Few	-
	Appearance of population	A considerable mixture of rather definite character combinations	A moderate mixture of definite character combinations	A relatively uniform population	A uniform population if present at all
Severe selection pressure in new environment	Biotypes	Few	Very few	One or none	One or none
	Potential biotypes	Moderate number	Few	Very few	-
	Appearance of population	A mixture of very few definite character combinations	A relatively uniform population	A uniform population if present at all	A uniform population if present at all
		Possibility of gene flow from neighbouring populations	Slight possibility of gene flow from neighbouring populations	No possibility of gene flow from neighbouring populations	No possibility of gene flow from neighbouring populations

which might interact with the pattern of variation, for example population size, pollination mechanism, and climatic, edaphic or biotic discontinuities. It was made clear that the propositions represented only a simplified abstract of what might be expected in nature.

The reproductive method of apomixis remains to be considered. Different population structures might result, depending on whether it is obligate or facultative, as Table 1.1. shows. In obligate apomixis no genetic variation would be expected either within or between populations, as all individuals will be genetically identical (barring mutation) as they have been cloned by an asexual process (Heslop-Harrison 1953). However, in contrast with the homozygosity expected from inbreeding, in many obligate apomicts their individuals would be highly heterozygous if the species has been created by interspecific hybridisation, as is often the case (Fryxell 1959). Further, Turrill (1949) suggests that the increase in heterozygosity bestows upon such apomicts a greater opportunity for phenotypic plasticity, and Thoday (1953) supports this view for heterozygotes in general. Turning to facultatively apomictic species, variation within populations (if it occurs) would be discrete rather than continuous, and marked distinctions might be expected between populations as a result of an occasional burst of segregation followed by selection for the genotype most fitted to a new habitat.

The above account of population variation and the breeding system is in the form of an hypothesis; evidence is required to support it. What is needed is a comparison of morphological variation within and between populations of closely related species which, however, adopt different breeding systems. Baker had little such evidence to call upon, but was able to provide some from his own work. He raised cultures from seed collected from wild populations of subspecies of Armeria maritima Willd. (Plumbaginaceae), namely A. maritima ssp.maritima (A.m. var. typica Lawr.), being dimorphic, self-incompatible and therefore outbreeding, and A. maritima ssp. californica (A.m. var. californica

Lawr.) and A. maritima ssp. sibirica (including A.m. var labradorica Lawr. and A.m. var sibirica Lawr.) both being monomorphic, self-compatible, and having a greater potential for inbreeding. These cultures showed the general trend that the dimorphic subspecies were much more variable than the monomorphic subspecies within them with respect to several morphological characters such as petal colour, ciliation of leaf margin, scape height and leaf index. For various reasons, some other characters did not show more variation within cultures of the outbreeders than of the inbreeders, but in the majority of cases they were significantly so; in no case did a self-compatible culture show greater variation than the outbreeder.

The Armeria cultures also showed a different pattern of interpopulation variation which could be related to the breeding system. In ssp. californica cultures were quite sharply differentiated by habit features and flowering date, but in ssp. maritima while there were some general differences between cultures there were usually some plants which could be transposed from one culture to another without appearing to be out of place.

The breeding systems of the different subspecies also corresponded to their ecological diversity; ssp. maritima showed a wide range of ecological preferences quite unknown in the monomorphic forms. It was also possible to distinguish climatic races in the monomorphic subspecies that were not evident in the widely distributed ssp. maritima.

A similar general relationship between variation and the breeding system was obtained in a much less comprehensive study of Limonium species (Plumbaginaceae) in the same paper. In addition, this genus contains apomictic species, and for morphological and chromosomal features they showed little variation within, but much variation between, their populations. Some of this data is provided later (Ch. 2, Table 2.2).

Further support for Baker's propositions has been provided by more recent studies. Rogers (1971) took measurements of ripe capsules of several

population samples of Papaver species, including P. rhoeas (self-incompatible) and P. dubium and P. lecoqii (both self-compatible). The pattern of variation within and between populations corresponded closely with the breeding system. By progeny testing, Rogers was also able to estimate the degree of inbreeding within the different populations, and relate this to their particular levels of variation. He also suggested that the observed ecological success of P. rhoeas compared with its self-compatible relatives was a result of its genetic heterozygosity, a similar observation to that of the outbreeding Armeria subspecies.

Using a very different technique, the electrophoresis of isozymes, Solbrig (1972) studied ecologically and morphologically similar species of Leavenworthia (Cruciferae). There was less variation of isozymic bands within the self-compatible populations than in the self-incompatible, while differences between populations were greater for the self-compatible than the self-incompatible. Another electrophoresis study, on animal species, by Selander and Kaufman (1973), compared colonising populations of the self-fertilising snail Ruminia decollata with those of the cross-fertilising Helix aspera. Although electrophoretic variation was observed within H. aspera populations, there was no variation within or between R. decollata populations. This latter observation was explained by suggesting that only a few individuals initiated the colonisation, and the subsequent spread by self-fertilisation rapidly led to homozygosity. Further details of these two electrophoresis studies are given in Chapter 5.1. They are of particular interest in relation to other work because they measure biochemical rather than morphological characters; these characters are under the control of one or two loci rather than many, and yet they yielded similar conclusions.

The above studies have been referred to because they provide supporting evidence for the hypothesis developed particularly by H.G.Baker. Inevitably this summary generalises the results and neglects some points of finer detail.

However it has become evident that more detailed consideration has to be given to the difference between complete inbreeding and 'heavy' inbreeding (usually one to ten per cent outcrossing). Work has shown that this apparently slight difference in breeding behaviour may have very different consequences to population structure. Baker himself (1953a) acknowledged this difference to some extent in his table and by reference to work by Harlan 1945). This study was of the facultatively cleistogamous Bromus carinatus complex and revealed swarms of local morphological races with few intermediates and little evidence of interbreeding. Tests on progenies of individual plants showed little segregation, indicating high homozygosity for individual members of the population; but differences between the progenies indicated that each race was not genetically uniform. It was suggested that cleistogamy interrupted by occasional outcrossing by a few chasmogamous panicles produced heterozygous hybrids which then produced new uniform genotypes by inbreeding.

An essentially similar population structure was described for the cleistogamous Festuca microstachys complex by Kannenberg and Allard (1967). Outcrossing was extremely rare (probably lower than one per 10,000 fertilisations) yet considerable genetic variation was present within and between populations; again each population consisted of a large number of different homozygous genotypes. A possible reason for this variation was that each population had evolved to produce a cohesive and harmoniously balanced collection of genotypes particularly suited to its local environment.

If the level of outcrossing is slightly greater, it seems that yet another pattern of variation emerges. Jain and Allard (1960) and Allard and Jain (1962) studying barly populations (Composite Cross V), estimated that this species outcrossed by only two per cent or so, yet after 18 generations of inbreeding to this degree the proportion of heterozygotes for some of a number of marker loci failed to decrease at the theoretically predicted rate.

It seemed that there was selection strongly favouring heterozygotes (heterozygote advantage) maintaining this variation. Another species, the lima bean, was studied by Allard and Workman (1963). Synthetic populations were found to adopt a minimum of 95 per cent inbreeding during eleven generations, reaching equilibrium with an again unexpectedly high number of heterozygotes.

Factors which encouraged heterozygosity despite inbreeding were discussed by Imam and Allard (1965) studying wild oats (Avena fatua L.). They demonstrated variation between and within populations of this predominantly inbreeding species (one to ten per cent outcrossing), and accounted for it by small-scale variability in the environment encouraging the survival of many different genotypes, each particularly suited to a certain'microniche' of the habitat. Tests indicated that many, if not all, individuals were still heterozygous at numerous loci despite this specific adaption. The overall variation within these wild oat populations was similar to that of the natural Festuca microstachys populations although the latter species showed a lower level of outcrossing.

The conclusions of studies on predominantly inbreeding species were summarised in a review by Allard et al.(1968). In their closing comments they say:

> "There is remarkable genetic diversity within natural and domestic populations of inbreeding species ..... any given population contains individuals of many different genotypes ..... and individuals are frequently heterozygous at many loci ..... Another feature is genetic differentiation between different populations. Clinal variation is frequently observed in association with factors of the physical environment. Superimposed on this is a patchwork or mosaic pattern of variation which reflects adaptation to the local environment .... It is apparent that population structure in inbreeding species is much more complicated than has been commonly supposed and that it probably does not take the same form in all inbreeding species or even in different populations of the same species. Population structure in

inbreeding species cannot be explained by focusing attention on any single factor among the complex of interacting genetic and ecological factors that are involved."

Evidence of other kinds and from other sources has been produced which tends to support this view of genetic variation in predominantly inbreeding species. For example Marshall and Allard (1970a, 1970b) studied natural populations of Avena barbaba (Brot.) by electrophoresis of isozymes, demonstrating that these inbreeding populations contain large amounts of biochemical genetic variation. In a morphological study of Arabidopsis thaliana by Jones (1971a, 1971b) the situation was approached from the opposite direction in that the pattern of variation in this presumed inbreeder could only be explained by invoking a slight amount of outcrossing with the genetic and ecological consequences described by Allard and others. Hayward and Jackson (1971) reported a similar state of affairs for Lolium temulentum L., that of ecologically influenced intrapopulational variation in an inbreeding species. Finally, a comparison by Hillel et al. (1973) of Triticum longissimum (predominantly inbreeding) and T. speltoides (predominantly outbreeding) showed that for many morphological characters there was greater variation both within and between populations of the inbreeder than of the outbreeder. Although the pattern of variation of the inbreeding species could be explained, the authors were unable to account for the unexpected result for the outbreeding T. speltoides.

These studies add further substance to the already well-documented theory of Allard's concerning the structure and variability of inbreeding populations, indicating that even predominantly inbreeding populations are genetically variable. Yet there has been equally persuasive evidence presented to support the claim that inbreeders are less variable within their populations than outbreeders, and more variable between their populations. This gives the impression that there are two opposing schools of thought on

the subject of population structure and the breeding system. However, I feel that there is not such a great conflict of opinion, for two reasons in particular.

Firstly, investigators of the inbreeding populations have often used for their material synthetic populations of crop plants in cultivation, or wild populations of grasses. Such populations are usually characterised by dense stands, high reproductive capacity, and may be wind pollinated when outcrossed. All these factors will affect the pattern of gene flow within and between populations and are not the same as those encountered by the other inbreeding species described, which may tend to be more sparsely distributed, or produce fewer seeds, or be insect pollinated. Allard himself (Allard 1965) referring to his own work on inbreeders is aware that his observations are quite different from those described for some other species. He suggests that this is because the species he has studied have been ecologically more successful than those of other workers, his criteria for success being abundance, world-wide distribution, diversity of habitat and colonising ability. By at least some of these criteria, inbreeding species of Armeria, Limonium and Papaver are not so ecologically successful as the inbreeding grasses, perhaps for the very reason that they are not so heterozygous.

The other major distinction between the different sets of conclusions is one of approach. Baker suggested an overall principle, while Allard was concerned mainly with inbreeding populations alone. The latter studied variation in several characters through a series of generations and compared it with computer predictions, while the former investigated characters at a fixed point in time, but provided information from a wider sample of populations and species. Hillel et al. (1973) in referring to studies on inbreeding species alone stated that:

> "These studies do not and cannot examine the direct effect of the mating system on genetic variations. We believe that such an

evaluation can be done by comparing two closely related species which differ in their mating system. Such pairs are not common."

I would further add that this close relationship should be ecological as well as genetic.

That the two approaches are not really at conflict is indicated by the fact that Rogers (1971) in his Papaver study was able to call upon both to interpret his data. However it seems very necessary in studies of variation to establish whether a population is completely or predominantly inbreeding, and to make some observations on its ecological success.

1. 3. THE EVOLUTIONARY POTENTIAL OF SPECIES ADOPTING DIFFERENT BREEDING SYSTEMS

The aim of analysing population structure and breeding system is not simply one of description; the genetic structure of a population will also be significant in determining its evolutionary potential. The influence of breeding system on this was realised early on, for example by Wright (1931) and Haldane (1932). An outbreeding population was considered to have greater evolutionary advantage than an inbreeder because although the former contained genes which were disadvantageous in existing circumstances, in a changing environment the population had the 'elasticity' to produce new gene combinations necessary for survival. An inbreeding population lacked the necessary heterozygosity and would perish.

This hypothesis was further developed by Mather (1943) who saw populations as being placed in a situation of conflict between adopting a state of high 'fitness' for a present environment or 'flexibility' necessary for the spread to new environments or for the adaption to changing ones. An inbreeding population would be at an evolutionary disadvantage because although it would possess fitness for its present environment, in the face of a changing environment it would be doomed to extinction because of its lack of genetic flexibility. An outbreeding population with its store of potential variability would be capable of adapting to change, although lacking in immediate fitness.

Despite this pessimistic view on the evolutionary future of inbreeding species, there are many cases where persistent self-fertilisers have had long-term success, some of which were described by Stebbins (1957). He suggested there were three reasons for this. Firstly, lack of suitable cross-pollination conditions would only permit self-fertilising species to reproduce. Secondly, self-fertilisation would assist long-distance dispersal, because only one individual would be required to found a new colony (originally proposed by Baker (1948)). Finally, it bestows upon the population an ability to build up large numbers of well-adapted individuals quickly, an advantage if new habitats appear. Fryxell (1959) adds a fourth advantage, that self-fertilisation brings about the rapid elimination of deleterious recessive genes by exposing them in the homozygous condition.

These earlier deductions on the evolutionary role of inbreeding were made on the assumption that such populations were uniform genetically, and highly homozygous. It has been indicated that this may not be so for the ecologically more successful inbreeding species, and the unexpectedly high levels of variation within their populations could also provide them with the potential for evolutionary success.

1. 4. THE FLEXIBILITY OF THE BREEDING SYSTEM

The impression may have been created that the breeding system of a population is a fixed characteristic. However, it is capable of short- and long-term change, and can vary from population to population because of factors such as population size, population density, pollen dispersal and seed dispersal. In the short-term, both regular and irregular internal fluctuations can occur for adaptive purposes (Heslop-Harrison 1966). For example, Löve and Dansereau (1959) considered that the pattern of variation and distribution in Xanthium strumarium subspecies is the consequence of alternation between outbreeding and inbreeding. A similar scheme is proposed by Nygren (1951) for Calamagrostis purpurea (Trin.) Trin. and Levin (1968) for

Lithospermum caroliniense (Walt.) MacMill. Compensatory adjustments in breeding system to regulate population structure are also an important consideration of Allard et al.(1968). It is not difficult to give a general explanation for the mechanisms causing such changes in the breeding system. The breeding system itself is a genetically controlled and adaptively adjusted character,and can respond to changes in circumstances which favour a different degree of outbreeding (Mather 1973). In the long-term, permanent and irreversible changes in the breeding system appear to be evolutionarily important to the process of speciation (Stebbins 1957, Baker 1959a). It is almost always the case that inbreeding is superimposed on outbreeding rather than the reverse (Baker 1959a 1959b 1966).

1. 5. CONCLUSIONS

If a general conclusion can be drawn from all the works cited, it is that every species and even every population is a special case, having unique properties reproductively and ecologically. In assessing the results obtained, the various authors have had to make qualifying statements to fit individual requirements. Therefore it is possible to construct only a very general theory of population structure and evolution in relation to the breeding system, and a very simple task to support or reject the theory with this or that evidence. Undoubtedly the main reason for this is that although the breeding system may be important in influencing population structure, it is by no means the only factor. It is just one aspect of the whole complexity of interaction of genetic and ecological components determining the variation in a species.

1. 6. THE OBJECTIVES OF THIS RESEARCH

In the following chapters I shall attempt to discover whether three species of Limonium, adopting different breeding systems, show variations within and between their populations that can be related to their method of reproduction. The variation measured will be of two groups of chemical substances found in leaves, namely flavonoids and isozymes, studied by the techniques of chromatography and electrophoresis respectively.

CHAPTER 2.

THE BREEDING SYSTEMS OF BRITISH LIMONIUM SPECIES, AND MORPHOLOGICAL VARIATION

2. 1. BREEDING SYSTEMS OF THE THREE SPECIES STUDIED

(a) LIMONIUM VULGARE Mill. (Sea Lavender)

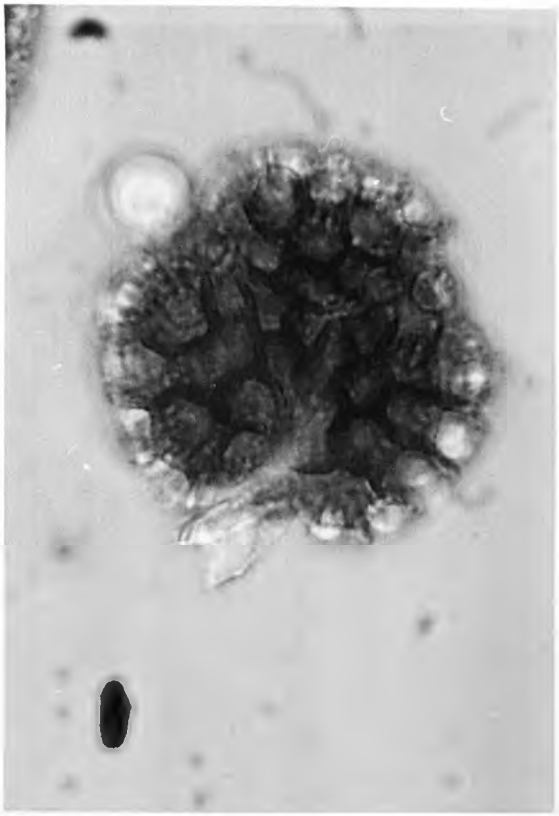
This species is self-incompatible, and shows pollen and stigma dimorphism (Baker 1948). The two forms are named A/cob and B/papillate (B/pap.), the letters referring to a particular structure of the pollen grain (see Plate 2.1 and 2.2) and the words describing the surface features of the stigmas (see Plates 2.3 and 2.4). In addition to the dimorphism, L. vulgare shows heterostyly; in A/cob plants the styles are longer than the stamens, and in B/pap. plants the styles are shorter than or equal to the stamens (Baker 1948).

The dimorphism is thought to be controlled by a supergene containing loci determining the morphological and physiological aspects of the self-incompatibility system (Baker 1966). Type A pollen will not germinate on cob stigmas, nor will B pollen germinate on pap., but A on to pap. and B on to cob will germinate successfully. This germination is shown in Plates 2.3 and 2.4. Several stigmas of both types which had been allowed to self-pollinate were examined, but no germinating pollen was found. This system of self-incompatibility backed up by heterostyly prevents self-fertilisation, and is a strong outbreeding mechanism.

(b) LIMONIUM HUMILE Mill. (Lax-flowered Sea Lavender).

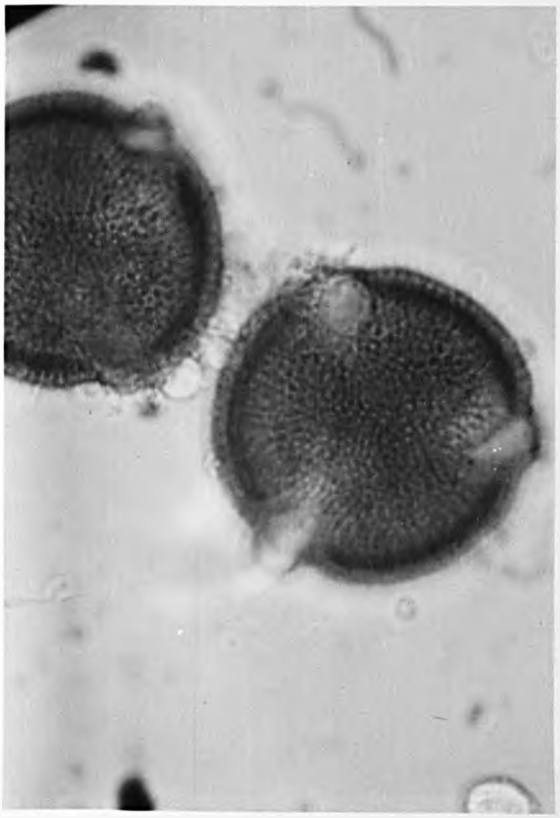
All plants of this species are monomorphic, with the self-compatible combination of A pollen and papillate stigmas (Baker 1953b). This self-compatibility was confirmed by examining stigmas from flowers which had been allowed to self-pollinate. Different plants showed between 56% and 84% of their pollinated flowers having stigmas bearing germinating pollen. The styles are also shorter than the stamens (Baker 1948) and the anthers are protandrous (Boorman 1967); my observations confirm this. The factors of self-compatibility and floral morphology allow L. humile to successfully

PLATE 2.1.



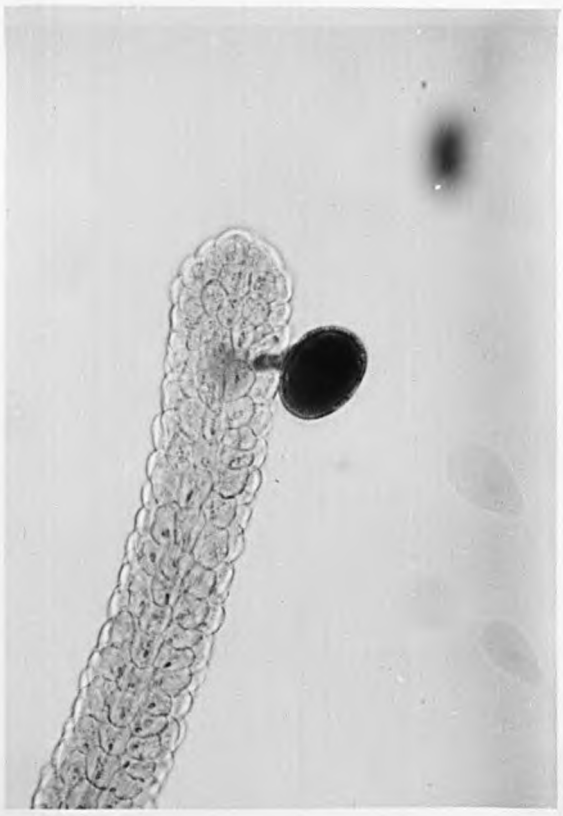
Type A pollen x620. L. humile
Note hexagonal pattern of surface pores.
(L. vulgare and L. humile)

PLATE 2.2.



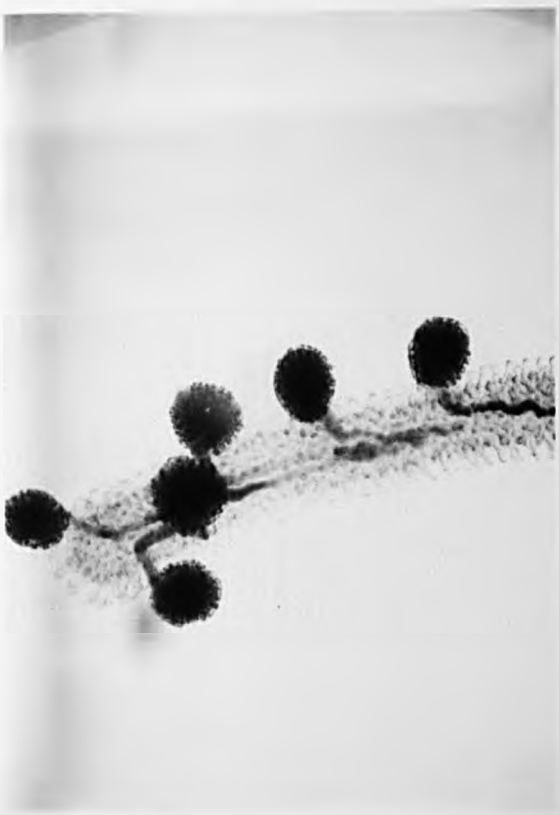
Type B pollen x620. L. vulgare
Note absence of surface pattern.
(L. vulgare only)

PLATE 2.3.



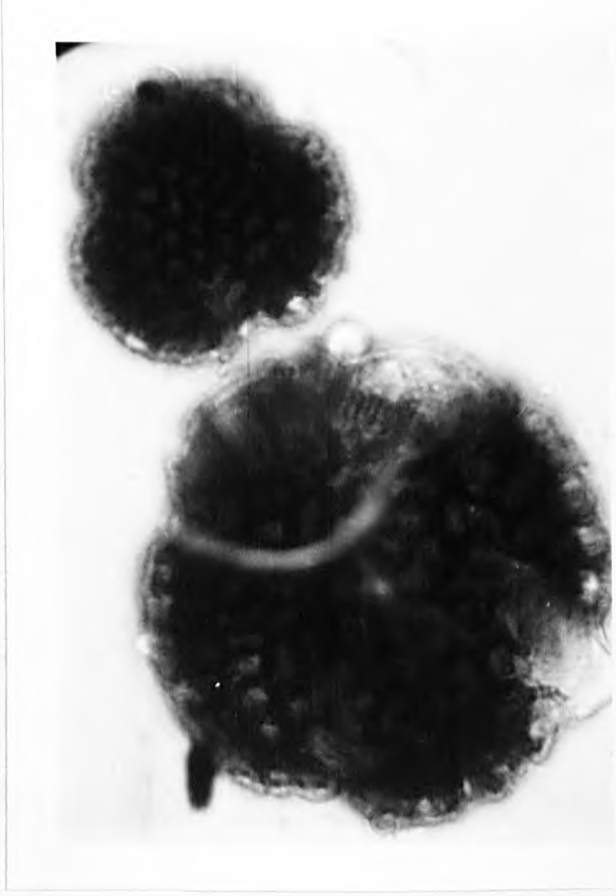
Type B pollen germinating on cob stigma x160. L. vulgare. Note smooth rounded stigmatic cells.
(L. vulgare only)

PLATE 2.4.



Type A pollen germinating on pap. stigma x120. L. vulgare. Note raised points on stigmatic cells.
(L. vulgare and L. humile)

PLATE 2.5.



Type A pollen of L. binervosum x620.
Note irregular size and shape
compared with Plate 2.1.

self-pollinate,making it possible for the species to inbreed.

(c) LIMONIUM BINERVOSUM (G.E.Sm.) C.E. Salmon, sensu lato (Rock Sea Lavender).

Although this species has the self-incompatible A/cob combination, it is apomictic and male-sterile (Baker 1950, 1954a). He demonstrated the apomixis by continuous emasculation. In the present study, pollen examined from several plants was irregular in size and shape (see Plate 2.5), and was not stainable in cotton blue/lactophenol (whilst pollen from the other two species was stainable), an indication of its infertility. Also, stigmas from flowers allowed to self-pollinate did not show any germinating pollen grains. However, there is a suggestion of a residual sexuality in L. binervosum because it is thought to form natural hybrids (De Fraine and Salisbury 1916; Baker 1950, 1953a), but no experimental evidence has been presented to confirm this, and only the first authors appear to have made unsuccessful attempts at producing hybrids, with L. bellidifolium (Gouan) Dum..

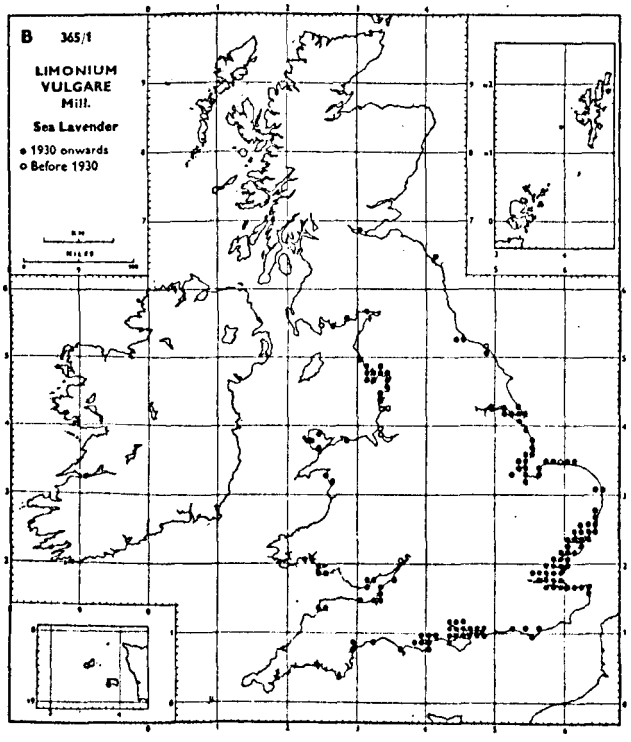
(d) General

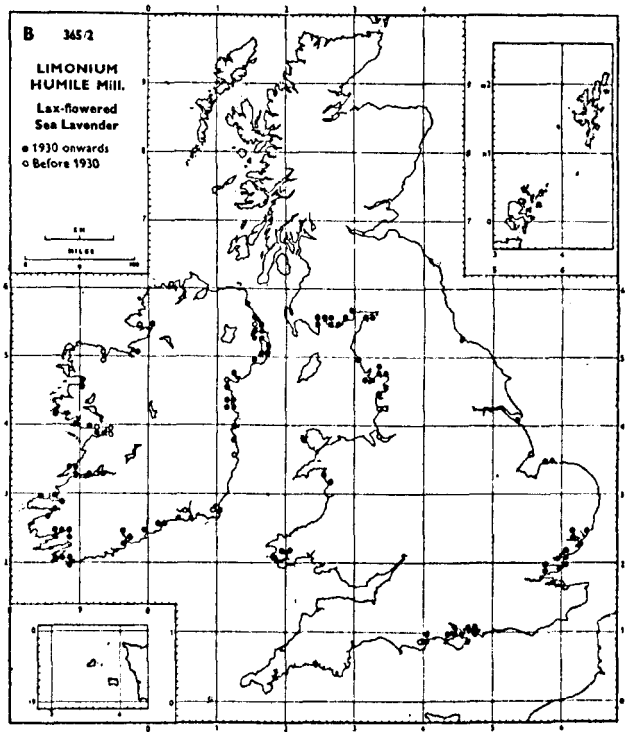
All three species are perennial and show powers of vegetative spread. A single plant of L.vulgare can spread over an area of several metres by horizontal underground shoots (Boorman 1967) but L. humile and L. binervosum can only produce vertical shoots, making their lateral spread much more restricted.

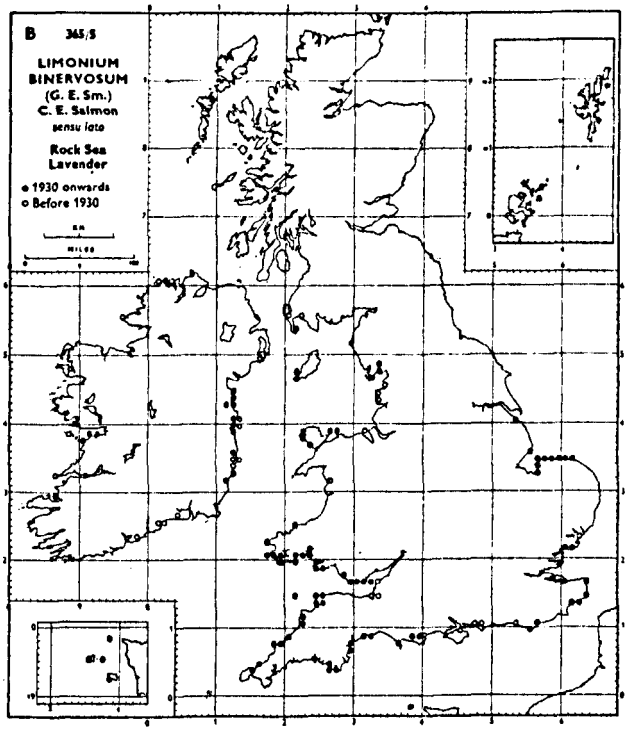
2. 2. DISTRIBUTION AND ECOLOGY

All three species have a coastal distribution; Fig.2.1 shows their British distributions, and Fig. 2.2 indicates their approximate distribution limits. They are all to be found in England, S. Scotland, Wales, and except for L. vulgare, Ireland. The European distribution limits of L. vulgare and L. humile were shown in Boorman (1967). Suggested explanations of the unequal distributions of L.vulgare and L. humile in the British Isles, involving factors of speciation, breeding system and long-distance dispersal, were given by Baker (1953c).

The distributions of LIMONIUM species of the British Isles.







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Taken from Perring F.H. & Walters S.M. (1962) Atlas of the British Flora. Nelson

L. vulgare and L. humile are found in muddy salt marshes, but L. humile is less common. A comprehensive study of their ecology has been made by Boorman (1966, 1967, 1971). Both are normally members of the 'General Salt Marsh' community, but L. humile is also found on the drift line in the West. Both species are sometimes also found in rocky crevices or shingle close to the shore.

Although L. binervosum is a coastal species, its characteristic habitats are stabilised shingle and rocky cliffs, above the high water line. Apart from an early study on L. binervosum at Blakeney Point, Norfolk (De Fraine and Salisbury 1916), little ecological work on this species has been published comparable with Boorman's on the other two. With the exception of a population at the Cefni Estuary, Anglesey (LB2), all populations investigated here were found on the 'type' habitats.

2. 3. DISCUSSION

Each of the three species has been shown to have a different breeding system. The self-incompatible L. vulgare must outbreed, while L. binervosum is apomictic. However, because L. humile is self-compatible, it does not necessarily follow that it is exclusively self-pollinating and therefore an inbreeding species. Cross-pollination is still possible, and Boorman (1967) named several insect visitors to L. humile. A natural hybrid between L. vulgare and L. humile, Limonium x neumanii Salmon has been noted (Baker 1953c; Boorman 1967) and I have observed forms intermediate between the two species, and have been able to produce a few artificially. If natural cross-pollination can occur between the two species, then it is not unreasonable to suppose that it occurs within L. humile.

In comparison with the self-incompatible L. vulgare, L.humile has a surprisingly low seed set. Although my counts on wild and cultivated material show that both species produce about 200 healthy flowers per inflorescence (each flower containing a single ovule), yet Boorman (1967)

stated that both species produced four to nine seeds per inflorescence. My counts approximately confirm this for L. humile. While a low seed set for the self-incompatible L. vulgare can be expected, it would be thought that the closely related self-compatible species L. humile would be capable of producing a higher number of seeds bearing in mind the similarity of the number of flowers per inflorescence. Although precise counts were not made, L. binervosum appears to produce considerably more seeds per inflorescence than the other two species.

It is possible to suggest from the comparatively low seed set and the discrepancy between pollination and pollen germination described earlier (see 2.1(b)) that there may be some partial self-incompatibility in L.humile, but this needs more study. This was prevented by the difficulty of maintaining L. humile under cultivation, and the problems of manipulating the small flowers. However, bearing in mind the possibility of partial self-incompatibility, and the existence of natural hybridisation, it is unwise to describe L. humile as a completely inbreeding species. Similarly, more study is needed of the breeding system of L. binervosum. Suggestions described in 2.1(c) of facultative apomixis and interspecific hybridisation require substantiation with studies of pollen viability and with controlled breeding experiments. Also, continual emasculation does not reveal very detailed information on the nature of the apomixis, and a cytological investigation of this is required. This latter study was not attempted because it was thought that this work was being done elsewhere (D. Ockendon, personal communication); unfortunately this could not be continued.

Therefore it is unwise to state the exact breeding systems of L. humile and L. binervosum. Indeed, many authors have found that the concept of a fixed breeding system for a particular species is unrealistic. However, it can

still be said that the three Limonium species have relative differences in their breeding systems, and their variation will be approached comparatively.

2. 4. POPULATIONS STUDIED

Table 2.1 summarises briefly the populations sampled; more detailed information is given in Appendix I. Fig. 2.2 shows the approximate locations of the sites, and the distribution limits of the three species.

Population LV17 deserves further mention because it consisted of about 10 large isolated clumps spread over $\frac{1}{4}$ of a mile, growing in crevices in an eroded cobbled estuary bank. All the leaves and inflorescences within and between clumps were remarkably similar morphologically and in flower colour, and all flowering spikes examined were A/cob. This was not the case for other L. vulgare populations (see Ch.2.5(a)), and it is possible that these clumps had been established by vegetative spread of a single plant, followed by fragmentation by erosion.

In one locality for each species, two adjacent populations were sampled with the intention of investigating ecological differences: LV2/LV3; LH1/LH2; LB3/LB4. For both L. vulgare and L. humile sites were selected within and without the distribution of each other, because of the possibility of hybridisation between the two affecting their variation. It was intended to take a sample of L. humile from Essex, but a suitable population could not be found.

The possibility of the existence of the hybrid L. x neumanii between L. vulgare and L. humile was borne in mind during sampling, but this was not given extensive investigation. However, in one locality, Scolt Head Island, it was clear that the population contained plants that were morphologically intermediate between the two species. In this case, these plants were treated separately, being designated 'wild putative hybrids' (LX18). Also a few hybrids were synthesised artificially,

TABLE 2.1.

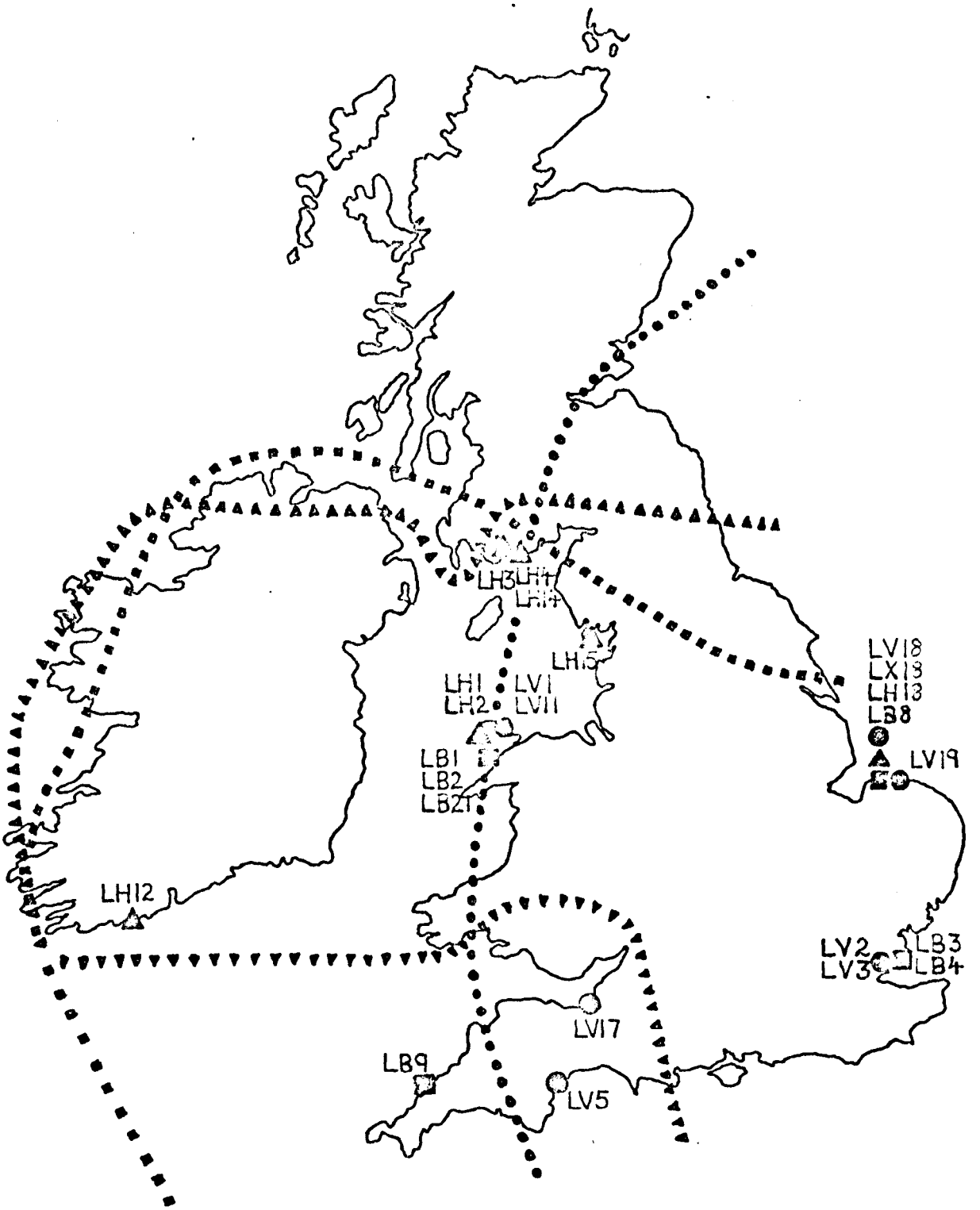
BRIEF SUMMARY OF LIMONIUM POPULATIONS STUDIED

POPULATION CODE	LOCALITY	COMMENTS
LV1	Traeth Dulas, Anglesey	Small isolated shaded plants on estuary bank
LV2	Fingrinhoe, Essex	Higher salt marsh
LV3	"	Lower salt marsh
LV5	Dawlish, Devon	Salt marsh, outside range of L.humile
LV17	Bridgwater, Somerset	Crevices in sea wall; may have spread vegetatively
LV18	Scolt, Norfolk	Salt marsh; L. humile present
LV19	Morston, Norfolk	Salt marsh; L. humile absent
LH1	Inland Sea, Anglesey	Salt marsh; L. vulgare absent
LH2	"	Crevices in rocks
LH3	Wigtown,Kirkcudbright	Just outside range of L. vulgare; estuary bank; shaded
LH4	Fleet Bay,Kirkcudbright	Just outside range of L. vulgare; mud and shingle
LH12	Roaring Bay, Co. Cork	Crevices in rocks; outside range of L. vulgare
LH15	Holme, Lancs	Sandy salt marsh; L. vulgare absent
LB1	Llanddwyn, Anglesey	Rocky cliff
LB2	Cefni, Anglesey	Sandbank in estuary above high water
LB3	Colne Pt., Essex	Exposed shingle
LB4	"	Overgrown shingle; plants shaded
LB8	Scolt, Norfolk	Exposed shingle
LB9	Padstow, Cornwall	Loose shale cliff

Fuller details are given in Appendix I.

FIGURE 2.2.

Map showing: (a) approximate positions of sites sampled.
(b) distribution limits of Limonium species.



● L.vulgare site .... N.W. limit of L.vulgare
▲ L.humile " ▲▲▲▲ " " " L.humile
■ L.binervosum " ■■■■ " " " L.binervosum

progeny LX1 and LX2, and were used for electrophoresis. LX1 had a female L. humile parent from W. Anglesey (LH1 site) and a male L. vulgare parent from E. Anglesey (LV11 site). Both parents were from pure stands. LX2 had a female L. humile parent from Kirkcudbright (LH3 site) and a male L. vulgare parent from Scolt (LH18 site). This latter plant may itself have been a hybrid, being from a mixed stand. Difficulty was experienced in cultivating the parents, and in producing and raising their hybrids, only 7 plants in all being finally obtained.

During the collection of wild population samples, because of the powers of vegetative spread, particularly in L. vulgare, care was taken not to re-sample the same plant; samples were taken approximately ten metres apart where necessary. Ten to fifteen leaves per plant were taken for chromatography, along with a cutting to cultivate for electrophoresis. Many cuttings of L. vulgare and L. humile did not root, so re-sampling at a later date was necessary. Usually 25 plants were sampled from each locality.

2. 5. THE BREEDING SYSTEM AND MORPHOLOGICAL VARIATION

(a) Introduction

Although the main aim of my work was to study chemical variation within and between populations of the three species, some observations on morphological variation were also made.

Baker (1953a) measured morphological variation in one population of L. vulgare and three of L. binervosum and related it to the breeding system. His results are given in Table 2.2. On a qualitative level, Boorman (1966) stated that L. vulgare had produced a number of forms which remained distinct in cultivation, which suggested they were genetically determined. These forms seemed to be the result of ecotypic differentiation. In contrast, L. humile was described as less variable, but a few extreme forms had been

TABLE 2.2.

MORPHOLOGICAL AND PHENOLOGICAL VARIATION IN *LIMONIUM* CULTURES

DATA FROM BAKER (1953a)

Species	Locality	Leaf Index ($\frac{L}{B}$)		Date of First Flowering (1951	
		Mean	Std.Dev.	Mean	Std.Dev. (Days)
L. vulgare	E. Yorks	2.64	0.53	4 Aug	16.4
L. californicum+	California	2.36	0.17	-	-
L. binervosum*	Sussex	3.89	0.27	27 July	4.0
L. lychnidifolium*	Jersey	1.67	0.09	30 July	4.1
L. binervosum*	Glamorgan	-	-	10 Aug	0.7
L. binervosum*	Tenby, Pembr.	-	-	10 Aug	0.6
L. transwallianum*	Giltar Pt., Pembr.	-	-	20 June	0.8

+ Self-compatible * Apomictic

L. transwallianum is thought to be a biotype of *L. binervosum*, given specific status because of its distinct morphology.

Note the high standard deviation in *L. vulgare* measurements compared with other species.

Note also the different flowering times of some *L. binervosum* cultures (including *L. transwallianum*)

named (Boorman 1967). Morphological differentiation between populations of L. binervosum has been known for some time (Salmon 1903) to the extent that it is sometimes possible to determine the origin of an herbarium specimen by its general appearance alone. The morphological variation between populations is such that some extreme forms have been given separate taxonomic status (Clapham et al. 1962) but it is believed (Baker 1954b) that these forms are no more than local derivations of L. binervosum, and that L. recurvum C E Salmon, L. transwallianum Pugsl. and L. paradoxum Pugsl. should be regarded as members of an apomictic complex of L. binervosum.

The above observations were supported during my sampling visits. Morphological variation within populations of L. vulgare could easily be seen, particularly in terms of leaf shape (which varied from lanceolate through elliptic to spathulate) and petal colour (ranging from pale lilac to dark purple). Populations contained both A/cob and B/pap. plants. Often in a well-established population component parts of coalesced clones could be identified from these features. Populations of L. humile did not show such variation within or between them. Populations of L. binervosum also appeared uniform, but variation was often evident between populations. No attempt was made to determine whether any of the L. binervosum samples resembled any of the named forms referred to above.

(b) Variation of Leaf Morphology in L. BINERVOSUM Populations.

Under natural conditions L. binervosum responds to its normally dry habitat by developing xerophytic features such as a rosette habit and small fleshy leaves. Cuttings from separate plants from several populations were cultivated in a 1:1 peat:sand mixture in a greenhouse and when watered freely produced larger leaves in quantity which still retained their fleshiness. The populations sampled are listed in Table 2.3..Fig. 2.3 shows tracings of a representative leaf from each population. Anglesey samples had short spathulate leaves, while those from Cornwall were slightly less spathulate

TABLE 2.3.

L. BINERVOSUM POPULATIONS UNDER CULTIVATION FROM WHICH MORPHOLOGICAL MEASUREMENTS WERE TAKEN

POPULATION CODE	LOCATION	NUMBER MEASURED	MONTHS CULTIVATION
LB21	Anglesey	20	17
LB3	Essex	20	33
LB4	Essex	17	33
LB8	Norfolk	36	10
LB9	Cornwall	25	16

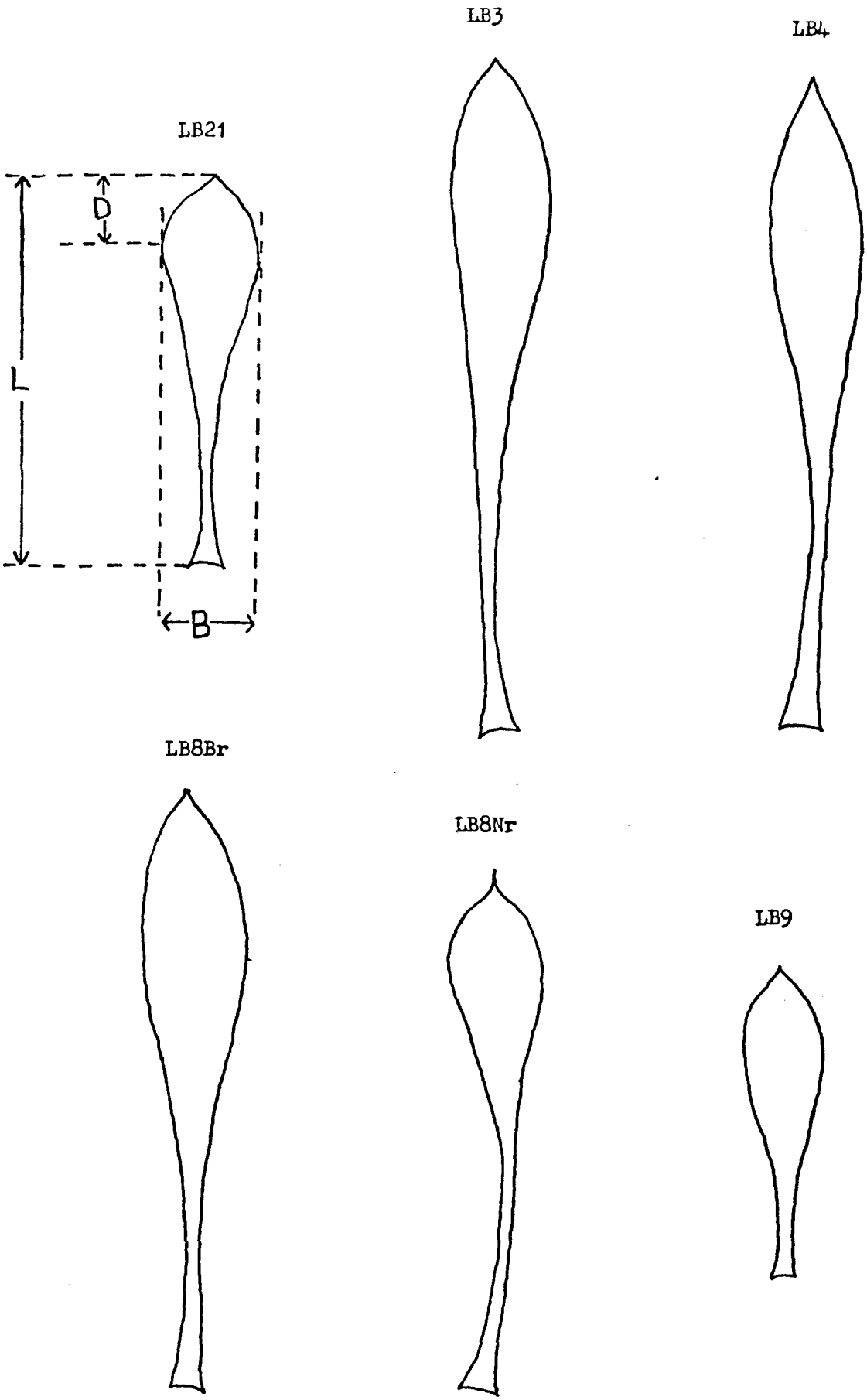
TABLE 2.4

WILD L. VULGARE AND L. HUMILE POPULATIONS FROM WHICH MORPHOLOGICAL MEASUREMENTS WERE TAKEN

POPULATION CODE	LOCATION	NUMBER MEASURED
LV5	Devon	20
LV17	Bridgwater	10
LV18	Norfolk	20
LX18	Norfolk	5
LH18	Norfolk	5
LH12	Ireland	20

Figure 2.3.

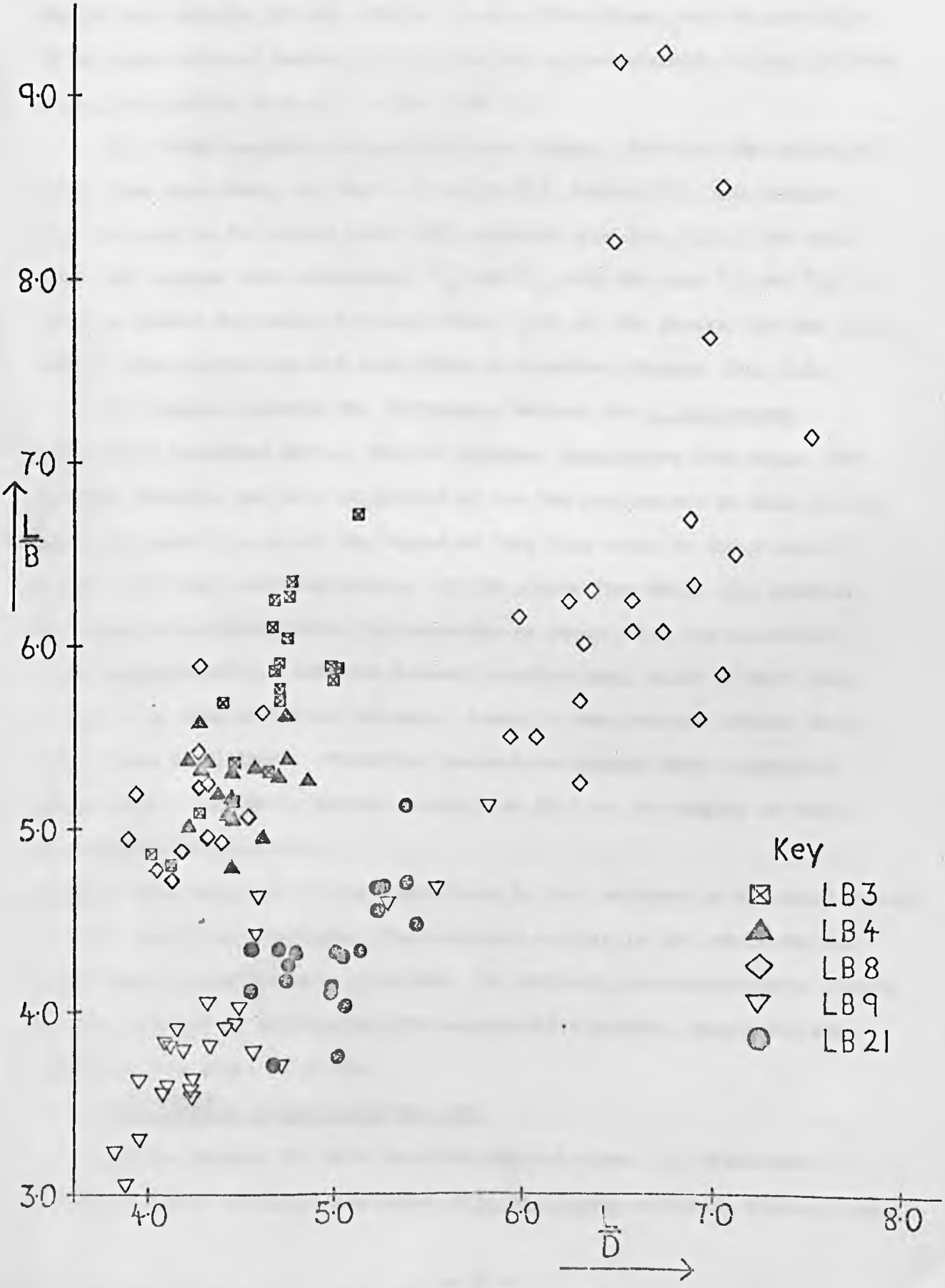
Outlines of cultivated L. binervosum leaves from different populations.



Each outline is typical of the plants from that population.
LB21 also shows the dimensions used in calculating leaf indices.
L = leaf length, B = breadth, D = distance of widest point from apex.

FIGURE 2.4.

Scatter diagram of leaf indices of cultivated L.Binervosum populations.



For key to origin of populations, see Table 2.3.

and also less fleshy. Leaves from Essex were longer and oblanceolate. Of the 36 Scolt plants, 13 were similar to those from Essex, but the remaining 23 had much narrower leaves with a spathulate or occasionally almost ensiform shape, but similar in length to the first 13.

An attempt was made to quantify these shapes. Five healthy leaves were taken from each plant, and the leaf length (L), breadth (B), and distance from the apex to the widest point (D), measured (see Fig. 2.3). For each leaf, two indices were calculated, $^{L}/_{B}$ and $^{L}/_{D}$, and the mean $^{L}/_{B}$ and $^{L}/_{D}$ for the five leaves determined for each plant. For all the plants, the two mean indices were plotted against each other on a scatter diagram, Fig. 2.4.

The diagram reflects the differences between the L. binervosum populations described above. The two adjacent populations from Essex, LB3 and LB4, overlap, but some separation of the two populations is seen in that some LB3 plants lie out of the region of LB4; this could be the result of slight ecological differentiation. 13 LB8 plants from Scolt also overlap the Essex populations, which is reasonable as these sites are relatively close geographically. LB9 from Cornwall scatters well clear of East Coast material, as does LB21 from Anglesey; there is some overlap between these two Western populations. Therefore the scatter diagram shows degrees of morphological similarity between populations that can be related to their geographical similarities.

Morphological variation within populations is low, as shown by the small spread of their individual scatters. The exception to this is LB8, where the two morphlogical types are well separated. In addition, the narrow-leaved plants to the top right of the diagram show a much wider scatter, suggesting more variation from plant to plant.

(c) Discussion of Variation Within LB8

Precise reasons for this variation are not clear. De Fraine and Salisbury (1916) described two forms of L. binervosum at nearby Blakeney Point,

a"typical" narrow-leaved form on stabilised shingle, and a broad-leaved form growing near the drift line. They suggested that the broad-leaved form was the result of hybridisation between L. binervosum and L. bellidifolium, a fourth species found both at Blakeney and Scolt (see Fig. 2.1). They supported this suggestion by morphological observations, but their attempts at artificial hybridisation were not successful. At the time of sampling LB8 this paper was not known. The 36 Scolt plants were collected from the same habitat, and were not consciously chosen for type. Variation in the sample was only noticed after the plants produced larger leaves in cultivation.

If the two forms are the same as those of De Fraine and Salisbury, it is difficult to accept that the broad-leaved form is of hybrid origin. This putative hybrid is not unique to Norfolk, as it resemples the populations from Essex where L. bellidifolium is absent, and it bears greater resemblance to the Western samples than the narrow form. A better candidate for the hybrid is the narrow form, because of its higher variability in the scatter diagram and its absence from other populations; it is difficult to see why it should be referred to as "typical" of the species.

Examining more closely the morphological information provided by De Fraine and Salisbury, no real case can be made for either form possessing intermediate characters between the other and L. bellidifolium. Of the 36 characters, 13 support the hypothesis that the broad form of L. binervosum is intermediate between the narrow form and L. bellidifolium, two characters that the narrow form is intermediate, and the remaining 21 that neither the broad nor the narrow is intermediate between its alternative and L.bellidifolium. There is also no direct experimental evidence of hybridisation between the two species.

Another explanation for the variation within the Scolt sample is that the normal apomictic reproduction of L. binervosum may have been temporarily replaced by a sexual event, producing new segregates which have been further

multiplied by a resumption of apomixis. This appears to have happened elsewhere in the British Isles in the production of the more extreme forms named in Clapham et al. (1962). Alternatively the narrow-leaved form may be an immigrant into an already established population of broad-leaved plants, or vice-versa. The morphological variation of L. binervosum in Norfolk populations needs more study before it can be discussed further.

(d) Variation of Leaf Morphology in L. VULGARE and L.HUMILE Populations.

Because of the difficulties in cultivating these two species, similar morphological studies to L. binervosum were not possible. A few wild populations were measured, listed in Table 2.4. As measurements were of wild material, environmental differences between plants within and between populations may have affected the scatter diagrams, and the measurements cannot be compared with those of L. binervosum. Leaf indices and scatter diagrams were prepared as before, and the scatter diagram is given in Fig. 2.5.

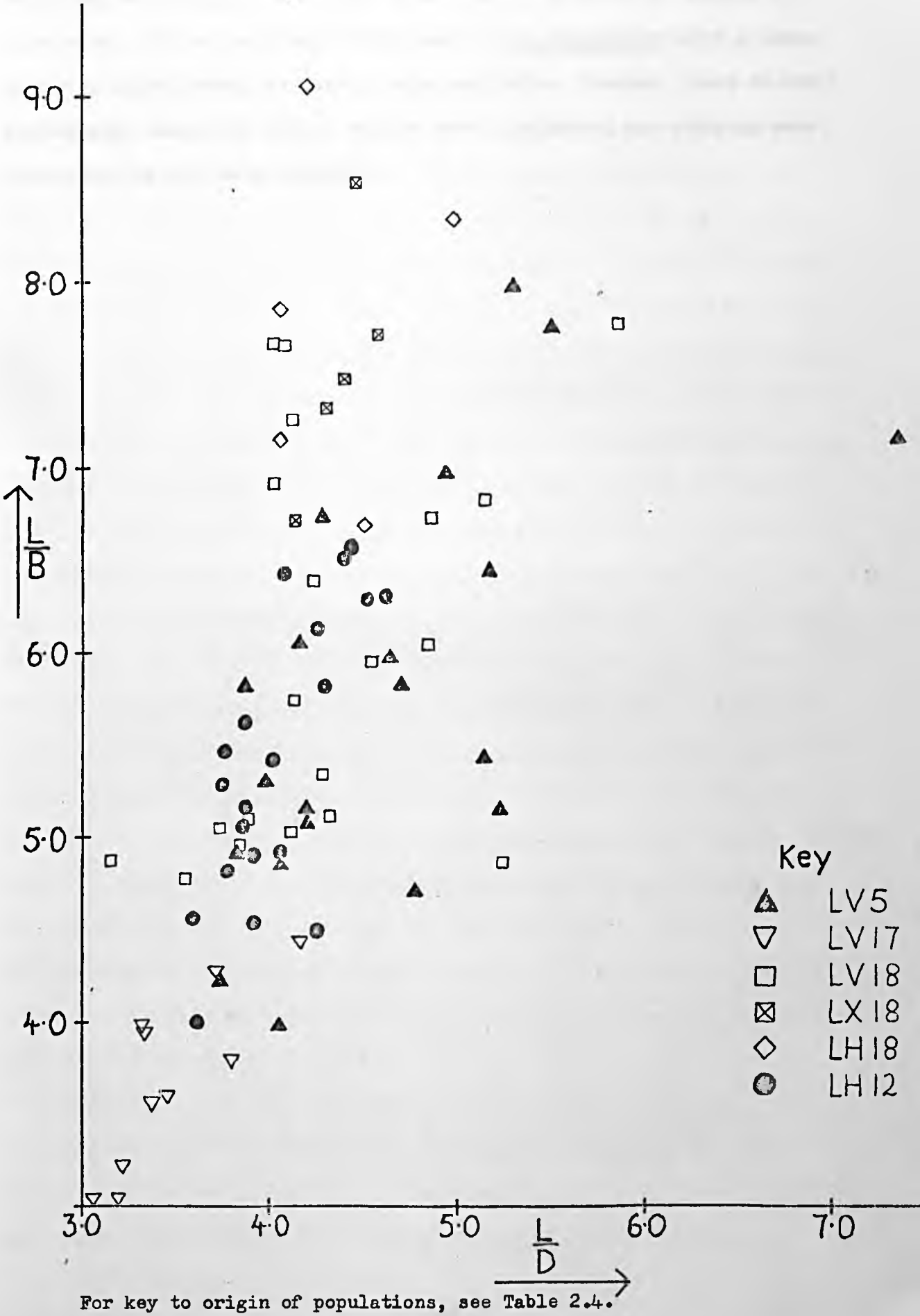
Populations LV5 and LV18, although from well-separated localities, show considerable overlap but a wide scatter, indicating variation within both populations, but showing that the two populations contain plants with similar shaped leaves. LH 12 also overlaps with these two populations, but has a smaller scatter, showing that there is less variation in leaf morphology within this population than the two L. vulgare populations. Because LH12 does not overlap with LH18 plants, it might seem that there are differences between these populations, but only five LH18 plants were measured. The unusual LV17 population (see Appendix I and Ch. 2.4) shows only slight overlap with other L. vulgare populations, and less scatter than them, but only ten plants could be sampled.

(e) Conclusion

Qualitative observations and quantitative data on morphology from this and other studies show that the variation within and between populations of Limonium species supports the relationship between breeding system and

FIGURE 2.5.

Scatter diagram of leaf indices of wild *L.vulgare* and *L.humile* populations.



For key to origin of populations, see Table 2.4.

morphology according to the theory of H G Baker described in Chapter 1. In my work, this is particularly the case for L. binervosum where a large number of plants under cultivation w as available. However, there is still considerable scope for further work on more populations and studying more characters for all three species.

CHAPTER 3.

THE STUDY OF CHROMATOGRAPHIC VARIATION, AND EXPERIMENTAL METHODS

3. 1. THE STUDY OF CHROMATOGRAPHIC VARIATION

(a) Survey of the Literature

In recent years, widespread use has been made of the technique of chromatography of tissue extracts in an attempt to solve various taxonomic and evolutionary problems, particularly in plant species. The pioneers of contemporary studies were R.E. Alston and B.L. Turner, who demonstrated the value of chromatography in analysing interspecific hybridisation in the genus Baptisia (Leguminosae) (Turner & Alston 1959; Alston & Turner 1962, 1963). They were able to study situations which could not be analysed morphologically because of the similarities of some of the taxa involved. There followed many other studies of a wide variety of plant genera; often the conclusions drawn from chromatography were supported by evidence from other sources, or else chromatography was used because other methods of comparing taxa could not be applied.

The chemical compounds which have been studied in most cases are referred to as phenolics, a collection of organic compounds with a wide variety of chemical structures but having in common a phenolic hydroxyl function. Chemical identification has shown that many of these phenolics belong to a particular group known as the flavonoids. Several classes of flavonoids are possible, and reviews of their structure and biosynthesis were given by Harborne (1967a, 1973) and Ribérau-Gayon (1972). However it is likely that some substances commonly appearing on chromatograms are non-flavonoid phenolics.

There are probably several hundred different phenolic compounds occurring naturally in various plants. In the case of flavonoids the variety of compounds is made possible by glycosylation, hydroxylation and esterification at several sites on a few basic structures. The steps in

biosynthesis of flavonoid compounds are thought to be controlled by specific enzymes, which are in turn determined by specific genes (Harborne 1967a). Therefore the presence of a particular flavonoid is considered to be a chemical marker indicating the presence of a specific gene, and a difference in flavonoid profile between two plants is a reflection of a genetic difference between them. This principle cannot be applied to all of the non-flavonoid phenolics because their inheritance and biosynthesis has not been so exhaustively studied.

The strongest proof of the genetic control of flavonoids comes from inheritance studies; if they are coupled with chemical identification, it may also be possible to construct biosynthetic pathways to the compounds. This was done by Fahselt and Ownbey (1968) for Dicentra (Fumariaceae) species and hybrids. It is thought that the presence of most flavonoids is determined by dominant genes, and their absence by recessive alleles (Alston 1965). A Mendelian study supporting this view was made by Belzer & Ownbey (1971) for Tragopogon (Compositae). Some other studies of artificial and natural interspecific hybrids also supporting this hypothesis were made by Levy & Levin (1971) on Phlox (Polemoniaceae) and Challice (1972) on Pyrus (Rosaceae). One group of flavonoids, the anthocyanins, responsible for flower colour, has been studied genetically in several taxa, and reviewed by Harborne (1967a).

As well as genetic control of presence and absence, there is some evidence that quantitative variation in flavonoids is in part genetically controlled. In Mendelian studies of Trifolium subterraneum, Francis & Wong (1966) discovered two recessive genes at separate loci which inhibited flavonoid production. Jana & Seyffert (1971) also suggested the existence of genes which affected the anthocyanin content of Matthiola incana. However it will be shown later that other factors besides heredity can influence the presence of flavonoids both qualitatively and quantitatively.

Although chromatography has often been useful in the study of

interspecific hybridisation and taxonomy, this has not always been the case. In contrast with Alston and Turner's work, Ockendon et al. (1965) found only limited interspecific differences between 30 Psoralea (Leguminosae) species, although this was a morphologically heterogeneous group. Similarly Abrahamson & Solbrig (1970) found that few apparent biochemical changes had resulted from speciation and polyploidy in 4 species and 3 ploidy levels of Aster (Compositae). Other cases where interspecific differences were lacking were described by Alston (1965). Therefore the universality of the value of chromatography to hybridisation and taxonomical studies needs some qualification. However if interspecific differences in phenolics do occur, they do run parallel with any morphological differences observed.

Where workers have taken the trouble to study a number of individuals of a species or population, intraspecific variation of phenolics has also been detected. Brehm & Alston (1964) investigated 21 plants of a population of Baptisia leucophaea Nutt. var. laevicaulis Gray. For a total of 24 spots on the chromatograms, 14 were variable in that they were not present in all of the plants; however, 7 of these variable spots were present more often than not, being found in 18 or more of the 21 plants. Intraspecific variation at a similar level was found by Brehm & Ownbey (1965) for populations of Tragopogon species. In all three species of Dicentra studied by Fahselt (1971) a large proportion of the flavonoids showed less than full occurrence. However, as in the case of interspecific differences, in some species intraspecific variation has not been observed. Levin (1967) showed intermittent expression of several compounds in Liatris spicata (L.) Willd., but all spots were consistent for L. aspera Michx.; Smith & Levin (1963) found little variation within Asplenium (Polypodiaceae) taxa.

During intraspecific studies, some workers have found that the same spot has varied from plant to plant in its size or intensity, revealing quantitative intraspecific variation. Brehm & Ownbey (1965) revealed such variation within

their Tragopogon populations. Similarly Asker & Frost (1969, 1970a) found quantitative differences in the same spots between different biotypes of apomictic Potentilla (Rosaceae). Quantitative variation between different varieties of wheat was measured densitometrically by Bose (1972) on material grown under controlled conditions, and some inheritance studies of intensity were also described.

In some cases where intraspecific chromatographic variation has been observed it has been possible to relate this to the geographical distribution of the material. Brehm & Alston (1964) detected "chemical races" of B. leucophaea var. laevicaulis in terms of presence/absence of a total of seven spots. Seeligman & Alston (1967) showed a similar level of geographical variation for Hymenoxys scaposa (DC.) Parker and H. acaulis (Pursh) Parker. In both these cases morphological variation was random and did not relate to the geographical origin of the material. However, negative results have also been reported. Bragg & McMillan (1966) did not detect chromatographic variation in Andropogon scoparius Michx. and A. gerardi Vitman that could be related to geography, although variation in chromatographic pattern was detected within and between populations. Other negative geographical studies were reported by Smith & Levin (1963) for Asplenium, and Fahselt (1971) for Dicentra canadensis.

As far as I know, no studies have been made which particularly relate chromatographic variation to the breeding system, although sometimes the breeding system has been suggested as one of several factors influencing the level of variation observed. Thus Brehm & Ownbey (1965) observed that the variation within and between Tragopogon populations was greater than that observed by Alston & Turner (1963) for Baptisia, and the former authors suggested that the differences between the two genera in their breeding systems might have been the cause of this. Brehm (1966) proposed that little or no qualitative variation in chromatographic spots could be expected from

an inbreeding species, and that variability could be expected from an outbreeder; however, this was not illustrated by specific examples. Chromatographic variation between different inbred lines of rye (Frost 1966), Galeopsis (Bose & Frost 1967) and barley (Frost & Asker 1973) was reported, and in genetic terms inbred lines might be compared with different wild populations of an inbreeding species. None of these references provide examples of comparable outbred samples. Differences in flavonoid profile have also been observed between different morphological types of apomictic Potentilla by Asker & Frost (1969, 1970a).

As the study of chemotaxonomy has developed, there has been an increasing feeling that some of the chromatographic differences between individuals, populations or species may not be the result of genetic differences alone, but may be caused by differences in environmental conditions or stage of physiological development. Erdtman (1963) stated that although chemical characteristics are genetically controlled,the presence of these compounds can be influenced by soil, season or climate. Experimental work to measure the influence of these factors has involved the use of culture solutions and growth cabinets, or else plants have been exposed to different but uncontrolled environments. Conflicting results were obtained for one genus. McClure & Alston (1964) showed that flavonoid components of two species of Spirodela (Lemnaceae) were consistently present under different conditions of light and nutrition, and that at most the variation created by the different conditions was quantitative. On the other hand, Ball et al. (1967) detected qualitative and quantitative chromatographic variation between different culture conditions in Spirodela although this study was more simple in terms of flavonoids surveyed and differences in culture conditions. Parks et al. (1972) suggested that the different conclusions may have been obtained because the two groups used different chromatographic methods. Parks et al. (1972) also used growth cabinets on Gossypium (Malvaceae), varying temperature, photoperiod and

nutrition. They found only minor variations in floral pigments, but found much in leaf flavonoids; however, the nature of this variation was not described.

Asker & Frost (1972, 1973) also used growth chambers, cultivating Potentilla and Avena respectively. In the former paper they list 12 out of a total of 63 spots which showed qualitative or quantitative changes with age, although most changes occurred within the first two months of growth from seed. No changes were associated with the onset of flowering. For Avena they compared young and old plants from the growth cabinet and the field, and concluded that both age and environment influenced chromatographic pattern. Other work, not with growth chambers, on Pyrus by Challice & Williams (1968) and on Aster by Abrahamson & Solbrig (1970) showed that developmental state and environmental conditions influenced the chromatograms obtained.

Despite these various findings, none of the authors urge complete disregard of wild sampled material, but recommended that samples should be taken carefully, and that the results should be treated with caution. Because of these environmental and developmental influences on chromatograms, and for other reasons related to difficulties with identification and repeatability of spots, there are several cases where spots have been neglected because they have been felt to be unreliable. Brehm & Ownbey (1965), Jaworska & Nybom (1967), Belzer & Ownbey (1971) and Frost & Holm (1971) discounted half or more of the total number of spots observed. Ising & Frost (1969) and Asker & Frost (1970a, 1970b, 1972) divided their spots into two groups, blues and non-blues, and concluded that the blue spots were of less taxonomic value because they were not always repeatable.

Another source of non-genetic variation may be encountered if the results from fresh and herbarium tissue of the same species is compared (Bate-Smith & Harborne 1971). Harborne (1968a) showed that storage of material, drying and extraction all produce artefacts by the hydrolysis of

ester and glycosidic linkages. However, short-term storage is probably not harmful, as indicated in experiments by Taylor & Campbell (1969) and Asker & Frost (1970a), and provided all material is subjected to the same treatment during storage and extraction results should be comparable.

Despite the many applications of flavonoid studies, relatively little attention has been directed towards describing the functions of these compounds within the plant. A better understanding of this would greatly enhance the value of flavonoid studies. At first they were regarded as secondary substances, in contrast to primary or essential metabolites such as enzymes (Brehm 1966), but more recently various functions for flavonoids have been suggested, and they should not be regarded simply as waste products conveniently deposited into cells (Harborne 1968b). Such functions are reviewed by Harborne (1967a), and the role of phenolics in general as substances for chemical defence against disease and predators is reviewed by Levin (1971). Other specific functions of flavonoids have been described, such as growth regulators (Galston 1969) phytotoxins (Muller & Chou 1972), and phenolics in general are involved in the process of lignification (Bate-Smith 1963). The fluctuations in phenolic content with age and environment indicated earlier may also indicate a functional role. However, despite these examples the knowledge of the precise necessity for the presence of any named flavonoid compound in a particular plant species is virtually non-existent. In general, much work remains to be done with flavonoids before it is possible to regard their function in plants as settled (Harborne 1973).

The validity of chromatographic results in the absence of positive chemical identification has been questioned several times. Such non-identification may lead to the inclusion of artefacts in the data obtained from chromatograms (Alston 1965; Harborne 1968a). To illustrate this the work of Fahselt & Ownbey (1968) is again referred to. By hydrolysis of their

extracts they revealed that several spots on their chromatograms were biosynthetically related to each other. In any study spots may be generated artificially by hydrolysis during extraction and running. Further, if the members of a group of spots are different glycosides of the same precursor, although a pair of individuals may differ by several spots this may be the result of only a single gene difference in the synthesis of the precursor.

Despite such dangers, Asker and Frost (1970b) concluded that comparisons between taxa are still feasible, and the number of successful studies where no attempt was made at identification bears witness to this view. Alston & Turner (1963) also claimed that non-identification by no means eliminated the possibility of obtaining a great deal of useful systematic information. Further, the task involved in the proper identification of a large number of complex organic compounds would be very great, as indicated by the methods described by Mabry et al. (1969) and Ribereau-Gayon (1972).

(b) Discussion

Much of the work referred to has had the common aim of drawing taxonomic conclusions from chemical information, but there have been many ways of doing this. Earlier work used paper chromatography, but more recently thin-layer chromatography has been favoured because it gives sharper spots and takes less time to run. On both media a variety of solvent systems have been used, with many different detection methods for the spots. Techniques for the extraction of phenolics from leaves have also varied, and some must be regarded as unsatisfactory because of their destructive nature.

Interspecific and intraspecific qualitative and quantitative variation has been studied, and has sometimes been related to taxonomic evidence of other kinds, or to the ecology and geography of the species. However there were also cases where such relationships did not exist. Little work has been done concerning intraspecific variation and the breeding system, and with a few exceptions, population studies have not been made, perhaps because the

running of a large number of chromatograms is time-consuming.

Evidence has been presented for the genetic control of chromatographic spots, but it has been shown that other non-genetic factors influence the spots at least quantitatively, such as the environment in which the plants were growing, and the conditions employed during extraction and chromatography. The dangers of non-identification of spots have been described, and the unreliability of some of the spots has been reported, causing workers to reject some information.

An important conclusion to be drawn from this review is that chromatographic data should be regarded with circumspection, and that genetic or taxonomic deductions can only be made after eliminating other sources of variation. Later in this chapter the techniques I have used will be described, with an attempt to eliminate artefacts in the results. However it will be clear that non-genetic variation cannot be entirely eliminated, but discussion of chromatographic variation and the breeding system is still possible on a comparative basis.

3. 2. THE USE OF INDICES IN ANALYSING CHROMATOGRAPHIC VARIATION

With the development of chemotaxonomy the need has arisen for mathematical methods of handling large quantities of chromatographic data, to express taxonomic relationships numerically. Several types of similarity index have been developed, some based on those described by Sokal & Sneath (1963). Most of these indices are calculated by numbering all the possible spots, scoring each plant with a plus or a minus for each spot, and then taking the plants in pairs and applying the appropriate formula to all the spot comparisons. An example of such an index applied to chromatographic data is the "Coefficient of Similarity", C_s, used for example by Jaworska & Nybom (1967)

$$C_s = \frac{p}{p + d}$$

where p = no. of positive matches
d = no. of differences

Other similarity indices resembling C_s have been used. For example, Ising & Frost (1969) applied three such indices to the same data and came to similar taxonomic conclusions in each case. I shall not use this type of index because with approximately 350 plants to compare with each other, this would create too much information to process and handle. Similarity indices will not be considered further, but their existence has prompted valuable discussion of the treatment of chromatographic data, particularly in connection with negative matches.

Runemark (1968) considered that those indices that gave reduced emphasis to, or totally ignored, negative matches were preferable, because a common absence of a compound was not thought to be taxonomically useful. This was perhaps too general a view, and Jaworska & Nybom (1967) distinguished between situations where only two units were studied, and where information was available for several. In the latter case the common absence of a pair should be considered as a true similarity. Runemark also criticised the use of positive matches in cases where spots occurred in all units. Asker & Frost (1970b) recognised this criticism but felt that with the quantity of information usually obtained from a chromatographic study, numerical expressions incorporating positive matches had to be used. Runemark's alternative was to present a table of all positive and negative matches and differences, which was unwieldy and did not clearly indicate relationships between units. Another alternative, considered by Taylor & Campbell (1969) was to give characters different weightings; however, although it was absurd to assume that all characters had equal significance, giving them equal weight was preferable to the difficult task of devising an objective method of weighting.

The similarity indices described apply to situations where spots are recorded in a simple presence/absence state. If the spot intensity is estimated in some way such as recording brightness or diameter, so-called quantitative

comparisons became possible. An index to measure such differences is based on Sokal & Sneath's(1963) "Taxonomic Distance", renamed "Biochemical Distance" and used by Jaworska & Nybom (1967) and others. It was defined as:

$$D^2_{jk} = \sum_{i=1}^{N} (A_{ij} - A_{ik})^2$$

Where N = Total no. of spots considered
A_{ij} = Area of ith spot in the jth plant
A_{ik} = " " " " " " kth "

This index gives greater weight to large differences than to small ones, because the differences are squared. This is of advantage because small differences may arise by chance or by errors in observation, and are likely to be less important than larger ones. Also, negative matches and fully positive ones, considered to be less important, do not contribute to the value of the index. In this form the index is not adjusted by the total number of spots used, which may differ between comparisons. Because in my data this number does vary from one comparison to another, I shall use a modification of this index (Sokal & Sneath 1963), which I shall call "Average Biochemical Distance":

$$d_{jk} = \sqrt{\frac{D^2_{jk}}{N}}$$

Where D_{jk} = Biochemical distance
N = Total no. of spots used in the particular comparison

This modification does not incorporate negative matches, but takes into account fully positive ones.

If several chromatograms are compared systematically with each other using one of the above indices, a matrix of comparisons is built up. The quantity of data obtained is difficult to interpret in this form, but the matrix can be further processed by cluster analysis (Sokal & Sneath 1963)

and a dendrogram constructed. This has been done in several cases, e.g. Ising & Frost (1969). However this simple "nearest neighbour" method, performed by inspection, means that once two or more units have clustered, their relationship with other units not yet clustered is no longer considered, and some of the information is lost. A way round this is the "weighted mean pair" cluster analysis of Gower (1967) which does consider all relationships.

None of the users of various indices applied significance tests for deciding when a difference between values was significant. Sokal & Sneath (1963) felt that ordinary tests applied to similarity indices were inappropriate because the distributions of the indices were not known. Also the characters used would be assumed to be independent, and this could only be established by chemical identification of the spots and a knowledge of their biosynthesis. As an approximate alternative Sokal and Sneath suggested the standard error of the binomial,but Runemark (1968) stated that such a calculation was mathematically untenable and the result meaningless.

Therefore at the present time an index with significance tests is not available. Nevertheless many useful conclusions have been drawn from indices and dendrograms using a comparative approach, considering the relative degrees of difference between the members of the group studied.

3. 3. EXPERIMENTAL METHODS

(a) Chromatography

(i) Plant Material The populations sampled for chromatography are listed in Appendix I, and the methods of sampling described in Chapter 2. At the time of collection the leaves were pressed and dried at 40°C and stored in the dark at room temperature until required.

(ii) Extraction of Phenolics The leaves from an individual plant were ground to a fine powder and 0.10g. weighed into a centrifuge tube. Extraction was with 1.0 cm^3. methanol containing 1% conc. HCl for 22-24 hours in the dark at room temperature. The extract was centrifuged and the clear dark green-brown

supernatant chromatographed immediately after extraction.

(iii) Chromatographic Separation Two-dimensional thin-layer chromatography was found to be preferable in terms of number of spots, sharpness of outline and speed of running. 20cm. x 20cm. glass plates were coated with MN 300 cellulose, spreader gap 0.4mm. The size of plate allowed for 16cm. movement of the solvent in both directions and this distance was scored off in the cellulose. A strip of cellulose 3mm. wide was removed from the edges of the plate, resulting in a straighter solvent front.

10 microlitres of extract were applied to the origin a drop at a time, drying between applications. Plates were run ten at a time in a rack inside a large glass tank; 30 or 40 plates could be run in a day, and wherever possible a whole population was processed simultaneously.

The most suitable solvent system was found to be that of Olden & Nybom (1968) except that I found that a more concentrated formic acid gave a better separation with Limonium. In more recent literature the second solvent was modified slightly (e.g. Asker & Frost 1969), but as several populations had already been run the proportions were not adjusted.

1st Direction: Formic acid: water 1 : 4 (vol.) approx 1 hr. 50 mins

2nd Direction: Benzene:Propionic acid:water 20:45:15(vol.) " 2 hrs.

The plates were dried thoroughly after development in each solvent.

(b) Examination and Recording of Plates. The most suitable detection methods were found to be, in this order:

(i) examination of the untreated plate in UV light

(ii) plate fumed with ammonia vapour and examined in UV light

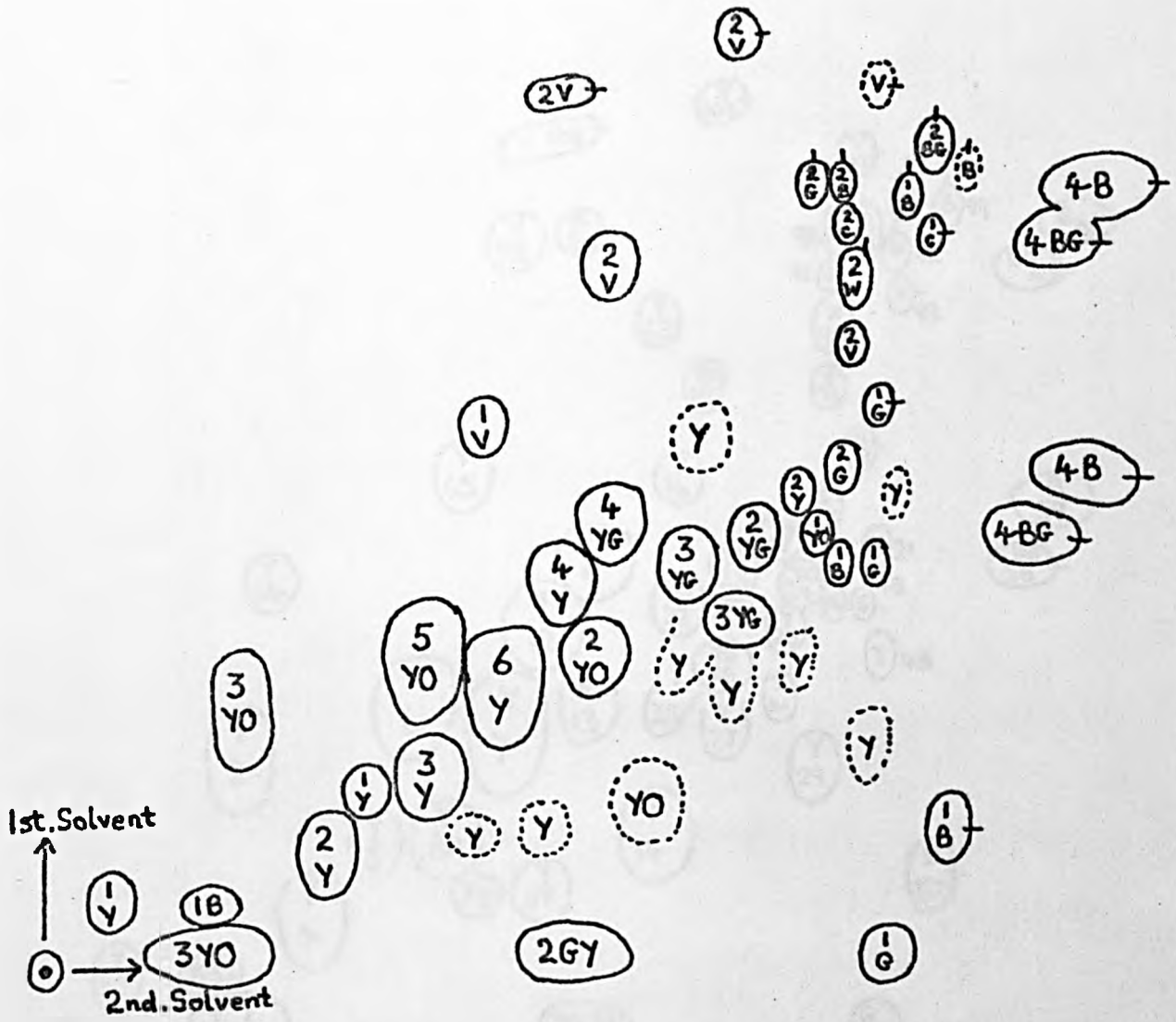
(iii) plate dipped in 'Flavone Reagent' (1% in methanol), dried, examined in UV light.

Many of the spots were visible by more than one of these methods, in which case the one that gave the best visibility was used. Each spot was circled with a 6B pencil. Inside the circle was written the colour of the spot and

FIG. 3.1

Chromatogram of a Single Plant

LV5/22



Y = yellow O = orange

G = green B = blue

V = violet W = white

= spot viewed in UV light only

= spot viewed in UV light + ammonia vapour

= spot viewed in UV light + flavone reagent

= spot very faint

number inside circle indicates approximate intensity

FIG. 3.2

Master Chromatogram of a Single Population

LV5

Spot colour and detecting reagent code as Fig. 3.1

This master corresponds to the data for the population LV5 on Table 3.1

Numbers indicate the spot number assigned to each spot

TABLE 3.1.

Chromatographic Data of a Single Population.

POPULATION LV5 11.8.72.

Spot No.	1	2	4	5	6	7	8A	9	11	12	13	14	16	17	18	21	22	23	24	25	26	27	29	30	31	32	37	38	41	47	48	52	57	58	59	60	61	64	65	67	69	71	73	82	84	90	91	92	93	95	96	98	99	108	109	117
LV5-																																																								
1	2	1	2	1	6	6	4	·	5	4	1	1		2	2	·	2	1	1		3	2		·	1			1	·				1	1	1	1	1					1		3		2	·	2	2	2	·	2	2	·
2	3	2	·	2	4	5	3	·	6	4	2	2	·	3	1	1	2	2	1		3	2	·	1	1	2		1	·		1		·	·	·	·	·	1	1	1		·	1	·	3		1	·	2	2	3		2		·
3	4	3	1	3	6	6	5	·	5	4	2	1		2	2	1	3	2	2		3	2	·	·	2	1		2	·	·	1			1	1	1	·	·	1	2	1		1	1	·	3		2		2		2		·
4	2	3	2	1	4	5	3	·	5	4	2	2	·	2	·	·	2	1		·	2	1	·	·	·	1		1	·				1	2	2	1	1	2	1	2	·	·	·	·	3		·	·	2	2	2		2	2	1
5	3	3	3	2	6	6	4	·	4	4	2	1	1	2	2	·	3	2		1	3	2	1	·	1	1		2	1		1			1	1	1	1	1	1	2	2	1	·	1	·	4	1	2	1	3	3	3		2	1	1
6	2	3	3	2	4	5	3	·	4	4	2	1		2	·	·	1	2			2	1		·	·			·			1	·	·	·				1	1			1		3	1	1	·	3	3	3		2	2	1
7	4	3	3	3	4	7	4	·	7	4	1	·		2	1	·	1	1	1		2	1		·	1	·		·	·		·	1	4	4	4	4	1	3	1	1	2	·		·	2		·	1	2	3	3	1	2	1	·
8	2	3	1	1	5	5	3	·	4	4	2	1	·	2	2	·	3	2		·	3	2		·	1	2		2		·	1	1	1	1				1	1			2	1	·	3	1	1	1	2	2	3		2	1	·
9	4	3	1	1	5	6	3	·	4	5	1	1	1	1	2	1	2	2	·		3	2	·	1	2			1	·		1			2	2	1	1	1	2	2		1	·	1		4		1	1		3	1		2	2	·
10	4	4	2	2	7	7	4	·	7	5	2	2	1	3	2	·	3	2	·		3	2	·	·	1			2	·				·	3	3	2	2	2	2	1		1	·	1	·	4	·	1		3	3	1	·	2	2	1
11	3	·	2	2	7	7	4	·	7	4	2	1		3	3	1	3	2	1		3	2		·	1			·	1		1		·	1	1	·	·	2	2	2			·	2	·	4		2	2	1	1	2		2	2	1
12	3	3	2	1	6	5	3	·	4	4	2	1	·	1	2	2	3	1	3		2	1		·	·	1		2	1	1			1	2	2	·	·	1	1	1	1	·	·	1	·	4		1	1	2		2		2	1	·
13	2	3	2	1	4	4	3	·	4	3	2	1		2	2	1	2	1	·		2	1		1	1	·		1		1	1	1	1	1	·	·									2		·	·	2	2	3		2	2	·
15	3	3	2	2	6	6	4	·	5	4	2	2		1	2	3	3	·	·		3	2	·	1	2	1		1	·	1	1		·	2	2	1	1	1	2	1		1	·	1	·	3		2	1	3	2	3		2	1	·
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19	3	3	3	2	5	6	3	·	4	5	2	2	·	2	1	·	2	3	2		3	2	·	·	1		·	1	·	1	1	·	2	2	2	2	2	1	3	2	1	1	1		·	4		·	2	3	3	3		2	2	1
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21	3	2	2	2	4	4	2	·	3	3	2	1	1	2	1	·	2	2		·	2	1		1	1			1						·	·	·	·		1	1		1	·		·	3	·	2	1	2		3	1	2	1	·
22	3	3	2	1	5	6	3	·	4	4	2	·	·	2	2	·	3	2	1		3	2	·	1	2	1	·	2	·	1	1	1	1	1	4	4	4	4		1	2		2	1		2	2	1	1	2	2	2	·	2	2	·
23	2	2	2	2	4	5	3	·	5	4	2	2		2	1	·	2	2		·	2	1		·	1	·		1	·			1	1	1	1			2	1			1		·	4		1	·	2	2	3		2	1	
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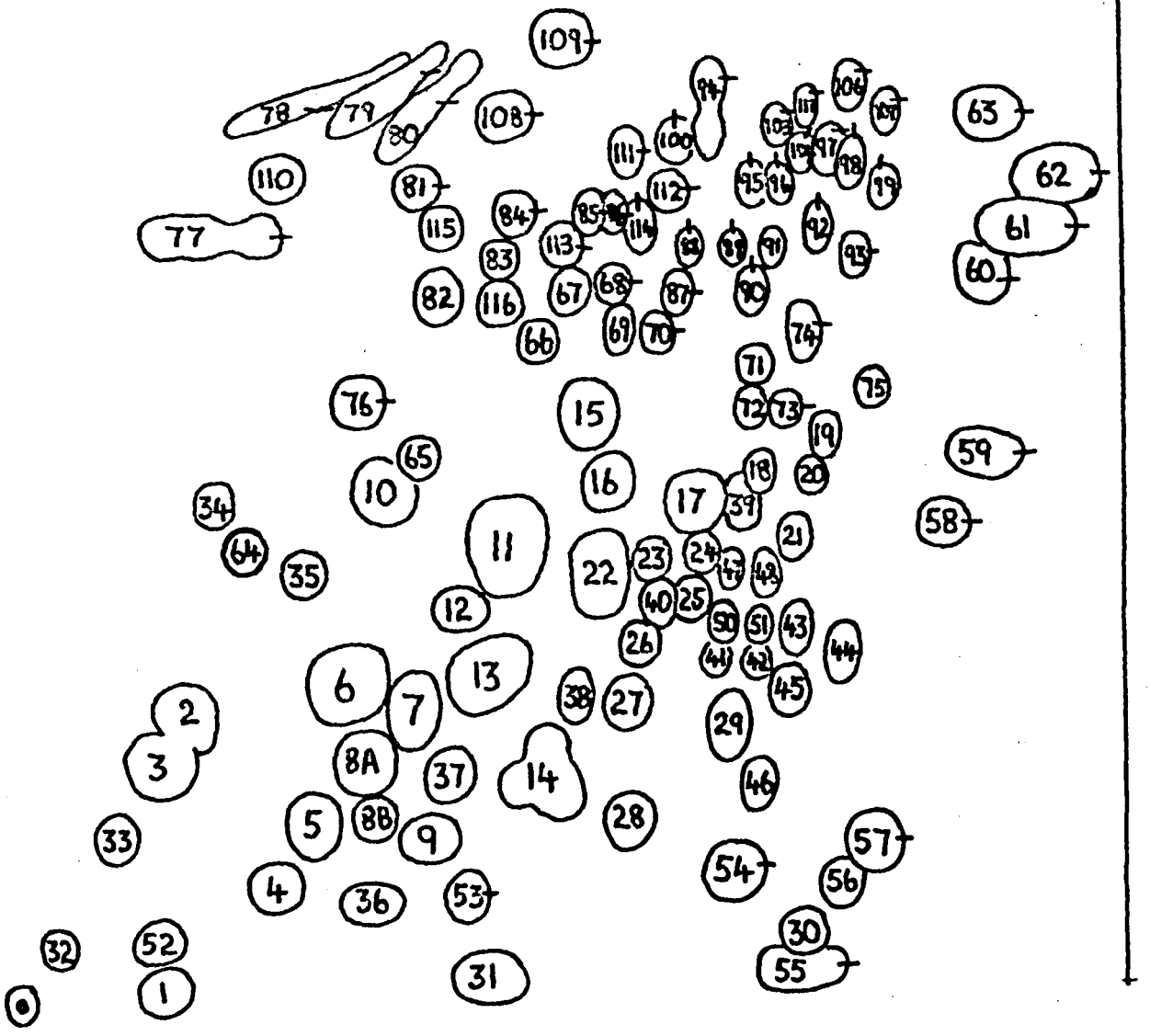
26	2	2	2	1	4	4	3	·	4	3	1	·	·	2	1	·	2	1	·		2	1		·	·			1			1	·	1	1	·	·		2				1		·	2		1		2		3	·	2	2	·
27	4	3	2	2	5	6	3	·	4	4	1	1	1	2	2	1	3	3	2		3	2	·	2	2	1		2	·		1	1	1	3	3	3	3		1	1		1	·		·	3		2	·	3	3	2	1	2	2	·
Total Intensity	25	25	25	25	25	25	25	25	25	25	25	25	17	25	25	25	25	25	16	8	25	25	14	25	25	15	4	25	16	5	16	13	22	25	25	22	22	11	24	23	7	14	25	11	20	25	7	25	22	24	21	25	9	25	23	23
	2-4	2-3	1-3	1-2	4-7	3-7	3-5	·	4-7	4-5	1-2	1-2	··1	1-3	·-3	·-2	2-3	1-2	·-2	·-1	2-3	1-2	·-1	·-2	·-2	·-2	·	1-2	·-1	1	·-2	·-1	·-1	1-4	1-4	·-4	·-4	1-2	1-3	1-2	1-2	·-2	·-1	1	·	2-4	·-1	1-2	·-2	2-3	2-3	2-3	·-1	2	1-2	·-1
Frequency	1	1	1	1	1	1	1	1	1	1	1	1	·68	1	1	1	1	1	·64	·32	1	1	·56	1	1	·60	·16	1	·64	·20	·64	·52	·88	1	1	·88	·88	·44	·96	·92	·28	·56	1	·44	·80	1	·28	1	·88	·96	·84	1	·36	1	·92	·92

FIG. 3.3

Master Chromatogram of all LIMONIUM Spots



Numbers indicate the spot numbers used in Appendix II and all other chromatograms

an estimate of its intensity compared with other spots on the same plate, on a scale of 1 for the weakest and 7 for the strongest. To maintain consistency in the relative intensity estimates, repeated comparisons between plates of a run were made. Very faint spots were outlined with a dotted circle. If necessary the detection method was indicated by a coded mark on the edge of the circle. To avoid subjective observations, plates were coded with a running number so that during observation the identity of the plant was unknown. A tracing of a typical chromatogram of an individual plant is given in Fig. 3.1.

A master chromatogram for a population was built up to include all possible spots. An example of a master chromatogram is given in Fig. 3.2. The spots on the master were numbered, and on a separate table each plant was scored for presence and intensity, or absence, of each numbered spot. An example of a table of a whole population is given in Table 3.1.

As the populations were processed a series of masters for various populations and species was acquired. From these an overall master was built up and spots were cross-identified by their position and colour in detecting reagents. The overall master is shown in Fig. 3.3. The spot scores for each population were transferred to one large table, see Appendix II. Each spot score was converted to a frequency in the particular population, and beneath the frequency is shown the intensity range for that spot. This table only gives information about whole populations; data for individual plants is lost in this method of recording, but to produce this information for over 350 plants would take too much space. At the top of the table, beneath the spot number, is shown the spot colour and the identifying method. Over all populations of all species a total of 115 spots was recorded.

(c) Chemical Nature of the Spots.

In the overall master of Fig 3.3, generally speaking the spots on the lower left, nos. 1 - 46, were absorbing in UV alone, and various shades of

fluorescent yellow in UV + ammonia and UV + Flavone Reagent. From several sources of information these properties suggest that the spots are flavone or flavonol glycosides, except for spots nos. 1 and 31 which are probably aglycones because they did not migrate in the organic solvent and fluoresced in UV alone. It is likely that some of the spots nos. 1 - 46 are derivatives of myricetin and quercetin because Harborne (1967b) showed that these two flavonoids in particular are found in Limonium species. The remainder of the spots, nos. 47 onwards, were a heterogeneous group of greens, blues and violets, and did not all have common reactions to the various detection methods. Some may be other types of phenolic compound, but no chemical identification of any of the spots was attempted.

3. 4. SOURCES OF ERROR

(a) Introduction

It was shown by other workers that differences between chromatograms may exist for non-genetic reasons, such as physiological age of the plant, or variations in the environment. It is not possible to control these factors when dealing with material gathered from wild populations. Others have also expressed doubts about the reliability of some spots in showing genetic differences for experimental reasons, and have rejected some of their data. I have also found difficulty in recording some of the spots, and feel that some need to be eliminated because their scores are unreliable in showing genetic differences between plants or populations.

(b) Difficulties of Identification

(i) Faint Spots For both the yellows and non-yellows some were faint and very difficult to score. Within a population run the same spot would be just visible in some plants but invisible in others, and the decision of its presence or absence was arbitrary. This was particularly so if the spot resembled the bluish-white background colour of the plate. Some of these faint spots, particularly nos. 16, 32-39, 42-46, were streaky and may have been

decomposition products of the stronger yellows. In the master chromatogram of Fig 3.3, the following spots were rejected because they were faint: nos 16, 28, 32-39, 42-46, 53-56, 62, 63, 66, 68, 70, 75, 86-88, 91, 93. However Appendix II shows that some of the retained spots were also faint in some populations, these being: nos. 9, 18, 25, 27, 29, 41, 47, 58, 57-61, 64, 69, 73, 84, 95, 97, 99, 103, 106. These were retained because in some plants or populations they were much stronger and could definitely be scored as present.

(ii) Cross-Identification In a few cases spots might have been confused with each other during the drawing up of the master chromatogram, because of their closeness and similarity in colour, particularly those furthest from the origin in the top right hand corner. To some extent the constant and reliable spots nos. 90, 92 and 98 could be used as markers, but spots nos. 100, 102, 104, 105, 107, 108, 111-117 had to be rejected because they could not be cross-related between runs with confidence.

(iii) Double Spots After examining many chromatograms I felt that some of the non-yellow spots had sometimes split into two adjacent spots and should be counted as one. These pairs were nos. 48/49, 67/69, 84/85, 95/96, 94/101. In each case the score of the pair was compounded if necessary, and the underlined member retained.

In L. binervosum extracts, spot no. 8 appeared double, and the two spots scored separately as 8A and 8B. In the other two species a single spot was observed at the same position as this pair, which was judged to be equivalent to 8A, although this may not have been so. I also felt that spots nos 13 and 14 in all three species were both compound spots, but because they were not sufficiently separated for this to be clear they were each treated as single spots.

(iv) Interference by the Solvent Fronts Spots nos. 62, 108-110 were obscured or distorted by the solvent fronts and were rejected. Some others were

observed in these regions but were never recorded for this reason.

(c) Repeated Runs

(i) Repeats of Extracts It would have been preferable to run every extract more than once, but because of the large number of plants involved this was not possible. Results of some extracts that were repeated within the same run are shown in Table 3. 2 (a). With the exception of two or three fainter spots out of 40 to 50 in each case, the spots were repeatable in terms of presence/absence. Also, particularly for the stronger yellow spots the relative intensity estimates were comparable. For example, if spot no. 7 was strongest in a chromatogram, it remained so in the repeat, and the other stronger yellows bore the same intensity relationship to each other. Spots which were rejected because of unrepeatability in the same run were nos. 16, 32, 87, 91, 110.

(ii) Repeats of Powdered Populations For populations LV1, LH3 and LB2, after the leaves were ground for a run, some of the dry powder remaining was stored in the dark in a refrigerator and extracted and run at a later date, meaning that a whole population after powdering was extracted and run twice. The results for LH3 are shown in Table 3. 2 (b). The pairs of scores for five randomly selected plants are shown, together with the overall scores for both runs. The following spots were rejected because they were inconsistent: nos 32, 38, 42, 43, 52, 56, 63, 67, 74, 83, 91, 93, 104, 110. For the stronger yellow spots there was reasonable agreement in the intensity estimates, certain spots being repeatedly judged to be stronger than others, such as nos. 6 or 7.

Similar results were obtained when the LV1 and LB2 re-runs were compared, but these were given less emphasis because they were the first two populations to be analysed, and during their first runs techniques of chromatography and recording were still being developed. In the table of results of Appendix II, the second run of all three populations was used.

TABLE 3.2.

Chromatographic Data of Repeated Runs.

Table 3.2(a). Repeats of extracts in the same run.

Spot No.	1	2	4	5	6	7	8A	9	11	12	13	14	16	17	18	21	22	23	24
LV5/7	4	3	3	3	4	7	4	•	7	4	1	•		2	1	•	1	1	1
	4	3	2	3	4	6	4	•	6	4	1	•		2	1	1	1	1	•
LV5/17	4	2	2	2	7	7	4		6	5	2	•	•	3	3	1	4	3	1
	3	2	2	2	7	7	3		6	5	2	•		3	3	1	4	3	•
LH1/12	4	3	3	2	5	5	3	1	3	3	3	2	•	1	2	2	3	2	3
	5	3	3	2	6	5	3	1	3	3	3	2	•	2	3	2	3	2	3
LH12/6	3	3	3	3	3	4	3	1	3	3	3	2	•	2	1	1	2	2	2
	3	4	3	3	3	5	3	1	4	4	3	2	•	2	1	1	2	2	2
LH12/7	4	4	3	3	6	5	3	1	4	4	3	2	•	2	3	2	2	2	3
	4	4	3	3	6	5	4	1	4	4	3	2		2	3	2	3	2	3

Spot No.	25	26	27	29	30	31	32	37	38	41	42	43	47	49	52	53	54	56	57	58	59	60	61
LV5/7		2	•		•	1	•		•	•					•				1	4	4	4	4
		2	•		•	1			•	•									•	1	1	1	1
LV5/17		4	•		2	3			•					2					•	•	•	•	•
		4	•		2	2			•					2					•	1	1	•	•
LH1/12	•	3	•	•	2	3			•	•			2	1					1	3	3	2	2
	•	3	1	•	2	3			•	•			2	2			1		3	3	3	1	2
LH12/6		2	•	1	1	1	1		•				4	1			2		2	1	1		
		2	•	1	1	1	1		•				4	1			1		2	2	2		
LH12/7		3	•	•	2	2			•				2	1		1	2		2	4	4	3	3
		2	•	•	2	1	•		•				2	2		1	2		2	4	4	1	1

Spot No.	62	63	64	65	67	69	71	73	74	82	83	84	87	90	91	92	93	95	98	99	103	104	109	110
LV5/7			1	3	1	1	2	•				•		2		•	1	3	3	1	•		1	
			1	2	2	1	2	•				•		3		1	1	2	2		•		•	
LV5/17				2	1		1	1				•		4		1	1	2	3		•		2	
				1	•		1	1				•		4		2	1	2	2		1		2	
LH1/12	2		1	3		1	3	1			1	1	•	3		1		1	2		•			
	2		2	3		2	4	1			1	1		4		1		1	2	•	1			
LH12/6			2	3		1	1	2				4		2	2			3	2			4		5
			2	3		1	1	1				4		2				3	2			4		5
LH12/7			4	2		2	2	1		2		2		3	•	1		1	1		1			2
			3	2		1	1	•		2		1		3		1		1	1		1			2

Table 3.2(b). Two runs of the same powdered population. (Spot numbers as above).

Spot No.	1	2	4	5	6	7	8A	9	11	12	13	14	16	17	18	21	22	23	24
LH3/5	5	3	3	2	5	5	3	1	4	3	2	2	•	2	2	2	2	2	4
LH3/5R	4	4	3	2	5	5	3	1	4	4	3	2	•	2	2	1	2	2	3
LH3/10	6	4	4	3	7	6	2	1	5	4	3	3	•	2	2	3	2	2	4
LH3/10R	5	4	4	3	6	6	3	1	4	4	2	2	•	2	2	1	2	2	3
LH3/15	3	3	3	3	4	4	3	1	3	3	2	2	•	1	2	1	2	2	2
LH3/15R	4	3	4	2	5	5	3	1	4	4	3	2	•	2	1	1	2	2	2
LH3/20	3	4	4	3	5	6	3	2	4	4	3	3	•	2	2	2	2	2	3
LH3/20R	4	5	4	3	5	5	3	1	4	4	2	2	•	2	2	1	2	2	3
LH3/25	5	3	3	3	6	5	3	1	4	4	2	2	•	2	3	2	2	2	4
LH3/25R	5	4	4	3	6	6	3	1	4	4	3	2	•	2	2	1	3	3	3
LH3 Total	25	25	25	25	25	25	25	25	25	25	25	25	25	25	25	25	25	25	25
Intensity	4-6	3-4	3-4	2-3	5-7	5-6	2-3	1	3-5	3-4	2-3	2-3	•	1-2	1-3	1-3	2-3	2	3-4
LH3R Total	25	25	25	25	25	25	25	25	25	25	25	25	25	25	25	25	25	25	25
Intensity	2-5	4-5	4	2-3	4-6	5-6	3	1	4	4	2-3	2-3	•	2	1-2	-2	2-3	2-3	-2

Spot No.	25	26	27	29	30	31	32	37	38	41	42	43	47	49	52	53	54	56	57	58	59	60	61
LH3/5		2	•	•	2	3		1		•			1	2	2		2	1	3	4	4	1	1
LH3/5R	•	3	•	•	2	2		•	•	•	•	•	1	1	2		1		2	2	2	1	1
LH3/10		3	•	•	2	2		2	•	•			2	2	1		2	2	3	4	4	1	1
LH3/10R	•	3	•	•	2	2	•	•	•	•	•		2	1	2		1		2	3	3	•	1
LH3/15		3	•	1	2	2		1	•	1			1	2	1		2	2	3	4	4	3	3
LH3/15R	•	3	•	•	2	3	•	•	•	•		•	2	1	2		1	•	2	3	3	1	1
LH3/20		4	•	1	1	1		1	•				1	2	1		1	2	3	4	4	2	2
LH3/20R		3	•	1	1	2	•	•	•				2	1	2		1		2	3	3	2	2
LH3/25		3	•		2	2		1	•		•		3	2		•	3	2	3	4	4	2	2
LH3/25R	1	4	•		2	2	•	•	•				2	1	•	•	3	1	2	4	4	2	2
LH3 Total		25	25	24	25	25		25	19	18		5	25	25	17	8	25	25	25	25	25	25	25
Intensity		2-4	•	-1	1-2	1-3		-1	•	-2		-1	1-3	1-3	-2	-1	-3	1-2	1-3	3-4	3-4	1-3	1-3
LH3R Total	22	25	25	22	25	25	18	25	25	13	16	10	25	25	25	8	25	11	25	25	25	25	25
Intensity	-1	3-4	•	-1	-2	1-2	•	•	•	-2	•	•	1-2	-2	-2	-1	1-2	-1	1-3	2-4	2-4	1-3	1-3

Spot No.	62	63	64	65	67	69	71	73	74	82	83	84	87	90	91	92	93	95	98	99	103	104	109	110
LH3/5		2	1	4	1	1	3	2						2	1	1		2	2		1			
LH3/5R			1	4	•	•	1	1	2					2	1	1	1	2	3					
LH3/10		1	2	4	2	1	3	3						3		1	1	2	2	1				
LH3/10R			2	4		1	2	3	•		•	•		2	•	1	1	2	3					
LH3/15		1	1	4	1	1	4	2				•		3	2	2	1	2	4					
LH3/15R			•	2		•	•	3						3	1	1	3	2	4					
LH3/20		1	1	4	1	1	3	2						3	1	2	1	2	4					
LH3/20R			•	3	•	1	•	3	•				•	3		1		2	4					
LH3/25		1	1	4	1	1	4	3			1	•		3	2	2	1	2	3					
LH3/25R			2	4		2	1	3			•	1	1	4	3	2	1	3	4					
LH3 Total		25	25	25	22	25	25	25			7	14	7	25	19	25	12	25	25	6	9	1		3
Intensity		-2	1-2	3-4	1-2	1-2	3-4	1-3			1-2	-1	•	3	1-2	1-2	2	2-4	2-4	1	3	2		1-2
LH3R Total			23	25	8	22	23	25	10		12	14	12	25	16	25	20	25	25	2				
Intensity			1-2	3-4	-2	-2	-2	2-3	-1		-1	-1	•	2-3	-2	1-2	-1	1-2	3-4	•				

Table 3.2(c). The same population sampled wild and under cultivation.

Spot No.	1	2	3	5	6	7	8A	8B	10	11	13	14	15	17	18	19	20	21	22
LB4 wild	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	16	17
(ex 17)	1-5	3-4	3-4	1-3	4-7	-4	2-4	-2	2-3	4-6	2-3	1-2	1-2	3-4	1-3	2-3	-2	-1	3-4
LB4 cultiv.	17	17	17	17	17	17	17		17	17	17	17	17	17	16	17	17	16	17
	1	3-4	3-4	2-3	3-4	3-4	3-4		2	5	2-3	2-3	1	4	-2	3	1-2	-1	2-3

Spot No.	25	26	27	29	31	40	41	44	45	47	52	53	54	55	57	58	59	60	61	63	65	68
LB4 wild	17	17	17	17	17	17	9	5	5	11	3	2	6	16	16	14	16	2	4	2	10	4
(ex 17)	1-2	1-3	•	1-2	-2	1-2	•	•	•	-2	•	1	-1	-2	-2	-2	-2	-2	-2	-1	-1	1-3
LB4 cultiv.	17	17		17	17	17	17	7	8	10					16		17					
	1-2	2-3		1-2	-1	1-2	-2	-1	-1	-1					-2		3					

Spot No.	69	70	73	74	77	78	79	80	81	82	84	87	89	90	91	92	93	94	95	97	98	100	103	106	113
LB4 wild	14	3	10	17	15	15	16	14	14	15	16	2	3	17	3	17	2	17	16	11	16	16	14	6	4
(ex 17)	1-4	1-2	1	2-3	2-3	1-2	1-4	1-4	1-3	-2	-1	•	-1	3-4	1	2	•	-3	-2	-2	2-3	-2	-4	1-2	1
LB4 cultiv.				17	17	17	17	17	17	15	17			17	17	17	14	17	17		17		15		17
				3-4	2-3	1-2	4-5	4-5	3	1-2	1-2			2-3	1-2	1-2	1-2	4-5	3-4		3-4		2-4		2-3

(iii) Repeats under Cultivation. Several populations of L. binervosum were maintained in cultivation after initial sampling, and in two cases, LB3 and LB4, the same plants have been chromatographed from the wild and after cultivation. Data for one such population is given in Table 3. 2 (c). There were several differences between such plants, which will be expressed numerically and considered further in Chapter 4. In particular, spots nos. 76 - 81 which were peculiar to L. binervosum appeared sporadically in some wild populations, but were strong and constant under cultivation. These particular spots were rejected in wild comparisons for this species. However, it is difficult to generalise as to spots which were found in all three species without information from cultivated populations of L. vulgare and L. humile for comparison.

In one run of cultivated material, LB3F2, at the time of harvesting some plants had produced spikes about to flower while others had not, but there were no consistent differences between chromatograms of the two types of plant. Also in this run leaves at three different stages of senescence (judged subjectively) were taken from one plant and ran separately. Normal leaves were green, but heavily senescent leaves were marked with yellow and red and were wrinkled and drying out. No differences were observed between the normal and senescent leaves in the stronger yellow spots, but intensity differences were found between fainter yellow spots and non-yellows. The senescent leaves also showed two additional spots, visible in daylight as pink, and probably anthocyanins.

(d) Discussion of Sources of Error. Although extracts were generally repeatable within the same run, other types of repeats did not show the same degree of reproducibility. The same leaf material ran on different occasions gave different results despite attempts to maintain the same experimental technique throughout. The reasons for this are not clear, but chemical changes in the leaves may have taken place during storage as was found by

other workers mentioned earlier in this chapter. Also the concentration of the compounds in the extracts may have been so sensitive to extracting and running conditions that no amount of care over techniques could provide conditions of different runs sufficiently identical not to influence the results.

In the comparison of wild and cultivated material it was shown that some spots were influenced by the environment in which the plants were growing. The developmental state of the leaves also affected the chromatograms that they produced. It was shown earlier that developmental state and environment had influenced chromatograms from other plant species. It was not possible to control these factors when sampling wild material, although the different experimental conditions were probably more extreme than those experienced by wild plants from different locations or within a population. However, if the factors influenced the fainter spots only quantitatively, this may have resulted in a qualitative difference being observed between plants. Also the environmental and physiological factors may have interacted with the experimental factors indicated above as producing differences between runs.

For these reasons and because of the difficulties in identification and recording of spots already outlined, I have had to reject a number of spots; some were rejected for more than one reason. These have been listed, and are asterisked in Appendix II, and will not be used in the analysis of variation within and between populations. A total of 58 spots for wild populations of the three species have been retained, 32 yellows and 26 non-yellows. Those retained were either strong and consistent whenever they appeared (mainly the yellow spots) or else they fluctuated quite widely in intensity between plants or populations (mainly the non-yellow spots). In making these rejections I may have discarded some useful information, but on the other hand I may have retained results that do not vary for genetic reasons; it has been very difficult to draw the line between useful and useless results.

I have shown that the estimated relative intensities of the stronger constant yellow spots can be relied upon to some extent, and use will be made of this later. However, because the overall results may reflect non-genetic differences between plants or populations, only very general conclusions can be drawn in the next chapter on relative amounts of chromatographic variation and the breeding system.

CHAPTER 4.

CHROMATOGRAPHIC VARIATION AND THE BREEDING SYSTEM IN LIMONIUM

4. 1. GENERAL INTRODUCTION TO THE RESULTS

Before describing indices of variation within and between the populations, it is useful to put the overall results given in Appendix II in perspective. For this introduction a spot is described as 'variable' if it is present in less than 95 per cent of the individuals of one or more populations of a particular species, a level chosen arbitrarily for convenience.

Table 4.1 lists the variable and the constant spots and how they are distributed amongst the three Limonium species. Of the 58 spots, a total of 27 are constant, and most (24) of these are yellows. Of the 31 variable spots, the majority (23)are non-yellows; therefore constant spots tend to be yellows while the variable ones are mainly non-yellows. Of the 27 constant spots, 15 are common to all populations of all species, 4 are found in L. vulgare and L. humile but not in L. binervosum, and 8 are found in L. binervosum alone. Therefore L. binervosum differs from the other two species by a total of 12 constant spots. In the populations studied here there are no constant spots which would distinguish any L. vulgare plant from any L. humile plant. Only 3 very variable spots are found in L. vulgare alone. None of the spots which had been rejected would distinguish L. vulgare from L. humile.

4. 2. VARIATION WITHIN POPULATIONS

(a) Introduction

Table 4.2 attempts to make generalisations about spots which are more 'variable' (see above) in one species than another. Many yellow (nos. 1 - 46) spots are constant in all species, and a few yellow spots are more variable in some L. vulgare populations than in populations of the other two species, but this is not so for all populations. No generalisations can be made about the non-yellow spots (nos. 47 onwards).

Examining the data casually for differences between populations within

TABLE 4. 1.

DISTRIBUTION OF CONSTANT AND VARIABLE SPOT NUMBERS AMONGST THE THREE *LIMONIUM* SPECIES

Spot Code Number	Common to all three Species		Present in *L. binervosum* only		Present in *L. vulgare* and *L. humile* only		Present in *L. vulgare* only		Present in *L. vulgare* and *L. binervosum* *	
	Constant	Variable	Constant	Variable	Constant	Variable	Constant	Variable	Constant	Variable
	1 22	4 59	3	106	9	24	-	50	-	84
	2 27	18 60	88		12	48		51		97
	5 31	21 61	10		23	64		72		
	6 90	25 65	15		30	71				
	7 92	26 69	19			99				
	8A	29 73	20							
	11	41 82	40							
	13	47 95	94							
	14	57 98								
	17	58 103								
Total No. of Spots	15	20	8	1	4	5	0	3	0	2
Total No. of Yellow Spots	13	7	7	0	4	1	0	0	0	0

Constant: Present in 95% or more of plants in all populations of a species

Variable: Present in less than 95% of plants of at least one population of a species

* No other categories observed

TABLE 4. 2.

Estimates of Relative Amounts of Variation in Different Species for Individual Spot Numbers

	More variable in L. vulgare than L. humile or L.binervosum	More variable in L. humile than L. vulgare or L.binervosum	More variable in L.binervosum than L. humile or L. vulgare	More variable in both L. vulgare and L. humile than L.binervosum	More variable in both L. vulgare and L.binervosum than L. humile	More variable in both L. humile and L.binervosum than L. vulgare	Variable in all three Species	Difficult to Classify
	18	-	26	25	4	-	41	50
Spot	21		58				60	51
	24		59				61	72
Code	29		65				64	84
	48		73				69	97
Number	57						82	99
	71						103	106
Total number of spots.	7	0	5	1	1	0	7	7
Total of yellow spots only.	4	0	1	1	0	0	0	0

Only the variable spots of Table 4. 1. are listed here.

These classes are only estimates made by simple inspection, but of the 7 yellow spots, note that 4 are more variable in L. vulgare than the other two species.

a species in the amount of variation is more difficult. In terms of frequency, spots nos. 21, 24 and 29 in particular show more variation in frequency between L. vulgare populations than between L. humile or L. binervosum populations. Only spot no. 25 shows marked variation between L. humile populations in that it is absent from LH12, and this spot varies in frequency between L. vulgare populations.

Because clear-cut distinctions in variation levels within and between populations are not immediately obvious, and because discussion of each spot individually would be a lengthy process, the data needs to be treated numerically. Three indices of variation within populations will be examined, the first two of which are applied to the data of Appendix II, and the third to part of the raw data of each population.

(b) 'Variable Spots/Total Spots' Index

For each population spots fall into two categories; constant spots, present in all plants, and variable spots, missing in one or more plants of that population. The fraction of all the spots that is variable can be calculated. The index ranges between 0 (no variation) and 1 (all spots variable). Note that 'variable' is defined slightly differently from that of Ch. 4.1. For this present index, it is better not to choose a level arbitrarily but to consider a spot as variable whatever its frequency.

(c) 'Polymorphic Index'

This was used by Marshall & Allard (1970a) in conjunction with electrophoretic data. It is defined by the equation:

$$P.I. \sum_{i=1}^{N} \frac{P_i(1 - p_i)}{N}$$

Where p_i = frequency of the ith spot in a population

N = total no. of spots in the population

The index is symmetrical about $p_i = 0.5$ and each spot contributes the

maximum to the index at this value. The maximum value of the index is 0.25, when every spot has $p_i = 0.5$ and the minimum is 0 when all spots score 1 or 0. The value will be multiplied by 4 for comparison with other indices.

(d) Kendall's 'W'

In Chapter 3. 4 it was shown that the relative intensity estimates of the stronger yellow spots were repeatable within runs, and to some extent between runs and in different environments, within the limits of simple observation. Other workers had shown for some other species that quantitative variation can be genetically controlled, although it is certainly influenced by the environment as well. Examination of the relative intensity estimates for the stronger yellows shows that in different plants, different spots are stronger in relation to others. In Table 3.1 plants LV5/1 and LV5/3 show that spots nos. 6 and 7 are strongest and of equal intensity, while in other plants, such as LV5/2 and LV5/4, spot no. 7 is stronger than no. 6. In LV5/12 and LV5/20, no. 6 is stronger than no. 7. Other differences can also be seen.

If the spots are ranked in decreasing order of intensity for each plate, differences between plates in extract concentration and differences in the judgement of intensity between plates and runs can be reduced. Relative intensity of different spots on the same plate was easily judged visually provided the spots were of similar colour and close together. 15 stronger yellow spots of 20 plants of LV5 have been ranked in decreasing order of intensity in Table 4.3 using information abstracted from Table 3.1. The degree of agreement between the spot rankings for these plants can be expressed numerically by calculating the "Coefficient of Concordance", W (Kendall 1962).

If there are m plants and n spots, then in general the sum of all the ranks of all the spots is $\frac{1}{2}$ mn(n+1), because they are composed of a sum of m sets each of which is the sum of natural numbers 1 to n. The mean value

TABLE 4. 3.

Rankings of Relative Spot Intensity for Stronger Yellow Spots in Plants of LV5.

SPOT NO.		2	4	5	6	7	8A	11	12	13	17	21	22	23	24	26
Plant No.	1	6	9	5	15	14	12	13	11	4	8	1	7	3	2	10
	2	8	1	7	13	14	11	15	12	6	10	3	5	4	2	9
	3	10	2	9	15	14	13	12	11	6	5	1	8	4	3	7
	4	11	9	4	13	15	10	14	12	8	7	2	6	3	1	5
	5	10	9	6	15	14	13	12	11	5	4	1	8	3	2	7
	7	10	9	8	13	15	12	14	11	5	7	1	4	3	2	6
	8	11	4	3	15	14	10	13	12	7	6	2	9	5	1	8
	9	11	6	5	14	15	10	12	13	4	3	2	8	7	1	9
	10	11	6	5	15	14	10	13	12	4	9	2	8	3	1	7
	11	1	7	6	15	14	12	13	11	5	10	8	9	4	2	8
	12	11	7	3	15	14	10	13	12	6	2	5	9	1	8	4
	15	10	6	5	15	14	12	13	11	4	3	9	8	2	1	7
	17	6	5	4	15	14	11	13	12	3	8	2	10	7	1	9
	18	9	8	7	14	15	11	13	12	3	6	2	5	4	1	10
	19	11	10	6	14	15	9	12	13	5	4	1	3	8	2	7
	20	9	3	5	15	13	12	14	11	8	10	2	7	4	1	6
	22	11	7	3	14	15	10	13	12	6	5	1	9	4	2	8
	23	10	9	8	13	15	11	14	12	7	6	2	5	4	1	3
	24	12	7	6	15	14	11	13	10	5	4	2	9	3	1	8
	25	10	8	7	15	14	12	13	11	4	6	1	5	3	2	9
Totals of Ranks		188	132	112	288	286	222	262	232	105	123	45	142	79	37	147
Deviation from Mean		+28	−28	−48	+128	+126	+62	+102	+72	−55	−37	−115	−18	−81	−123	−13
(Deviation from Mean)^2		784	784	2304	16384	15876	3844	10404	5184	3025	1369	13225	324	6561	15129	169
Estimated Overall Ranking		10	7	5	15	14	11	13	12	4	6	2	8	3	1	9

Data taken from Table 3. 1; 15 numbered spots ranked in decreasing order of intensity

To calculate W:

$$\text{Mean of column totals} = \tfrac{1}{2}m(n+1) = \tfrac{1}{2} \cdot 20 \cdot 16 = 160$$

$$W = \frac{12S}{m^2(n^3 - n)} = \frac{12.\ 81118}{400.(3375-15)} = .724$$

Where S = sum of squares of deviations of column totals from mean of 160.

For further explanation, see Chapter 4. 2(d).

of the sums for each column total is then $\frac{1}{2}m(n + 1)$. The maximum sum of squares of the deviations about this mean of all the individual column totals has been shown to be $\frac{1}{12} m^2(n^3 - n)$, this applying when all of the rankings are in absolute agreement. If S represents the sum of squares of the actual deviations of the column totals about the mean value of $\frac{1}{2}m(n + 1)$, then the Coefficient of Concordance is

$$W = \frac{12S}{m^2(n^3-n)}$$

All populations were processed as described for LV5 in Table 4.3. 15 of the stronger yellow spots were used because they were the maximum number available that were of similar colour, usually strong and close together and therefore easily estimated, and were present in all plants of all populations of L. vulgare and L. humile. Because of the chromatographic differences between L. binervosum and the other two species described in Chapter 4.1, a slightly different but comparable set of spots had to be used for this species. Only 20 plants were used from each population because some extracts in a run were slightly weak and therefore relative intensities were more difficult to judge, and because some populations did not have 25 samples. Ties were not admitted, therefore if more than one spot scored the same intensity they were ranked in the same numerical order each time. 'W' was then calculated for each population as a measure of agreement between rankings of different plants. If the agreement was not perfect the coefficient was less than unity, down to 0 for minimum agreement. For comparison between this and other indices, the values of W were subtracted from 1.

(e) Discussion of the Indices

Each index attempts to measure variation within the populations, but each expresses it in different ways. The 'Variable Spots/Total Spots' indicates how many of the spots are variable, but does not take into account whether it is the same spots that vary in each population. This is also the

case for 'Polymorphic Index'. In these two indices the system of expressing each spot total separately ignores any associations which may occur between spots in particular plants; it was suggested in Chapter 3. 1 that biosynthetic relationships may exist between different spots.

Although the 'Variable Spots/Total Spots' index shows the proportion of variable spots, it does not measure by how much the individual spots vary, whether they are relatively common or rare within the population. However, the 'Polymorphic Index' does take this into account. Further, because a spot is considered most variable at a frequency of 0.5, this latter index is perhaps closer to the idea of genetic variation, where a character is felt to be more variable if it is possessed by half of the individuals rather than either nearly all or very few of them.

The first two indices are overall, coarse estimates of variation within populations, which do not take into account individual plants or spots. To do this, indices like the 'Coefficient of Similarity'(Chapter 3. 2) would have to be used, involving many calculations and producing too much data to handle easily. At least the two indices used give a single figure for each population, making the levels of variation within different populations easy to see. The 'Coefficient of Concordance', W, overcomes some of the disadvantages of the previous two indices by taking into account individual plants and spots. However, it cannot measure qualitative variation, and it would be difficulty to apply it to all the spots because constant, closely-positioned and similar-coloured spots can only be used. Applying the other two indices to the 15 spots used for 'W' would show no variation within the populations.

At the present time I know of no suitable statistical tests of significance for these indices, and there are no other suitable indices which do have tests. The reasons for the difficulties in devising such tests have been explained in Chapter 3. 2. Therefore the figures from the indices will

be examined for relative, overall trends.

(f) Discussion of Numerical Estimates of Variation Within Wild Populations.

Table 4. 4 lists the calculated values of the three indices using the data of Appendix II. The ranges of these indices are displayed in Fig. 4. 1. LV17 and LB4 have been separated from the other populations because of their unusual circumstances (see later). Table 4. 4 also includes the indices for the two repeat runs of the powdered leaves from two populations, LH3R and LB2R. Neither of these populations show the same index value as the first run, although spots thought to be unreliable had been eliminated from the calculations. However, the value of the repeat index usually lies close to the original value in comparison with other values, and at least remains within the range of other populations of the same species in most cases. The first run of LV1 has not been processed for the first two indices because it was used for developing chromatographic techniques.

Comparing the ranges of indices for L.vulgare and L.humile populations, for all indices L. vulgare populations have higher values and therefore show more variation within them than L. humile populations, except for LV17 which lies within the range of L. humile. Populations of L. binervosum are not so consistent in their indices. In terms of variable spots and Polymorphic Index they overlap with the other two species; however some have lower values for W than L. humile, and they do not overlap with L.vulgare except for LV17.

In the first two indices it is population LB4 which causes this species to overlap with L.vulgare; if LB4 is deleted, then L.vulgare (excluding LV17) becomes generally more variable than L.binervosum. The higher value for LB4 for these two indices is the result of a higher proportion of weak, variable non-yellow spots; the stronger yellow spots do not contribute much to the qualitative variation in any of the species. Possibly the higher qualitative variation within LB4 is a reflection of its environment. Plants of this population were growing among taller species and were probably experiencing

TABLE 4. 4.

INDICES OF CHROMATOGRAPHIC VARIATION WITHIN POPULATIONS

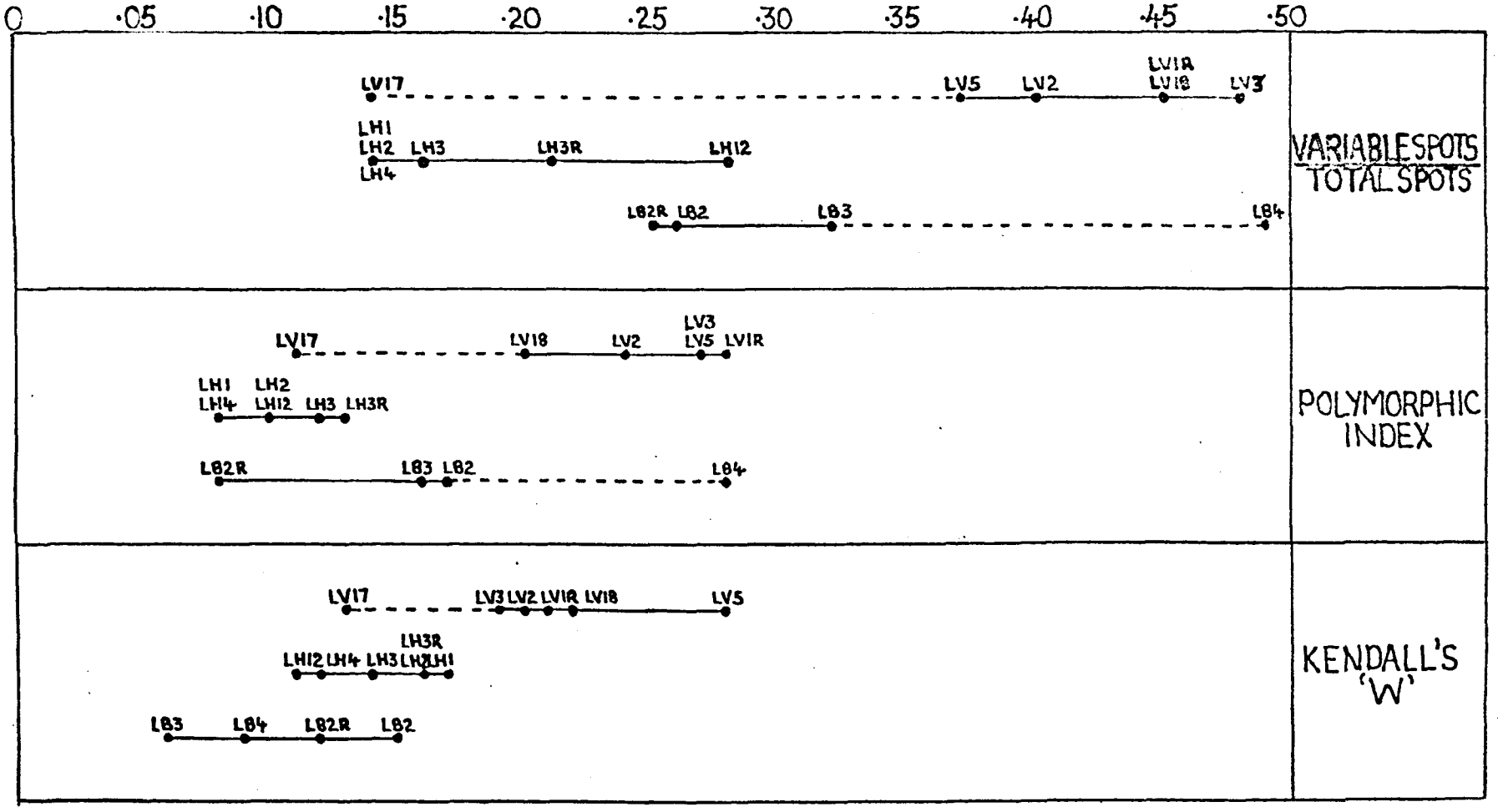
Population Code	No. of Plants	No. of Spots	Variable Spots: Total Spots	Polymorphic Index *	Kendall's W +
LV1R	23	45	.45	.28	.21
LV2	25	45	.40	.24	.20
LV3	25	44	.48	.27	.19
LV5	25	43	.37	.27	.28
LV17	10	42	.14	.11	.13
LV18	25	45	.31	.20	.22
LH1	25	44	.14	.08	.17
LH2	25	44	.14	.10	.16
LH3	25	44	.16	.12	.14
LH3R	25	43	.21	.13	.16
LH4	30	44	.14	.08	.12
LH12	25	44	.28	.10	.11
LB2	24	38	.26	.17	.15
LB2R	25	40	.25	.08	.12
LB3	25	41	.32	.16	.06
LB4	25	45	.49	.28	.09

* Value multiplied by 4 + Value subtracted from 1

In all indices, minimum variation = 0 maximum variation = 1

Figure 4.1.

Ranges of indices of variation within populations for the three Limonium species.



Data from Table 4.4.

greater competition for light, water and minerals than other populations; therefore environmental differences between individual plants were probably greater than in other populations and resulted in more variable expression in the quantities of weaker spots. Two other lines of evidence support this suggestion. Firstly, LB4 does not show an anomalously high W value, which attempts to eliminate overall quantitative differences between plants; and secondly, it will be seen later (Ch. 4.5) that under cultivation variation within LB4 is reduced to a similar level to that of its neighbour LB3.

(g) Variation Within Populations and the Breeding System

Considering first the raw data, if there was more genetically determined chromatographic variation within L.vulgare than in L. humile or L. binervosum because of their differences in breeding system, this should be shown as qualitative variation in the stronger yellow flavonoid spots. This is because studies of these compounds showed them to be under genetic control in other species,and to vary qualitatively in some cases. However,nearly all of this group of spots in the three Limonium species are constantly present in all populations. The few exceptions to this for L. vulgare were indicated in Table 4.2. Although only four yellow spots show this,and are only absent from a few plants, this may be significant. However the general lack of qualitative variation for flavonoids within this species may not be unusual because similar results were described for other outbreeding species in Ch. 3. 1.

While qualitative variation for these stronger yellow flavonoid spots is low, W shows the important feature that the spots do vary quantitatively in the three species in an order agreeing with Baker's anticipations described in Ch.1, generally L.vulgare being the most variable and L.binervosum the least. It is reasonable to assume that these relative differences are genetically caused, because W should eliminate environmental influences. Quantitative variation has been shown to be under genetic control in some other species.

When the information for both the yellow and non-yellow spots is analysed

numerically L. vulgare populations are more variable than L.humile, but the latter populations still show some variation and it is difficult to say whether this is genetic, environmental or experimental in origin. An exception to this comparison between the two species is LV17. This should be an outbreeding population and be more variable than L. humile populations, but it is not more variable. It was suggested (Chapter 2. 4.) that this population was established by the vegetative spread of a single plant, and the chromatographic and morphological (Chapter 2. 5.) evidence supports this. This would in turn imply that the observed values of the indices around those of LV17 are not caused by genetic factors.

Populations of the apomictic L. binervosum are expected to be the least variable, but they show almost as much qualitative variation as L. vulgare; again the cause of this variation is not clear. If it is genetic, then L. binervosum may be facultatively apomictic within its populations, but this does not concur with results of W and morphological information. Alternatively, like some other apomicts, L. binervosum may be highly heterozygous and capable of phenotypic plasticity in a heterogeneous environment; this suggestion would particularly fit the case of LB4 where there were probably greater environmental differences between plants than in other populations.

Considering the L. humile results in the context of Allard's findings that heavily inbreeding species may be variable because of a high selective advantage of heterozygotes, it is not possible to distinguish heterozygosity from homozygosity in the chromatographic results. If the theory was true for L. humile then this species would be as variable as L. vulgare, which is not so. Another finding was that extreme inbreeding in a population may split it up into a series of different genotypes. Again this cannot be shown chromatographically for L. humile; the plants of a population cannot be classified into a series of types either for spot presence/absence, or relative spot intensity.

(h) Variation Within Populations and Other Factors

It is also relevant to ask if the levels of variation can be related to any facts about the geographical or ecological origins of the populations. For Polymorphic Index and W, LH12 from Ireland and farthest from L. vulgare is to some extent less variable than other L. humile populations, as is LH4 which is also just out of the range of L. vulgare. The L. vulgare population farthest from L. humile, LV5, is also more variable than other L. vulgare populations to some extent. However, none of these distinctions is clear, and also no generalisations can be made about L vulgare populations in relation to their distribution limits. More samples of L. humile from the East Coast are needed before a similar comparison can be made for this species. With the possibility of hybridisation and introgression with L. humile in LV18, a highly variable population might be expected, but the indices do not show this; in any case it has been shown that the two species are chromatographically very similar.

Ecological comparisons can be made particularly between LH1 and LH2, LB3 and LB4, and LV1 and LV2. In the first two cases, the latter population might be expected to be more variable because of greater heterogeneity in the second environment. In the case of the L. humile pair differences between them are small and inconsistent, but for the two L. binervosum populations an effect is noticeable as already stated, although in this case the differences are probably mainly not genecological. For the L. vulgare pair where LV2 is from a higher part of the marsh than LV3, no important differences are detectable.

(i) Wild Putative Hybrids

It was only possible to chromatograph five plants, from Scolt Head Island (LX18), because most plants did not possess sufficient leaves. The plants did not show any unusual chromatographic features such as additional spots or spots absent, therefore their results are not reported.

4. 3. VARIATION BETWEEN POPULATIONS

(a) Average Biochemical Distance

This index has been discussed in Chapter 3. 2. It has been used previously to compare spot sizes of different plants, but here it will be used to compare spot frequencies between different populations. It is derived from Biochemical Distance

$$D^2_{jk} = \sum_{i=1}^{N} (P_{ij} - P_{ik})^2$$

Where P_{ij} = frequency of the ith spot in the jth population

P_{ik} = " " " ith " " " kth "

N = total number of spots used in the comparison

The Average Biochemical Distance is defined as

$$d_{jk} = \sqrt{\frac{D^2_{jk}}{N}}$$

D^2_{jk}, and therefore d_{jk}, can be calculated directly from the frequencies of the spots as set out in Appendix II. d_{jk} has a minimum value of 0 (no difference between populations) and a maximum of 1 (all spots fully present in one population and absent in the other).

The same 58 spots were used as in variation within populations, and a matrix of comparisons constructed. The matrix was processed by two methods of cluster analysis, the 'Nearest Neighbour' method in which the clusters are drawn up by inspection (Sokal & Sneath 1963) and the 'Weighted Mean Pair' (Gower 1967).

(b) Rank Correlation Coefficient

In the calculation of Kendall's W, the sums of the ranked spot intensities for each column can themselves be ordered to give an estimate of the overall ranking of the relative spot intensities for the whole population (Table 4.3). The measure of agreement between these rankings for

TABLE 4. 5.

ESTIMATED OVERALL RANKINGS OF STRONGER YELLOW SPOTS FOR TWO POPULATIONS LV3 and LV5

Spot No.	6	7	11	12	8A	2	26	22	4	17	5	13	23	21	24
Population Ranking															
LV5	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1
LV3	14	15	13	9	12	11	4	7	10	6	8	5	3	1	2

P = 1 + 0 + 0 + 3 + 0 + 0 + 5 + 2 + 0 + 1 + 0 + 0 + 0 + 1

The method of obtaining the overall ranking for LV5 is given in Table 4. 3.

Rankings for all wild populations are given in Table 4. 6 .

To calculate γ :

$$\gamma\,(\text{LV5-LV3}) = 1 - \frac{2P}{\frac{1}{2}n(n-1)} = 1 - \frac{2.13}{\frac{1}{2}.15.14} = .752$$

For further explanation see Chapter 4. 3(b).

TABLE 4. 6

ESTIMATED OVERALL RANKINGS OF STRONGER YELLOW SPOTS IN WILD POPULATIONS

Spot No.	2	4	5	6	7	8A	11	12	13	17	21	22	23	24	26
LV1 (1st run)	11	9	8	13	15	12	14	10	6	5	3	2	1	7	4
LV1 (2nd run)	12	11	8	14	15	10	13	9	6	5	2	3	1	7	4
LV2	9	10	4	15	14	13	12	11	6	5	2	8	3	1	7
LV3	11	10	8	14	15	12	13	9	5	6	1	7	3	2	4
LV5	10	7	5	15	14	11	13	12	4	6	2	8	3	1	9
LV17	11	8	7	15	14	10	12	9	4	3	5	6	1	13	2
LV18	11	7	3	15	14	12	13	10	8	4	2	9	1	6	5
LH1	13	12	11	15	14	6	10	9	8	2	3	5	1	4	7
LH2	13	11	10	15	14	5	12	9	7	4	2	6	1	3	8
LH3 (1st run)	13	11	9	15	14	8	12	10	6	4	3	2	1	7	5
LH3 (2nd run)	13	12	8	15	14	9	11	10	6	5	1	4	3	2	7
LH4	13	12	6	15	14	10	11	9	7	1	3	5	2	4	8
LH12	13	11	9	15	14	8	12	10	6	3	2	4	1	7	5

Spot No.	2	3	5	6	7	8A	11	13	17	19	20	21	22	40	26
LB2 (1st run)	10	9	1	15	11	7	14	5	13	8	4	2	12	3	6
LB2 (2nd run)	11	10	7	15	8	9	14	6	13	5	4	2	12	1	3
LB3	12	10	5	15	9	8	14	7	13	6	3	2	11	1	4
LB4	13	12	6	15	9	8	14	7	11	5	2	1	10	3	4

The method of obtaining these overall rankings is given in Table 4. 3.

any pair of populations can be expressed by the 'Rank Correlation Coefficient', γ (Kendall 1962), defined as

$$\gamma = 1 - \frac{2P}{\frac{1}{2}n(n-1)}$$

Where P = total of 'positive' scores

n = total number of spots used

The value of P is obtained by ordering the spot numbers in sequence of decreasing intensity for the first population, and then recording the relative spot intensities of the second population in the same numerical sequence as the first. Then for the second population, taking each spot in turn the number of spots greater in intensity lying to the right of each spot are counted up. This total is P, a measure of how much the second ranking is out of sequence with the first. An example of how P and γ are obtained is given in Table 4.5. γ has a maximum value of 1 (complete agreement between rankings) and a minimum of -1 (complete disagreement).

The estimated overall rankings of the fifteen stronger yellow spots in the different wild populations are given in Table 4.6. A matrix of rank correlation coefficients between the L. vulgare and L. humile populations together and the L. binervosum populations separately was drawn up. A slightly different set of 15 spots had to be used for L. binervosum, as explained in Chapter 4. 2(d), therefore comparisons between this species and the other two could not be made. Cluster analysis using the nearest neighbour method only was performed on the matrices of rank correlation coefficients.

(c) Discussion of the Indices

Only one index, the average biochemical distance, has been found which can be applied to all of the data for comparisons between populations, and this has been discussed in Chapter 3. 2. It seems equally as valid to apply it to spot frequencies in different populations as to spot intensity estimates in different plants. Indices like the coefficient of similarity cannot be

applied to data of whole populations.

The rank correlation coefficient is an attempt to measure quantitative variation between populations for the stronger yellow spot intensities. If the average biochemical distance index was applied to these fifteen spots alone, the distances between all populations of L. vulgare and L. humile together and L. binervosum separately would be zero, so only quantitative variation is measured. By converting the relative spot intensities to rankings it is hoped that differences between runs in experimental conditions would be eliminated. There are no suitable significance tests available for either index, so comparisons must be made on a relative basis. Both indices have two disadvantages in common. They do not take individual plants into consideration; and they are influenced by the amount and nature of the variation within populations.

(d) Discussion of Numerical Estimates of Variation Between Populations.

Table 4.7(a) gives the matrices of both average biochemical distance (upper right) and rank correlation coefficient (lower left) for all wild populations. In the three repeated populations, the second runs have been used for comparisons.

Average biochemical distances have also been calculated between the two repeated populations LH3-LH3R and LB2-LB2R, the values being .125 and .349 respectively (Table 4.7(b)). The second value is high, but this may be because LB2 was one of the earliest runs and experimental techniques had not been perfected; this figure is therefore given less emphasis. Although the distance for the other repeat is lower, it is still greater than the smallest distance between any two populations (LH3-LH4, .106), suggesting that some of the differences between populations may be the result of differences between runs. Rank correlation coefficients between the repeated runs also sometimes show greater differences than between populations, particularly for LB2 (LV1-LV1R, .905; LH3-LH3R, .810; LB2-LB2R, .733), but no coefficients between

TABLE 4. 7.

INDICES OF VARIATION BETWEEN WILD POPULATIONS

4. 7(a) Matrix of average biochemical distances and rank correlation coefficients between wild populations

	LV1	LV2	LV3	LV5	LV18	LV17	LH1	LH2	LH3	LH4	LH12	LB2	LB3	LB4
LV1		.227	.263	.274	.247	.310	.261	.248	.256	.253	.275	.600	.636	.580
LV2	.562		.205	.275	.221	.315	.296	.250	.282	.301	.333	.619	.629	.587
LV3	.771	.752		.257	.278	.330	.339	.314	.328	.339	.343	.598	.604	.562
LV5	.562	.848	.752		.246	.258	.325	.319	.302	.311	.300	.616	.570	.550
LV18	.676	.771	.714	.733		.207	.235	.242	.223	.243	.221	.623	.627	.582
LV17	.714	.505	.638	.467	.657		.281	.306	.288	.303	.232	.651	.667	.616
LH1	.714	.505	.638	.543	.581	.543		.112	.153	.127	.223	.648	.675	.616
LH2	.733	.581	.676	.619	.581	.505	.886		.160	.157	.279	.643	.666	.616
LH3	.771	.676	.771	.714	.600	.524	.791	.848		.106	.261	.646	.667	.613
LH4	.752	.567	.676	.619	.695	.581	.810	.771	.867		.238	.637	.665	.606
LH12	.886	.562	.695	.600	.676	.676	.829	.829	.810	.791		.642	.658	.588
LB2													.418	.314
LB3												.905		.243
LB4												.829	.886	

Upper right = Average biochemical distance (smaller distance = greater similarity)

Lower left = Rank correlation coefficient (higher coefficient = greater similarity)

In the rank correlation coefficients, comparisons between L. binervosum and the other two species were not possible because different spots were used- see text

4. 7(b) Comparisons between repeated runs of the same powdered leaves of some wild populations

	Biochemical distance	Rank Correlation Coefficient
LV1 - LV1R	-	.905
LH3 - LH3R	.125	.810
LB2 - LB2R	.349	.733

Figure 4.2.

Dendrogram of weighted mean pair cluster analysis of average biochemical distance between wild Limonium populations.

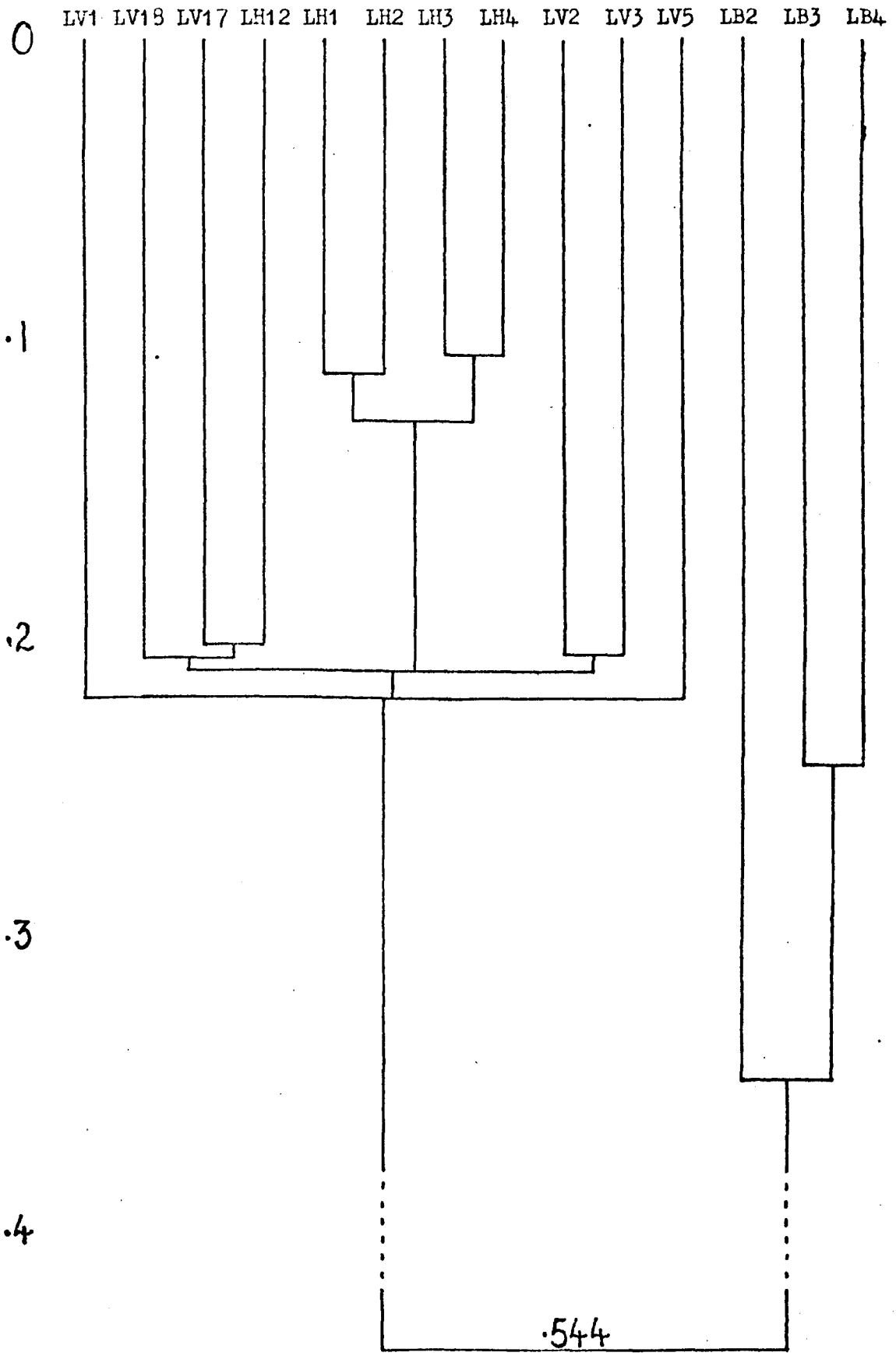


Figure 4.3.

Dendrogram of weighted mean pair cluster analysis of average biochemical distance between wild Limonium populations (excluding LV17).

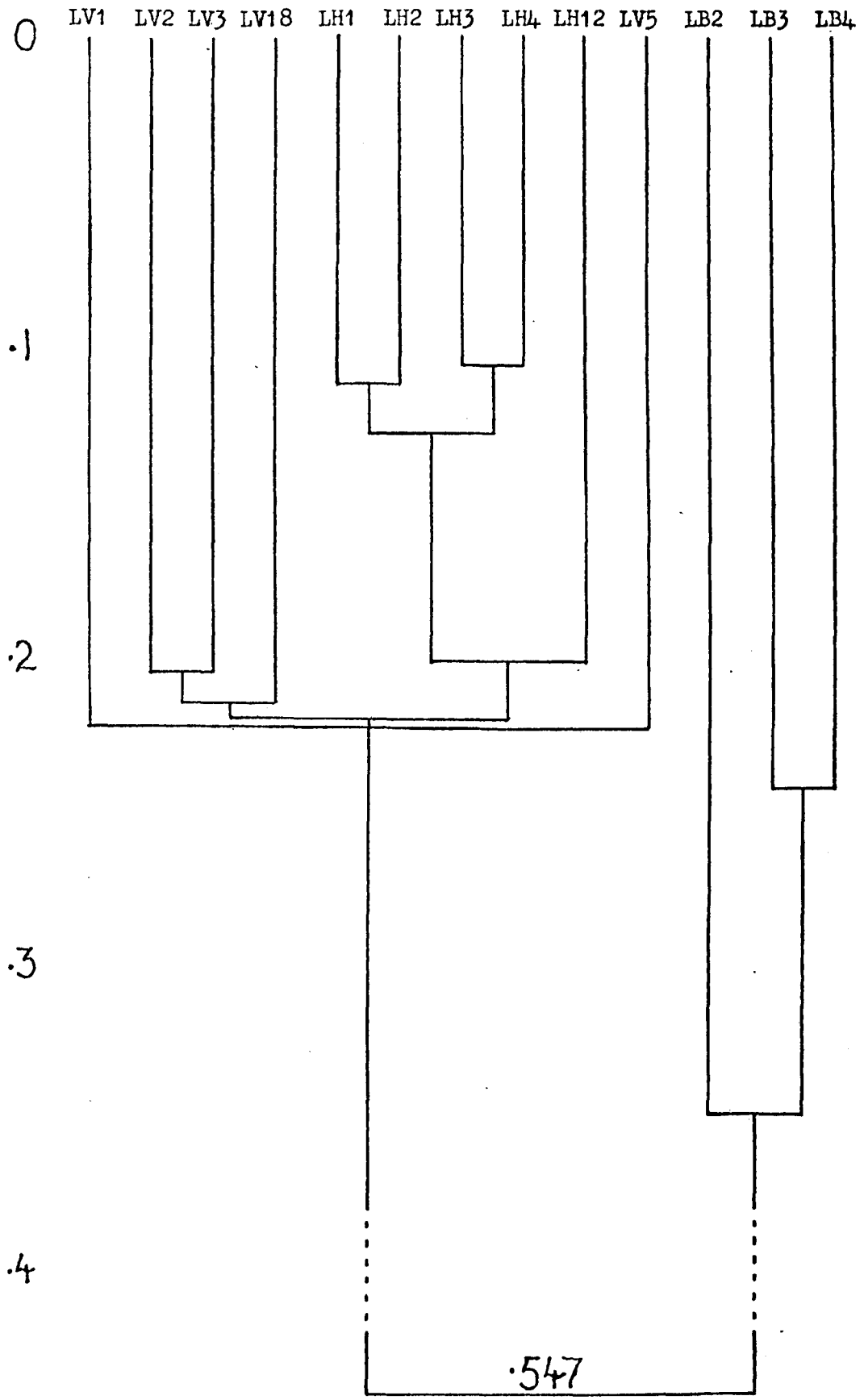
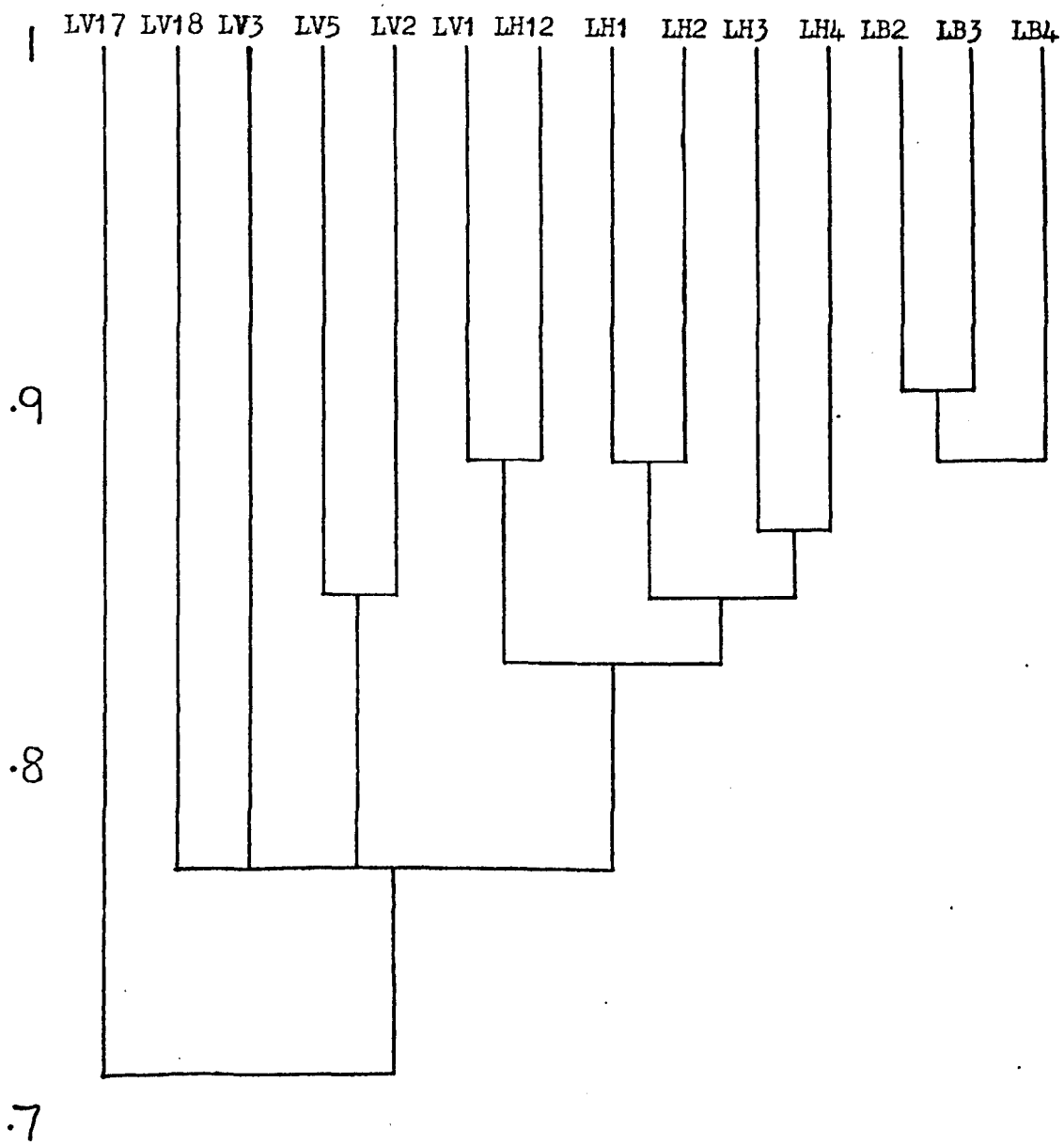


Figure 4.4.

Dendrogram of nearest neighbour cluster analysis of rank correlation coefficient between wild Limonium populations.



populations exceed .905, and the majority are less than .810, showing that this index does have a greater repeatability. However, for both indices the suggestion that differences between all populations were the result of differences between runs alone cannot be confirmed unless all populations are repeated, and the drawing up of a matrix of comparisons tries to eliminate these experimental differences. If all differences between populations were the result of differences between runs alone then all values would be in the region of .125 for d_{jk} and .833 for γ

Comparisons were also made between some L. binervosum populations sampled in the wild and then cultivated in the greenhouse; full details are given in Chapter 4. 4(d). For LB3(wild) - LB3 (cultivated) d_{jk} = .271 γ = .752 and for LB4, d_{jk} = .244, γ =.791. The qualitative variation (d_{jk}) is high in relation to comparisons between different wild populations; the effect is not so marked for the quantitative variation of the fifteen stronger yellow spots. Therefore differences detected between populations may be environmentally rather than genetically determined particularly for d_{jk}, although this has only been proved for L. binervosum and under the very different ecological conditions of the field and the greenhouse.

Fig 4.2 gives the dendrogram resulting from the weighted mean pair cluster analysis of the average biochemical distance matrix. It showed little difference from the dendrogram obtained by the simpler method of nearest neighbour analysis, but it will be used as it is considered to be more accurate. The most closely associated populations are those of L. humile, except for LH12, clustering in the region of 0.11 - 0.13. L. vulgare populations do not cluster until 0.20 - 0.22. A curious grouping is that of LV17 and its lack of variation within the population may have created this false similarity to LH12, and the fact that LV17 and LV18 were chromatographed in the same run may be the cause of their similarity. In Fig. 4.3 the data has been re-analysed with the omission of LV17, and this gives the more reasonable

result that LH12 joins other populations of L. humile before joining L. vulgare, and that LV18 joins L. vulgare and L. humile populations almost simultaneously which can be related to its occurrence with L. humile in the field.

In general the dendrograms of d_{jk} show that there are greater differences between L. vulgare populations than between L. humile. Variation between L. binervosum populations is even greater than the other two species, even for LB3 and LB4 which were adjacent populations.

It was only possible to analyse the matrix of rank correlation coefficients by the nearest neighbour method, but the weighted mean pair method would probably have yielded a very similar dendrogram. The dendrogram (Fig 4.4.) shows similar relationships of LH1 through to LH4 as in the qualitative analysis, but some unexpected similarities occur between the well-separated LV2 and LV5 populations, and LB2 and LB3, and between the unrelated LV1 and LH12. LV3 is removed from its neighbour LV2, an unexpected difference. Therefore rank correlation coefficients of relative spot intensities of the stronger yellow spots do not always agree with the average biochemical distance figures for all the spots in their conclusions. However there is no reason why they should because they are measuring different kinds of variation, and are calculated in different ways. The unexpected associations between some individual populations may be spurious as they occur at similar levels to those of variation between repeat runs of the same population and between repeats under cultivation. The main agreement between the two types of index is that they both show greater variation between L. vulgare populations than L. humile.

(e) Variation Between Populations and the Breeding System

If there was greater chromatographic variation between inbreeding populations than outbreeding ones, as anticipated by Baker, it should be shown in the stronger yellow spots; L humile would have spots present in all plants of some populations, but absent in other populations. This was not found,

except that LH12 does not have spot no. 25, present in other populations. In the numerical analysis of qualitative data, L. humile and L. vulgare show the reverse of expectation. Perhaps this is due to the nature of the variation within populations; for there to be lower variation between L. vulgare populations by this method, variation within populations must be in the same spots and at a similar level, which is not so in the data of Appendix II. Because L. humile is less variable within its populations, the consequence is less variation between populations. L. binervosum has expectedly high qualitative variation between its populations, but this may not be genetic. It could be caused by environmental differences between populations, or be influenced by the relatively high variation within LB4.

The analysis of quantitative variation between populations is also difficult to relate to their breeding systems alone. Variation between L. vulgare populations is generally higher than that of both the other species, again perhaps because levels of intrapopulational variation were similar for L. humile and L. binervosum, and lower than L. vulgare.

(f) Variation Between Populations and Other Factors.

Detailed consideration of individual populations may be taking the coarse numerical estimates beyond their limits of reliability. The pairs of populations that are separated by ecological differences alone, LV2-LV3, LH1-LH2 and LB3-LB4, cluster with each other before joining any other populations of their species for qualitative variation, so it appears that ecotypic differentiation for Limonium species cannot be demonstrated chromatographically; variation between these populations is probably only due to differences in environmental factors causing phenotypic variation. Also levels of repeats of the same population were similar to those above. Quantitative differences between the adjacent populations are not consistent enough to draw conclusions from; no reliable quantitative relationships can be seen between geographically separate but ecologically similar populations.

However, within L. vulgare and L. humile, for d_{jk} populations LV1-LV5 and LH3-LH4 which are geographically separated but ecologically similar do cluster together at similar levels to the adjacent pairs; therefore it appears that ecological differences between populations yield similar degrees of qualitative biochemical separation as geographical ones. Three other populations geographically well-separated are LV17, LH12 and LV18, but these cluster together before joining other populations. Reasons for this have already been suggested in Chapter 4. 3(d).

4. 4. CHROMATOGRAPHY OF CULTIVATED L BINERVOSUM POPULATIONS.

(a) Introduction

The cultivated populations of L. binervosum described in Chapter 2.5 and Table 2.3 were subjected to chromatographic analysis using the same methods as for wild populations. The overall results for these populations are given in Appendix III. Table 4.8 explains the symbols used to indicate all the runs of the cultivated material.

(b) Spots Used

When present in L. binervosum, the same selected spots as Appendix II were used, with the following exceptions. Spots nos. 77-81, present sporadically and faintly in wild material and rejected for wild comparisons, were strong and easily detected under cultivation, and also contributed important information about differences between some populations, therefore these spots were used in the calculations. Spots nos. 4, 58, 69, 76 and 106, used in the wild calculations, showed noticeable differences between repeat runs of cultivated LB3, and were therefore rejected.

(c) Repeated Runs

The main repeated runs were those of LB3, run three times during cultivation, re-sampling the same plants on each occasion. Some spots were rejected on the basis of these repeats, as above. For comparison of these runs, indices of variation within and between different runs of the same

population are given in Table 4.9 and 4.10(b) respectively. For LB3 repeats the indices of variation within the different repeats are not identical, despite the rejection of spots, but the indices do show similar levels of variation in each run. Differences between runs are seen most clearly in Table 4.10(b), showing that even under cultivation the runs are not entirely repeatable. The differences may reflect differences in growth conditions from one season to the next, or uncontrollable experimental differences between runs. However, the largest average biochemical distance value between these repeats, LB3F2-LB372, is only .171, which is lower than all of the distances between different cultivated populations (except LB372-LB472, .111, which were adjacent populations). The rank correlation coefficients between relative intensity estimates of different runs of LB3 were also higher than those between different populations under cultivation. This suggests that numerical differences between different cultivated populations are at least in part the result of genetic differences. Of the three LB3 cultivated runs, LB372 was used for comparisons with other populations.

After discovery of two morphological types of LB8, chromatographic differences between the two forms were found. Because of the importance of this finding, after the main run of the whole population, five plants each of the two forms were re-run on two further occasions. The same qualitative and quantitative differences between the two forms were found each time, showing that these differences under cultivation were repeatable.

(d) Differences Between Wild and Cultivated Populations

Populations LB3 and LB4 were run both sampled directly from the wild and from under cultivation. The indices of variation within the wild populations have been re-calculated using the same spots and the same plants as the cultivated runs, so that valid comparisons can be made. Re-calculated indices of variation within populations are included in Table 4.9. Comparing LB372 and LB472 with their wild counterparts, the LB3 indices are almost identical,

TABLE 4. 8.

EXPLANATION OF SYMBOLS USED TO INDICATE L. BINERVOSUM POPULATIONS CHROMATOGRAPHED

Pop. Code	Date Run	No. Plants	
LB2R	16.2.70	25	Sampled direct from wild-growing material
LB3	12.5.70	25	" " " " " "
LB4	26.5.70	25	" " " " " "
LB3W	12.5.70	25	Same as LB3 run, but slightly different spots used for comparison with cultivation
LB4W	26.5.70	25	" " LB4 " " " " " " " " " " " "
LB3F1	22.6.70	20	LB3 plants grown on in cultivation, and then chromatogrammed.
LB3F2	14.5.71	20	" " " " " " " " " "
LB372	29.5.72	20	" " " " " " " " " "
LB472	29.5.72	17	LB4 " " " " " " " " " "
LB21	2.6.72	20	Re-sampling of LB2 site, plants grown on in cultivation before chromatography
LB8Br	3.6.71	13	Broad-leaved plants described in Ch. 2 grown on in cultivation before chromatography
LB8Nr	3.6.71	23	Narrow- " " " " " " " " " " " " " "
LB8All	3.6.71	36	Not a separate run, but combined data of LB8Nr and LB8Br
LB9	30.11.71	25	Plants grown on in cultivation before chromatography

In the first group (LB2 ⟶ LB4W) the symbols refer to runs of populations which had been growing wild and were chromatogrammed using wild-growing leaves. In the second group (LB3F1 ⟶ LB9) the populations were grown on under cultivation and sampled and chromatogrammed on the running date.

TABLE 4. 9

INDICES OF VARIATION WITHIN WILD AND CULTIVATED *L. BINERVOSUM* POPULATIONS

Pop. Code	No. of Plants	No. of Spots	Variable Spots: Total Spots	Polymorphic+ Index	Kendall's ‡ W
LB2R	25	40	.25	.08	.12
LB3	25	41	.32	.16	.06
LB4	25	45	.49	.28	.09
LB3W*	20	43	.23	.14	.06
LB4W*	17	46	.41	.23	.09
LB3F1	20	45	.24	.14	.06
LB3F2	20	43	.26	.13	.04
LB372	20	44	.23	.11	.07
LB472	17	44	.14	.07	.05
LB21	20	46	.28	.16	.11
LB8Br	13	43	.23	.16	.08
LB8Nr	23	33	.18	.06	.06
LB8A11	36	43	.46	.32	.17
LB9	25	47	.26	.13	.14

\+ Value multiplied by 4 ‡ Value subtracted from 1

In all indices, minimum variation = 0 maximum variation = 1

*To enable direct comparisons between wild and cultivated plants, indices have been re-calculated using the same plants and same spots as the cultivated material.

For explanation of symbols for various populations, see Table 4. 8.

but in LB4 the variation within populations is considerably reduced under cultivation. This supports the earlier theory that variation in the wild LB4 population is the result of variation in the population's natural environment; reduction in Kendall's W is not so marked, but it was relatively low in the wild population. The biochemical distances between wild and cultivated populations (Table 4.10(b)) are also high in relation to comparisons of different wild populations, showing that differences in the environment considerably influence the chromatographic results; however, wild and cultivated biochemical distances are often lower than distances between different populations cultivated under the same conditions. It is more difficult to make generalisations about the rank correlation coefficients, but they give the impression that this estimate is more reliable in comparing populations from different environments than d_{jk}.

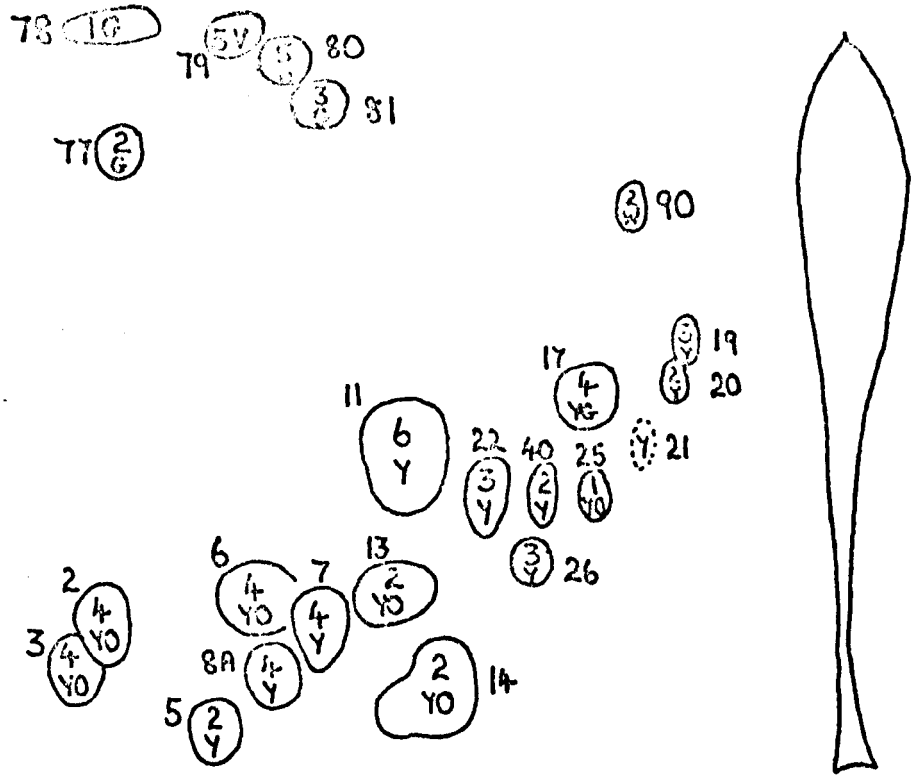
(e) Variation Within Cultivated Populations.

The indices are given in Table 4.9. With the exception of LB8, most of the populations have approximately 25 per cent of their spots as variable, and this variation is of the fainter, non-yellow spots. Polymorphic indices and W are also low compared with wild populations. For all indices the most variable population is LB8. Chapter 2.5(b) described two morphological types within this population, and when the plants are separated into two groups LB8Br and LB8Nr corresponding to leaf morphology, variation is comparable to other L. binervosum populations under cultivation. Apart from this mixed population, LB21 is the most variable qualitatively and quantitatively, but it is difficult to find a reason for this.

(f) Variation Between Cultivated Populations.

Even without the use of indices, certain obvious chromatographic differences were observed between populations; Figs. 4.5 - 4.10 show chromatograms of representative individuals from the various populations, but with only the stronger spots that show major differences drawn in. These spots

FIG. 4.5. LB3/16



Figs. 4.5 - 4.10. Chromatograms of typical plants from each cultivated L.hirservosum population. Only spots showing differences included. Inside spot - approximate intensity and colour; outside spot-number of that spot. Solvent fronts omitted. On right - outline of typical leaf of that plant.

FIG. 4.6. LB4/20

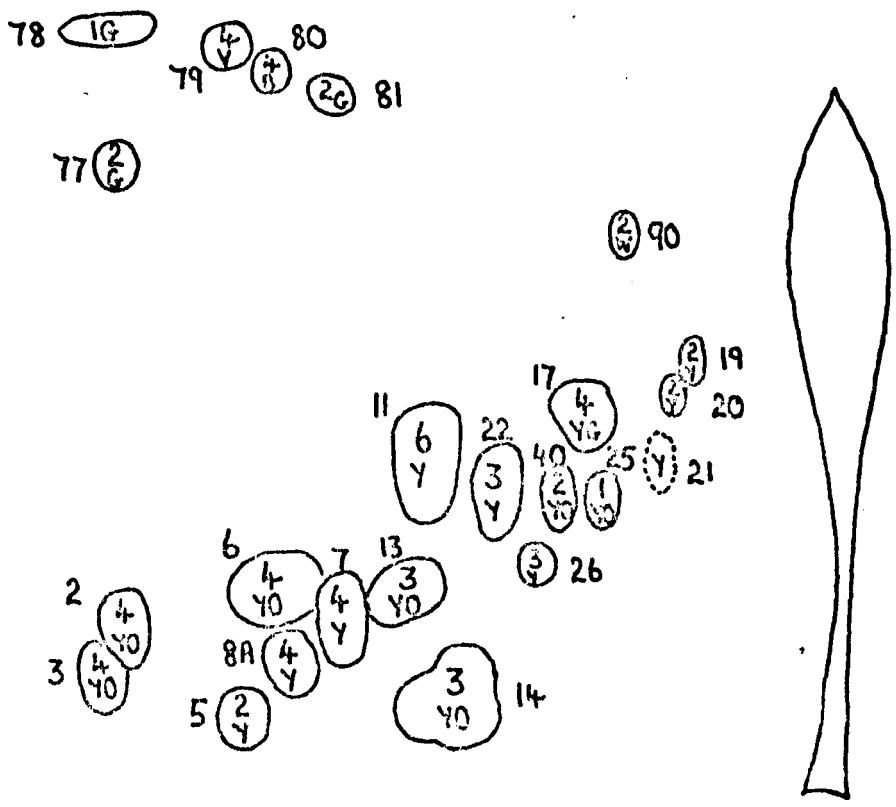


Fig. 4.6. Note similarity to Fig. 4.5; plants from adjacent populations.

FIG. 4.7. LB21/6

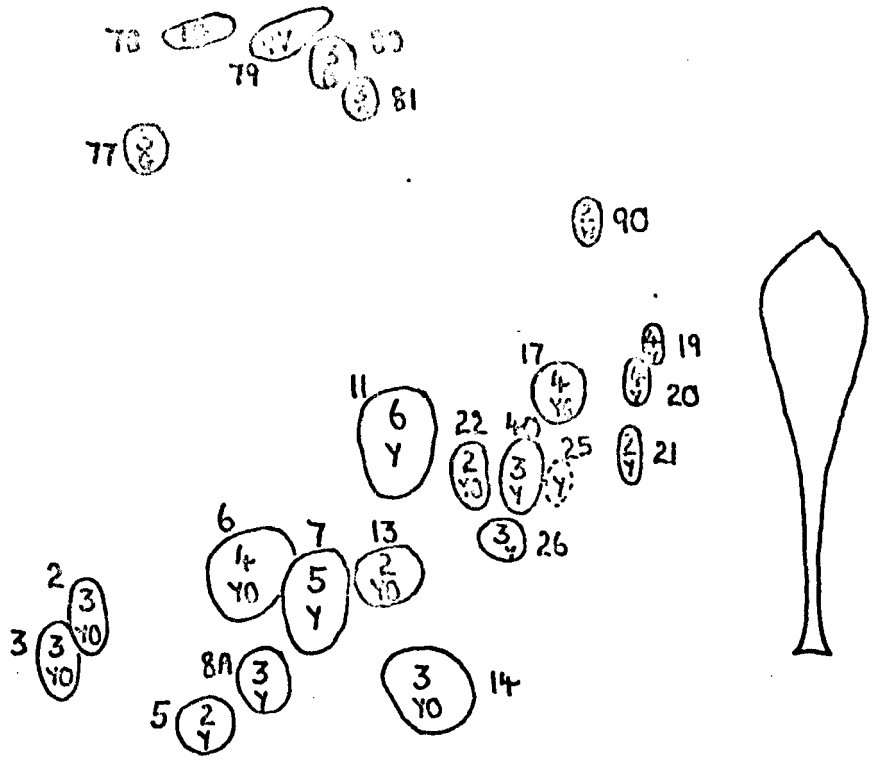


Fig. 4.7. Note spot 7 stronger than 6, stronger than 8A - compare Figs. 4.5, 4.6, 4.9 , where this group is equal in intensity. Spots 19 and 20 stronger than in other plants. Figs. 4.7 and 4.8 are plants from N. England.

FIG. 4.8. LB9/16

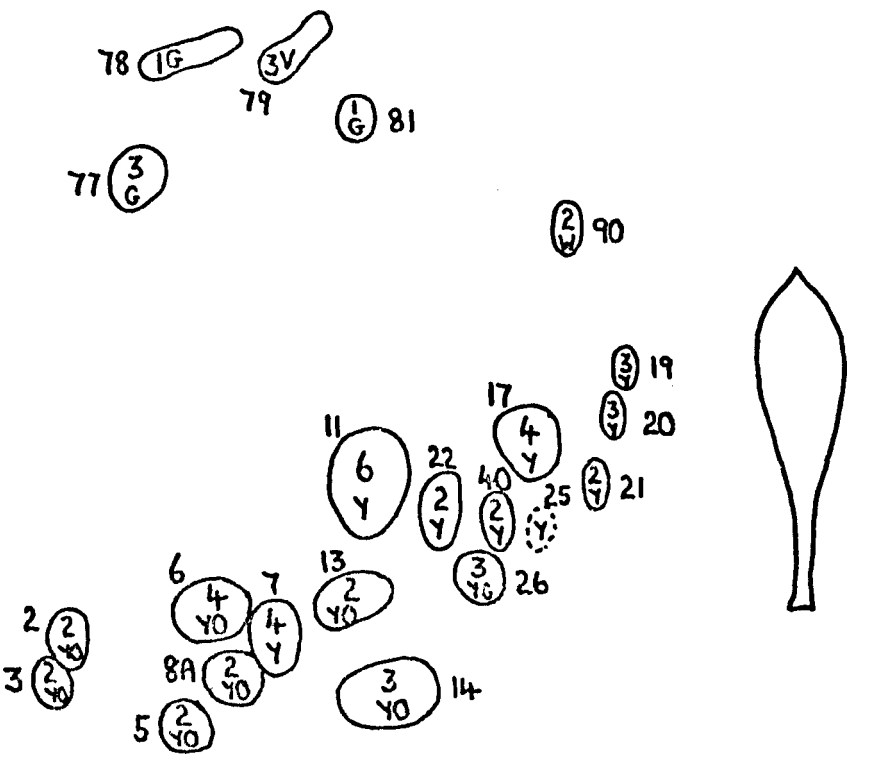


Fig. 4.8. Note spots 6 and 7 same intensity, 8A weaker - some similarity to above. 2 and 3 weaker than other plants. 80 absent.

FIG. 4.9. LB2/21

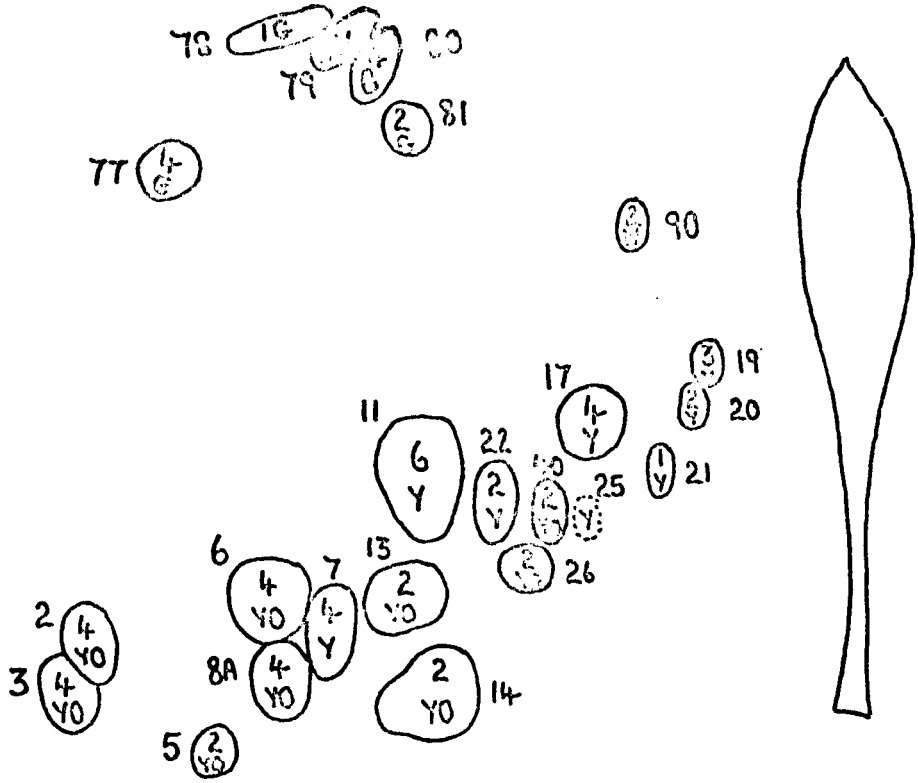


Fig. 4.9. Note similarity to Figs. 4.5 and 4.6, also plants from E. England, but difference from 4.10, morphologically distinct plant in same population.

FIG. 4.10. LB8/32

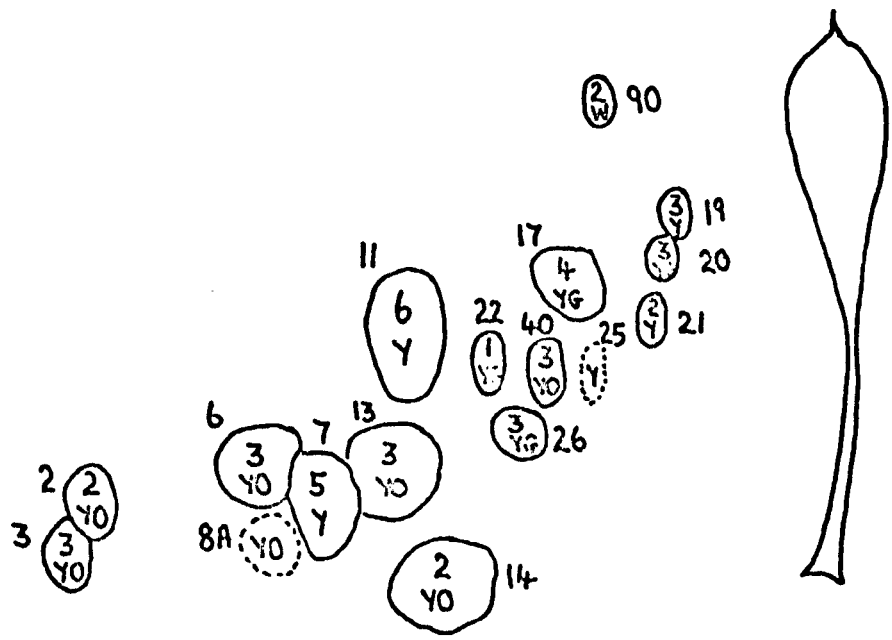


Fig. 4.10. Note absence of spots 5, 77 - 81. 8A weak, 7 stronger than 6 - these are marked differences from other plants.

TABLE 4. 10.

MATRICES OF AVERAGE BIOCHEMICAL DISTANCES AND RANK CORRELATION COEFFICIENTS BETWEEN CULTIVATED AND WILD L. BINERVOSUM POPULATIONS

4.10(a) Variation between Cultivated L. BINERVOSUM Populations

	LB372	LB472	LB21	LB8Br	LB8Nr	LB9
LB372		.111	.210	.298	.545	.326
LB472	1		.195	.318	.531	.337
LB21	.543	.543		.289	.480	.298
LB8Br	.848	.848	.657		.496	.351
LB8Nr	.505	.505	.733	.657		.536
LB9	.524	.524	.829	.562	.562	

4.10(b) Variation between Wild and Cultivated Runs

	LB3W	LB3F1	LB3F2	LB372		LB472	LB4W
LB3W		.280	.303	.271	LB472		.244
LB3F1	.657		.088	.132	LB4W	.791	
LB3F2	.791	.752		.171			
LB372	.752	.829	.848				

For both tables:

Upper right = Average biochemical distance
(smaller distance = greater similarity)

Lower left = Rank correlation coefficient between rankings
(higher coefficient = greater similarity)

For explanation of population codes, see Table 4. 8.

TABLE 4. 11.

ESTIMATED OVERALL RANKINGS OF STRONGER YELLOW SPOTS IN CULTIVATED L. BINERVOSUM POPULATIONS

	2	3	5	6	7	8A	11	13	17	19	20	21	22	40	26
LB3F1	14	13	8	12	7	11	15	9	10	6	4	1	5	2	3
LB3F2	13	12	3	15	10	9	14	6	11	8	5	1	7	2	4
LB372	14	13	5	12	11	9	15	7	10	8	3	1	6	2	4
LB472	14	13	5	12	11	9	15	7	10	8	3	1	6	2	4
LB8Br	13	11	1	14	12	9	15	7	10	8	5	2	6	3	4
LB8Nr	11	10	1	12	14	2	15	9	13	8	7	4	3	6	5
LB9	10	8	7	12	13	6	15	2	14	11	9	1	4	3	5
LB21	11	9	4	12	14	7	15	3	13	10	8	2	1	5	6

For explanation of population codes, see Table 4. 8.

The method of obtaining these overall rankings is given in Table 4. 3.

Figure 4.11.

Dendrogram of weighted mean pair cluster analysis of average biochemical distance between cultivated L. binervosum populations.

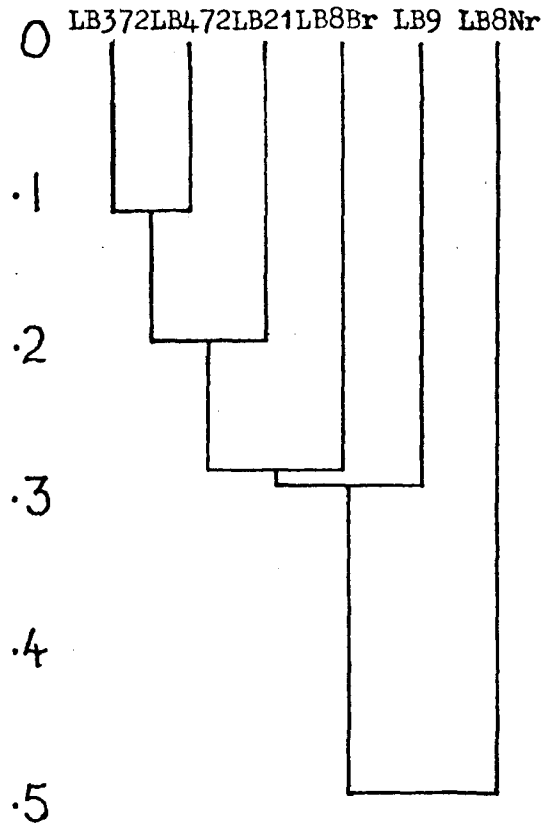
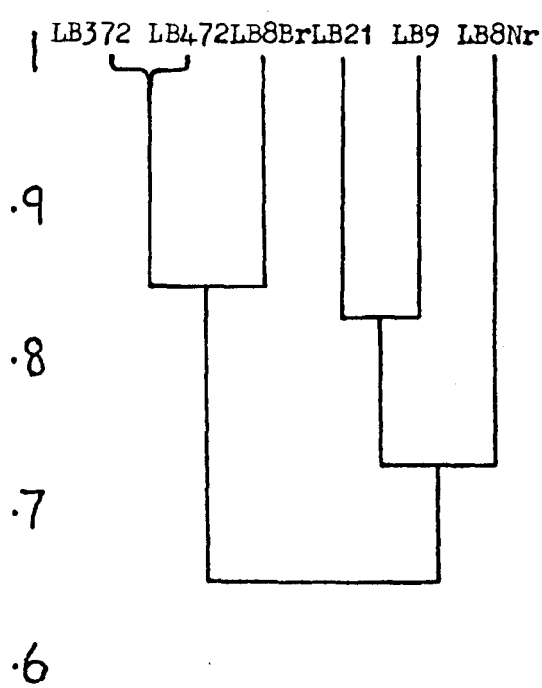


Figure 4.12.

Dendrogram of nearest neighbour cluster analysis of rank correlation coefficient between cultivated L. binervosum populations.



shown do not vary at all in terms of presence/absence within the populations, and the stronger yellow spots vary in relative intensity between different plants by the amounts given for W. The chromatograms show marked qualitative and quantitative differences between populations. For example, spot no 80 is absent from all LB9 plants, and nos 77 - 81 absent from allLB8Nr plants. Differences in relative intensities of the strong yellow spots occur between populations: no 7 is stronger than no 6 in plants from LB21 and LB8Nr, but as strong as no 6 in other populations. No 8A is usually as strong or slightly weaker than 6 for most populations, but in LB8Nr it only occurs faintly. It is not unreasonable to suppose that these differences between populations are genetic. These results are closely comparable with the differences observed between apomictic biotypes of Potentilla by Asker & Frost (1969, 1970a).

The average biochemical distances and rank correlation coefficients between populations have been calculated as before, and Table 4. 10 (a) gives the matrices for these coefficients. Table 4. 11 gives the rankings of relative spot intensities of the stronger yellow spots for the different populations from which rank correlation coefficients are derived. A dendrogram of weighted mean pair cluster analysis of the average biochemical distance matrix is given in Fig. 4. 11. Fig 4. 12 gives the dendrogram of nearest neighbour cluster analysis of the rank correlation coefficient matrix.

The two geographically closest populations are LB3 and LB4; when sampled wild they showed some differences between them, but under cultivation there is only little separation of the two populations. As this is caused by qualitative variation of the weaker, non-yellow spots, the small biochemical distance may be the result of slight genetic differentiation under different natural ecological conditions, or experimental variation. The former alternative is supported by the raw data, where some spots, for

example no. 103, show differences not exhibited by other cultivated populations. Chapter 2.5 also showed some slight morphological differentiation between these populations.

The morphological and gross chromatographic differences between LB8Nr and other L. binervosum populations are also reflected in the dendrograms, this population being chromatographically well removed from others. Apart from this and LB3-LB4, the average biochemical distance dendrogram does not reflect the morphological and geographical differences between populations particularly well. However the rank correlation coefficients for the stronger yellow spots do conform with these differences; the eastern populations LB3, LB4 and LB8Br and the western populations LB21 and LB9 cluster separately, and then LB8Nr joins the western populations which it more closely resembles morphologically.

(g) Variation and the Breeding System in Cultivated L. BINERVOSUM

Without comparisons with other Limonium species under cultivation and adopting different breeding systems, little can be said about the breeding system and the amounts of variation within populations. At least the variation within populations appears to be low, as expected from an apomictic species, and where it does occur (LB8) it is discontinuous. Biochemical variation between populations does conform to expectations from Baker's theory; under cultivation, notable differences do occur between populations. If it is envisaged that a population is built up by apomictic reproduction of a suitable genotype, and then a rare sexual event produces new genotypes capable of colonising new areas, then the further apart the populations the greater the genetic difference between them. If it is true to say that the chromatographic variation between cultivated populations reflects genetic variation, then the results are consistent with the variation between populations expected from L. binervosum. The unusual composition of LB8 could be explained by the occurrence of a rare sexual event perhaps with

hybridisation, or by re-colonisation from another source, as indicated in Chapter 2.5.

4.5. SUMMARY AND CONCLUSIONS

In wild populations of Limonium species, most of the qualitative chromatographical variation is in the weaker, non-yellow compounds, rather than in the yellow (presumably flavonoid) substances. If genetic variation is influenced by the breeding system in the way that Baker suggests, and if the flavonoids are under genetic control, it would be expected that the yellow spots would show more qualitative variation within populations of the outbreeding L. vulgare than the other two species. With a few exceptions, this is not so. Either the two suppositions just made are incorrect, or else the presence of the flavonoid compounds in Limonium is under such a great selective pressure that a plant lacking such a compound or having an extra one is at a disadvantage, and does not survive. This requires that flavonoids have specific functions in the plant, rather than being secondary substances, but these functions have not yet been identified precisely. Other chromatographically invariant species have been described in other studies.

However, more quantitative variation for 15 stronger yellow spots is detected within populations of the outbreeding species than either the inbreeding or apomictic species. If it is accepted from repeats and from studies of cultivated material that this quantitative variation is mainly genetic in origin, then the relationship between chromatographic variation within populations and the breeding system holds for this type of variation. Genetic control of quantitative variation in other species was reported earlier, but this still needs demonstrating conclusively for Limonium by inheritance studies. Neither quantitative nor qualitative variation for the stronger yellow spots relating to that shown by Allard and others for inbreeding species is detected. Variation between wild populations of the different species for these spots cannot be related to

breeding system theory in a simple way.

When the data for all 58 selected spots from wild populations is analysed numerically, L. vulgare populations show more variation within them than do populations of L. humile, but not necessarily more than L.binervosum. In this latter species, the qualitative variation for all these spots is reduced under cultivation, suggesting that this high variation within wild populations is the result of environmental factors. Although this might also be found for the other two species if grown under cultivation, none of the populations were growing naturally in such apparently unfavourable conditions as LB4, the most variable wild population. In all populations this variation is mainly in the non-yellow spots, and although it is recorded qualitatively, it may be quantitative, varying above and below the threshold levels of detection.

The variation between wild populations for all the spots for the three different species cannot be related to theoretical expectation from their breeding systems; in fact, the trend is the reverse. It seems that the amount of variation within populations and how it is distributed influences the indices of variation between populations. Differences between populations also appear to be created by environmental factors and by uncontrollable variation in experimental conditions between runs. When environmental differences are removed, as in the cultivated L. binervosum populations,chromatographic variation between populations can be detected corresponding to breeding system, geographical distribution and morphological variation.

Throughout this account various difficulties in this chromatographic study have been stressed, and I feel that for Limonium species chromatography is not a useful or convenient method of detecting any genetic variation that may be present within or between populations sampled in the wild. The technique can yield valuable information if material is cultivated under uniform conditions, and if it is supported by other work such as morphological studies, as was shown for L. binervosum.

CHAPTER 5.

ENZYME VARIATION AND THE BREEDING SYSTEM IN *L. VULGARE* AND *L. HUMILE*

5. 1. THE STUDY OF ENZYME VARIATION

The study of enzymes by the method of electrophoresis represents a major extension to the techniques available for the genetic analysis of individuals, populations and species. The underlying principle behind electrophoretic studies is that the amino acid composition of enzymes, like all proteins, is determined genetically. The nucleotide sequence of DNA ultimately specifies the way in which the variety of amino acids are linked together to form a protein. In turn, the physical properties of the protein are dictated by its amino acid composition. A change in the nucleotide sequence - a mutation - may change the amino acid composition of the protein and therefore its physical characteristics. One of these characteristics is the overall electrical charge of the molecule; such a change can be detected by electrophoresis. A mutation may also cause the tertiary structure of the protein molecule to be altered, again affecting its electrophoretic mobility. Not all mutations give rise to a change in the amino acids specified, and not all amino acid substitutions result in a change in physical properties. It is estimated that only between one-third (Shaw 1970) and one-half (Lewontin & Hubby 1966) of all substitutions are detected electrophoretically.

Where substitutions are detectable, then if a locus specifying an enzyme in a diploid organism is heterozygous, more than one form of the same enzyme may be detected, but if the locus is homozygous only one of the possible forms is exhibited. Multiple molecular forms of the same enzyme are known as "isozymes" or "isoenzymes". The precise definition of an isozyme has been the subject of much discussion, and the variety of opinion was considered by Brewer (1970). One of these definitions useful for my purpose is: 'multiple

molecular forms of enzymes derived from the same organism, sharing a catalytic activity'.

If an isozyme system is under simple genetic control, then it must obey the laws of Mendelian inheritance. Many studies of both animals and plants and covering a diversity of enzyme systems have shown this to be so. A review of some of the literature describing the inheritance of some isozyme systems in different plants was made by Scandalios (1969). Also, Brown and Allard (1969a, 1969b) demonstrated this for several loci in maize, and Marshall & Allard (1969) did the same for Avena barbata. In animals proof of genetic control by inheritance studies has been given in such diverse species as Drosophila pseudoobscura (Hubby & Lewontin 1966) and Zoarces viviparus (a fish) (Hjorth 1971). Because the genetic control of isozymes has been so widely accepted, then providing the electrophoretic patterns are simple, breeding experiments have not been necessary as proof of their inheritance. This is particularly useful in situations where controlled crosses are not possible, as was the case with Limulus (Selander et al. 1970).

Usually the alleles of an enzyme locus are found to be codominant, both alleles being expressed in the heterozygous condition; Fig. 5. 1(a) illustrates this. It is not uncommon for isozyme loci to produce more than two bands in the heterozygous condition, shown in Fig. 5. 1(b). This is the result of each band from each homozygote being constructed from two identical subunits, and in the heterozygote a subunit from each of the two alleles combines to produce a molecule of intermediate mobility, referred to as a 'hybrid' band or 'heteropolymer' (Brewer 1970). More complex patterns than Fig. 5. 1. can be obtained if each band is composed of more than two subunits, or if multiple alleles exist, or if the species is polyploid. The various possibilities and their banding patterns were described by Shaw (1964). It is also possible for a homozygous locus to produce multiple bands which will not segregate; for example, lactate dehydrogenase does this in several species,

including man (Harris 1970). Such multiple bands in the homozygote can be caused by different levels of polymerisation of identical subunits, or 'homopolymers' (Brewer 1970). But here again, different alleles of the same locus usually produce banding patterns subject to genetic interpretation despite their complexity.

Sometimes it is found that more than one locus is present in a species for a particular enzyme, resulting in more than one group of bands on an electrophoresis gel; these loci may or may not be linked. For example, in maize Brown & Allard (1969a) found two unlinked esterase loci and Scandalios (1967) found two linked alcohol dehydrogenase loci; Jelnes (1971) found two linked esterase loci in Ephestia kuehniella Z. Breeding experiments were essential to determine whether the observed banding patterns were caused by one or more loci, and if more than one, whether they were linked.

The majority of isozyme loci have been shown to possess codominant alleles, but there are examples where this is not the case. Wilcox (1966) found that in chicken alkaline phosphatase the slow allele was recessive to the fast. Other cases of recessiveness were found by Johnson (1964) - esterase in Drosophila; Law (1967) - alkaline phosphatase and leucine aminopeptidase in chickens; and West & Garber (1967) - esterase in Phaseolus. Another possibility is that of 'silent' alleles, where one allele produces no band at all. Examples of this were discovered by Gahne (1963) - cattle phosphatase; and Krimbas & Tsakas (1971) - Dacus oleae esterase.

Interspecific hybridisation studies have shown that a particular isozyme system may be determined by homologous genes in closely related species. West & Garber (1967) and Wall (1968) demonstrated that several categories of enzyme loci were homologous in Phaseolus species. This was also shown for Cucurbita species by Wall & Whitaker (1971). Berger (1970) found an allelic identity of 34 per cent in two species of Drosophila; homologies were particularly clear in some dehydrogenases, where 'hybrid' bands were seen.

These results are of significance to the Limonium hybrids examined later on.

Also relevant to the electrophoretic study of Limonium is the fact that its species are tetraploid in the British Isles. Assuming that inheritance is tetrasomic (which is not definite, but seems likely according to Choudhouri (1942))then a fully heterozygous individual of such a species could possess four different alleles at a locus, and produce four enzyme bands (neglecting the possibility of hybrid bands). More complex situations are possible if each band is composed of more than one subunit, because 'hybrid' bands would be produced in heterozygotes. There have been relatively few isozyme studies on polyploid species to confirm these suppositions, but from their results Desborough and Peloquin (1967) proposed a system of three alternative alleles specifying three monomers for an esterase locus in tetraploid potatoes. The active isozyme molecule was thought to be a tetramer, which would result in a maximum of fifteen different possible isozyme bands. A situation of six catalase alleles in maize was described by Scandalios (1969). Each active band was a tetramer, and heterozygotes showed hybrid bands. Although normal plants are diploid, the endosperm is triploid, and in this tissue the relative intensity of the multiple bands could be interpreted by considering the dosage of each allele present. In both these studies further experiments were required to eliminate other hypotheses explaining the phenotypes observed.

Isosyme variation within a species can exist for developmental as well as genetic reasons. Several studies have shown this, for example Chen et al. (1970) found changes in several enzymes with leaf age in Xanthium, and Downton & Slatyer (1971) found this for Atriplex. Also the electrophoretic mobility of the same enzyme band may vary in different tissues, as shown in maize pollen, scutellum and leaves by Efron (1970). Mitra et al. (1970) demonstrated developmental and tissue variations in ten different Dianthus enzymes, but the bands were reproducible under different growth conditions.

However, environmental influences on isozymes have been shown to exist by McCown et al. (1969b, 1970) and Van Lear & Smith (1970).

In spite of these environmental and developmental influences on enzyme pattern, it is generally accepted that isozymes are a much more reliable indication of genetic variation than are some other biochemical characters, for example flavonoids. This is particularly so if the same tissue or organism at the same stage of development is studied, which is always the case. This reliability combined with the possibility of recording many isozyme loci simultaneously has enabled geneticists to obtain a much more comprehensive picture of an individual's genome that was previously possible. In many species, particularly animals, electrophoretic screening is a simple procedure and a large number of individuals and enzyme loci can be surveyed; therefore the ease and effectiveness of electrophoresis has permitted significant advances in population genetics. This has shown that substantial amounts of genetic variation for isozymes exist within natural outbreeding populations. A suitable way of expressing this has been in terms of the overall proportion of loci showing polymorphism in a given population, and the proportion of loci which show heterozygosity in an average individual (Lewontin & Hubby 1966). A recent summary of all but the latest works on animal species was made by Frydenberg & Simonsen (1973). Marshall & Allard (1970a) and Singh & Jain (1971) produced comparable data for the plant genus Avena.

With this revelation of genetic variation within natural populations many people have asked what are the forces that maintain it. It was suggested by Kimura (1968, 1969) and King & Jukes (1969) that a large proportion of the mutations which created this variation are selectively neutral or nearly neutral, and that the variation is therefore maintained by genetic drift. Their conclusions were based on genetic load considerations, and on new estimates of mutation rates. Shaw (1970) provided electrophoretic evidence for this "neutral" theory. However, since then projects designed specifically

to test this hypothesis have suggested the opposite. In artificial Drosophila populations studied through several generations and established with abnormal gene frequencies, they tended to return to the frequencies observed in the original natural population (Berger 1971). Different population cages established with small numbers of flies reached similar allele frequencies (Sing et al. 1973). An excess of heterozygotes was found in natural and artificial populations (Richmond & Powell 1970; Powell 1973, 1974). In an earlier artificial study the degree of polymorphism increased with the amount of variation in the environment (Powell 1971). All these Drosophila experiments indicated that enzyme polymorphisms are selectively advantageous, and not neutral and therefore subject to random drift as was earlier proposed.

A recent analysis by Bryant (1974) of earlier data of others attempted to correalate their enzyme heterozygosities with specific components of variability in the environment. In contrast to this where the environment was uniform and the demand for adaptive variation was reduced, relatively low levels of enzyme variation have been found, for example in natural populations of the subterranean mammals the Mole Rat (Nevo & Shaw 1972), and Pocket Gopher (Nevo et al. 1974). Population sizes were too large for this low variation to be explained by inbreeding, and genetic drift was also unlikely because the same allele was fixed in different populations. Further evidence and discussion against the "neutral" theory using other species has been provided by Johnson (1971, 1972) and Sing & Brewer (1971). For a penetrating consideration of the selection and drift theories and their evidence see Lewontin (1974). However to determine the precise advantage of enzyme multiplicity to the organism requires a much better understanding of their in vivo functioning. A move towards this has been made by Krimbas & Tsakas (1971) and Ayala & Powell (1972) but much work has still to be done in relating a specific enzyme variation to a particular adaptive function within the organism.

Whatever the conclusions of these lines of research, one of the factors

which may influence isozyme variation levels within populations is the main subject of this thesis - the breeding system. For example, Solbrig (1972) analysed three self-compatible and three self-incompatible Leavenworthia species for between four and six enzyme systems. Assessing variation in terms of banding pattern, he found that populations of self-compatible species were less variable than those of self-incompatible species. Some of his data are given in Table 5. 1. There was also a tendency for differences between self-compatible populations to be greater than between self-incompatible populations.

These results supported the general prediction on population structure and the breeding system made by Baker, but Solbrig was not prepared to attribute all the differences between species and populations to this hypothesis because he was aware of the work on other inbreeding species by Marshall & Allard (1970a). They studied eight populations each of two heavily inbreeding (95 - 98 per cent selfing) species of Avena, and revealed variation within populations in enzyme bands. A. fatua and A. barbata were estimated to have 54 and 31 per cent respectively of their loci polymorphic, figures similar to those of unrelated outbreeding species. Variation was also expressed as polymorphic indices of each enzyme system and population. Averaged over all enzyme systems and populations, A. fatua had a polymorphic index of 0.06 and A. barbata 0.03, the maximum of this index being 0.25. They suggested (Marshall & Allard 1970b) that particularly for A. fatua this polymorphism despite heavy inbreeding was maintained by heterozygote advantage. Although these results did not support Baker's hypothesis, reasons were outlined in Chapter 1 for considering Allard's work on certain inbreeding grasses as a special case. Also in this work on Avena there were no comparisons between closely related outbreeding species.

A different type of polymorphism within wheat, another inbreeding species was observed by Sing & Brewer (1969). All of the enzyme systems studied showed multiple enzyme bands, but the phenotypes did not segregate on selfing. The isozymes were thought to have arisen not by homopolymers, but by gene

TABLE 5. 1.

WITHIN POPULATION VARIABILITY OF SPECIES OF LAEVENWORTHIA. PERCENTAGE OF PLANTS DIFFERING FROM THE MODAL TYPE OF BANDING FOR PARTICULAR ENZYMES TESTED. DATA FROM SOLBRIG (1972).

Species	No. of Plants	Enzyme System				
		EST	LAP	APH	MDH	ADH
uniflora	52	2.82	0.00	0.00	0.00	0.00
exigua	336	2.65	0.00	1.30	2.85	0.00
alabamica *	102	14.70	1.00	0.00	28.98	0.00
crassa *	44	43.47	28.57	0.00	0.00	61.50
stylosa *	209	62.00	65.00	32.81	4.08	0.00

* Self-incompatible species.

EST = esterase; LAP = leucine aminopeptidase; APH = acid phosphatase; MDH = malic dehydrogenase; ADH = alcohol dehydrogenase.

duplication made possible by polyploidy with allelic variation between these duplicate loci. This is an alternative route to heterozygosity which can be adapted by polyploids whose genetic variation may be reduced by inbreeding.

A further significant contribution to enzyme variation and the breeding system was made by Selander & Kaufman (1973). A self-fertilising land snail Ruminia decollata had successfully colonised southern North America from the Mediterranean. A survey of over 750 snails from many American localities failed to reveal any heterozygosity for a total of 25 enzyme systems, and there was also much homozygosity in samples from the native Mediterranean region. Populations from this latter region had unique combinations of alleles indicating variation between populations, but only two out of nine lines revealed any variation within populations. These results were compared with as yet unpublished data on outbreeding Helix aspera populations also colonising North America from Europe, but which had maintained full variability. The parallelism between R. decollata and self-fertilising colonising plants was noted, and the authors suggested that observations similar to those on this species might be expected from autogamous plants.

This brief summary of electrophoresis studies indicates that the technique is potentially very appropriate for studying genetic variation in Limonium species and populations in relation to the breeding system. However, isozyme studies have been shown to be subject to some disadvantages, listed by Shannon (1968) and also mentioned in several of the other works referred to. For example, the in vitro enzyme phenotype may not be an accurate reflection of its in vivo state. Also the use of a particular histochemical test does not necessarily mean that the enzyme detected performs the same biochemical function within the organism. It was shown above that it seems likely that enzyme polymorphisms are selectively advantageous, although the precise selective factors maintaining this variation are not clear. Finally it has also been stated that not all enzyme polymorphisms are detectable by

electrophoresis.

5. 2. MATERIALS AND METHODS

(a) Populations Studied

Table 5. 2 shows the populations and numbers of plants scored for the various enzymes. In addition to these whole populations various other plants and synthetic hybrids were run, including some cultivated L. humile. Those plants which had been sampled growing wild and had survived cultivation showed the same enzyme phenotypes when repeated.

(b) Extraction of Enzymes

Only a few enzyme systems could be surveyed in great numbers because of the practical difficulties and time associated with the preparation of reasonably pure enzyme extracts from fresh green leaves. Other studies have reported that the presence of phenolic compounds in plant tissues causes irreversible damage to enzymes when extraction is attempted, and several techniques have been devised to avoid this (Loomis & Battaile 1966, Tucker & Fairbrothers 1970, McMullan & Ebell 1970). It was also found that crude "squeezates" of Limonium leaves discoloured rapidly and did not yield detectable enzymes when electrophoresed. The method of extraction devised to avoid this was a modification of that of McCown et al. (1968); some of the modifications were a consequence of the remarks of Loomis & Battaile (1966).

2.5g of clean fresh green leaves were cut into small pieces and ground in a mortar with liquid N_2. The leaf powder was mixed with 6g purified 'Polyclar - AT' (GAF, Manchester) which had already been soaking with 20 cm^3 0.1 M 'Hepes' buffer pH 7.3 and 2 cm^3 20 mM dithiothreitol. This mixture was stood in an ice bath for one hour and then squeezed through muslin to obtain the liquid. The residue was re-extracted with a further 5 cm^3 'Hepes' and 1 cm^3 dithiothreitol. The total liquid was centrifuged at 25,000 rpm (approx. 30,000g) in an MSE 'Superspeed 50' refrigerated centrifuge for 30 minutes. A small amount (about 0.5g) of 'Polyclar - AT' was included in

TABLE 5. 2.

LIMONIUM POPULATIONS STUDIED BY ELECTROPHORESIS

Population Code	Locality	No. of plants available	No. of plants scored		
			ES	LAP	6PGD
LV11	Anglesey	10	10	10	10
LV5	Devon	20	19	20	17
LV17	Bridgwater	11	11	11	-
LV18	Scolt	17	16	17	10
LV19	Nr. Scolt	10	10	10	-
LH12	Ireland	12	12	12	-
LH14	Scotland	13	13	13	-
LH15	Lake District	16	16	16	-
LH18	Scolt	7	7	7	-
LX18	Scolt	8	8	8	5
LX1	Synthetic	2	2	2	2
LX2	Synthetic	5	5	5	5

Not all plants available were scored for some enzymes for two reasons:

(i) 6PGD staining technique was not developed until after *L. humile* populations had been run,

(ii) extracts were occasionally weak or the stained gel was blurred in some regions.

the centrifuge tubes. The supernatant was passed through a 0.22μ 'Millipore' filter and placed in a dialysis tubing which had previously been soaked in 10 mM EDTA and rinsed with water. At this stage the volume of extract was approximately 12 cm^3. Processing five samples simultaneously, this stage in the extraction could be reached in six hours.

Each extract was then dialysed for six hours in a refrigerator against gel buffer which had been diluted to ten per cent normal strength. During this time the dialysis buffer was changed twice, and the dithiothreitol in the tubing replenished. After dialysis the extract was retained in the visking tubing and concentrated against 'Aquacide' (Calbiochem) overnight (12 hours). After this procedure the final volume of extract was reduced to about 1 cm^3.

Despite care in extract preparation the concentration of the enzymes within the extracts was subject to variation. The extracted enzymes were unstable and yielded diffuse isozyme bands if they were not electrophoresed immediately after the extraction process. This extraction method failed to yield any detectable enzymes for L. binervosum. Other methods, including crude 'squeezates', and the phenoxyethanol technique of Karig & Wilson (1971) also yielded negative results when L. binervosum was extracted. It is not possible to provide any reasonable explanation for this, and only results for L. vulgare and L. humile can be presented.

(c) Electrophoresis

Many of the wide variety of buffer systems available were tried, and the most suitable was found to be the discontinuous system of Scandalios (1969) p.76. A 12 per cent starch (Connaught) gel was used, and the electrophoresis apparatus was that available commercially for horizontal electrophoresis by Shandon-Southern Ltd. The power supply was manufactured by Stogate.

Each extract was absorbed onto a 10 mm x 5 mm rectangle of Whatman No 3 filter paper and this sample inserted into a 10 mm slit in the gel. 10 samples

could be accommodated on the gel used. A constant voltage supply of 350v (35-20 mA) was applied, and after 15 minutes the filter paper rectangles were removed, giving a sharper final separation. The run took about 8 hours, during which time the borate front had travelled approximately 10 cm from the origin. During the run an ice tray was placed on top of the gel to reduce overheating, and the whole apparatus was placed in a refrigerator. The completed gel was sliced horizontally and stained.

(d) Staining

(i) α-Esterase (Es) The method of Shaw & Prasad (1970) was found to be most effective, except that only α-naphthyl acetate was used as the substrate. Other substrates (β-naphthyl acetate, α-naphthyl butyrate) were also tried, but yielded no difference from or improvement over α-naphthyl acetate. The staining solution yielded variable results, because it sometimes decomposed rapidly, staining the whole of the gel surface and making the enzyme bands difficult to visualise.

(ii) Leucine Aminopeptidase (LAP) The method of Scandalios (1969) was used.

(iii) 6-Phosphogluconate Dehydrogenase (6PGD) Early attempts were unsuccessful, but later the method of Shaw & Prasad (1970) yielded results; therefore not all plants were surveyed for this enzyme.

It was only possible to assay a large number of plants for the three above enzymes. Other enzymes tested for included:

(iv) Acid Phosphatase Although the staining method of Scandalios (1969) revealed activity, the banding pattern was complex and faint, and differences between individuals and species could not be detected. On some occasions the staining method did not work at all; therefore this assay was abandoned.

(v) Peroxidase The method of Shaw & Prasad (1970) yielded a complex banding pattern with much streaking, so this assay was abandoned.

(vi) Glucose-6-Phosphate Dehydrogenase Early attempts were unsuccessful, but in a later trial several diffuse regions of activity were observed. It may

have been possible to improve this technique, but time was limited and the necessary reagents expensive.

(vii) Other Enzymes The following assays did not yield any bands: alkaline phosphatase, β-galactosidase, catalase, lactate dehydrogenase, malic dehydrogenase, alcohol dehydrogenase. This does not mean that these enzymes were absent from the plants; they could have been lost during extraction, or different electrophoresis or staining conditions may have been necessary for their visualisation. A general protein stain (amido black) was also tried, but this showed one single band common to all plants of both species. This protein was also seen as a reddish band in the LAP stain.

(e) Recording of Gels

Sketches of all gels were made at the time of development, and photographs were taken of those gels which had developed clearly enough. Earlier runs were also recorded densitometrically, but it was later decided that this was not necessary for the three enzymes studied in detail.

5. 3. RESULTS AND DISCUSSION

(a) Esterase Results

Several regions of the gels showed Es activity, but the faster region stained most reliably, and only this region was studied. A slower constant broad band common to both species, visible in the photographs, made a useful reference point for comparisons between runs.

(i) *L. humile* All plants from all populations showed the same Es phenotype, which consisted of four bands, the second of which was always the strongest. Plate 5. 1. shows photographs of representative samples. This common phenotype was designated 'phenotype C'. Although the relative band intensity within different plants was always the same, because of the fluctuations in extract concentration and variable development of gels, it was not possible to decide whether absolute quantitative variation in band intensity existed between plants. Plants from different populations run on the same gel confirmed the

existence of a common phenotype throughout the species. Fig. 5. 2 (a) gives an explanatory sketch of this phenotype.

(ii) L. vulgare All plants showed a series of four bands comparable to L. humile but migrating to a slower position - L. vulgare plants were one band "out of phase" with L. humile. An explanatory sketch is given in Fig. 5.2 (a). Therefore between both species there was a total of five bands; L. vulgare showed bands 1 - 4, L humile bands 2 - 5. However L vulgare plants showed different relative band intensities from L. humile; while in L. humile band 3 was always strongest, in L. vulgare at least two types could be distinguished. Either band 2 was the strongest and bands 1, 3 and 4 were all fainter, designated phenotype A; or bands 2 and 3 were strongest and of approximately equal intensity, with bands 3 and 4 fainter, designated phenotype B. Plate 5. 1 shows photographs of the two L. vulgare phenotypes and their differences from that of L. humile. There is a suggestion from these photographs that there may be further subdivision of the L. vulgare phenotypes, but the gels were not clear enough to justify this. Table 5. 3 gives the numbers of the various phenotypes in the different Limonium populations.

(iii) Wild 'hybrids' Eight plants, coded LX18, were judged to be morphologically intermediate between the two species, and were growing in a mixed population at Scolt. No differences in Es phenotype could be detected between these putative hybrids and 'pure' L. humile plants, and these plants were classed as phenotype C.

(iv) Synthetic hybrids Two crosses were made, both of which only yielded a small number of progeny (two and five respectively). At the time of hybridisation only a few plants were available in cultivation, and it was not possible to set up the most useful crosses in terms of enzyme phenotype. Further it was later discovered that one of the (morphologically) L. vulgare parents (from Scolt) was probably itself a hybrid as judged by its Es phenotype (type C, not found in any other L. vulgare plants, with band 3 the strongest).

Figure 5.1.

Genetic control of some simple isozyme phenotypes.

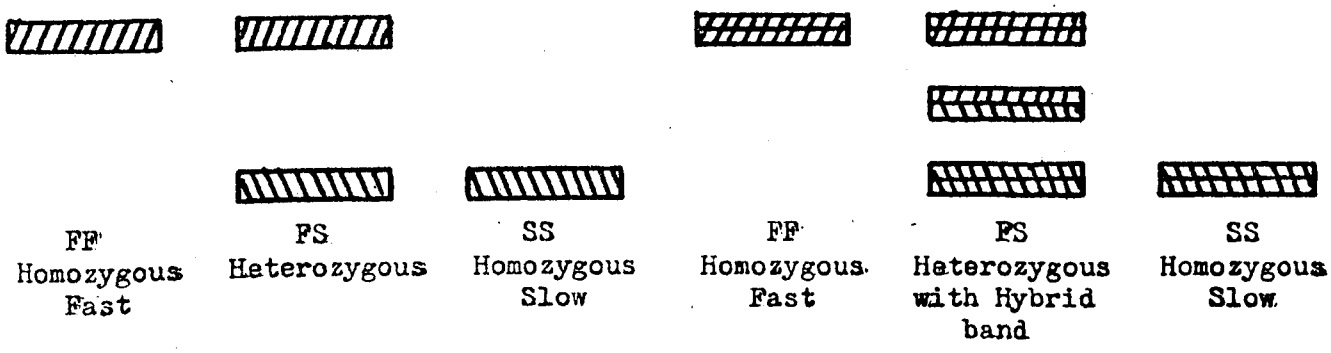
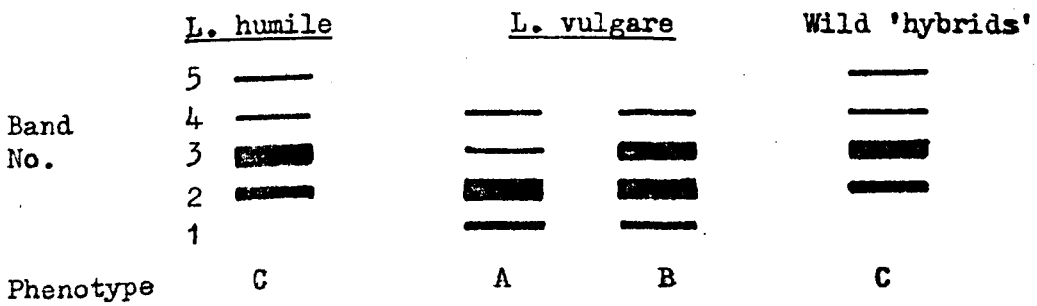


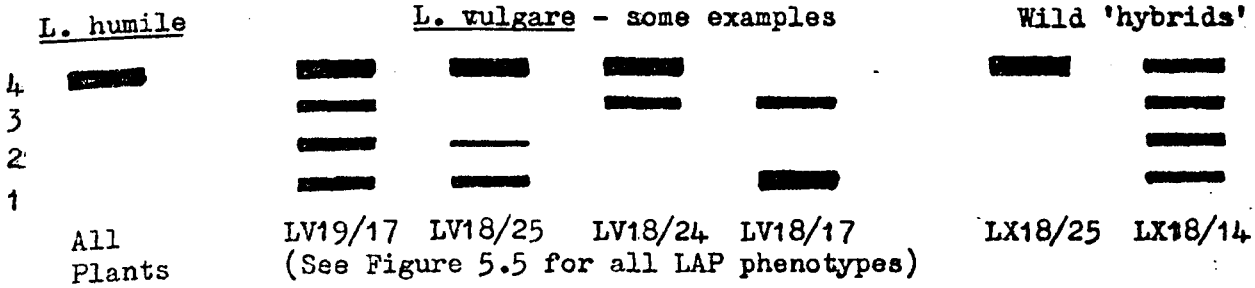
Figure 5.2.

Enzyme phenotypes detected in Limonium.

(a) Esterase



(b) Leucine Aminopeptidase



(c) 6-Phosphogluconate Dehydrogenase

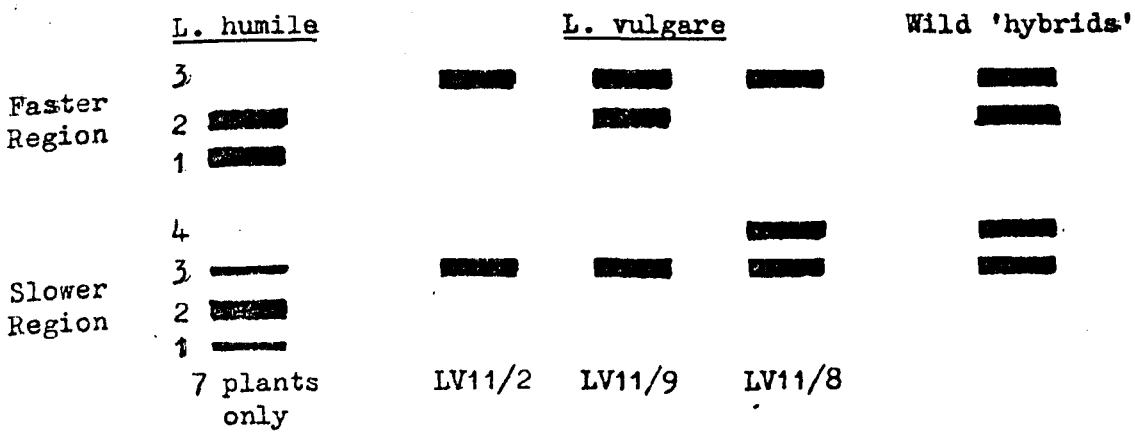
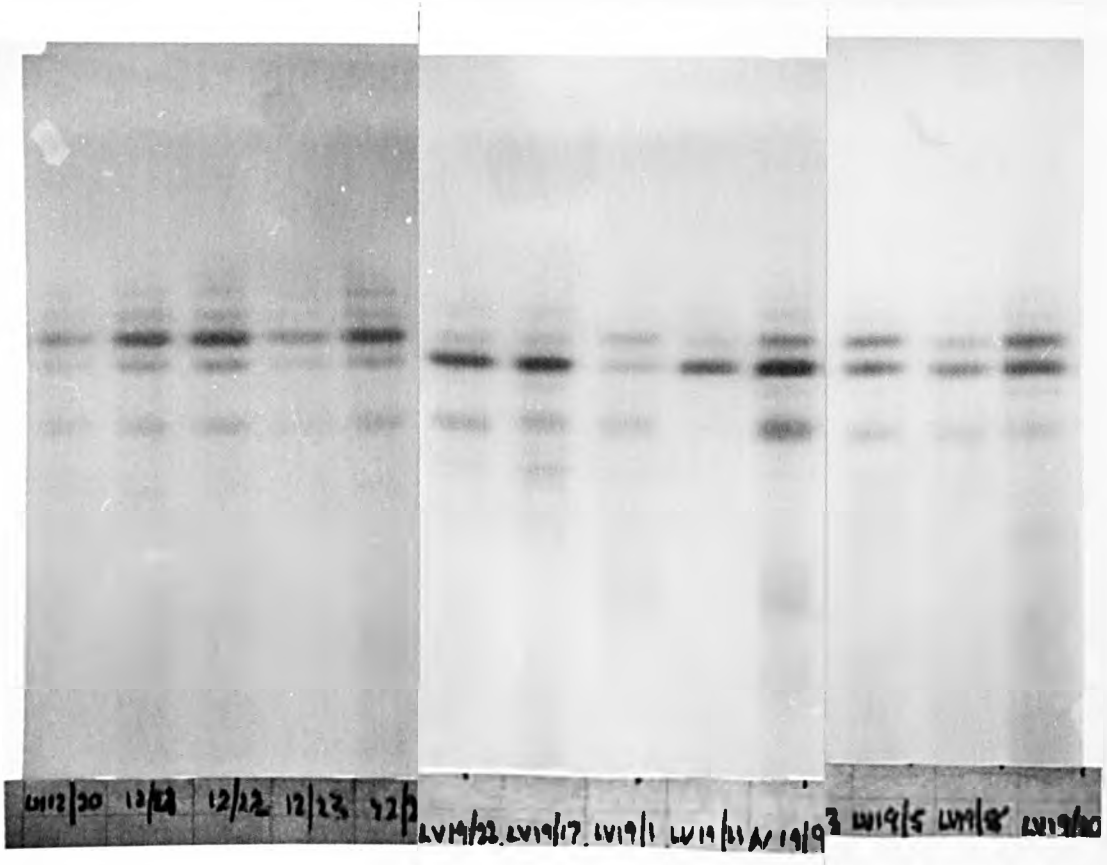


PLATE 5.1.

Esterase phenotypes of L. humile and L. vulgare.



1	2	3	4	5	6	7	8	9	10	11	12	13
C	C	C	C	C	A	A	B	A	A	B	A	B

Nos. 1-5 - L. humile; note the similarity between plants. This phenotype is also seen in 'hybrids'.

Nos. 6-13 - L. vulgare, showing the range of phenotypes.

Beneath the numbers are the phenotype letters. Note the constant, slower broad band enabling comparisons between runs to be made.

TABLE 5. 3.

NUMBERS AND FREQUENCIES OF ESTERASE PHENOTYPES IN *LIMONIUM* POPULATIONS

Population Code	Locality	No. of Plants	Number of Phenotype A	B	C	Frequency of Phenotype A	B	C
LV11	Anglesey	10	7	3	-	0.70	0.30	-
LV5	Devon	19	16	3	-	0.84	0.16	-
LV17	Bridgwater	11	11	-	-	1.00	-	-
LV18	Scolt	16	9	7	-	0.56	0.44	-
LV19	Near Scolt	10	7	3	-	0.70	0.30	-
LH12	Ireland	12	-	-	12	-	-	1.00
LH14	Scotland	13	-	-	13	-	-	1.00
LH15	Lake District	16	-	-	16	-	-	1.00
LH18	Scolt	7	-	-	7	-	-	1.00
LX18	Scolt	8	-	-	8	-	-	1.00
LX1	Synthetic	2	-	-	2	-	-	1.00
LX2	Synthetic	5	-	2	3	-	0.40	0.60

For sketches of these phenotypes see Fig 5. 2. Note that all *L. humile* plants show phenotype C, and *L vulgare* are either A or B, except LV17 - all A

TABLE 5. 4.

CONTINGENCY TABLE AND CHI-SQUARED OF ESTERASE PHENOTYPES IN *L. VULGARE* POPULATIONS

Population Code	Phenotype A Observed	Expected	Phenotype B Observed	Expected	Total
LV11	7	7.1	3	2.9	10
LV5	16	13.5	3	5.5	19
LV18	9	11.3	7	4.7	16
LV19	7	7.1	3	2.9	10
Total	39		16		55

Chi-squared = 3.21 3 degrees of freedom. Therefore p = 0.3 - 0.5

Therefore there is no significant difference between populations in the numbers of phenotype A.

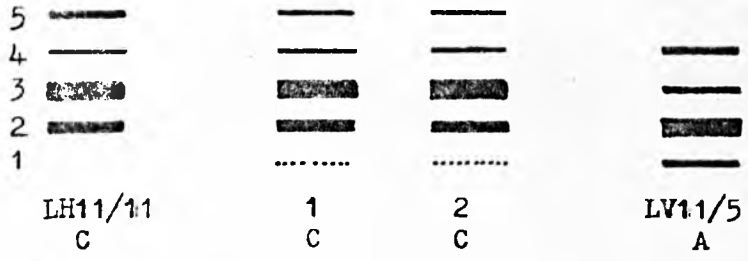
Note that LV17 (all phenotype A) has been omitted.

Figure 5.3.

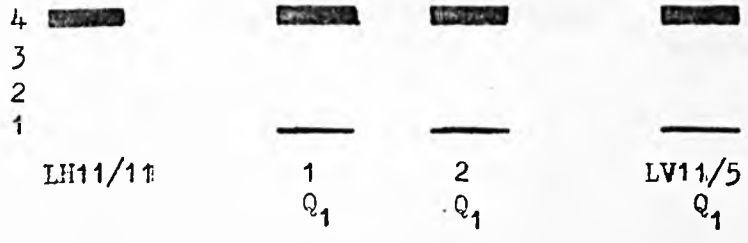
Enzyme phenotypes of LX1 synthetic hybrids and parents.

LH11/11♀ x LV11/5♂ = LX1

(a) Esterase



(b) Leucine Aminopeptidase



(c) 6-Phosphogluconate Dehydrogenase

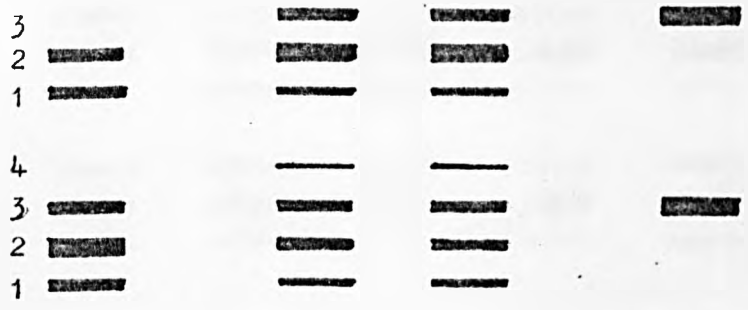
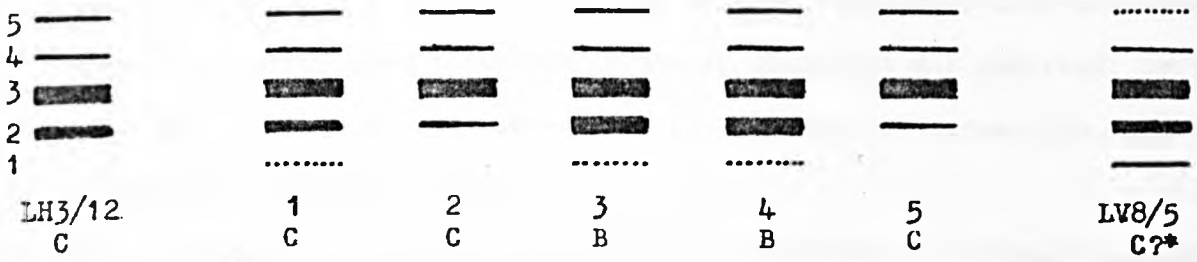


Figure 5.4.

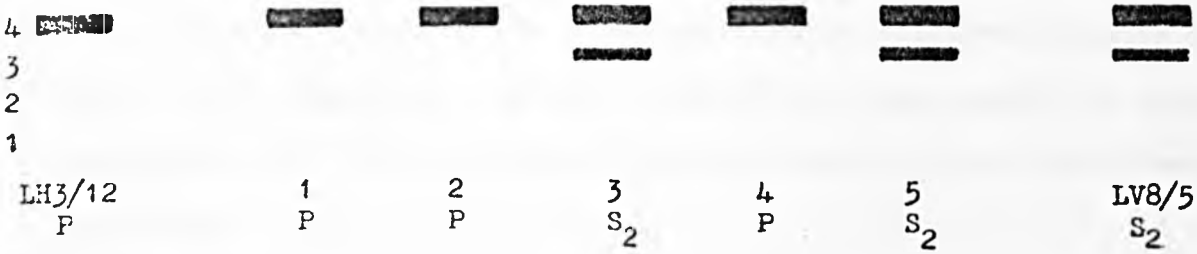
Enzyme phenotypes of LX2 synthetic hybrids and parents.

LH3/12♀ x LV8/5♂ = LX2

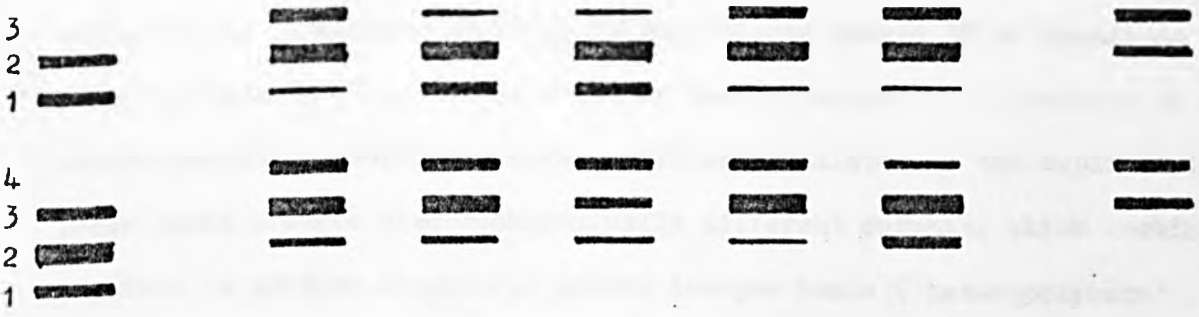
(a) Esterase



(b) Leucine Aminopeptidase



(c) 6-Phosphogluconate Dehydrogenase



* From the Es phenotype, it is possible that this plant is itself a hybrid.

Sketches of the parental and F_1 phenotypes of all enzymes studied are given in Figs 5. 3 and 5. 4. The LX1 progeny, of parents from pure stands, showed Es phenotypes in both plants similar to those of wild putative hybrids, with band 3 the strongest. In the five LX2 progeny, where the male parent was a suspected hybrid, some variation in the F_1 phenotype was observed, two plants being of phenotype C, the other three of phenotype B, suggesting that segregation had taken place.

(b) Discussion of Esterase Results. It is not easy to suggest a genetic explanation for the Es results in the two species without further crosses. There are two problems to be solved, namely the cause of the multiple banding in both species, and the cause of the variation in relative intensity of the bands within L. vulgare. A genetic explanation of the results for this enzyme is necessary for the description of genetic variation within and between populations.

Considering first the multiple banding, a total of 48 plants from four different natural populations of L. humile all showed the same phenotype, C, while the 55 (excluding LV17) L. vulgare plants showed 39 of phenotype A and 16 of B (Table 5. 3.). It is possible that phenotype C is produced by fixed heterozygosity in duplicate genes. Different alleles of the duplicated Es locus could produce electrophoretically different subunits which combine together to produce oligomeric active isozyme bands ('heteropolymers' according to the terminology of Brewer 1970). The known tetraploidy of L. humile could provide a basis for this apparently non-segregating variation, but inheritance would have to be diploid to prevent segregation. Disomic inheritance of isozymes in tetraploid species is known; Humphreys & Gale (1974) showed this to be so for peroxidase in Papaver dubium. Alternatively the different bands could be made of the same active subunit at different degrees of polymerisation ('homopolymers'). This alternative hypothesis would not require the existence of fixed heterozygosity. Whatever the explanation, in

previous studies multiple bands have been of equal intensity or varying in a simple relationship to each other. In L. humile they were not, being ordered in decreasing intensity Nos. 3, 2, 4, 5. Cases like this where non-segregating isozymes had unequal banding intensity were referred to by Brewer (1970) and were explained by assuming that the association of subunits is, for some reason, non-random.

The same explanation for the multiple banding in L. humile must be applied to L. vulgare, and also be compatible with the observation that the latter species shows variation between plants in the relative intensities of its bands. Under the 'heteropolymer' hypothesis it would be necessary to propose the existence of further alleles specifying different oligomers which polymerise to form a different preponderance of banding. The 'homopolymer' hypothesis would require a separate locus which influenced the degree of association of identical subunits, with one allele specifying that a preponderance of band 2 is formed (phenotype A) and another determining that equal intensities of bands 2 and 3 be formed (phenotype B). The lack of phenotype C in L. vulgare plants also needs explanation. It could be sufficiently rare in this species not to have been detected in the population samples studied, or else unique to L. humile. If the latter is the case, this could mean that phenotype A plants of L. vulgare could be dominant over phenotype B, the homozygous recessive phenotype. One assumption that can definitely be made is that from the natural and synthetic hybrid plants phenotype C of L. humile is dominant over L. vulgare phenotypes, since the hybrids are phenotype C (except for LX2 which showed some segregation because one of the parents was itself a hybrid - see 5. 3(a) (iv) above).

Without assuming any genetic hypothesis, the numbers of the various phenotypes in the Limonium populations are given in Table 5. 3. The unusual status of LV17 has been described previously, and this was reflected in the Es results, where all plants sampled from this population were of phenotype A.

The other population samples of L. vulgare appeared to show a decrease in the frequency of the A phenotype with increasing proximity to L. humile. LV5, out of its range, had the highest frequency of phenotype A, while LV18, from a mixed population, had the lowest. It is tempting to suggest that under the hypothesis of the previous paragraph, and regarding LV18, LX18 and LH18 as a single interbreeding population, the phenotype A 'allele' is reduced in LV18 because it has been lost to L. humile where the phenotype C 'allele' is dominant over others. However, a statistical test is required to decide whether the numbers of phenotypes A and B are significantly different between L. vulgare population samples. It might be thought that Fisher's Exact Method for calculating Chi-squared of a contingency table of phenotype numbers is most suitable, but this is a complicated procedure. According to Cochran (1954) it is permissible to use an ordinary chi-squared test in this case, and this is presented in Table 5. 4. LV17 has been excluded. If the null hypothesis is that there is no significant difference between L. vulgare population samples in the numbers of phenotype A, $p = 0.3 - 0.5$, indicating that the null hypothesis should be accepted. Therefore this variation between L. vulgare population samples is not significant, and the trend of phenotype A to reduce in contact with L. humile may be artificial.

(c) Leucine Aminopeptidase Results

Extracts showed some streaking, possibly caused by some decomposition as a result of uncontrollable temperature rises during electrophoresis (also observed by Beckman et al. 1964 in maize LAP). Tests on extracts of various ages also revealed that the strongest band was particularly unstable and decomposed to give a diffuse faster region. In most plants a band which was designated band 4 was present. Many L. vulgare plants showed additional bands which were numbered according to the scheme sketched in Fig. 5. 2(b). Re-runs of the same extract and of plants maintained under cultivation showed that the presence of the various bands was repeatable.

(i) L. humile All plants from all populations sampled possessed only band 4, classed as phenotype P. Plate 5.2 shows photographs of this universal L. humile phenotype. It was checked by running plants from different populations side by side on the same gel.

(ii) L. vulgare A total of four bands was detected among these plants, numbered 1 to 4. Therefore band 4 was common to both species. If band 4 was present, it was nearly always the most intense, while other bands sometimes differed in intensity between individuals. When re-runs of cultivated material were possible, these intensity fluctuations were repeatable. Because the slower bands were sometimes weak,if a diffuse stronger band 4 was present, it was difficult to determine the exact phenotype of the individual with confidence. Population LV11 also presented difficulties because bands 1 - 3 were faint. Plate 5.2 shows photographs of some of the LAP phenotypes observed in L. vulgare and indicates the nature of the difficulty sometimes encountered in determining the presence of weaker bands. Fig.5.5 shows sketches of the various phenotypes found, together with the numbers of each phenotype found in each population. Note that some phenotypes show the same bands but with different intensities, such as Q_1 and Q_2 where band 1 is weaker in the former.

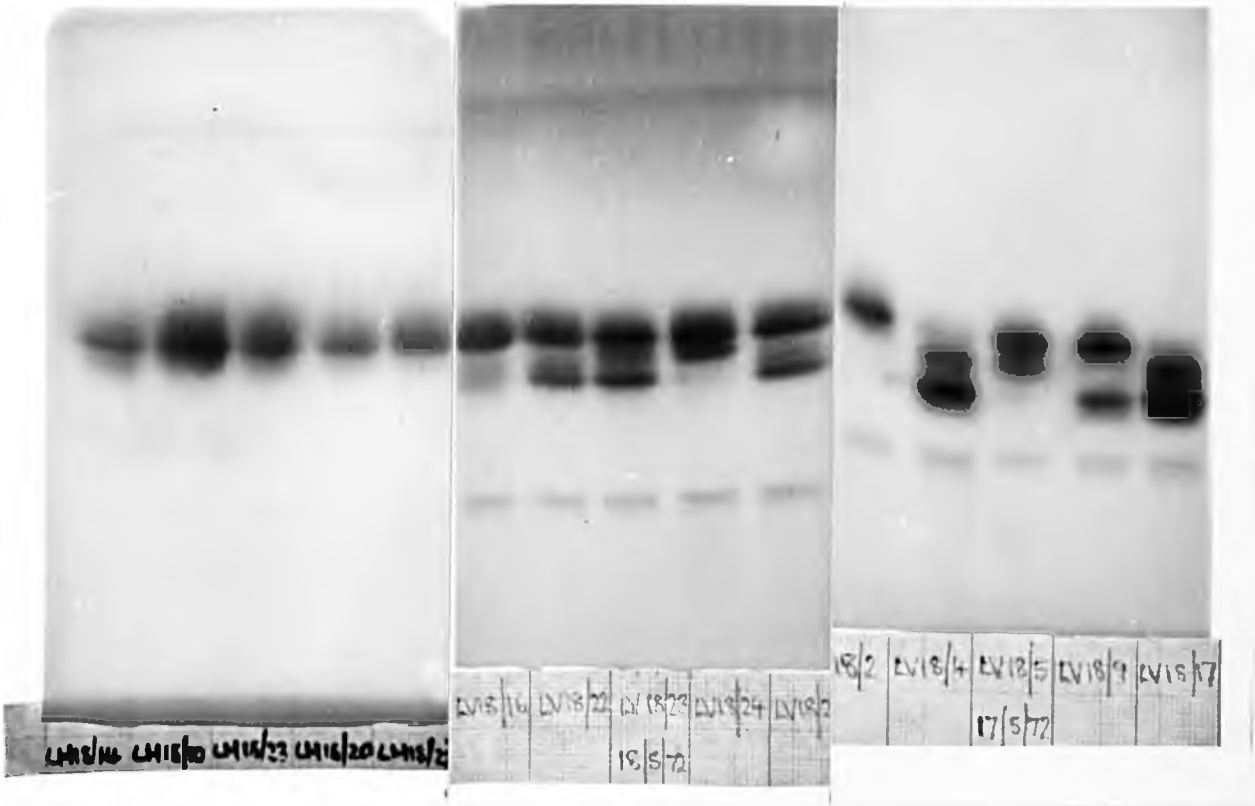
(iii) Wild 'Hybrids' All plants had band 4, and a few had some additional bands; one plant LX18/14, had all four bands, phenotype X_1. Fig. 5.5. shows sketches of these phenotypes.

(iv) Synthetic Hybrids Sketches of the results of crosses are given in Figs 5.3 and 5.4. Crossing an S_2 plant (- - 34) of L. vulgare with phenotype P of L. humile gave S_2 and three P phenotypes (LX2), suggesting segregation of the factor determining the presence of band 3. The results of wild and synthetic hybrids indicate that genes determining the LAP isozymes in the species are homologous.

(d) Discussion of Leucine Aminopeptidase Results The simplest genetic explanation is that the actual presence of the four bands is controlled by

PLATE 5.2.

Leucine aminopeptidase phenotypes of L. humile and L. vulgare.



1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
P	P	P	P	P	P	U_1	T_2	S_2	U_1	P	V	S_2	Q_2	V

Nos. 1-5 - L. humile. All plants showed this phenotype.

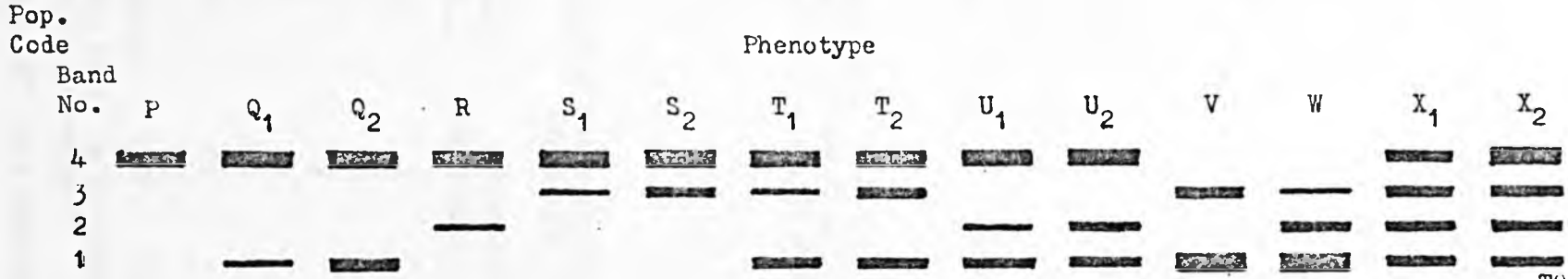
Nos. 6-15 - L. vulgare. Beneath each slot number is the phenotype letter.

Note slots 7, 8, 10, 12, 15 are difficult to score for band 2. Note also the variation of intensity of band 3 e.g. 8, 13, 14.

The slower constant faint band is protein (lost in first photograph due to destaining). The distortion of slots 10 and 11 is due to edge effects on the gel during electrophoresis.

Figure 5.5.

Leucine aminopeptidase phenotypes of Limonium populations.



Pop. Code	P	Q_1	Q_2	R	S_1	S_2	T_1	T_2	U_1	U_2	V	W	X_1	X_2	TOTAL
LV17	11														11
LV11	3	2		2	1		2								10
LV5	8								7	4		1			20
LV18	4		1			2		1	5	1	2			1	17
LV19	2					2								6	10
LX18	5				2								1		8
LH-	ALL PLANTS														

TABLE 5. 5.

LEUCINE AMINOPEPTIDASE BAND FREQUENCIES, AND PHENOTYPE NUMBERS AND RATIOS, IN L. VULGARE POPULATIONS

Population Code		LV11	LV5	LV17	LV18	LV19	LX18
No. of Plants		10	20	11	17	10	8
Band Frequency	4	1.00	0.95	1.00	0.88	1.00	1.00
	3	0.30	0.05	0.00	0.35	0.80	0.38
	2*	0.20	0.60	0.00	0.41	0.60	0.13
	1	0.40	0.60	0.00	0.65	0.60	0.13
Frequency of phenotype P		0.30	0.40	1.00	0.24	0.20	0.63
No. of different phenotypes		5	4	1	8	3	3
Phenotype ratio		0.50	0.20	0.09	0.47	0.30	0.38

*The frequency of band 2 is unreliable because of difficulties of observation
All L. humile plants from all populations were of phenotype P.
Phenotype ratio = No. of different phenotypes ÷ No. of plants in population sample.

TABLE 5. 6.

CONTINGENCY TABLE AND CHI-SQUARED OF LEUCINE AMINOPEPTIDASE PHENOTYPES IN L. VULGARE POPULATIONS

Population Code	Phenotype P		All other Phenotypes		Total
	Observed	Expected	Observed	Expected	
LV11	3	3	7	7	10
LV5	8	6	12	14	20
LV18	4	5	13	12	17
LV19	2	3	8	7	10
Total	17		40		57

Chi-squared = 1.71. 3 degrees of freedom. Therefore p = 0.5 - 0.7

Therefore there is no significant difference between populations in the numbers of phenotype P.

Note that LV17 (all phenotype P) has been omitted.

four codominant alleles at the same locus. Since L. vulgare and L. humile are tetraploid, this would permit all four alleles to be present in an individual, a situation which has been observed, phenotype X. This hypothesis requires that inheritance of the LAP locus is tetrasomic but one of the hypotheses to explain the Es demanded disomic inheritance. Also this LAP hypothesis only explains the presence of the bands, and not the fact that particularly bands 1 - 3 when present vary quantitatively between plants in a repeatable fashion. Also under this hypothesis allele dosage effects should be observed; in multiple-banded plants band 4 should be weaker than in plants with band 4 only, and so on for other bands. This was not the case: for example, see phenotype X_2.

It is also possible that the LAP isozymes are under the control of more than one locus. Studies of maize LAP (Beckman et al. 1964, Scandalios 1969) revealed a series of closely situated bands determined by several loci. Perhaps in Limonium band 4 is controlled by one locus common to both species, and bands 1 - 3 are controlled by a separate locus found in L. vulgare only but capable of being expressed in hybrid plants. In this case there would have to be a silent allele for bands 1 - 3 at this second locus to explain the presence of phenotype P in L. vulgare as well.

It is clear that the genetic control of the LAP isozymes found in Limonium cannot be explained with the present data; breeding experiments are necessary to solve this problem. One reasonable assumption that can be made is that phenotype P is representative of a homozygous condition, but because of the lack of a general genetic explanation for the phenotypes, combined with the difficulty of identifying some of the bands, variation within and between L. vulgare populations is difficult to express and measure. One way is in terms of the different phenotypes for each population, given in Fig. 5. 5; another method is to express the occurrence of each separate band as a frequency in the population sample. This has been done in Table 5. 5.

The LAP results for L. humile are comparable to those for Es in that all plants from all populations showed the same phenotype, in this case phenotype P. Population LV17 also showed this LAP phenotype in all samples. However, there was variation within all the other L vulgare populations in that more than one LAP phenotype was observed. There is also variation between these populations in terms of the frequency and category of the various phenotypes detected, and with respect to the frequencies of bands 1 - 3 (Table 5. 5 and Fig. 5. 5). On the other hand, a common feature of the different L. vulgare population samples is the relatively high proportion of phenotype P, and the high frequency of band 4. The two features are not unrelated, but Fig. 5. 5 shows that most of the plants other than phenotype P also possess band 4; only three plants of the 57 L. vulgare plants (excluding LV17) lacked this band. It is difficult to give a reason for this. Under the genetic hypothesis of a single locus with four alleles, a total of 15 different phenotypes would be expected, and larger samples would be required to detect all theoretically possible phenotypes, particularly as some band "alleles" are rarer than others. The high frequency of band 4, together with its often greater intensity than other bands might be a further indication that it is under the control of a separate locus from other bands, but it would be difficult to fit phenotypes V and W (band 4 absent, band 1 strong) into this scheme.

Because there are several phenotype classes scoring zero in some populations, the simplest statistical test to perform on the LAP data is to compare the numbers of phenotype P against all other phenotypes in each population sample. This is shown in Table 5. 6. On the null hypothesis that there is no significant difference between populations in the numbers of phenotype P, $p = 0.5 - 0.7$, meaning that the hypothesis is accepted, and that there is no significant variation between populations in the numbers of phenotype P in each sample.

Another way of measuring variation between populations is in terms of actual numbers of phenotypes detected in each population. Because the sample sizes are different, this has to be expressed as a proportion of the total number of plants sampled. This has been done in Table 5. 5. There appears to be an increase in this variation within L. vulgare populations with increasing proximity to L. humile, population LV18 from Scolt having a phenotype number: sample size ratio of 0.47, and LV5 (a pure population out of the distribution of L. humile) having a ratio of 0.20. Why and how L. humile could be influential on this cannot be explained without more knowledge of the genetic control of the phenotypes.

(e) 6 - Phosphogluconate Dehydrogenase Results

This enzyme was found to decompose rapidly, and only the freshest of extracts yielded recordable phenotypes. Because the technique of enzyme assay was only perfected after L. humile populations had been run, only a few cultivated plants of this species were investigated. All L. vulgare samples were also run from cultivation except LV5.

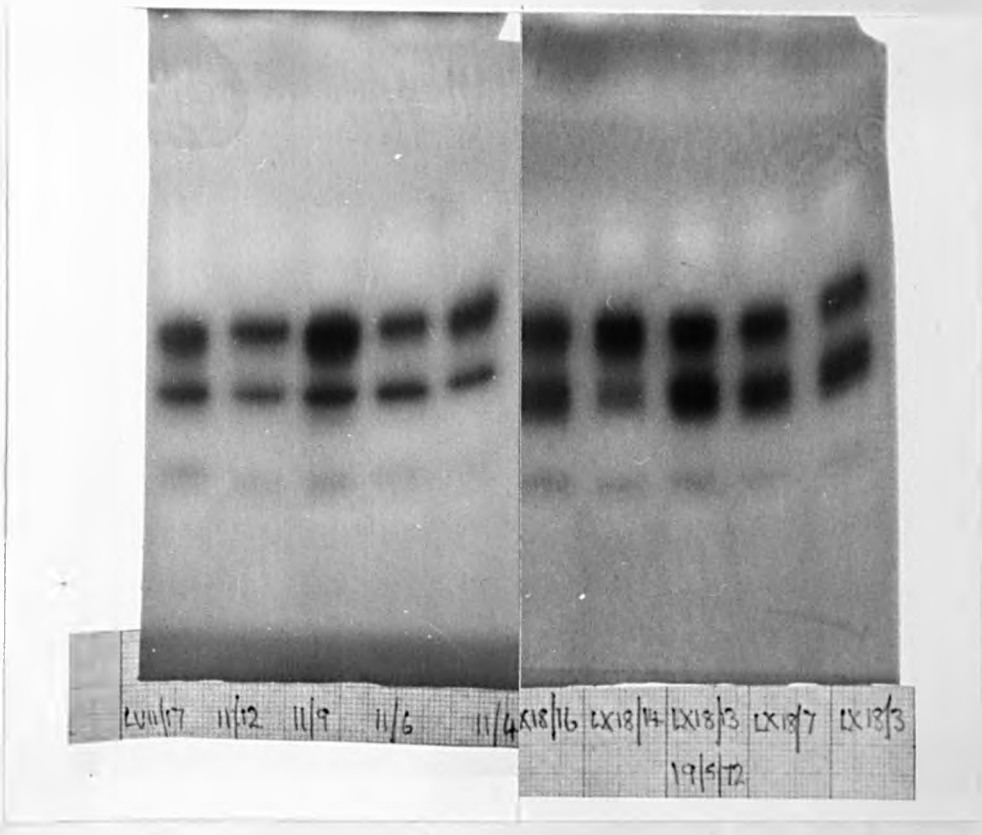
(i) L. vulgare Two well-separated regions of 6PGD activity were detected, and in the light of other dehydrogenase work, it is probable that the two regions are each controlled by a separate locus. The two regions were designated the 'faster region' and the 'slower region'. Within each region a maximum of two bands was observed, and for each region the two bands were designated S(slow)and F (fast). Plate 5. 3 shows photographs of these regions and their banding; Fig 5. 2(c) provides explanatory sketches.

(ii) Wild 'Hybrids' All 5 plants appeared to show both S and F bands in both regions; additional fainter bands adjacent to the stronger ones may have been present, but this could not be seen clearly. Plate 5. 3 shows photographs of this banding. One plant, LX18/14, had a faint slower region, which was repeatable.

(iii) Other Plants Runs were made in which wild 'hybrids', synthetic hybrids and

PLATE 5.3.

6 - Phosphogluconate dehydrogenase phenotypes of L. vulgare and wild 'hybrids'.



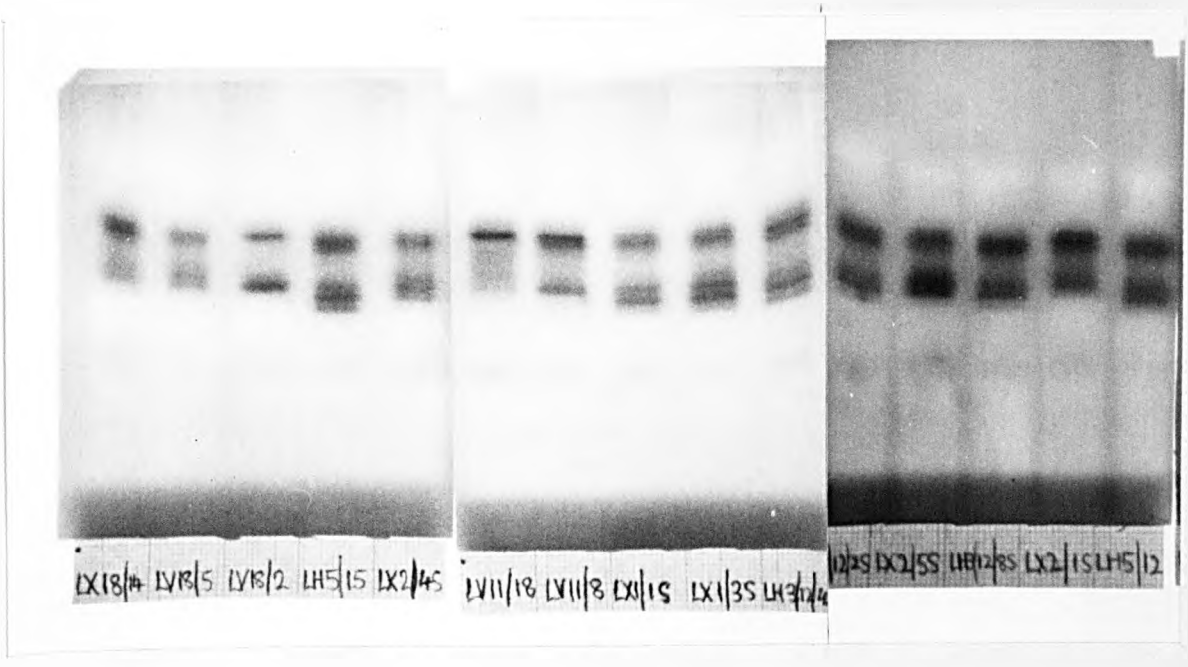
	1	2	3	4	5	6	7	8	9	10
Faster Region	SF	F	SF	F	SF	SF	SF	SF	SF	SF
Slower Region	S	S	S	S	S	SF	SF	SF	SF	SF

Nos. 1–5 - L. vulgare. Nos. 6–10 - wild 'hybrids'.

Beneath the slot numbers are descriptions of the phenotype for each region. In wild 'hybrids' all plants show two bands in both regions. The sharpest, no. 10, suggests there may be additional fainter bands.

PLATE 5.4.

6 - Phosphogluconate dehydrogenase phenotypes of various cultivated plants.



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Slots 6 and 7, LV11 from Anglesey. Slots 2 and 3, LV18 from Scolt. Slots 4 and 15, LH5 from Lake District. Slots 10, 11, 13, progeny of selfed LH3/12 plant from Scotland. Slot 1, wild 'hybrid' from Scolt (faint slower region repeatable and atypical). Slots 8 and 9, synthetic hybrids made from plants from pure stands in Anglesey. Slots 5, 12, 14, synthetic hybrids from LH3/12 and wild 'hybrid' from Scolt. For interpretative drawings see Figs. 5.2(c), 5.3(c), 5.4(c). Note similarity between 'pure' L. humile from different sources (slots 4, 10, 11, 13, 15). Note greater complexity of L. humile phenotypes than L. vulgare (slots 2, 3, 6, 7). Note differences between LX2 progeny (5, 12, 14) suggesting segregation. Note differences between wild (slot 1) and cultivated hybrids (slots 5, 8, 9, 12, 14).

TABLE 5. 7.

PHENOTYPE NUMBERS AND ALLELE FREQUENCIES OF 6-PHOSPHOGLUCONATE DEHYDROGENASE ISOZYMES IN L. VULGARE POPULATIONS

Population Code		LV11	LV5	LV18
Number of Plants		10	17	10
FASTER	FF	5	11	8
REGION	FS	5	5	2
	SS	0	1	0
Frequency of F allele		0.75	0.79	0.90
SLOWER	FF	1	0	0
REGION	FS	1	2	8
	SS	8	15	2
Frequency of F allele		0.15	0.06	0.40

The allele frequencies for the two regions have been calculated according to the genetic hypothesis described in Chapter 5. 3 (f). Only three L. vulgare populations were surveyed for this enzyme.

TABLE 5. 8.

CONTINGENCY TABLES AND CHI-SQUARED OF
6 - PHOSPHOGLUCONATE DEHYDROGENASE PHENOTYPES IN L VULGARE POPULATIONS

5. 8 (a) Faster Region

Population Code	FF Phenotype Observed	FF Phenotype Expected	FS + SS Phenotypes Observed	FS + SS Phenotypes Expected	Total
LV5	11	11	6	6	17
LV11	5	6.5	5	3.5	10
LV18	8	6.5	2	3.5	10
Total	24		13		37

Chi-squared = 1.98 2 degrees of freedom therefore p = 0.3 - 0.5

Therefore there is no significant difference between populations in the numbers of FF phenotype

FS and SS had to be grouped together because of the small numbers of SS.

5. 8 (b) Slower Region

Population Code	SS Phenotype Observed	SS Phenotype Expected	SF + FF Phenotypes Observed	SF + FF Phenotypes Expected	Total
LV5	15	11.5	2	5.5	17
LV11	8	6.8	2	3.2	10
LV18	2	6.8	8	3.2	10
Total	25		12		37

Chi-squared = 14.56 2 degrees of freedom therefore p = less than 0.001

Therefore the difference between populations in the numbers of SS phenotype is significant.

SF and FF had to be grouped together because of the small numbers of FF.

pure L. vulgare and L. humile plants (all cultivated) could be compared. Some of these are photographed in Plate 5.4. These runs revealed a greater complexity of banding in the faster and the slower regions for both L.humile and synthetic hybrids than was seen in L.vulgare and wild 'hybrids'. Taking into account all types of plant, a total of three banding positions was found in the faster region, and four in the slower region. Sketches of these positions are given in Fig. 5. 2 for wild material, and Figs 5. 3 and 5. 4 for synthetic hybrids.

(f) Discussion of 6 - Phosphogluconate Dehydrogenase Results

Considering L. vulgare alone, a simple genetic explanation of its phenotypes is possible; that each region is controlled by a separate locus, and that each locus has two codominant alleles specifying bands of different mobilities. Under this hypothesis the phenotype frequencies and the allele frequencies for the two loci are given in Table 5. 7. Breeding experiments are necessary to confirm this hypothesis.

Whether or not this is correct, there is variation within L. vulgare population samples for 6PGD phenotype. The raw data did not show any association between the faster and slower phenotypes of individual plants. Variation also exists between L. vulgare populations in terms of phenotype frequency for both regions, although this is not so marked if the allele frequencies are considered, except that the F allele for the slower region of LV18 has a much higher frequency than in the other two populations.

To decide whether these differences between populations are significant, a chi-squared test was used, as described in 5. 3(b). Because some classes of phenotype for each region were rare or absent in some samples, for the faster region the numbers of FS and SS plants were grouped together, and for the slower region the FF and FS plants were grouped. The tests showed (Table 5. 8) that for the faster region, the numbers of FF plants did not differ significantly between population samples ($p = 0.3 - 0.5$), but for the

slower region the numbers of SS plants did differ significantly between populations, $p < 0.001$. In the latter case, this difference is particularly due to the high proportion of FS plants in LV18. However it should be borne in mind that LV11 and LV18 are only the part of population samples which had survived after cultivation. Therefore the proportions of the phenotypes may be even less a reflection of the whole population than the original sample if the loss of the various phenotypes during cultivation was non-random.

With the limited data it is more difficult to provide a genetic explanation for the more complex L. humile 6PGD phenotype. In all, seven presumably 'pure' individuals from various sources were run, and these all appeared to give the same phenotype, sketched in Fig. 5. 2(c), of three slow bands and two faster ones. If it is assumed that they have fixed heterozygosity by gene duplication, then for each region there could be two alleles producing bands of different mobility. In the slower region hybrid bands are formed by the association of subunits, while in the faster region there are no subunits and therefore no hybrid bands are formed. This hypothesis is highly speculative.

An even more complex situation is observed in the wild and synthetic 'hybrids'. None of these phenotypes can be derived by the simple addition of the phenotypes of the two pure species; possibly there is some interaction between the genomes of the two species. A further difficulty is that the wild 'hybrids' do not exactly resemble the synthetic hybrids in 6PGD phenotype. All these problems require solution by inheritance studies.

5. 4. ELECTROPHORESIS RESULTS AND THE BREEDING SYSTEM

It has been shown for the outbreeding L. vulgare that phenotype variation, and very probably genetic variation, occurs within populations for the three enzymes studied. These findings correspond with the hypothesis of Baker described in Chapter 1 which referred mainly to morphological variation and the breeding system. He also anticipated that this variation

within outbreeding populations would tend to obscure clear-cut distinctions between populations, particularly when the characters were not connected with ecologically significant variation by linkage or pleiotropy. In the present isozyme results, although there was some variation between populations of L. vulgare in actual phenotype frequencies, and this may have been associated in some way with the occurrence of L. humile, simple statistical tests showed that this was generally not significant. Further, different population samples contained plants which showed the same phenotype, again indicating overlap between populations. Therefore isozyme variation between populations of L. vulgare also corresponded to Baker's anticipations concerning morphological variation expected between outbreeding populations.

One of Baker's prerequisites was that the morphological variation between populations was not connected with ecological variation. As Limonium species in general are ecologically restricted (and this was indicated by the work of Boorman referred to in Chapter 2) differences between populations as a result of this factor are likely to be relatively small. If this assumption is true, then the present isozyme results for L. vulgare could be interpreted as providing rather tenuous evidence to reject the 'neutral' theory of isozyme variation discussed earlier in this chapter. This is because if the pattern of isozyme variation in ecologically similar populations did not have a selective value, different populations would be expected to show different levels of variation and different proportions of phenotypes as a result of genetic drift. However it has been shown that phenotype variation between populations was not significant - for example phenotype P and band 4 of LAP showed relatively high frequencies in all L. vulgare populations. This sort of electrophoretic information has been used elsewhere as evidence to reject the 'neutral' theory (e.g. Lakovaara & Saura 1971, Nevo et al. 1974).

For L. humile, there was a lack of variation within and between populations for the two enzyme systems surveyed in number. This lack of

variation within populations is expected if L. humile fully exploits its ability to inbreed, but from the enzyme phenotypes it appears that this invariability may be expressed in different ways; for Es by fixed heterozygosity or by varying degrees of polymerisation of identical subunits, and for LAP by simple homozygosity. In Es, the inflexibility created by the probable lack of segregation and recombination has been countered by producing a variety of forms of the same enzyme within a single individual.

Concerning variation between populations, Baker suggested that where variation was rendered discontinuous by nearly complete inbreeding, a stable pair of populations would be either identical, or relatively clearly distinguishable. The isozyme results exhibit the former alternative, and there are two possible reasons for this. Firstly, L. humile is ecologically even more restricted than L. vulgare, and differentiation between populations for ecological reasons alone might be expected to be low. The second reason relates to the suggested method of speciation and subsequent establishment of L. humile in the British Isles (Baker 1953c). One or a few individuals may have arrived from North America soon after speciation, and established populations in this country by self-fertilisation. Barring mutation, this would create a series of genetically identical individuals and populations by the 'founder effect' (e.g. Mayr 1970). This method of colonisation resulting in a lack of isozyme variation within and between populations is exactly comparable with that of Ruminia decollata described earlier (Selander & Kaufman 1973). Only when L. humile meets L. vulgare (for example at Scolt Head Island, LX18) is it possible for genetic variation to be incorporated into L. humile by hybridisation and introgression. It should also be noted that the lack of variation in L. humile does not correspond with the pattern of variation observed by Allard and others in some other inbreeding species.

Although the enzyme phenotypes require inheritance studies, there is a large body of evidence from previous work to support the contention that the

variation in enzyme phenotypes found in Limonium is genetically controlled, and therefore that L. vulgare exhibits genetic variation and segregation while L. humile does not. This assumes that all isozyme variation is electrophoretically detectable, and it is possible that some cryptic variation does occur in L. humile for Es and LAP. However, the fact that the closely related L. vulgare does show variation for the same enzymes makes it unlikely in this case. In some previous work, the observed isozyme patterns have been relatively simple to interpret genetically without the need for breeding experiments, but in this case the genetical basis for the isozyme patterns is not immediately obvious. Other cases of complex banding systems have been encountered, for example by Beckman et al (1964), Desborough & Peloquin (1967), Endo (1971) and Jelnes (1974).

For L. vulgare populations, the results were examined to see if there were any associations between the different phenotypes for the three enzymes in a particular plant, and also to see if any phenotypes were characteristic of a particular pollen and stigma combination. No such relationships were observed.

Quite different results may have been obtained had it been possible to survey other enzyme systems. The present experiments have investigated only an infinitessimally small fraction of the whole genome of Limonium individuals. Unfortunately, much needed information from L. binervosum populations was unobtainable.

CHAPTER 6.

DISCUSSION AND CONCLUSIONS

During the discussion of the results of chromatography and electrophoresis in Chapters 4 and 5 respectively, attempts were made to relate the observed variation to hypotheses described in Chapter 1 concerning the variation expected within and between populations adopting different breeding systems. Table 6.1. considers together the findings of the chromatographic, electrophoretic and also morphological studies of variation within and between populations of the three Limonium species. The species have been ranked 3, 2, 1 in descending order of relative variation for each character measured. Where this order agrees with that expected from predictions described in Chapter 1 it has been asterisked. Table 6.1. only gives very general conclusions, and some simplification has been necessary; chapter sections where more information is given are indicated in the table.

Table 6.1. attempts to answer two important questions in particular about the research; further questions arise from these. Firstly, does the type of variation studied yield results that correspond with those expected from the breeding system - does chromatographic and electrophoretic variation within and between Limonium populations relate to their degree of inbreeding or outbreeding? The table and previous discussion show that within populations, variation measured by both biochemical methods does conform with general expectations. Therefore in this respect the hypotheses are supported. Concerning variation between populations, there is less concurrence between expectation and observation in the chromatographic measurements, but the electrophoresis results for L.vulgare and L. humile do compare well with predictions from their breeding systems. In addition, the lack of enzyme variation between L. humile populations can be accounted for if the founder effect, not considered in Chapter 1, is

TABLE 6. 1.

RESULTS OF VARIOUS METHODS OF MEASUREMENT OF VARIATION AND THE BREEDING SYSTEM IN THE THREE LIMONIUM SPECIES

	Variation Within Populations				Variation Between Populations			
	Chromatographic		Electrophoretic	Morphological	Chromatographic		Electrophoretic	Morphological
	Qualitative Polymorphic Index	Quantitative Kendall's W			Qualitative Biochemical Distance	Quantitative Correlation Coefficient		
L. vulgare	3 **	3 * some overlap with L. humile	3 **	3 few results	2	3	2 *	1 few results
	4.2(g)		5.4	2.5(a)&(d)	4.3(e)		5.4	2.5(d)
L. humile	2 =*	2 * some overlap with L.binervosum	1 ** no variation	2 few results	1	2 =	1 * no variation	-
L.binervosum	2 =* some variation remains	1 * some overlap	-	-	3 *	2 =	-	-
L.binervosum (Cultivated)	1 * some variation remains	1 *	-	1 **	3 * correlates with geography and morphology	3 *	-	3 **
	4.4(e)			2.5(a)&(b)	4.4(f)			2.5(b)

Key: 3 = high variation, 1 = low variation, ** = good agreement with expectation from Baker's hypotheses expressed in relative terms for each index separately. * = moderate " " " " " "

Other numbers refer to chapter sections where more detail may be found.

assumed to be influencing the structure of these populations.

The inconsistencies in chromatographic variation between populations can be explained in two ways. Either the predictions concerning variation between populations are incorrect, or else chromatography should be regarded as an unreliable method of measuring genetic variation between populations. This will be discussed after further examination of the nature of chromatographic variation.

The second major question that can be raised is whether the two methods of measurement of variation agree with each other in their general levels; if there is more chromatographic variation in one species than another, then is there also more electrophoretic variation? This question is not quite the same as the first, because any variation observed need not be related to the breeding system. Note in passing that the two types of variation were computed using different criteria. Electrophoretic variation considered individual enzymes and plants, while chromatographic indices were derived by grouping plants and spots together. However, the complete absence of detectable enzyme variation in L. humile means that there is bound to be more electrophoretic variation in L. vulgare whatever way it is calculated. Table 6.1. shows that within the limits of the reliability of comparisons in the absence of electrophoresis results for L. binervosum, the two methods of measuring variation both within and between populations agree in their general trend. Both electrophoresis and chromatography show more variation within and between L. vulgare populations than L. humile populations. However, there is one way in which the two methods of measurement differ, not shown in the table. Electrophoresis of L. humile showed zero variation in enzyme phenotype between plants and populations, but in chromatography all L. humile populations did show some variation within and between populations.

It is also of interest to see if the two types of biochemical

variation agree in their trends at a population level within a particular species. It is only possible to make comparisons of four L. vulgare population samples, because of the complete lack of electrophoretic variation in L. humile, and because LV2 and LV3 did not survive cultivation for electrophoresis. In Table 6.2. the populations have been ordered in increasing variation for the different measures. The table shows that no general trend can be observed either between the two chromatographic measures, or between different enzyme systems, or between electrophoresis and chromatography in general, with the important exception that LV17 was the least variable in all cases. Turning to this population in detail, the actual data showed that LV17 always held the same level of variation as L. humile populations both chromatographically and electrophoretically. This observation will be returned to later.

To clarify the above general discussion, it is necessary to determine more precisely the nature of the variation anticipated, and the nature of the variation observed. Is the expected variation within and between populations in fact genetic in nature; and is the observed chromatographic and electrophoretic variation directly genetically controlled? In answer to the former point, although the mathematical predictions from theoretical models were specifically concerned with genetic variation, in practice it has sometimes been difficult to study the variation of actual genes and their alleles in plant populations, and the hypotheses of Baker did not refer to genetic variation but to phenotypic variation. The relationship between the two was not made clear, except that in the experimental evidence provided (mainly morphological measurements of Armeria), it was assumed that most adaptive variation is controlled by multiple gene differences. There was also some attempt to distinguish between characters determined by a few and by a number of genes, but no evidence of their actual genetic control was presented. With the exception

TABLE 6. 2.

RELATIVE LEVELS OF BIOCHEMICAL VARIATION WITHIN L. VULGARE POPULATIONS

Measurement of variation	Least variable population →			Most variable population
Polymorphic Index	LV17	LV18	LV5	LV11
Kendall's W	LV17	LV11	LV18	LV5
Es	LV17	LV5	LV11	LV18
LAP	LV17	LV18	LV11	LV5
6PGDH Fast	-	LV5	LV18=	LV11=
6PGDH Slow	-	LV5	LV18=	LV11=

For each method of measuring variation, the populations have been arranged in increasing order of variation. Data for Polymorphic Index and Kendall's W from Table 4. 2; Es Table 5. 3 (frequency of Phenotype A); LAP Table 5. 5. (frequency of Phenotype P); 6PGDH Table 5. 7. (frequency of FF and SS phenotypes respectively).

of electrophoretic studies, the same is true for other work described to support the general hypotheses. For practical reasons the assumption had to be made that phenotypic variation gives a true reflection of genetic variation, although this may not be so.

The genetic control of biochemical variation can now be considered further. In the case of chromatographic variation, evidence from other sources (e.g. Brehm & Alston 1964, Fahselt 1971) was presented that the presence of single chromatographic spots can be genetically controlled, but that it was the stronger yellow flavonoid spots that reflected genetic variation by their simple presence and absence. In Limonium no such variation could be found, except between cultivated L. binervosum populations and in a few isolated L. vulgare plants. Instead, most variation was found in the intensity of these spots and in the generally fainter non-yellow spots. Although it is possible that this quantitative variation is genetically controlled, it is likely that this control, like that of some morphological variation, is polygenic, and that quantitative chromatographic variation is influenced by variation in the physical environment.

At this stage, the low variation in LV17 should be recalled. It had been previously suggested that this population had been established by vegetative spread. The electrophoresis results support this, because all plants were identical in this respect, whereas there was enzyme variation within other L. vulgare populations. If it is true to say that LV17 had been established vegetatively, then all chromatographic variation observed in this population would be non-genetic in origin. Since LV17 and L. humile populations had similar levels of chromatographic variation, then this could in turn imply that the chromatographic variation detected within L. humile populations was also mainly non-genetic. This argument would also apply to L. binervosum populations if they have

been established by apomixis. Therefore chromatographic variation bears a similar relationship to the breeding system as does morphological variation; the correspondence is not clear, and can be related to both genetic variation and environmental influences.

Therefore the above argument can be used to explain the observed residual chromatographic variation in L. humile populations compared with their lack of electrophoretic variation. When environmental influences can be reduced, as in the case of cultivated L. binervosum populations, variation within populations was lowered when compared with the wild counterparts. However, the variation within these populations was not then zero, and other sources of variation such as experimental technique had to be invoked in order for the chromatographic variation within populations to be consistent with apomixis.

However, chromatographic variation between these populations did yield valuable information. Both wild and cultivated L. binervosum populations showed greater chromatographic differences between them than those found between the other two Limonium species as a whole. The differences between cultivated L. binervosum populations were also as much as those between species of other genera (e.g. Alston & Turner 1962, Levin 1967, Cruise & Haber 1972), and were similar to those found by Asker & Frost (1970a) between apomictic Potentilla argentea biotypes. Therefore the chromatographic variation between cultivated L. binervosum populations correlates with that of other apomictic species and with that anticipated from its breeding system. In Limonium, greater chromatographic differentiation between populations of an apomict... is permitted than that between related sexually reproducing whole species. However, even this variation does not provide direct access to gene differences between populations. In general, chromatography of Limonium populations sampled wild yielded ambiguous results which could not confidently be related

purely to either the breeding system or genetic variation.

On the other hand, there is little doubt that the electrophoresis results do accurately reflect genetic variation in Limonium, therefore the relationship between electrophoretic variation and the breeding system must be regarded as significant. Considering the results of L. humile, although Baker did not give any consideration to the founder effect, he did mention the alternative that a pair of inbreeding populations would either be identical or relatively clearly distinguishable.

However, there is another hypothesis that would explain the lack of electrophoretic variation in L. humile. While this lack may be a consequence of its breeding system and mode of establishment in this country (compare with Selander & Kaufman 1973), it could be that LAP and Es in L. humile happened to be enzymes which simply did not vary. Some enzymes studied in both inbreeding (e.g. Marshall & Allard 1970a) and outbreeding (e.g. Lewontin & Hubby 1966) populations have been invariant. However, in the present work the closely related L. vulgare does show variation in these homologous enzymes, and studies on putative and synthetic hybrids showed that variation can be incorporated into L. humile by hybridisation and introgression. Therefore it is likely that some genetic variation of LAP and Es in this species would have been detectable had it been present.

The lack of enzyme variation between L.humile individuals is of interest when related to the current views outlined in Chapter 5, on the selective advantage of genetic polymorphism in natural populations. Supporters of this hypothesis would expect any existing opportunity for variation in enzymes to be favoured, and Allard showed theoretically and experimentally that even a small amount of outbreeding in an inbreeding species can encourage genetic heterozygosity for selective reasons. This was demonstrated

electrophoretically in Avena. Therefore it is difficult to accept that the lack of enzyme variation in L. humile is entirely due to inbreeding and the founder effect, although this may be the major reason. A further factor which could reduce variation in the species may be its ecological requirements. It is ecologically restricted, having a less frequent overall distribution, and also a smaller tidal range, than L. vulgare where the two species occur together. However in salt marshes where it does occur it is successful, sometimes being a codominant species. Perhaps the predominance of inbreeding, faithfully reproducing a successful genotype for a specific ecological niche, has a greater selective advantage than any outbreeding, which may produce genetic variation and heterozygosity but provide less specific adaptation. Further, it has been shown that even outbreeding need not create isozyme variation if a specific genotype is required, e.g. by Nevo et al.(1974). The Es results for L. humile may indicate a strategy to produce plasticity despite a lack of segregation and variation, by producing invariant multiple bands. The greater ecological success of L. vulgare could be attributed to its self-incompatibility and its necessity to outbreed, maintaining genetic variation. Rogers (1971) suggested a similar correlation between breeding system and ecological success in Papaver species.

Therefore the lack of enzyme variation in L. humile in comparison with L. vulgare can be seen as an interaction of the factors of breeding system, method of colonisation and ecological success, although it should be stressed that only a small number of enzyme systems were studied. This same general explanation might apply to the relatively low chromatographic variation found between L. humile populations, but here there are environmental complications of an even greater magnitude than those influencing chromatographic variation within populations.

As it is observed that there is electrophoretic variation in L. vulgare why is there not also more observed genetically controlled variation of the yellow flavonoid spots? These spots are synthesised by enzymes, and if LAP and Es can vary, why cannot flavonoid synthesising enzymes? Perhaps this is partly the result of flavonoids being controlled by dominant genes, meaning that heterozygotes cannot be detected. Any homozygous recessives that were produced would lack a particular spot and perhaps suffer severe selection pressure, because the presence of a certain group of flavonoids may be in some way essential for the continued existence of the plant; however the function of specific flavonoids within plants is not known. Possibly the few homozygous recessives that have survived and were sampled are represented by the small number of plants found to be lacking in one of the stronger yellow spots (e.g. LV3 spot No. 12; LV18 spot No. 4; LV1, LV2, LV3 spot No. 23). Cases where flavonoids have not varied in other species have been reported e.g. Ockendon et al. (1965), Levin (1967).

As a brief conclusion to this general discussion, there is no biochemical evidence which directly contradicts the general hypothesis of Baker. In the case of chromatographic variation, instances where the results are ambiguous cannot be used to reject the hypothesis, since these results can be attributed to environmental influences. The electrophoresis results, although limited in their quantity and scope, provide direct genetic evidence to support the hypotheses.

A broader question for discussion is that of the relative usefulness of chromatography and electrophoresis as general genetic research tools. In theory, both techniques should be of value in the measurement of genetic variation, but it has been shown that there are practical difficulties associated with chromatography, particularly in intraspecific studies. This is because the amounts of the compounds

studied are influenced by variations in the environment, or because the compounds simply have not varied detectably. Several authors report that their conclusions from chromatographic studies of intraspecific variation are "tentative" (e.g. Bragg & McMillan 1966, Abraham & Solbrig 1970, Douglas & Taylor 1972) and there may have been others who should have done so. Only in a few cases has genetic variation been positively demonstrated by breeding experiments (e.g. Belzer & Ownbey 1971). Therefore early conclusions about the universal reliability of chromatography have needed to be re-examined. These doubts have probably contributed to the recent decline in the number of publications using this technique. However, chromatography has come into its own in the study of interspecific hybridisation, particularly when used in conjunction with morphological evidence (e.g. Parups et al. 1966, Olden & Nybom 1968, Harborne & Williams 1973). In this context it has also been useful to the present work in conjunction with electrophoresis in indicating a close biochemical genetic similarity between L. vulgare and L. humile. This further suggests that Baker's (1953c) scheme for the origin of L. humile from L. vulgare involving at least seven intermediate species and two transatlantic crossings requires further investigation.

However, in general terms even the useful contributions to the study of variation made by chromatography pale into insignificance when compared with the major advances in genetic and evolutionary research made possible by electrophoresis. A small proportion of these have been referred to in Chapter 5, and a recent review of the progress was made by Lewontin (1974). Electrophoresis has been applied to a wide variety of physiological, genetic and evolutionary problems in many plant and animal species, and has a potential for much more research in the future. It gives direct access to the genotype of individuals, and should therefore be of great value in further studies on the lines of this thesis.

However, general progress using electrophoresis has not been made without the consumption of large quantities of time and financial and technical resources, and in this respect chromatography has been more economical. In the present work a chromatographic result from a single plant represented about 3 hours work, and used simple and inexpensive chemicals and apparatus, while electrophoresis required about 10 hours work per plant and required more costly facilities. One of the demands on time and resources by electrophoresis was caused by the difficulties of preparing reasonably pure enzyme extracts from fresh leaf tissue; an additional disadvantage was the absence of detectable enzymes in L. binervosum. Fortunately these practical problems have not been so important in other work using animal tissues.

SUGGESTIONS FOR FURTHER RESEARCH

Firstly, preliminary work in Chapter 2 showed that the degree of inbreeding and outbreeding of the three species needs to be established more precisely, particularly in L. humile. Although the variety of reproductive mechanisms enabling plants to possess a wide range of breeding systems has been known for some time, only recently have natural populations received a detailed study to establish their precise degrees of inbreeding or outbreeding e.g. Brown & Allard (1970), Levin (1972), Ellis (1973), and similar studies should be directed to Limonium. In these and other examples the reverse approach to the present work has been taken - morphological or biochemical evidence has been assumed to be an indicator of genetic variation and therefore of the breeding system.

Also relevant to determining the breeding system of natural Limonium populations is the consequence of their mode of dispersal to the genetic relationships between individuals. How is a population established? Is this achieved by a few plants building up a local population by dispersing seed over a short distance within the same salt marsh, or by the continuous

arrival of many seeds carried greater distances by currents and tides from one or more populations already established elsewhere? These methods of colonising a developing salt marsh, as well as the perennial habit of all three species, will influence the genetic relationships of the colonists, and therefore their degree of inbreeding or outbreeding.

More work needs to be done of the present kind. A particular salt marsh or cliff side may contain hundreds or thousands of individuals, and it can be argued that a sample of around 25 plants from a locality is insufficient. However, it would have been difficult to process many more than this number from each region in the time available. From the present results, more leaf chromatography might not have provided more useful information, but electrophoresis of more plants and enzyme systems, had this been practicable, would have given more reliable estimates of genetic variation, and would have provided information comparable to that from some animal species on the degrees of genetic polymorphism and heterozygosity in the population. Also some areas of the British Isles have not been sufficiently sampled, in particular the English South Coast and Ireland.

One possibility that was not explored was the chromatography of Limonium flower pigments. In view of their well-established genetic control in other species, and because of the observed variation between L. vulgare plants in petal colour (Chapter 2.5. (a)) this line of research could have yielded useful results.

Finally, to study thoroughly the relationship between variation and the breeding system it is necessary to take closely related species with similar ecological habits but adopting diverse reproductive methods. Although it is generally agreed that the breeding system is an important factor in influencing the variation and evolution of a species, surprisingly little work meeting all the above criteria has actually been undertaken to investigate the relationship. The breeding system has been invoked to

explain results of observed variation without it being realised that the experimental evidence is still relatively sparse. Therefore the most important 'further research' which is necessary is more work, both morphological and biochemical, on other plant genera.

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NOTE: The literature survey refers to relevant publications that were available up to DECEMBER 1974.

APPENDIX I

DETAILS OF *LIMONIUM* POPULATION SAMPLES

Pop. Code	Date of Sample	G.R.	Locality	No. of plants C'graphed	No. of plants Elec'resed
LV1	13.8.69	SH 486885	Traeth Dulas, Anglesey	23	2
LV11	17.10.70	"	" " "	-	8
LV2	16.8.69	TM 052156	Fingrinhoe, Essex	25	1
LV3	"	"	" "	25	-
LV5	24.7.72	SX 980792	Dawlish Warren, Devon	25	20
LV17	19.8.71	ST 261430	Combwich, Somerset	10	10
LV18	12.8.71	TF 810460	Scolt Hd. Is., Norfolk	20	17
LV19	11.8.71	TG 010445	Morston, Norfolk	-	10
LX18	12.8.71	TF 810460	Scolt Hd. Is., Norfolk	5	8
LH1	12.8.69	SH 278787	4 Mile Bridge, Anglesey	25	-
LH2	14.8.69	"	" " " "	25	-
LH3	5.9.69	NX 472573	Wigtown Bay, Kirk'b'shire	25	1
LH4	"	NX 573545	Fleet Bay, Kirk'b'shire	30	-
LH14	5.7.71	"	" " "	-	13
LH15	13.7.71	SD 426786	Holme, Lancashire	-	16
LH12	15.8.71	-	Roaring Bay, Co. Cork, Ireland	25	12
LH18	12.8.71	TF 810460	Scolt Hd.Is., Norfolk	5	7
LB1	9.8.69	SH 385636	Llanddwyn Is., Anglesey	25	-
LB2	12.8.69	SH 400662	Cefni Est., Anglesey	25	-
LB21	17.10.70	"	" " "	20	-
LB3	16.8.59	TM 103123	Colne Point, Essex	25	-
LB4	"	"	" " "	25	-
LB8	9.6.70	TF 812462	Scolt Hd. Is., Anglesey	36	-
LB9	31.7.70	SW 850764	Trevose Head, Cornwall	25	-

CONTINUED OVER

APPENDIX I - Continued

Pop. Code	Brief Description of Habitat	Comments
LV1	Estuary bank, S.M. plants + *Juncus*, mud over sand	Isolated, small plants, shaded
LV11	" " " "	Resampling of same site
LV2	Upper salt marsh, *Aster* rare	Adjacent populations, ecologically different
LV3	Lower salt marsh, *Aster* frequent	
LV5	Salt Marsh	Outside distribution of *L. humile*
LV17	Muddy crevices of old sea wall	All plants A/cob; perhaps spread vegetatively
LV18	Developing salt marsh	*L. humile* present, only 'type' plants sampled
LV19	Salt Marsh	*L. humile* absent
LX18	Developing salt marsh	Morphologically intermediate, probably hybrids
LH1	Salt Marsh	Just outside distribution of *L.vulgare*; adjacent populations, ecologically different
LH2	Crevices in rocks	
LH3	Estuary bank	Isolated plants, shaded
LH4	Clumps in bare mud/coarse shingle	10 miles LH3; just outside *L.vulgare*
LH14	" " " " "	Resampling of same site
LH15	Sandy salt marsh	
LH12	Crevices in rocks	*L. vulgare* absent from Ireland
LH18	Developing salt marsh	*L. vulgare* present, only 'type'sampled
LB1	Rocky cliff	
LB2	Sandbank above drift line	Unusual habitat; plants vigorous
LB3	Stabilised shingle, 60% bare	Adjacent populations ecologically different; LB4 heavy competition shading
LB4	Older shingle covered with vegetation	
LB8	Raised shingle bank	Mixture of forms
LB9	Cliff of loose shale	

APPENDIX II

Frequencies of Phenolic Spots in all Wild Populations

Spot No.			1	2	3	4	5	6	7	8A	8B	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
Colour			Y	Y	Y	YG	YG	YO	Y	Y	Y	Y	Y	YG	YG	YO	YO	Y	Y	YG	G	YO	Y	Y	Y	YG	Y	Y	YG	Y
Detected By			F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F
																			*											
Date Ran	Pop. Code	No. Plants																												
30.11.69	LV 1	23	1	1		1	1	1	1	1		1		1	1	1	1		·83	1	·39			·91	1	·96	1	·22	1	1
			2-5	2-4		2-3	2-3	3-5	4-6	3-4		•-1		5-6	2-4	2-3	1-2		•-1	1-3	•-1			1-2	1-2	1-2	1-4	1-2	2-3	•-1
14.4.70	LV 2	25	1	1		1	1	1	1	1		1		1	1	1	1		·88	1	1			·92	1	·96	·68	·32	1	1
			2-4	2-3		2-3	1-3	4-6	4-5	3-4		•-1		3-4	2-4	2-3	1-2		•-1	2-3	1-3			1-2	2-4	1-3	1-5	1-2	2-4	2-3
21.4.70	LV 3	25	1	1		1	1	1	1	1		1		1	·96	1	1		·64	1	·88			·32	1	·96	·12	·88	1	1
			2-3	2-3		2-3	1-3	3-5	3-5	3-4		•-1		3-5	2-3	1-3	•-2		•-1	2-3	•-2			•-1	2-3	2-3	1	•-2	1-3	1-3
11.8.72	LV 5	25	1	1		1	1	1	1	1		1		1	1	1	1		·68	1	1			1	1	1	·64	·32	1	1
			2-4	2-3		1-3	1-2	4-7	5-7	3-5		•-1		4-7	4-5	1-2	1-2		•-1	1-2	•-2			•-2	2-3	1-2	•-2	•-1	2-3	1-2
9.3.72	LV17	10	1	1		1	1	1	1	1		1		1	1	1	1		·70	1	1			1	1	1	1		1	1
			4-5	2-4		2-4	2-3	5-6	3-6	3-4		•		3-5	3-4	1-3	2-3		•-1	1-2	2-3			2-3	2-3	1-3	4-5		2-3	1-2
9.3.72	LV18	20	1	1		·95	1	1	1	1		1		1	1	1	1		·85	1	1			1	1	1	·90	·10	1	1
			3-5	3-4		2-3	2	5-7	4-6	3-4		•-1		4-5	3-4	2-3	1-2		•-1	2-3	2-3			1-3	3-4	1-2	2-5	1-2	2-4	1-2
24.2.70	LH 1	25	1	1		1	1	1	1	1		1		1	1	1	1		1	1	1			1	1	1	·96	1	1	1
			2-5	3		2-3	•-1	4-6	3-5	3		1		2-3	2-3	2-3	1-2		•	1-2	2			1-3	2-3	1-2	1-4	•-1	2-4	1-2
24.3.70	LH 2	25	1	1		1	1	1	1	1		1		1	1	1	1		1	1	1			1	1	1	1	1	1	1
			2-5	3		3	3-4	4-6	4-6	2-3		1		3-4	3-4	3	2		•-1	1-3	2-3			1-2	3-4	2-3	2-4	1-2	3-4	1-2
2.6.70	LH 3	25	1	1		1	1	1	1	1		1		1	1	1	1		1	1	1			1	1	1	1	·88	1	1
			3-5	4-5		4	2-3	5-6	5-6	3		1		4	4	2-3	2-3		•-1	2	1-2			•-1	2-3	2-3	•-2	•-1	3-4	•-1
10.3.70	LH 4	30	1	1		1	1	1	1	1		1		1	1	1	1		·97	1	1			1	1	1	1	1	1	1
			3-5	3-4		3-4	2-3	4-6	4-6	3-4		1-2		3-4	3-4	2-3	2-3		•	1-2	2-3			1-3	2-3	2-3	2-4	•-1	3-4	1-2
17.11.71	LH12	25	1	1		1	1	1	1	1		1		1	1	1	1		1	1	1			1	1	1	1		1	1
			4-6	4		3-4	3	5-6	4-5	3		•-1		4	3-4	2-3	2		•	2	1-3			2	2-3	2	2-4		2-3	•
16.2.70	LB 2	25	1	1	1	1	1	1	1	1	1		1	1		1	1	1		1	1	1	1	1	1			1	·84	1
			3-6	2-5	2-3	1	1-2	4-6	2-4	2-3	•-1		2	4-6		2-3	2	2		4-5	2-3	2-3	2-3	1-2	4			2	2-3	1-2
12.5.70	LB 3	25	1	1	1		1	1	1	1	1		1	1		1	1	1		1	1	1	1	1	1			1	1	1
			1-2	3-4	3-4		2-3	5-7	3-4	3-4	1-3		3	5-6		3	1-2	2		4	2-3	2-5	1-2	•-3	4			1-2	2-3	1-2
26.5.70	LB 4	25	1	1	1		1	1	·96	1	1		1	1		1	1	1		1	1	1	1	·84	1			1	1	1
			1-5	3-4	3-4		1-3	4-7	•-4	2-4	•-2		2-3	4-6		2-3	1-2	1-2		3-4	1-3	2-3	•-2	•-1	3-4			1-2	1-3	•-2

Spot No.			28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55
Colour			Y	Y	G	GY	Y	Y	Y	Y	Y	Y	Y	YG	Y	Y	Y	Y	Y	Y	Y	B	G	B	B	B	B	B	B	WB
Detected By			F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	A	A	A
			*				*	*	*	*	*	*	*	*			*	*	*	*	*			*			*	*	*	*
Date Ran	Pop. Code	No. Plants																												
30.11.69	LV 1	23		·61	1	1	·30	·52					1			·65						·83		·74	·39	·26	·87		·30	
				1	1	1-2	•-1	•-1					•-1			•-1						1-3		1-2	1-2	1	•-1		1	
14.4.70	LV 2	25		·24	1	1		·12				·92	1			·84					·68	·64	·92	·04	·56	·44	1	·04	·76	
				•-1	1-3	2-4		•				•-1	•-1			•-1					•-1	1-2	1-2	1	•-2	•-1	1-2	1	•-1	
21.4.70	LV 3	25		·56	1	1	·04	·04				·84	1			·52					·44	·32	·52		·08	·08	1		·80	
				•	1-3	1-3	•	•				•-1	•-1			•-1					•	1	•-1		1-2	•-1	1-3		•-1	
11.8.72	LV 5	25		·56	1	1	·60					·16	1			·64						·20	·64				·52			
				•-1	•-2	•-2	•-2					•	•-2			•-1						•-1	•-1				•-1			
9.3.72	LV17	10		·80	1	1	·10	·50				·90	1			·20				·60		1	·50					·20	·80	
				•-1	1-2	1-2	1	•-1				•	•-1			•				•		1-3	1-2					•	•-2	
9.3.72	LV18	20		·50	1	1	·35	·25				·55	1			·45				·90		·95	·85		·20			·70	1	
				•	1-3	1-3	•-1	•-1				•	•-2			•				•		•-2	•-2		1-2			•-1	•-2	
24.2.70	LH 1	25		1	1	1				·52			1			·44						1	1					·08	·68	
				•	2-3	2-4				•			•			•						1-2	1-2					•	•-1	
24.3.70	LH 2	25		1	1	1				1		1	1			·92	·76	·16	·36			1	1				·80	·16	·76	
				•-1	2-3	2-3				•		•	•-1			•-1	•-1	•	•			1-2	1-2				•-1	•	1-2	
2.6.70	LH 3	25		·88	1	1	·72					1	1			·52	·64	·40				1	1				1	·32	1	
				•-1	•-2	2	•					•	•-2			•-2	•	•				1-2	•-2				•-2	1	1-2	
10.3.70	LH 4	30		1	1	1						1	1			·53	·27	·27		·10		·1	1				1		·97	
				1-2	1-3	2-3						1-2	•-1			•-1	•	•		•		2	1-2				•-2		1-2	
17.11.71	LH12	25		·96	1	1	1					·84	1			·08						1	1				·40	·92		
				•-1	•-2	1-3	•					•	•			•						1-2	1-2				•-1	•-2		
16.2.70	LB 2	25		1		1			·44						1	1				1		·96								1
				1-2		3-4			1						2	•-1				1		1-2								1
12.5.70	LB 3	25		1		1					·20				1	·72		·60		·24		·08					·32	·36	1	1
				•-1		1-2					•				1-2	•		•		•		2					•-1	•-1	•-3	1-3
26.5.70	LB 4	25		1		1					·16			·16	1	·48			·20	·28		·72					·20	·20	·40	·96
				•-2		1-2					•			•	1-2	•			•	•		•-2					•	•	•	•-2

For key see separate page.

APPENDIX II Continued

Spot No.	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117
Colour	BV	B	BG	B	BG	B	V	G	V	V	V	V	B	BV	G	V	Y	G	G	G	G	G	G	V	B	G	V	O	G	G	V	WG	G	G	W	G	B	G	B	G	B	V	BG	B	WY	B	G	B	YG	V	B	G	V	V	V	B	G	G	BG	G	G	V
Detected By	F	A	A	A	A	A	A	A	F	F	F	A	F	A	F	F	A	A	F	A	A	A	A	A	A	F	F	A	A	A	A	U	U	U	F	U	A	A	U	U	A	U	U	U	A	A	A	A	A	A	A	A	F	A	A	A	U	F	F	A
	*						*	*		*	*	*		*					*	*	*	*	*	*	*				*		*	*	*	*		*		*			*			*	*	*			*	*			*	*	*	*	*	*	*	*	*	*	*	*
Pop.Code																																																																								
LV 1		·78	1	1	·96	·96	·48		·17	·96		·17		·87		1		1										·52	·39	·39			1	·74	·96	·96		·48		·30	1	·26			·74																						
		1	1-3	1-3	1-3	1-3	•-2		1	1-3		1		1-2		3-4		1-3										1-2	1	1			3-4	1	1	1		1		1	1-2	1			1-2																						
LV 2		·20	·88	1	1	1	·96	·96	·44		1			·12		·96		1											·60		·68			1	·92	1			·88			1	·60														·56	·48								
		•	•-2	3-4	2-4	1-4	2-4	2-4	1		1-2			1		1-2		1-2											1-2		•-1			3-4	1-2	1-2			1-2			2-3	•-1														1-2	2								
LV 3		·04	1	1	·96	·96	·80	1	·04		·96			·16		·32	·08	1											·84		·28			1	·80	1			·32	·52		1	·52														·96	·96								
		1	1-2	2-4	1-4	1-4	1-4	1-4	1		1-3			•-3		1-3	1	2-3											•-1		•-1			2-4	•-2	2			•-1	•-1		2-3	1-2														1-3	1-2								
LV 5		·88	1	1	·88	·88			·44	·96		·92		·28		·56		1										·44	·80					1	·28	1	·88		·96	·84		1	·36														1	·92								·92
		•-2	•-4	•-4	•-4	•-4	•-4		1-2	1-2		1-2		•-2		•-2		•-1										1-2	•					2-4	•-2	•-2	•-2		2-3	2-3		2-3	•-1														2	1-2								•-1
LV17		1	1	1	1	1	1		1		·40			1		1		1										·60	·10	·50		·10		1	·90	1	·70		·10			·90			·30										·50	·50		·50			1		
		1-2	2-4	3-4	•-3	1-3	1-5		2-3		2			•-2		3-5		1-2										2-5	•	•-1		•		1-3	•-2	1	•-1		1-2			•-1			•-1										•-1	•-1		•			•-2		
LV18		1	1	1	1	1	1		·95	1	·30	·45		1		1		1										·30	·20	·40		·40		1	1	1	·85		1			·65			·50										·40	·20		·50	·25		1		
		•-2	2-4	3-4	1-3	1-3	2-5		2	2-4	•-1	•-2		1-2		2-5		1-2										1-2	•	•-2		•-1		2-3	•-2	1-2	•-1		•-2			•-1			•-2										•-1	•-1		•-1	•		•-2		
LH 1		1	1	1	1	1	1		·80	1	·20			·96		1		1										·80		·36		·56		1		1			1			·84			·88																				
		1-2	2-3	2-3	1-2	1-2	1-2		1-2	2-3	•			•-2		2-3		1										•-2		•-1		•-1		2-4		1-2			1			1-2	1-2		1-2																				
LH 2		·36	1	1	1	1	1	1	·60	·48	1	·32		·64		1		1										·12		·12				1	·76	1	·40		1			1	·72			·68																				
		•-2	1-2	2-3	2-4	1-2	1-3	2-3	•-1	•-1	2-4	•-2		•-2		1-4		1-2										1-2		•-1				2-4	1-2	1-2	•-1		1-2			2-3	•-2			•-1																				
LH 3		·44	1	1	1	1	·40		·92	1		·88		·32		·92		1	·40									·48		·56		·48		1	·64	1	·80		1			1	·08																				
		•-1	2-3	3-4	3-4	•-3	•-2			•-2	3-4			1-2		•-2		•-2										•-1		•-1		•-1		2-3	•-2	1-2	•-1		2			3-4	1																				
LH 4		·67	1	1	1	1	1		·87	·90	1			·93		·97		1										·40		·53				1	·70	1	·23		1			1	·03			·77																				
		1-2	2-3	3-4	3-4	1-3	1-3		•-2	•-2	2-4			•-2		•-2		2-4										•-2		•-2				3-4	•-2	2	•-2		1-2			2-3	•			•-2																				
LH12			1	1	1	·84	·84			1	1	·32			·96		1		1										·84		·88				1	·52	·96			·96			·92	·68				·96										·20	·88		·16	1	·08	·16	·72
			1-2	1-4	1-4	•-3	•-3			3-4	1-3	1			•-1		1		•-2										1-3		•-2				2-3	•	1-2			•-2			1	1				•-4										•	1-3		•	•-2	•	•	•-1
LB 2		·96	·84	1		1				·96		·68	1				1	·52			·20			·72		·96		·44	·44	1		1	·16	1				·48		·92	1	·96			·96	·60																		
		1	1	1-2		1-2				2		1	3-4				2-3	•-1			2			1-2		1-2		1	1	3-4		2	1	1				1-2		1	1	1-4			1-2	1-2																		
LB 3		1		·76						·96		·52	·76				·16	1			1	1	1	1	1	·72		·60	·60		·32	·64	·64	1	·40	·96	·72	·96	·68	·48	·92	1		·84	·96		·28		·08		·76																
		1-3		•-2						•-2		•-1	1-4				•	2-3			2-3	1-2	3	2-3	2-3	1-3		•-3	•-2		1-2	•-1	•-1	3-4	•-2	1-2	•-2	2-3	•-2	•-2	•-3	3-4		•-2	1-2		•-2		•		•-2																
LB 4		·92	·84	·96	·20	·32		·20		·60		·32	·80	·28			·64	1		·36	·80	·80	·92	·76	·76	·84		·96	·96		·16	·16	·16	1	·28	1	·20	·96	·96		·56	·92		·80	·96		·84		·40	·04		·52		·16	·16	·28						
		•-2	•-2	•-2	1-2	1-2		1-2		1-2		1-3	1-4	1-2			1-2	2-3		1-3	2-3	1-2	1-4	1-4	1-3	•-2		•-1	•-1		1	1	1	3-4	•-1	2	•-1	1-3	•-2		•-2	2-3		•-2	•-2		•-4		1-2	•		1-2		1-2	•-1	1-2						

For key see next page.

KEY TO APPENDIX II & III

Spot No. This refers to the numbered spots on the master chromatogram of Fig. 3.3.

Colour

Y = yellow
O = orange
G = green
B = blue
V = violet
W = white

Detecting Reagents

F = plate viewed under UV light after dipping in Flavone reagent.
A = " " " " " " fuming with NH_3 vapour.
U = " " " " " without any treatment.

Scores Large numbers indicate frequency of the particular spot in each population.
(1 = spot present in all plants of the population;
space = spot absent in all plants).

Smaller numbers beneath each spot frequency indicate intensity range of the spot between different plants of the population.
(o = very faint; 1 to 7 = faint to very strong - see Chapter 3.3 (b).

* This indicates spots rejected for purposes of calculating variation within and between populations - see Chapter 3.4.(b) and 3.4. (c).

APPENDIX III

Frequencies of Phenolic Spots in Cultivated L. Binervosum Populations

Spot No.		1	2	3	4	5	6	7	8A	8B	10	11	13	14	15	17	18	19	20	21	22	25	26	28	29	31	40	41	43
Colour		Y	YO	YO	Y	Y	YO	Y	Y	Y	YO	Y	YO	YO	Y	YG	G	Y	YG	Y	Y	Y	YG	Y	Y	YG	YO	Y	Y
Detected By		F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F
					*																								
Pop Code	No. Plants																												
LB3F1	20	1	1	1		1	1	·90	1	1	1	1	1	1	1	1	1	1	1	·95	1	1	·90		·75	1	1	1	
		1-4	3-4	3-4		2-3	4	1-3	3-4	•-2	2-3	4-5	2-3	2	1-2	3-4	•-2	3	•-2	•-2	•-3	•-2	•-2		•-1	1-3	•-2	•-2	
LB3F2	20	1	1	1	·20	·95	1	·95	1	1	1	1	1	1	1	1	·45	1	1	1	1	·10	1		·70	·90	·95	·95	·05
		2-3	4	4	•	•-2	5	3-4	3-4	1-2	2	5	2-3	2	1-2	4	•-1	3	2	•-1	2-3	•	1-3		•-1	•-1	1-2	•-1	•
LB372	20	1	1	1		1	1	·95	1		1	1	1	1	1	1	·80	1	1	·90	1	1	1		1	1	·95	1	·45
		1-2	3-4	3-4		2-3	3-4	3-4	3-4		2	5	2-3	2-3	1	3-4	•-2	2-3	1-2	•-1	2-3	•-2	2-3		•-1	•-1	1-2	•-2	•
LB472	17	1	1	1		1	1	1	1		1	1	1	1	1	1	·95	1	1	·95	1	1	1		1	1	1	1	·60
		1	3-4	3-4		2	3-4	3-4	3-4		2	5	2-3	2-3	1	4	1-2	3	1-2	•-1	2-3	•-2	2-3		1-2	•-1	1-2	•-2	•-1
LB8Br	13	1	1	1		1	1	1	1	1	1	1	1	1	1	1	·30	1	1	1	1	·05	1		·40	·75	1	1	
		1-3	3-4	3-4		•-1	4-5	3-4	3-4	1	2-3	6-7	2-3	2	1	4	•-1	3	2	1	3	1	2-3		•-1	•-1	2	•-1	
LB8Nr	23	1	1	1			1	1	·85	1	1	1	1	1	1	1		1	1	1	1	·15	1		1	·70	1		
		1-3	3	3			3-4	4-5	•	•-1	1-2	6-7	3	2-3	1	4		3	2-4	2	2	1-2	3		1	•-1	3		
LB21	20	1	1	1	·65	1	1	1	1	·15	1	1	1	1	1	1	·10	1	1	1	1	·60	1	·25	1	1	1	1	·90
		2	2-3	2-3	•	1-2	3-4	4-5	2-3	•	1-2	5-6	1-2	2-3	1	4-5	2	3-4	3-4	1-2	2	•-1	3	•-1	1-2	1	2-3	•-1	1-2
LB9	25	1	1	1	·35	1	1	1	1	·90	1	1	1	1	1	1	·95	1	1	1	1		1	1	1	·90	1	·90	·35
		1-2	2	2	•	2	3	3-4	2	1-2	1	5	1-2	2-3	1	4	•-2	3	2-3	•-2	1-3		2-3	•-1	•-2	•-1	1-3	•-1	•

Spot No.		44	45	46	47	55	57	58	59	65	69	74	76	77	78	79	80	81	82	83	84	87	89	90	91	92	93	94	95	98	99	100	103	104	106	109	110	111	112	113
Colour		Y	Y	Y	B	WB	B	BG	B	V	BV	G	G	G	G	V	B	G	V	O	G	WG	G	W	G	B	G	B	G	BG	B	WY	B	YG	B	V	V	B	G	G
Detected By		F	F	F	F	A	A	A	A	F	F	A	A	A	A	A	A	A	F	F	A	A	U	U	F	U	A	A	U	U	U	U	A	A	A	A	F	A	A	A
		*	*	*		*		*			*		*							*		*	*		*		*				*	*		*	*	*	*	*	*	*
Pop Code	No. Plants																																							
LB3F1	20	·75			·20	·05	·30	·85	1	·20	1	1	1	·70	·70	·75	·70	·70	1		·60		·20	1		·95	·40	1	1	·20		·70	·25		·85					
		•			1	•	•-2	•-1	1-3	•-1	1-2	1-3	1-2	1-3	•-1	2-3	1-2	•-2	1-2		•-2		•-1	2-3		1	•-1	1-3	•-2	•-1		1	•-2		1-3					
LB3F2	20				·45	·05			1	·20	1	1	·70	1	1	1	1	1	1		·70			1		·35	·55	1	1	·95					1		·05			
					•-1	•			1-3	•	1-4	1-2	•-2	2-4	1-2	3-4	3-4	1-2	1-3		•-1			1-2		•-1	•	3-4	1-2	•-2					•-2		2			
LB372	20	·20	·60		·30		·95		·85			1		1	1	1	1	1	·85		·95			1	·95	1	·95	1	1	1			·25	·25						1
		•	•		•-1		•-2		•-2			2-3		2-3	1-2	3-5	3-5	2-3	1-2		1-2			1-2	1-2	1-2	1-2	4-5	2-3	2-4			2	2						1-2
LB472	17	·40	·45		·60		·95		1			1		1	1	1	1	1	·90		1			1	1	1	1	1	1	1			·90	·90						1
		•	•		1		1-2		1-2			3		2-3	1-2	4-5	4-5	2-3	1-2		1-2			2	1-2	1-2	1-2	4-5	2-3	3-4			2-4	2-4						1-3
LB8Br	13				·50				1	·25	1	1	·90	1	1	1	1	1	1		·75		·05	1	·45	1		1	1	·75	·25		·05		·70	·05	·15	·60	·60	·25
					1-2				1-3	•	1-2	1-2	1-2	3	1-2	2-3	3	2	2-3		1		•	1-2	•-2	•-1		2-3	1-2	•-3	•-1		2		•-2	1	1	•-2	•-2	•-2
LB8Nr	23	·15		·10	·90				·95		·70	1							·05		1		·15	1	·80	·80	·35	1	1	1	·25		1		·15		·05	·30	·30	·15
		•		•	1-2				•-2		1-2	2-3							2		•-2		•	1-2	•-1	•-2	•-1	2-3	2-3	2-3	•-1		2-3		•-1		2	2	•-1	1
LB21	20		·35	·50	·90		·95		·95		·20	1		1		1	1	1	·45	·45	·95	·85		1	·90	·95	1	1	1	1			·70	·70						·65
			•	•-1	1-3		1-2		1-2		•-1	3		2-4		3-4	3-4	2-3	1-2	1	1-2	1-2		1-2	1-2	1-2	2	4	1-2	2-3			2-3	2-3						1-2
LB9	25		·15	1	·80		·70	1	1	·10		1	·25	1	1	1		1	·10		·10	·35		1		1	1	1	·15				·10			1				
			•	•-1	•-2		•-1	•-2	•-2	1		3-4	•	2-4	1	3		1-2	•-1		1-2	•-1		1-2		1-2	•-2	3-4	•				1-2			•-2				

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