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Study of electrophoretic variability in *Epipactis helleborine* (L.) Crantz, *E. palustris* (L.) Crantz and *E. microphylla* (Ehrh.) Swartz (fam. Orchidaceae)

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Abstract

Allozymic variation in seven enzymes coded by eight loci was studied in seven populations of terrestrial *Epipactis* orchids, including four populations of *E. helleborine,* one population of *E. palustris* and two of *E. microphylla,* from Latium (Central Italy).

Heterozygosity estimates reveal that *E. helleborine* (EH) is the most variable species (HET = 0.233; P = 0.59; A = 1.8), followed by *E. palustris* (EP) (HET = 0.085; P = 0.29; A = 1.3) while *E. microphylla* (EM) is monomorphic for all the loci examined.

It is suggested that the two EM populations examined, with a total lack of genetic variability, could originate from the same genetically impoverished ancestral population. Also the low variability amount of EP can be ascribed to genetic drift, probably due to a founder event.

Nei's identity coefficients were also calculated. These show a high similarity in EH ($\bar{I}$ = 0.987) and EM ($\bar{I}$ = 1.000) populations. Average identity value between EP and EM is 0.289, between EP and EH is 0.336 and between EM and EH is 0.784. These data reveal that *E. palustris* presents a remarkable genetic, besides geographical and ecological, differentiation.

Introduction

In plants, enzyme and protein electrophoresis has been found particularly useful to study the degree of genetic variability within and among the various taxa, as well as to investigate the phylogenetic relationships in plant systematics (Gottlieb, 1977). Some authors (Hamrick *et al.,* 1979; Brown, 1979; Gottlieb, 1981) have reviewed heterozygosity data of plants and have related them to numerous parameters, such as mating type. These data show that outbreeders generally present a higher genetic variability level than inbreeders.

Terrestrial orchids are present in Italy with about a hundred species grouped in 31 genera (Pignatti, 1982). Most of them practice the outbreeding mediated by pollinating insects (Dressler, 1981), with a particular mechanism that allows the sticky pollinia of a flower to adhere to an insect and then to be released during a successive visit to another flower. Orchids present a relatively easy formation of interspecific and intergeneric hybrids, and often of hybridogen populations. Some European species as *Limodorum abortivum* (L.) Swartz, *Neottia nidus-avis* (L.) L. C. M. Rich. and *Cephalanthera damasonium* (Mill.) Druce, which rarely present flowers in complete anthesis, are inbreeders as a rule, while *Ophrys apifera* Huds. is thought to be

an inbreeder only in English populations (Walters & Briggs, 1969).

The European orchids, which are part of the subfamily Orchidoideae (fam. Orchidaceae), have been studied in various ways (morphologically, karyologically, etc.) but, to our knowledge, an electrophoretic approach has not yet been tried. We have carried out a study concerning the distribution in this plant group of the gene frequencies of some enzyme markers and here we present the data obtained in three *Epipactis* species.

This genus is present in Italy with seven species, at least three of which (the (very) rare *E. muelleri* Godfr., *E. leptochila* Godfr. and *E. persica* (Soó) Nannf.) are inbreeders, as is shown by the presence of a rudimentary rostellum or by its complete absence, and by the pulverulent pollinia. *E. palustris* (L.) Crantz, *E. helleborine* (L.) Crantz, *E. atrorubens* (Hoffm.) Besser and *E. microphylla* (Ehrh.) Swartz are outbreeders, even though some populations of *E. microphylla* with pulverulent pollinia seem to tend to autogamy (Senghas, 1970; Pignatti, 1982).

The aim of this work is to study levels of genetic variability within and among *E. palustris, E. helleborine* and *E. microphylla* populations. Moreover, we have calculated Nei's identity coefficients and genetic distances to estimate the phylogenetic relationships among the three species.

Materials and methods

Samples were collected in the area of Lucretili Mounts (Latium, Central Italy) (Fig. 1), proposed as Regional Natural Park, characterized by a remarkable environmental variety, in which orchids are present with more than 45 species grouped in 16 genera (De Angelis & Lanzara, 1987). *E. helleborine* and *E. microphylla* were collected in nearly

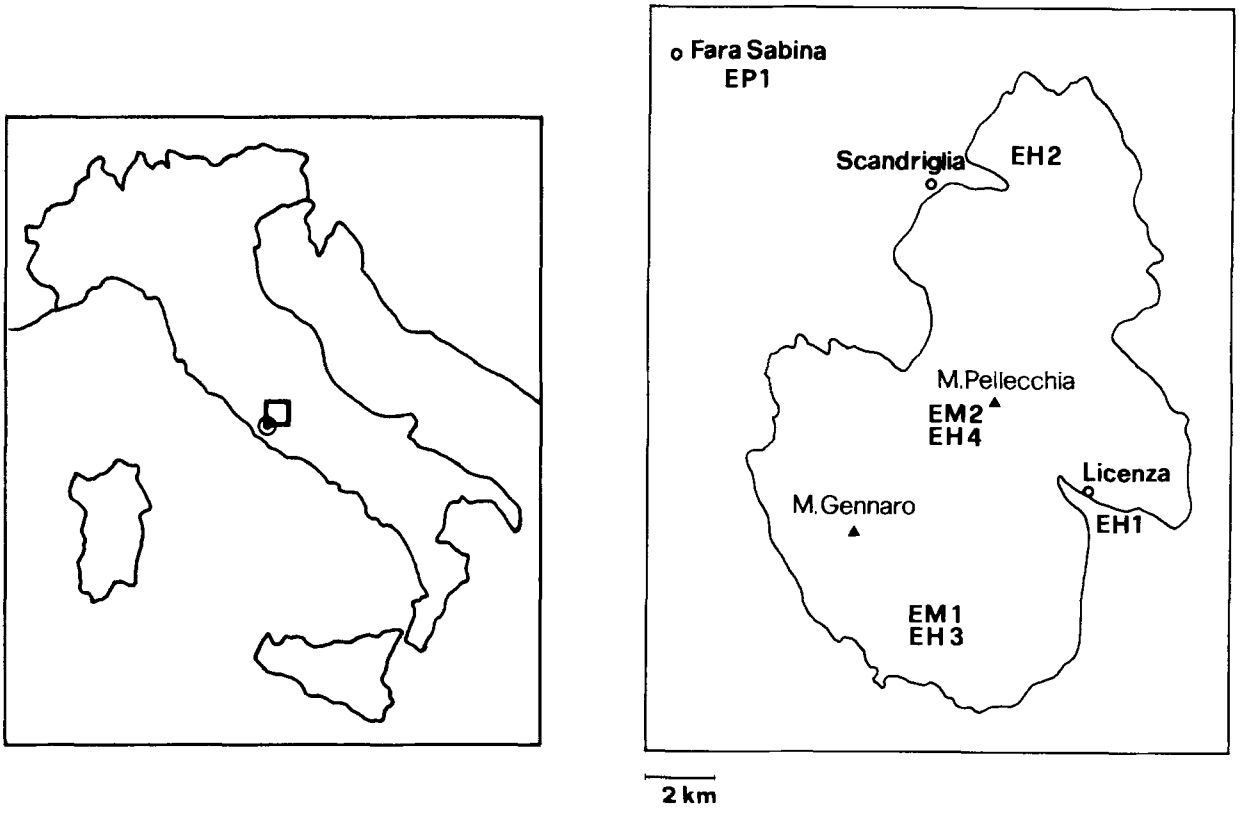

Fig. 1. Sampling sites for the seven populations of *Epipactis* studied in the area proposed as Regional Natural Park of the Lucretili Mounts in Latium (Italy). For details see text and Table 1.

pure beech forest (EH2, EH3, EH4, EM1, EM2) or mixed forest (EH1). *E. palustris* (EP1), a species of humid environments, rather rare in Latium and in Italy, was found in a colony on the border of a recently built road, in which water penetration was present (Table 1).

As regards geographical distribution, EH and EM are eurasiatic species (EH: paleotemperate, EM: europ. caucas.), while EP belongs to the circumboreal flora.

While *E. helleborine* and *E. palustris* have rich inflorescences, *E. microphylla* usually presents an inflorescence with a few, rather widely spaced, flowers. In sampling the last species, we considered neither the differential morphology of the pollinia (pulverulent or sticky) nor other morphometric characteristics of the flower.

Seven populations of *Epipactis* spp. were examined (Table 1). These included four populations of *E. helleborine* (EH), one population of *E. palustris* (EP) and two of *E. microphylla* (EM). Sample sizes ranged from 9 to 51 (Table 2). In almost no case could we say which was the effective population size, only *E. palustris* was estimated as approximately 200 individuals grouped in a 30 m^2 area. Samples, consisting of one flower for every plant, stored at +4 °C in a plastic envelope, were examined within five days.

The composition of extraction buffer was: 10 ml of 0.5 M Tris-HCl pH 8, 40 μl β-mercaptoethanol, 10 mg EDTA (Na_2). Flowers were added to 100 μl of this buffer in the wells of a plastic plate and homogenized with a glass rod. The obtained mixture, adsorbed in a Whatmann 3 MM filter paper wick, was inserted into a 12.5% starch gel.

Two buffer systems were used:

(A) A Histidine/EDTA:Tris/Citrate buffer system pH 7, according to King & Dancik (1983) was used for the following enzymes: 6-phosphogluconate dehydrogenase (*6Pgdh*), Shikimic dehydrogenase (*Skdh*), Isocitrate dehydrogenase (*Icdh*), Malate dehydrogenase (*Mdh*) and Phosphoglucomutase (*Pgm*).

(B) A Tris/Citrate: Li/Borate buffer system pH 8.2, according to Crawford & Smith (1982) was used for Phosphoglucose isomerase (*Pgi*) and Malic Enzyme (*Me*).

Gels, 0.7 cm. thick, were cut into several slices and enzymes were stained according to Shaw & Prasad (1970) and Harris & Hopkinson (1976). The *Skdh* staining mixture was described by Weeden and Gottlieb (1980). Superoxide dismutase (*Sod*), Leucine aminopeptidase (*Lap*), Aspartate amino transferase (*Aat*) and Hexokinase (*Hk*) were initially investigated but successively abandoned for some difficulties in resolution.

European spontaneous orchids require symbiotic mycorrhiza for germination and, sometimes, for growing. For this reason it is very difficult to cultivate them, despite attempts by many orchidists. As a result we could not study the transmission of the characters from parents to F_1. Genotypes were deduced from electrophoretic phenotypes. The three species were run on the same gel to verify the correspondence of the isozyme patterns.

Table 1. Epipactis populations in Latium (Italy) examined in this paper.

Species	Designation	Location	Habitat
E. helleborine (L.) Crantz	EH1	Licenza (Rome)	Mixed forest
E. helleborine (L.) Crantz	EH2	Scandriglia (Rieti)	Nearly pure beech forest (sparsely mixed with *Acer*)
E. helleborine (L.) Crantz	EH3	M. Gennaro (Rome)	Nearly pure beech forest (sparsely mixed with *Acer*)
E. helleborine (L.) Crantz	EH4	M. Pellecchia (Rome)	Nearly pure beech forest (sparsely mixed with *Acer*)
E. microphylla (Ehrh.) Swartz	EM1	M. Gennaro (Rome)	Nearly pure beech forest (sparsely mixed with *Acer*)
E. microphylla (Ehrh.) Swartz	EM2	M. Pellecchia (Rome)	Nearly pure beech forest (sparsely mixed with *Acer*)
E. palustris (L.) Crantz	EP1	Fara Sabina (Rieti)	Humid environment at roadside

Results

Genetic variability within populations

Table 2 reports the gene frequencies of the eight markers studied in seven populations of *Epipactis*. *6Pgdh* was practically undetectable in *E. palustris*. *E. microphylla* showed a three-banded pattern for Malic enzyme (*Me*), in which the anodal band, more intense, had the same electrophoretic migration as the single one of *E. helleborine*. For every polymorphic locus the most common allele in EH was designated 100, while the other alleles were named with numbers referred to their migration relative to 100. The Mdh_1 locus specifies the isozyme with the most anodal migration.

Table 2 shows that in EH three loci are monomorphic (*6Pgdh, Skdh, Me*), in EP five loci are monomorphic (*Skdh, Pgm,* Mdh_1, Mdh_2, *Me*) and in EM the eight examined loci are monomorphic. EP and EH were fixed for alternative alleles at *Skdh* and *Me* loci, EP and EM were fixed for alternative alleles at *Skdh,* Mdh_1, Mdh_2 and *Me* loci.

Table 3 shows the genetic variability estimates in

Table 2. Allele frequencies at 8 gene loci in seven populations of *Epipactis* (n = number of plants examined).

Locus	Allele	*E. helleborine*				*E. palustris*	*E. microphylla*	
		EH1	EH2	EH3	EH4	EP1	EM1	EM2
6Pgdh	100	1.000	1.000	1.000	1.000	-	1.000	1.000
	n	23	12	13	16		33	9
Skdh	100	1.000	1.000	1.000	1.000	-	1.000	1.000
	120	-	-	-	-	1.000	-	-
	n	28	34	16	20	22	26	9
Icdh	90	-	0.056	-	0.146	0.068	-	-
	100	1.000	0.944	0.974	0.833	0.932	1.000	1.000
	110	-	-	0.026	0.021	-	-	-
	n	25	27	19	24	22	32	9
Pgm	95	0.426	0.397	0.304	0.360	-	-	-
	100	0.574	0.603	0.696	0.640	1.000	1.000	1.000
	n	27	34	23	25	22	33	9
Mdh_1	90	0.437	0.177	0.295	0.417	-	-	-
	100	0.563	0.823	0.705	0.583	-	1.000	1.000
	102	-	-	-	-	1.000	-	-
	n	24	31	22	24	22	33	9
Mdh_2	62	-	-	-	-	-	1.000	1.000
	87	0.039	0.050	-	-	1.000	-	-
	100	0.903	0.750	0.864	0.895	-	-	-
	113	0.057	0.200	0.136	0.104	-	-	-
	n	26	10	22	24	22	33	9
Pgi	100	0.420	0.574	0.458	0.568	0.627	-	-
	120	0.180	0.250	0.313	0.318	-	1.000	1.000
	140	0.400	0.176	0.229	0.114	0.373	-	-
	n	25	34	24	22	51	33	9
Me	92	-	-	-	-	1.000	-	-
	100	1.000	1.000	1.000	1.000	-	1.000	1.000
	n	25	33	23	22	51	16	9

Epipactis populations. A locus is considered polymorphic if the most frequent allele is not above 0.99. The percentage of polymorphic loci (P) ranges form 0–0.63; the heterozygosity (HET = $1 - \Sigma x_i^2$, averaged over all loci) ranges from 0–0.248; the average frequency of observed heterozygotes (H_0) ranges from 0–0.229; the mean number of alleles per locus (A) ranges from 1–1.88 and the mean number of alleles per polymorphic locus (A_p) ranges from 2–2.5. Comparing EP and EH indexes with those reported by Gottlieb (1981) for outbreeding plants (HET = 0.086; P = 0.37) one can see that EP presents similar values (HET = 0.085; P = 0.29), while EH is definitely superior (HET = 0.233; P = 0.59). EH and EP show a lower number of alleles per locus (A) (1.82 and 1.29 respectively) than do outbreeders (2.9).

As the observed heterozygosities are lower than the expected ones (Table 3), we compared the two values by means of the Fixation Index (F) (Wright, 1969; Workman & Niswander, 1970). Table 4 reports the F values calculated for the polymorphic loci in EH and EP populations, EM being monomorphic for all loci. Both positive and negative values (deficiency and excess of heterozygotes, respectively) are present, but the Chi-squared test revealed no significant deviations from Hardy-Weinberg equilibrium.

Table 3. Estimates of genetic variability in seven populations of *Epipactis*.

Species and populations	Loci examined	P	HET	H_0	A	A_P
EH1	8	0.50	0.224	0.187	1.75	2.5
EH2	8	0.63	0.231	0.222	1.88	2.4
EH3	8	0.63	0.227	0.187	1.75	2.2
EH4	8	0.63	0.248	0.229	1.88	2.4
	mean EH	0.59	0.233	0.206	1.82	2.4
EP1	7	0.29	0.085	0.070	1.29	2
EM1	8	0	0	0	1	-
EM2	8	0	0	0	1	-
total mean		0.38	0.145	0.128	1.51	2.3

P = frequency of polymorphic loci (0.99 criterion),
HET = Heterozygosity ($1 - \Sigma x_i^2$) averaged over all loci,
H_0 = Average frequency of observed heterozygotes,
A = Mean number of alleles per locus,
A_p = Mean number of alleles per polymorphic locus.

Table 4. Fixation indices (F) of the polymorphic loci in *E. helleborine* and *E. palustris* populations.

Locus	Populations				
	EH1	EH2	EH3	EH4	EP1
Icdh	-	−0.047	−0.060	0.120	−0.079
Pgm	0.168	0.326	0.383	0.132	-
Mdh_1	0.069	0.223	0.017	0.142	-
Mdh_2	−0.067	−0.226	−0.161	0.335	-
Pgi	0.303	−0.071	0.219	−0.130	0.246

Relationships among populations and species based on isozyme data

Table 5 reports the genetic diversity apportionment calculated according to Nei (1975) in the *Epipactis* group. Gst values, indicating the quote attributable to differentition among populations, range from 0.069–1, with a mean value of 0.586. The lower value indicates that in *Icdh* the differentiation is almost entirely present within populations (only 7% among populations) while the higher values (1.000) indicates that the diversity is entirely present among populations, as *Skdh* and *Me* are fixed for alternative alleles in some populations.

Table 6 shows the apportionment calculated

Table 5. Genetic diversity levels among *Epipactis* spp. populations.

Locus[1]	H_T	H_S	D_{ST}	G_{ST}
Skdh	0.244	0	0.244	1.000
Icdh	0.091	0.085	0.006	0.069
Pgm	0.362	0.294	0.068	0.189
Mdh_1	0.494	0.252	0.242	0.490
Mdh_2	0.649	0.126	0.523	0.806
Pgi	0.643	0.439	0.204	0.317
Me	0.408	0	0.408	1.000
Mean	0.413	0.171	0.242	0.586

[1] Only polymorphic loci are considered

Table 6. Genetic diversity levels among four *E. helleborine* populations.

Locus[1]	H_T	H_S	D_{ST}	G_{ST}
Icdh	0.123	0.115	0.008	0.065
Pgm	0.429	0.426	0.003	0.007
Mdh_1	0.436	0.412	0.024	0.055
Mdh_2	0.238	0.233	0.005	0.021
Pgi	0.621	0.603	0.018	0.029
Mean	0.369	0.359	0.012	0.033

[1] Only polymorphic loci are considered.

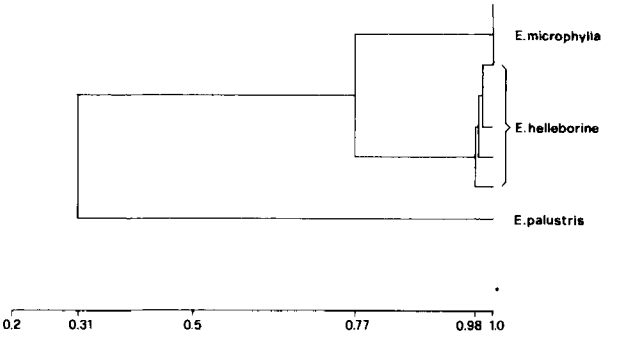

Fig. 2. Allozyme-based dendrogram of *Epipactis* species and populations, based on unweighted pair-group arithmetic average (UPGMA) cluster analysis of genetic identity values of Table 4.

within *E. helleborine* species. The $\bar{G}_{st}$ value indicates that only a small quantity (3.3%) of the genetic diversity is present among populations, being almost entirely present within populations (96.7%).

Table 7 reports the identity coefficients (I) and the genetic distances (D) calculated according to Nei (1972). I values are very high among populations of the same species (0.979–1.000) while they are lower for pairwise comparisons in different species (0.289–0.805). The average identity value between EP and EM is 0.289, between EP and EH it is 0.336 and between EM and EH it is 0.784.

Figure 2 reports the allozyme-based dendrogram of *Epipactis* species and populations, based on unweighted pair-group arithmetic average (UPGMA) cluster analysis of the genetic similarity coefficients of the Table 4 and shows the phylogenetic relationships among the three examined species.

Table 7. Coefficients of Nei's genetic identity (above diagonal) and distance (below) between populations of three *Epipactis* species.

	EH1	EH2	EH3	EH4	EP1	EM1	EM2
EH1	-	0.979	0.991	0.984	0.339	0.755	0.755
EH2	0.021	-	0.994	0.984	0.341	0.802	0.802
EH3	0.009	0.006	-	0.992	0.343	0.805	0.805
EH4	0.016	0.016	0.008	-	0.322	0.773	0.773
EP1	1.080	1.080	1.070	1.130	-	0.289	0.289
EM1	0.281	0.221	0.217	0.257	1.240	-	1.000
EM2	0.281	0.221	0.217	0.257	1.240	0	-

Discussion

The observed total lack of isozyme variability within and between the two *E. microphylla* populations examined (Table 3) could likely be attributed to the fact that EM1 and EM2 originated from the same genetically impoverished ancestral population. Probably some founding event reduced the genetic variation of this species.

It has been observed that a tendency to autogamy could exist for some *E. microphylla* populations presenting pulverulent pollinia (Senghas, 1970; Pignatti, 1982). In some inbreeding species a remarkable variation among populations can be observed, due to differences in allelic frequencies at a number of loci (Brown, 1979). As some other inbreeders, as *Limnanthes, Tragopon, Typha* and *Xanthium* (Gottlieb, 1981), presenting neither intra nor interpopulational variation, show data close to those we obtained for EM, one could speculate that inbreeding may have played some role in determining such a pattern in EM too.

EM presents most loci monomorphic for the most common allele at EH, only one novel allele (Mdh_2 62%) and a reduced genetic variation in comparison to EH. This is precisely what is observed for progenitor-derivative species pairs of *Coreopsis* and *Lasthenia* (Crawford & Smith, 1982; Crawford *et al.*, 1985), *Stephanomeria* (Gottlieb, 1981) and others. Nevertheless, other data supporting the hypothesis of a relatively recent origin of EM from EH are not available.

Of the other two species studied, both outbreed-

ers, the heterozygosity of *E. palustris* (0.085) is similar to the mean value reported by Gottlieb (1981) for outbreeders (0.086 ± 0.017). It is quite low in comparison to the value of *E. helleborine* (0.233). However, these values are respectively lower and higher than the mean value obtained examining approximately 30 species of the Orchidaceae family ($\overline{HET} = 0.156 \pm 0.018$; $\bar{P} = 0.431 \pm 0.040$) (Scacchi, unpublished data).

The different HET and A values of *E. helleborine* and *E. palustris* may be explained if we consider that, while the former is widespread on the large sampling area, the latter, besides being rare, is gathered in a population restricted to 30 m^2. For this populations we can suppose a genetic drift effect which may be due to a recent founder event.

Table 5 shows that a major portion of the genetic variation in the *Epipactis* group ($\bar{G}_{st} = 0.586$) occurs among populations, indicating a remarkable differentiation (Hartl, 1980). This value is not so high as that observed for another species complex (Sytsma & Schaal, 1985) because the loci fixed for alternative alleles in the various populations are few and because the major portion of the variability in EH ($\bar{G}_{st} = 0.033$) is within populations (Table 6). This value agrees with the low $\bar{G}_{st}$ values (0.071) reported for outbreeders by Loveless and Hamrich (1984).

These results are confirmed by Nei's identity coefficients (Table 7). $\bar{I}$ mean value for comparisons in EH populations (0.987) is close to the value reported by Gottlieb (1981) for outbreeders ($\bar{I} = 0.956$), while the average for all pairwise comparisons in *Epipactis* ($\bar{I} = 0.672$) is similar to the one reported for different cogeneric species ($\bar{I} = 0.670$) by the same author. These data also reveal a larger similarity between EM and EH living in the same habitat ($\bar{I} = 0.784$), than between EP-EM ($\bar{I} = 0.289$) and EP-EH ($\bar{I} = 0.336$). The genetic distance calculated according to Nei (1972) based on the average difference between the last two $\bar{I}$ values ($\bar{I} = 0.312$, $\bar{D} = 1.16$) reveals that approximately one electrophoretically detectable allelic substitution per locus occurred in the separate evolution of EP and the other two species. *E. palustris*, besides presenting a different geographical distribution and a high ecological differentiation (as shown by the particular habitat it occupies), presents also a remarkable genetic differentiation.

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References

Brown, A. H. D., 1979. Enzyme polymorphism in plant populations Theoret. Popul. Biol. 15: 1–42.

Crawford, D. J. & Smith, E. B., 1982. Allozyme variation in *Coreopsis nuecensoides* and *C. nuecensis* (Compositae), a progenitor-derivative species pair. Evolution 36: 379–386.

Crawford, D. J., Ornduff, R. & Vasey, M. C., 1985. Allozyme variation within and between *Lasthenia minor* and its derivative species, *L. maritima* (Asteraceae). Am. J.Bot. 72: 1177–1184.

De Angelis, G. & Lanzara, P., 1987. Le Orchidee spontanee dei Monti Lucretili. Ed. Regione Lazio, Roma.

Dressler, R. L. 1981. The Orchids, Natural history and classification. Harvard University Press, Cambridge, Massachusetts, and London, England.

Gottlieb, L. D., 1977. Electrophoretic evidence and plant systematics. Ann. Missouri bot. Gard. 64: 161–180.

Gottlieb, L. D., 1981. Electrophoretic evidence and plant populations. Progr. Phytochem. 7: 1–46.

Hamrick, J. L., Linhart, Y. B. & Mitton, J. B., 1979. Relationships between life history characteristics and electrophoretically detectable genetic variation in plants. Ann. Rev. Ecol. Syst. 10: 173–200.

Harris, H. & Hopkinson, D. A., 1976. Handbook of enzyme electrophoresis in human genetics. North Holland Publ. Company, Amsterdam, Oxford, New York.

Hartl, D. L., 1980. Principles of population genetics. Sinauer Associates, Sunderland, Massachusetts.

King, J. N. & Dancik, B. P., 1983. Inheritance and linkage of isozymes in white spruce (*Picea glauca*). Can. J. Genet. Cytol. 25: 430–436.

Loveless, M. D. & Hamrick, J. L., 1984. Ecological determinants of genetic structure in plant populations. Ann. Rev. Ecol. Syst. 15: 65–95.

Nei, M., 1972. Genetic distances between populations. Am. Natur. 106: 283–292.

Nei, M., 1975. Molecular population genetics and evolution. North Holland Publ. Company, Amsterdam, Oxford, New York.

Pignatti, S., 1982. Flora d'Italia. Edagricole, Bologna.

Senghas, K., 1970. Übersicht zur Systematik und Taxonomie der Gattung *Epipactis*. In: Senghas, K. & Sundermann, H., Probleme der Orchideengattung *Epipactis*. Jber. naturw. Ver Wuppertal 23: 26–37.

Shaw, C. R. & Prasad, R., 1970. Starch gel electrophoresis of enzymes. A compilation of recipes. Biochem. Genet. 4: 297–320.

Sytsma, K. J. & Schaal, B. A., 1985. Genetic variation, differentiation, and evolution in a species complex of tropical shrubs based on isozymic data. Evolution 39: 582–593.

Walters, M. & Briggs, D., 1969. Les plantes: variations et évolution, Hachette, Paris.

Weeden, N. F. & Gottlieb, L. D., 1980. Isolation of cytoplasmic enzymes from pollen. Plant Physiol. 66: 400–403.

Workman, P. L. & Niswander, J. D., 1970. Population studies on Southwestern Indian tribes. II. Local genetic differentiation in the Papago. Amer. J. hum. Genet. 22: 24–49.

Wright, S., 1969. Evolution and the genetics of populations. Vol. II. The theory of gene frequencies. Univ Chicago Press, Chicago and London.