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Population Genetics of *Echium plantagineum* L.— Target Weed for Biological Control

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Abstract

Eight Australian and two European populations of *E. plantagineum* were surveyed for their genetic structure at 16 variable isozyme loci. On average, the Australian and European populations possessed 2·7 and 2·6 alleles per locus, a gene diversity of 34 and 35% and heterozygosity of 32 and 29% respectively. Estimates of the outcrossing rate in one Australian population were 61 and 73% for mean single-locus and multi-locus methods respectively. The levels of genetic diversity detected in this species consistently exceed those detected in a range of other species that occupy a similar stage in succession or that have similar breeding systems. Moreover, contrary to expectation, genetic diversity was equally great in the colonial populations in Australia as in European-source populations. If this high level of isozyme diversity reflects the diversity likely to be found in other parts of the genome, attempts to achieve substantial biological control may require the use of many different control agents.

Introduction

Echium plantagineum L. (Paterson's curse) is an extremely successful weedy species of western Mediterranean origin that has spread widely throughout the world, becoming particularly well established in countries with Mediterranean-type climates (Piggin 1982). It was first introduced into Australia during the middle of the 19th century both deliberately, as a garden ornamental, and accidently as a contaminant of seed and stock feed. Further introductions almost certainly continued to occur over many years (Piggin 1977, 1982). Since its introduction, *E. plantagineum* has spread extensively to become a serious weed of pastures in south-eastern and south-western Australia. In these situations it is generally believed to reduce both the quality and carrying capacity of infested pastures. Furthermore, it has been implicated in the poisoning of sheep, cattle, pigs and horses (Delfosse and Cullen 1981). For these reasons this species is currently the subject of a biological control program involving the use of a variety of leaf-eating and stem-boring insects from the plant's native range in the Mediterranean region (Delfosse and Cullen 1981).

Many aspects of the biology of potential control agents, of target species and of the interaction of the two have been implicated in the success or failure of biological control programs. For example, particular attention has been paid during the process of selecting suitable control agents to searching environments in the plant's home range that are climatically homologous to those in which the species has achieved weedy status in its new environment (Wapshere 1981). This increases the likelihood of synchronization of developmental patterns in the target species and the potential controlling agent and hence helps to ensure a maximum effect. Experience in the

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application of biological control to weed problems around the world has also shown that the genetic structure of populations of the target species may have a marked effect on the success of individual control programs. In particular, populations that were presumed to be genetically uniform or possess low levels of diversity have been controlled more effectively and more often than those species which are highly variable (Burdon and Marshall 1981). To date, this has also been found to be true in respect to Australian biological control programs. Here the most successful programs have involved the weeds *Alternanthera philoxeroides*, *Chondrilla juncea*, *Opuntia* spp. and *Salvinia* spp.—all species that reproduce primarily by agamospermous or clonal means.

E. plantagineum presents a marked contrast to these species. In Australia it is an insect-pollinated, self-compatible species in which genetic variation for flower colour and soluble proteins is known to occur. In a study of the multilocus structure of a single population of *E. plantagineum* growing at Gundagai, N.S.W., Brown and Burdon (1983) found a high level of genetic diversity. Of 23 isozyme loci examined, more than half were polymorphic with an average number of three alleles per locus, a gene diversity of 38% and a heterozygosity value of 35%. This population in fact consisted of a large array of different multilocus genotypes. At present we do not know whether this result is typical of populations of *E. plantagineum* growing in Australia nor what relationship it bears to similar populations of this species growing in their home environment. However, the answers to these questions will help develop an understanding of diversity in this weedy species and whether such studies will be useful in devising biological control strategies.

Table 1. Geographic location of the Australian and European populations of *E. plantagineum* studied

Population No.	Site	Latitude	Longitude
	Main range Australian populations		
1	Wagga Wagga	35°07′S.	147°22′E.
2	Gundagai	35°04′S.	148°07′E.
3	Burrinjuck Junction	34°45′S.	148°26′E.
4	Binalong	34°40′S.	148°38′E.
	Outlying Australian populations		
5	Canberra	35°19′S.	149°13′E.
6	Tharwa Junction	35°22′S.	149°13′E.
7	Lake George	35°09′S.	149°22′E.
8	Braidwood	35°27′S.	149°48′E.
	European populations		
9	Collioure (France)	42°32′N.	3°05′E.
10	Vilademat (Spain)	42°08′N.	3°05′E.

As a consequence, the present study was designed to address some of these questions. Estimates of the level of genetic diversity occurring in eight distinct Australian populations of *E. plantagineum* were determined and compared both with themselves and with similar estimates for two populations growing in Europe. These results were complemented by an assessment of the mating system of *E. plantagineum* at one of the Australian sites.

Materials and Methods

Plants

Eight populations of *E. plantagineum* growing in various parts of southern New South Wales and the Australian Capital Territory and two populations from Europe were studied in this survey. Four of the Australian populations were growing within the main range of *E. plantagineum* which stretches in an arc from western South Australia through Victoria and southern New South Wales to northern New South Wales (Piggin 1977). The other four Australian populations were more isolated ones growing in the general vicinity of Canberra, south of the arc of the main distribution. In the case of each of the Australian populations (Table 1), 50 flowering stems were collected at random on the day immediately preceding electrophoretic analysis. The flowering stems were wrapped in moist paper towelling and transferred to the laboratory. There they were placed in water overnight. The following morning each stem was labelled and an immature flower bud removed for analysis. Labelling individual stems ensured that, if necessary, individual plants could be sampled again the following day to obtain a complete data set.

A limited amount of seed was available from two populations of *E. plantagineum* growing in southern Europe. At these two sites, Collioure near Perpignon in French Catalan and Vilademat near Gerona in Spanish Catalan (Table 1), seed had been collected separately from 19 and 40 individuals respectively. In April 1985, seed from each of these parental lines was sown in 10-cm pots filled with a sandy loam. The resultant plants were grown in a heated glasshouse (25/20°C day/night) with natural lighting. After 3 months the plants elongated and came into bud. At this stage immature flower buds were removed for electrophoretic analysis. As soon as a complete data set was obtained the plants were destroyed.

Electrophoresis

Single immature flower buds were prepared for horizontal, starch-gel electrophoresis (Brown and Burdon 1983) using one of three buffer systems (system A: electrode buffer—0·4 M sodium citrate, pH 8·0; gel buffer—5·0 mM histidine, pH 8·0; system B: electrode buffer—0·3 M borate, 0·1 M sodium hydroxide; gel buffer—3·0 mM citrate, 15·2 mM Tris; system C: electrode buffer—75·0 mM lithium hydroxide, titrated to pH 8·5 with boric acid; gel buffer—6% electrode buffer, 94% 9·1 mM citrate, 65 mM Tris). For system A, electrophoresis was conducted for 5 h, and for systems B and C it was allowed to proceed until the borate front had migrated 9 cm from the sample slot. Each gel was then cut horizontally into three slices and the anodal portion of the gel was assayed for the following enzymes: acid phosphatase (ACP; EC 3.1.3.2), aconitate hydratase (ACO; EC 4.2.1.3), glucosephosphate isomerase (PGI; EC 5.3.1.9), phosphoglucomutase (PGM; EC 2.7.5.1) and shikimate dehydrogenase (SDH; EC 1.1.1.25)—assayed on system A; alcohol dehydrogenase (ADH; EC 1.1.1.1), NAD(P)H dehydrogenase (syn. menadione reductase MDR; EC 1.6.99.2) and endopeptidase (ENP; EC 3.4.22.?)—assayed on system B; NADH diaphorase (syn. dihydrolipoamide reductase DIA; EC 1.6.4.3), methylumbelliferyl esterase (EST; EC 3.1.?) and glutamate oxalate transaminase (syn. aspartate aminotransferase GOT; EC 2.6.1.1)—assayed on system C. Enzyme assays are as cited in Collins *et al.* (1984), except that the fluorescent esterase assay is that of Mitton *et al.* (1979).

Measurements of Genetic Diversity

The plants were scored for their genotype at 16 polymorphic loci including 12 previously studied (Brown and Burdon 1983). For each population and each locus, estimates of gene diversity (h), heterozygosity (H) and fixation (F) were computed using standard formula (see Brown and Weir 1983).

Mating System Estimation

In late November 1984, 15 fruiting clusters were collected separately from each of 20 randomly selected plants growing in one of the eight Australian populations (Tharwa) of *Echium plantagineum*. From each fruiting cluster (each being the product of a single flower and containing a maximum of four seeds), a single seed was removed and used in the subsequent mating system analysis. Prior to electrophoretic analysis these seeds were chipped to promote germination and placed on moist filter paper for 48 h at 25°C. After this time crude extracts were prepared in the manner described above. Of the enzyme assays used in the analysis of genetic diversity, only five (namely ADH, MDR, PGI, PGM and SDH) proved effective for the enzyme extracts derived from the germinating seeds.

Estimates of outcrossing rate were made using the procedure described by Brown *et al.* (1975) for single-locus data. Genotypic classification was converted to a diallelic basis by lumping all alleles but

the commonest allele into a single class. For the *Pgi1* locus, three estimates were made, based on three different alleles. An outcrossing estimate was also made on a multilocus basis using the procedure of Green *et al.* (1980).

Results

Table 2 lists, for all sites, the allelic frequencies for the three loci *Dia*, *Sdh* and *Pgi1*. Alleles are designated a, b, c, or d in decreasing order of electrophoretic mobility and maintain conformity with previous studies of electrophoretic variation in *E. plantagineum* (Burdon *et al.* 1983; Brown and Burdon 1983). At the *Pgi1* locus, the two new alleles *e* and *f* are intermediate in mobility between *c* and *d*. Multiple loci on the same zymogram (e.g. ACO) are enumerated in order of decreasing mobility. In the case of PGI, the *Pgi2* locus is for the isozyme near the origin and corresponds to the *Pgi* locus of our previous papers.

The data for three loci in Table 2 are presented as these loci are representative of the degree of variation occurring the different *E. plantagineum* populations. (Data for the 13 remaining loci are available on request from the authors.) Among the Australian populations, allele frequencies are quite different at all three loci in the Tharwa population (No. 6). In that population, the *Dia a* allele was common ($P = 0{\cdot}55$) while it was absent or very rare in all other populations. The frequencies of the *Sdh a* and *Pgi1 a* alleles were also markedly higher in population 6 than in any other. A similar divergence was found in the case of the *Pgi1 b* allele in population 2. Comparison of the Australian and European populations showed that for these three loci, all populations were dominated by the same alleles (more strictly, the same electromorphs).

Values for the gene diversity (h) index for each locus in all the Australian and European populations of *E. plantagineum* are given in Table 3. In the Australian populations the mean of these values ranged from 0·07 for *Aco1* to 0·65 for *Pgi1*, while in the European populations the mean h values ranged from 0 for the *Aco1* and *Dia* loci to 0·57 for the *Sdh* locus. Within the Australian populations there were several cases of marked differences in h values (e.g. at the *Mdr* locus) although for many other loci h values were remarkably consistent across all populations (e.g. *Est2*, *Pgi1*, *Pgm1*).

Estimates for individual populations of the average number of alleles per locus, the mean population gene diversity ($\bar{h}$), the observed level of heterozygosity (H) and the fixation index value (F) are given in Table 4. Despite considerable variation in the size, geographic position (marginal versus central) and known history of the Australian and European populations all of these estimates of genetic variation showed similar values. This similarity held in fact for all populations studied regardless of their origin. Only for fixation index values was there any consistent difference between the European and Australian populations. Larger positive fixation values in the former populations indicate the occurrence of a greater frequency of homozygotes. This difference may, however, reflect the different growing conditions for the two groups of populations used to assess this genetic parameter. Plants from the European populations were grown individually in pots from seed and were thus not subject to competition and selection during vegetative growth. In contrast genetic assessment of the Australian populations utilized mature plants growing naturally in the field.

Table 2. Gene frequencies at three loci in eight Australian and two European populations of *E. plantagineum*

Enzyme locus	Allele	Gene frequencies for Australian populations									Gene frequencies for European populations		
		1[A]	2	3	4	5	6	7	8	$\bar{p}$[B]	9	10	$\bar{p}$[C]
Dia	*a*	0·01	–	–	–	–	0·55	–	–	0·07	–	–	–
	b	0·95	0·98	0·94	0·93	0·82	0·31	0·98	0·54	0·81	1·00	1·00	1·00
	c	0·04	0·02	0·06	0·07	0·17	0·14	0·02	0·46	0·12	–	–	–
	d	–	–	–	–	0·01	–	–	–	–	–	–	–
Sdh	*a*	0·40	0·38	0·31	0·26	0·39	0·71	0·38	0·40	0·40	0·47	0·56	0·54
	a^-	–	–	–	–	–	–	–	–	–	–	0·06	0·04
	b	0·56	0·60	0·60	0·74	0·61	0·29	0·62	0·58	0·58	0·45	0·34	0·37
	c	0·04	0·02	0·09	–	–	–	–	0·02	0·02	0·08	0·04	0·05
Pgi1	*a*	0·02	0·05	0·02	0·01	0·04	0·45	–	0·02	0·08	–	–	–
	b	0·38	0·58	0·16	0·36	0·16	0·11	0·26	0·16	0·27	0·37	0·27	0·31
	c	0·42	0·17	0·50	0·29	0·45	0·20	0·41	0·38	0·35	0·16	0·08	0·10
	d	0·18	0·20	0·32	0·34	0·33	0·24	0·33	0·44	0·30	0·37	0·55	0·49
	e	–	–	–	–	0·02	–	–	–	–	–	–	–
	f	–	–	–	–	–	–	–	–	–	0·10	0·10	0·10

[A] 1, Wagga Wagga; 2, Gundagai; 3, Burrinjuck Junction, Hume Highway; 4, Binalong; 5, Canberra; 6, Tharwa Junction, Monaro Highway; 7, Lake George; 8, Braidwood; 9, Collioure, France; 10, Vilademat, Spain.

[B] Mean gene frequencies for the Australian populations. [C] Mean gene frequencies for the European populations.

Table 3. Gene diversity index values (h_i) of eight Australian and two European populations of *E. plantagineum*

Enzyme locus	h_i values for Australian populations[A]									h_i values for European populations[A]		
	1	2	3	4	5	6	7	8	Mean	9	10	Mean
Aco1	0·11	0·31	0·06	0·04	0	0	0	0·04	0·07	0	0	0
Aco2	0·39	0·11	0·54	0·50	0·54	0·08	0·47	0·30	0·37	0·62	0·42	0·52
Aco3	0·35	0·29	0·35	0·43	0·56	0·04	0·32	0·42	0·34	0·41	0·14	0·28
Aco4	0·04	0·08	0·21	0	0	0·18	0·27	0·17	0·12	0·05	0	0·03
Acp	0·50	0·45	0·30	0·43	0·13	0·11	0·44	0·58	0·37	0·47	0·41	0·44
Adh	0·16	0·10	0·35	0·34	0·11	0·16	0·23	0·27	0·22	0·41	0·35	0·38
Dia	0·10	0·04	0·11	0·13	0·30	0·58	0·04	0·50	0·22	0	0	0
Enp	0·62	0·46	0·67	0·67	0·70	0·32	0·40	0·73	0·57	0·67	0·57	0·62
Est1	0·33	0·39	0·15	0·42	0·43	0·04	0·66	0·46	0·36	0·52	0·59	0·55
Est2	0·55	0·47	0·40	0·49	0·43	0·48	0·44	0·35	0·45	n.s.[B]	n.s.	n.s.
Mdr	0·48	0·48	0·27	0·43	0	0·41	0	0·49	0·32	0·58	0·28	0·43
Pgi1	0·65	0·59	0·62	0·67	0·66	0·68	0·66	0·64	0·65	0·31	0·61	0·46
Pgi2	0·21	0·02	0·21	0·11	0·27	0·16	0·10	0·10	0·15	n.s.	n.s.	n.s.
Pgm1	0·46	0·49	0·40	0·50	0·44	0·65	0·49	0·54	0·50	0·15	0·14	0·14
Pgm2	0·41	0·44	0·37	0·25	0·17	0·20	0·31	0·37	0·31	0·53	0·49	0·51
Sdh	0·52	0·50	0·54	0·38	0·48	0·41	0·47	0·50	0·48	0·57	0·56	0·57

[A] As for Table 2. [B] n.s., not scored.

Table 4. Estimates of the average number of alleles per locus, the gene diversity, the observed level of heterozygosity and fixation index values for eight Australian and two European populations of *E. plantagineum*

	Values for Australian populations[A]										Values for European populations[A]			
	1	2	3	4	5	6	7	8	Mean	Total	9	10	Mean	Total
Average No. of alleles per locus	3·06	2·69	2·69	2·63	2·63	2·56	2·38	3·13	2·72	3·56	2·57	2·64	2·61	2·86
Diversity (h)	0·37	0·33	0·35	0·36	0·33	0·28	0·33	0·40	0·34	0·39	0·38	0·33	0·35	0·381
Heterozygosity (H)	0·35	0·32	0·34	0·33	0·29	0·26	0·33	0·36	0·32	0·32	0·31	0·28	0·29	0·294
Fixation index (F)	0·05	0·00	0·02	0·07	0·11	0·08	−0·01	0·10	0·06	—	0·17	0·15	0·16	—

[A] As for Table 2.

The distribution of alleles among all 10 populations (Fig. 1*b*) showed no indication of a marked genetic disjunction between those from Australia (Fig. 1*a*) and those from Europe. In all, 36% (21/58) of the alleles detected were found in all populations.

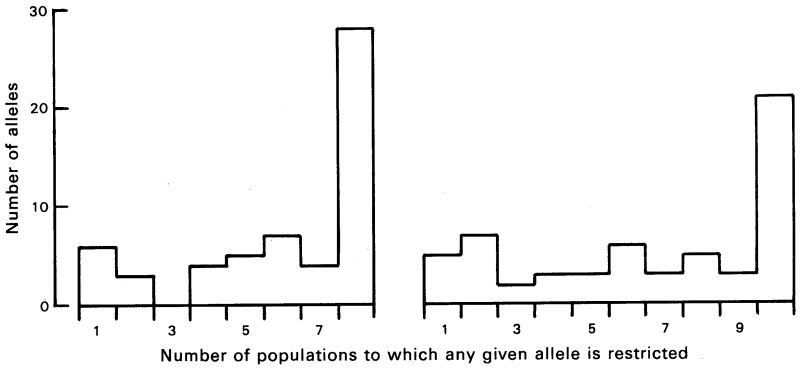

Fig. 1. Frequency distribution of alleles among the eight Australian populations (left) and the eight Australian and the two European populations (right) of *E. plantagineum*.

(This excludes results from the *Est2* and *Pgi2* loci which were not scored in the European material.) The remaining alleles were evenly distributed as to the number of populations in which they occurred. The maximum number of alleles found in any particular number of populations was seven (12·5%) which were restricted to various combinations of two of the populations. Four of the seven alleles that were restricted to two populations were found in the European populations only.

Table 5. Outcrossing estimates (*t*) in the population of *E. plantagineum* growing at Tharwa Junction in 1984

Enzyme locus	Outcrossing rate (s.e.)	Common pollen allele frequency
	(*a*) Single-locus estimates	
Adh	0·51 (0·12)	0·83
Mdr	0·42 (0·13)	0·61
Pgi1[A]	0·94 (0·14)	0·37
	0·73 (0·08)	0·30
	0·69 (0·09)	0·18
Pgm2	0·54 (0·07)	0·81
Sdh	0·70 (0·14)	0·60
Weighted mean	0·61	
	(*b*) Multilocus estimate	
	0·73 (0·04)	–

[A] Three outcrossing estimates based on the *a*, *c* and *d* alleles of the *Pgi1* locus respectively.

Single and multilocus estimates of the rate of outcrossing occurring in the population of *E. plantagineum* growing at Tharwa Junction are given in Table 5. Differences in the single locus estimates derived from different loci reflect, in large

part, differences in the number and frequency of alleles present at each locus in this population. This in turn affects which outcross events are detected unambiguously by heterozygosity for one non-maternal allele. The multilocus estimate, based on the combined data of all the single locus estimates, is higher (0·73) than that of the weighted mean of single locus estimates (0·61). The allele frequencies estimated for outcrossing pollen (Table 5) were consistent with the independent estimates of the frequency for these alleles in the original survey.

Discussion

Population Genetic Structure of E. plantagineum

Populations of *E. plantagineum* occurring in both Australia and Europe are genetically highly variable. While differences were observed between different populations in the occurrence and frequency of particular alleles (Table 2), gene diversities (h) and observed levels of heterozygosity were similar across all populations (Tables 3 and 4). Even populations that were markedly isolated from all others (e.g. Braidwood) showed the same level of genetic diversity as did populations that lay within the main distribution of *E. plantagineum* in south-eastern Australia (e.g. Gundagai, Wagga Wagga). To date, detailed information concerning the genetic structure of *E. plantagineum* populations is only available for that population occurring on river flats of the Murrumbidgee river at Gundagai, N.S.W. in 1980 (Burdon *et al.* 1983) and 1982 (Brown and Burdon 1983). The allele frequencies, gene diversity and levels of heterozygosity reported here for that population are in close agreement with those previous results.

Comparison of the present results with average levels of genetic diversity observed in other plant species indicates that *E. plantagineum* is remarkable in many respects. Indeed, Barrett and Richardson (1986) have pointed out that results of an earlier study of a single population of *E. plantagineum* (Brown and Burdon 1983) suggested that this population was among the most diverse of any which has been studied electrophoretically. That this is true for the species as a whole is confirmed by a comparison of the present data with that given by Loveless and Hamrick (1984). In a review of 163 studies of electrophoretic variation in plants these authors obtained, for a number of ecological classifications of these species, estimates of the total diversity occurring in a species (h_T), the average diversity of all constituent populations ($\bar{h}$) and the G_{ST} ratio ($=1-\bar{h}/h_T$) which estimates the degree of differentiation of individual populations relative to the total diversity. The averages for some of these variables clearly differ from the present findings concerning *E. plantagineum* populations in both Australia and Europe. Although *E. plantagineum* is a typical weedy, early successional annual species, values of both h_T and $\bar{h}$ either in Australia ($h_T = 0{\cdot}388$; $\bar{h} = 0{\cdot}34$) or Europe ($h_T = 0{\cdot}381$; $\bar{h} = 0{\cdot}35$) were markedly higher than the equivalent mean values determined by Loveless and Hamrick (1984). In addition, the G_{ST} values for *E. plantagineum* (0·12 and 0·08 respectively) were much lower, then those determined by Loveless and Hamrick for early successional (0·41) or annual (0·43) plants. Indeed, the results reported here for *E. plantagineum* would appear to be much more typical of species occurring in the late stages of succession, or perennial species, rather than of weedy colonizers.

Using paternity analysis, Ellstrand and Marshall (1985) measured the minimum pollen migration into a local population of *Raphanus sativum*. In some instances, 18% of seed were attributed to interpopulation gene flow in this insect-pollinated

colonizing outbreeder. Such rates are sufficient to act as a homogenizing force to suppress the population differentiation which might otherwise occur under colonization.

Mating System of Predominant Outcrossing

Estimates of the outcrossing rate of the *E. plantagineum* population occurring at Tharwa Junction indicate that outcrossing (mainly mediated by *Apis mellifera*) is the predominate mode of fertilization. However, although outbreeding was predominant the level of self-fertilization was still appreciable (approx. 30%). Differences between the average single locus and the multilocus estimates of outcrossing rate may have resulted from the departure of the population from the assumptions of a mixed-mating model, most likely due to micro-differentiation in the stand. Under a mixed mating system with an outcrossing rate of 0·73, the expected inbreeding coefficient would be 0·16. The estimates of the fixation index in Table 4 are generally lower than this expectation in the Australian populations. Such a discrepancy could arise from selection in favour of heterozygotes (Burdon *et al.* 1983). However, more detailed estimates of outcrossing rate throughout the season are needed to determine the extent to which this parameter fluctuates. The general relationships between breeding system and the genetic parameters h_T, $\bar{h}$ and G_{ST} found by Loveless and Hamrick are evident for *E. plantagineum*. albeit with more extreme values. This may be due to the particular sample of enzymes surveyed.

Effect of Intercontinental Migration of Genetic Structure

Comparative data concerning the level of genetic diversity found in weedy plant populations growing in new environments and that in their original home ranges are rare. In general, such data show that the former populations possess only a small proportion of the genetic diversity of the latter (Brown and Marshall 1981). Such observations have generally been explained in terms of the establishment of new populations through the migration of a very limited number of individuals from the source area (Brown and Marshall 1981; Barrett 1982; Brown and Burdon 1987). However, the present data indicate that depauperate levels of variation are not always present in colonizing species.

In the *E. plantagineum* populations studied here, values for the average and total gene diversity of Australian populations (colonial populations) were virtually the same (Table 4) as those of the two European populations (source populations). Furthermore, even within Australia, populations known to be geographically isolated (e.g. Braidwood) were just as diverse as those occurring in the centre of distribution range (e.g. Gundagai). These findings suggest that any limitations placed on the diversity of *E. plantagineum* populations by long-distance colonization and its accompanying genetic bottlenecks are rapidly overridden by the effects of the open system of sexual recombination.

From the point of view of the current proposal to control *E. plantagineum* in Australia by the introduction of part of the plant's natural phytophagous insect fauna (Delfosse and Cullen 1981), this study reinforces the inclusion of *E. plantagineum* in a general grouping of genetically highly variable plants that are likely to require a particularly concerted effort to control (Burdon and Marshall 1981). If this high level of isozyme diversity reflects the level of variation likely to be found in other parts of the genome (e.g. insect and disease resistance), then it seems very likely

that many alleles controlling various forms of resistance to *E. plantagineum* insect pests are likely to be currently present in many Australian populations. Even if the size of populations is initially reduced substantially by insect attack, the presence of these resistances, the flexibility of the breeding system and the plant's high reproductive capacity may well result in a rapid population resurgence as the frequency of resistant individuals in populations climb steeply.

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