



Isozyme Evidence for Natural Hybridization in *Phlomis* (Lamiaceae): Hybrid Origin of the Rare *P. × margaritae*

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Received: 31 March 1999 Returned for revision: 25 August 1999 Accepted: 13 September 1999

Phlomis × margaritae is a rare and sterile hybrid taxon found in a single endangered population in southern Spain. It was previously described as a morphologically intermediate hybrid, putatively between *Phlomis purpurea* and *P. composita*. The present study used allozymes as molecular markers to assess the hybrid identity of *P. × margaritae*. Ten putative loci from seven enzyme systems were resolved: five were monomorphic and fixed across all taxa studied and the rest (*Aat-1*, *Aat-2*, *6-Pgdh-2*, *Pgi* and *Pgm*) were polymorphic in at least one taxon. The two parental taxa are fixed for different alleles at *6-Pgdh-2* and show distinct allelic frequency differences for four other loci. *Phlomis × margaritae* displays fixed heterozygous phenotypes for four of the five polymorphic loci, these being composed of combinations of the alleles found in the parental taxa. No unique alleles were detected in *P. × margaritae*. We conclude that this taxon is of hybrid origin and confirm the identity of the parental taxa involved. It is further suggested that this population is composed of individuals that are recent F₁ hybrids that have not undergone backcrossing or introgression. Global conservation measures are necessary for the whole hybrid system in this location since further continuous assessment could reveal the evolutionary input of hybridization in *Phlomis*.

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Key words: Endangered species, genetic diversity, isozyme variation, hybrid species, *Phlomis × margaritae*, plant conservation, population genetics, Lamiaceae, Spain.

INTRODUCTION

Speciation may occur in plants through hybridization either at a diploid or polyploid level, and there are many studies in the literature dealing with hybrid plant evolution (for a recent review see e.g. Arnold, 1997). However, hybridization and introgression may have different evolutionary consequences, not always leading to the formation of new species (Arnold, 1992). Furthermore, Rieseberg (1997) showed that there are a few confirmed examples of homoploid hybrid species (recombinational species) in flowering plants. Therefore, detecting whether or not reproductive isolation exists between F₁ hybrids and parental species is necessary to understand the evolutionary significance of hybridization (Rieseberg and Ellstrand, 1993). On the other hand, the time when hybrids are examined relative to their history would provide valuable information regarding the spread of introgression (Rieseberg and Ellstrand, 1993) and would give sense to hybrid fitness (reproductive success) studies since a primary concern of these should be the fitness of stabilized and fertile hybrid lineages rather than F₁ generations or early segregating hybrid classes (Rieseberg, 1997).

The use of molecular markers is now widespread in many aspects of plant biology and systematics. With regard to hybridization, morphological characters are of limited value

for the study of introgression due to phenotypic plasticity, intermediacy or additivity (Gallez and Gottlieb, 1982). Rieseberg and Wendel (1993) have argued in favour of molecular markers as a tool for the study of hybrids because: (1) there are a large number of independent characters; (2) levels of non-heritable molecular variation are low; and (3) such characters are neutral. Complementarily, many recent studies (e.g. Gemmill *et al.*, 1998; Godt and Hamrick, 1998) also use isozyme markers to evaluate the genetic resources and variability of plants for conservation purposes.

The genus *Phlomis* L. is composed of over 100 species distributed throughout Eurasia and northern Africa with two main centres of diversification in Anatolia (Turkey) and Iran (Azizian and Moore, 1982) in which hybridization seems to be frequent at least in some areas of its distributional range (e.g. Huber-Morath, 1982). In the Iberian Peninsula, four species of *Phlomis* can be found, plus a complex array of hybrid forms that have been known since the early 20th century; these have recently been revised by Mateu (1986), although hybridization was never confirmed. *Phlomis crinita* Cav. and *P. lychnitis* L. are sympatric in the south and southeast of Spain (Betic Mountains) where they supposedly hybridize to produce several hybrid taxa. All of these hybrids were included within *P. composita* Pau by Mateu (1986), however their ecology, biology and genetics are largely unknown. At least some of the hybrids are stabilized and fertile and show a widespread distribution

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across southern Spain. Furthermore, all members of this hybrid complex are homoploid, maintaining the diploid level of the parental species ($2n = 20$) showing high levels of bivalent formation and regular segregation of chromosomes during microsporogenesis and fertility (Aparicio, 1997 and unpubl. res.).

Phlomis × *margaritae* Aparicio and Silvestre (bicolour flowers) was described by Silvestre and Aparicio (1986) as a morphologically intermediate hybrid between fertile forms of the diploids *P. composita* (yellow flowers) and *P. purpurea* L. (pink flowers). *Phlomis* × *margaritae* is also a diploid taxon but is apparently sterile as evidenced by irregular microsporogenesis, low pollen viability, no observed seed set and high predation susceptibility (Aparicio, 1997). Furthermore, it has been considered at risk of extinction (Aparicio, 1993) and listed as 'rare' among the threatened plants of southern Spain (Hernández-Bermejo *et al.*, 1994).

The first step in the study of hybridization and its consequences is to document the hybrid nature of the plant in question (Crawford, 1985). Therefore, as a starting point to evaluating the evolutionary input of hybridization in *Phlomis* in the Iberian Peninsula, the aims of this study were to: (1) confirm or reject the hybrid nature of *P. × margaritae*; (2) deduce its probable parental taxa; (3) elucidate whether the population of *P. × margaritae* was composed exclusively of F_1 individuals or if there is, at present, genetic documentation of backcrossing in the form of individuals belonging to introgressed generations; and (4) identify individual genotypes of *P. × margaritae* plants for conservation and further research purposes.

MATERIALS AND METHODS

At present, only one population of *Phlomis* × *margaritae* is known and it is sympatric with both putative parental taxa (*P. composita* and *P. purpurea*). The population is located within a preserved area in Cádiz province in southern Spain (Parque Natural de la Sierra de Grazalema); its exact location and general features are published elsewhere (Aparicio, 1993, 1997). All the plants are tall perennial herbs with the capacity to root sprout, hence they usually form a dense grouping of ramets (patches). In this study, physically separate patches were treated as separate genets. It was previously estimated (Aparicio, 1993) that about 50 individuals of *P. × margaritae* exist at this site. Populations of the two parental taxa are sympatric here: approx. 100 individuals of *P. composita* and several thousand *P. purpurea* individuals. There is some indication of ecotypic restriction in that the *P. composita* and *P. × margaritae* populations are located at the top of a slope whereas the *P. purpurea* population mainly extends downwards.

Thirty physically separated patches of *P. × margaritae* were randomly selected and individually marked for long term identification. Young leaves were collected from them for enzyme electrophoresis. Additionally, young leaves from 30 patches of *P. composita* and 28 individual plants of *P. purpurea* were also collected. This sample size should ensure that alleles with moderate to high frequencies are represented (Marshall and Brown, 1975).

All leaves were packed and kept refrigerated until protein extraction was undertaken in the laboratory, usually within 24 h. Small pieces of leaves (~100 mg) were ground with 0.15 ml (three drops) of the extraction buffer of Werth (1985) containing 0.17% of β -mercaptoethanol; crude extracts were then absorbed onto Whatman No. 3 paper wicks which were stored at -70° until use (within 2 months).

Protein electrophoresis of the extracts was conducted generally following the methods of Wendel and Weeden (1989). Nineteen enzyme systems were assayed for activity, in 12% (w/w) horizontal starch gels containing 2.5% of sucrose. Nevertheless, scoreable information was obtained for only seven enzyme systems: Aspartate Amino Transferase (AAT), Isocitrate Dehydrogenase (IDH), Malate Dehydrogenase (MDH), Malic Enzyme (ME), Phosphoglucoisomerase (PGI), 6-Phosphogluconate Dehydrogenase (6-PGDH) and Phosphoglucomutase (PGM). Three buffer systems were used to resolve these enzymes: IDH, ME, PGI, 6-PGD and PGM were resolved in a 6.1/6.5 (electrode/gel buffer pH) morpholine-citrate system (Nickrent, 1986); MDH was resolved in a 7.5/7.5 tris-citrate system (Soltis *et al.*, 1983), and AAT in a pH = 8.1/8.1 lithium-borate/tris-citrate system (Ridgeway *et al.*, 1970). The loci were numbered (and the alleles within them were labelled with letters) starting from the most anodally migrating form.

Measures of genetic diversity, including percentage of polymorphic loci (P , where the locus is considered polymorphic when the frequency of the most common allele is 99% or less), number of alleles per locus (A), number of alleles per polymorphic locus (A_p), observed heterozygosity (H_o), expected heterozygosity under Hardy-Weinberg equilibrium (H_e), the fixation index or inbreeding coefficient (F) of Wright (1951), and the genetic identity index (Nei, 1972) were calculated using a Genetic Data Analysis program (Lewis and Zaykin, 1997). The effective number of alleles per locus (polymorphic loci only) was calculated following Kimura and Crow (1964) as

$$n_e = \frac{1}{\sum_{i=1} p_i^2}$$

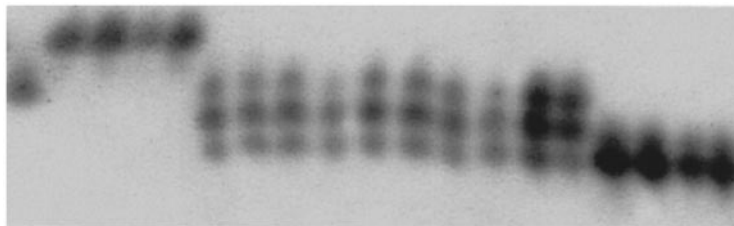
with p being the frequency of the polymorphic allele i .

RESULTS

All the electrophoretic phenotypes for both parental species and the hybrid taxon could be interpreted in terms of the genes and alleles expected for a $2n$ ploidy level, and Mendelian segregation. Furthermore, banding patterns were consistent with the expectations of subunit structure given in May (1998). Several enzyme systems such as Shikimate dehydrogenase, Glucose-6-phosphate dehydrogenase, Leucine amino peptidase and Glutamate dehydrogenase provided inconsistent or confusing zymograms and consequently were not included in the study. However, seven enzyme systems, encoded by ten putative loci were scoreable and provided consistent information for all taxa (Table 1).

TABLE 1. Genotype frequencies of all loci resolved for three *Phlomis* taxa

Locus	Genotype	<i>P. purpurea</i> (n = 28)	<i>P. × margaritae</i> (n = 30)	<i>P. composita</i> (n = 30)
<i>Aat-1</i>	<i>aa</i>	0.179	0	0
	<i>ab</i>	0.143	0	0
	<i>bb</i>	0.678	1.000	1.000
<i>Aat-2</i>	<i>aa</i>	0.930	0	0
	<i>ab</i>	0.070	1.000	0
	<i>bb</i>	0	0	1.000
<i>Idh</i>	<i>aa</i>	1.000	1.000	1.000
<i>Mdh-1</i>	<i>aa</i>	1.000	1.000	1.000
<i>Mdh-2</i>	<i>aa</i>	1.000	1.000	1.000
<i>Me</i>	<i>aa</i>	1.000	1.000	1.000
<i>6-Pgdh-1</i>	<i>aa</i>	1.000	1.000	1.000
<i>6-Pgdh-2</i>	<i>aa</i>	0	0	1.000
	<i>ab</i>	0	1.000	0
	<i>ac</i>	0	0	0
	<i>bb</i>	0.893	0	0
	<i>bc</i>	0.107	0	0
	<i>cc</i>	0	0	0
	<i>Pgi</i>	<i>aa</i>	0	0
	<i>ab</i>	0	0	0.100
	<i>ac</i>	0	0	0
	<i>bb</i>	0	0	0.033
	<i>bc</i>	0.071	1.000	0
	<i>cc</i>	0.928	0	0
<i>Pgm</i>	<i>aa</i>	0.750	0	0.166
	<i>ab</i>	0.250	1.000	0.333
	<i>ac</i>	0	0	0.200
	<i>bb</i>	0	0	0.166
	<i>bc</i>	0	0	0.133
	<i>cc</i>	0	0	0

FIG. 1. *Pgi* zymogram showing the *a* and *b* alleles in *Phlomis composita* (rows 1–5), the *c* allele in *P. purpurea* (rows 16–19) and the *bc* heterozygous phenotype in *P. × margaritae* (rows 6–15).TABLE 2. Allelic frequencies for polymorphic loci in the surveyed taxa of *Phlomis*

Locus	Allele	<i>P. purpurea</i>	<i>P. × margaritae</i>	<i>P. composita</i>
<i>Aat-1</i>	<i>a</i>	0.250	0	0
	<i>b</i>	0.750	1.000	1.000
<i>Aat-2</i>	<i>a</i>	0.965	0.500	0
	<i>b</i>	0.035	0.500	1.000
<i>6-Pgdh-2</i>	<i>a</i>	0	0.500	1.000
	<i>b</i>	0.946	0.500	0
	<i>c</i>	0.053	0	0
<i>Pgi</i>	<i>a</i>	0	0	0.916
	<i>b</i>	0.035	0.500	0.083
	<i>c</i>	0.964	0.500	0
<i>Pgm</i>	<i>a</i>	0.875	0.500	0.433
	<i>b</i>	0.125	0.500	0.400
	<i>c</i>	0	0	0.167

TABLE 3. Genetic diversity measures in *Phlomis*

	<i>n</i>	P	A	A _p	n _e	H _o	H _e	<i>F</i>
<i>P. purpurea</i>	28	0.50	1.50	2.0	1.23	0.0642	0.0848	0.2453
<i>P. × margaritae</i>	30	0.40	1.40	2.0	1.80	0.4000	0.2033	−1.0000
<i>P. composita</i>	30	0.20	1.30	2.5	1.36	0.0766	0.0790	0.0305

n, Sample size; P, % polymorphic loci (99% criterion); A, no. alleles/locus; A_p, no. alleles/polymorphic locus; n_e, mean effective no. of alleles per polymorphic locus (Kimura and Crow, 1964); H_o, observed heterozygosity; H_e, expected heterozygosity under Hardy-Weinberg equilibrium; *F*, fixation index (Wright, 1951).

Five loci (*Idh*, *Me*, *Mdh-1*, *Mdh-2* and *6-Pgdh-1*) did not show variation among or within taxa and were monomorphic and fixed for the same allele. MDH electrophoretic phenotypes always consisted of a three-banded pattern which was interpreted as two monomorphic loci with an additional interlocus heterodimeric band, as has been observed for this enzyme in other plant species (see Raspé *et al.*, 1998). The remaining five loci were polymorphic for at least one taxon.

The parental taxa showed a fixed difference at *6-Pgd-2* and allelic frequency differences at the remaining four polymorphic loci: *Aat-1*, *AAT-2*, *Pgi* and *Pgm* (Table 1). Of the 18 alleles detected across all enzyme loci, ten are shared between the two parental taxa whereas 14 are found in *P. × margaritae*. Indeed, this taxon displayed fixed heterozygous phenotypes for four of the five polymorphic loci, these being composed of combinations of the alleles found in the parental taxa (see Fig. 1). No unique alleles were detected in *P. × margaritae*. Greater allelic diversity was seen in *P. purpurea* than in *P. composita* owing to the presence of polymorphic *Aat* (two loci) and *6-PGD-2* (Tables 1 and 2). Unlike the other polymorphic loci that show fixed heterozygous phenotypes, *P. × margaritae* presents a homozygous 'bb' genotype at *Aat-1* as is also found in *P. composita*.

Intrapopulational genetic variability measures were determined for all three taxa and the results are shown in Table 3. A genetic identity value (Nei, 1972) of 0.677 was obtained for the *P. purpurea* and *P. composita* comparison. The values calculated for *P. × margaritae* and its two putative parents were 0.901 and 0.868, thus indicating that this taxon is only slightly more closely related to the former parental taxon.

DISCUSSION

The hybrid status of *P. × margaritae*, first proposed based upon morphological and cytological criteria by Silvestre and Aparicio (1986), is confirmed in this electrophoretic study. This taxon shows fixed heterozygous phenotypes composed only of alleles found in *P. purpurea* and *P. composita*, which occur sympatrically with this hybrid. The genetic identity value measured for the two parental taxa (0.67) is quite similar to average values obtained for outcrossing diploid plants (Gottlieb, 1981). Genetic identity values between these parentals and *Phlomis × margaritae* are high and approximately equal: the slightly higher value with *P. purpurea* may be due to the fact that *P. × margaritae* uniquely shares alleles at three loci (*Aat-2* 'a', *6-Pgd-2*

'b' and *Pgi* 'c') with *P. purpurea* as opposed to only one with *P. composita* (*6-PGD-2* 'a'). These data support the contention that *Phlomis × margaritae* is an F₁ hybrid and that it has not undergone significant amounts of backcrossing to either parent. As in many studied angiosperms (Gallez and Gottlieb, 1982; Nason *et al.*, 1992), the absence of unique alleles and the presence of alleles of both parental species further suggests a historically recent origin of a hybrid taxon with no advanced generations or backcrossings.

Fixation index (*F*) values (Wright, 1951) provide a measure of the degree of inbreeding that may result from differences in the breeding system within each population and taxon. With random mating under Hardy-Weinberg equilibrium *F* = 0, and with complete inbreeding *F* = 1. Negative values can result from selection favouring heterozygotes or negative assortive mating (Olmstead, 1990), that is, where individuals tend to mate with phenotypically different individuals to produce heterozygous genotypes (Hartl, 1987). Our results show that parental taxa in this population are mostly outcrossing; however, partial selfing resulting from substructuring may be occurring in the extensive population of *P. purpurea* as evidenced by *F* = 0.245. Factors such as non-random dispersal of seeds and pollen (Levin and Kerster, 1974) can result in matings between relatives (Olmstead, 1990). However, these results obtained for the parental taxa must be interpreted with caution, as more studies on genetic variability of other populations throughout their range should be carried out. In the case of *P. × margaritae*, the *F* value of −1 indicates an excess of heterozygotes, double the number expected. This is probably not due to negative assortive mating, but instead to the fact that sexual reproduction in *P. × margaritae* is not actually taking place and because of the lack of genetic variation among individual plants. In fact, all individuals of *P. × margaritae* show an almost perfect combination of alleles and frequencies of the parental species. The original hybridization probably produced this highly heterozygous F₁ genotype which has since been vegetatively maintained and spread by sprouting. If this hybridization event occurred just once, the population of *P. × margaritae* is actually an ecological genet with no genetic variation. Alternately, the hybrid may have been formed on more than one occasion with gametic contribution by genetically similar parents. The lack of genetic variation in this taxon supports, at least at present, the hypothesis of a single origin; however, other more fine-scale molecular markers (e.g. RAPDs) should be used to confirm this result (see Wolfe *et al.*, 1998).

Although recombinational speciation is not the only evolutionary consequence of natural hybridization (Arnold, 1992), stabilization of the hybrids could theoretically, and in practice, lead to the formation of new species (Rieseberg, 1997). This stabilization should involve chromosome rearrangements and the development of reproductive isolation barriers (Grant, 1971). In our case, previous studies in *P. × margaritae* showed that microsporogenesis was mostly an irregular process with the formation of a variable number of univalents and bivalents at diakinesis. Unexpectedly, *P. × margaritae* was also observed to produce full pairing of chromosomes, with the production of tetrads being the norm at the end of microsporogenesis (Aparicio, 1997). Therefore, despite the fact that no seeds of *P. × margaritae* have been collected to date, occasional production should not be ruled out. At this point differences between high and complete hybrid sterility should be underlined: the production of just one seed, by hybrid plants regarded as sterile, which can form a vegetatively-propagating adult is of major biological and evolutionary importance in the overall hybrid fitness and hybridization outcome (Stebbins, 1959; Grant, 1971).

With regard to conservation, knowledge of levels and distribution of genetic resources in rare, endangered and endemic species is essential in designing population and genetic conservation action plans (Olmstead, 1990; Hamrick et al., 1991; Gemmill et al., 1998). But, on the other hand, such a population genetic approach is only efficient for conservation purposes after the identification of the demographic framework of the taxa (Schemske et al., 1994). One aim of this study was to identify genotypes of individually marked plants of *P. × margaritae* to develop an individual conservation programme for the whole genetic variability of *P. × margaritae* in this unique site, being an example of a recent and infrequent hybridization in *Phlomis*. However, as discussed above, no genetic variability was detected in this hybrid taxon. Therefore, we believe that the results of the present study stress the necessity of global conservation measures from non-natural extinction of the whole hybrid system relying on the fact that evolution of such hybrid systems depends on whether repeated opportunities for natural hybridization are provided or not (Arnold and Hodges, 1995). This unique hybrid zone represents a valuable opportunity, with this work as a starting point, to study the evolutionary input of hybridization in *Phlomis*. Furthermore, repeated sampling over the years could reveal progressive changes in the degree or direction of hybridization and introgression, if any (Rieseberg and Ellstrand, 1993).

ACKNOWLEDGEMENTS

This study has been supported by the Spanish DGES (Dirección General de Enseñanza Superior) project PB96-1362. The manuscript has benefitted from the valuable review of Dr. D. Nickrent (who also provided his laboratory manual) and from the comments of Dr. J. Arroyo, Dr. G. Nieto, and Dr. R. Zárate, to whom we are grateful. We are also indebted to B. Garrido y R. Hidalgo who provided some useful laboratory skills, and to the authorities of

Sierra de Grazalema Natural Park for the permission to work in the area.

LITERATURE CITED

- Aparicio A. 1993.** Planes de recuperación de especies vegetales amenazadas en el Parque Natural de la Sierra de Grazalema (Cádiz-Málaga). *Acta Botanica Malacitana* **18**: 199–221.
- Aparicio A. 1997.** Fitness components of the hybrid *Phlomis* × *margaritae* Aparicio and Silvestre (Lamiaceae). *Botanical Journal of the Linnean Society* **124**: 331–343. Erratum. 1999. **130**: 303.
- Arnold ML. 1992.** Natural hybridization as an evolutionary process. *Annual Review of Ecology and Systematics* **23**: 237–261.
- Arnold ML. 1997.** *Natural hybridization and evolution*. New York: Oxford University Press.
- Arnold ML, Hodges A. 1995.** Are natural hybrids fit or unfit relative to their parents? *Trends in Ecology and Evolution* **10**: 67–71.
- Azizian D, Moore DM. 1982.** Morphological and palynological studies in *Phlomis* L. and *Eremostachys* Bunge (Labiatae). *Botanical Journal of the Linnean Society* **85**: 249–281.
- Crawford DJ. 1985.** Electrophoretic data and plant speciation. *Systematic Botany* **10**: 405–416.
- Gallez GP, Gottlieb LD. 1982.** Genetic evidence for the hybrid origin of the diploid plant *Stephanomeria diegensis*. *Evolution* **36**: 1158–1167.
- Gemmill EC, Ranker TA, Ragone D, Perlman SP, Wood KR. 1998.** Conservation genetics of the endangered endemic Hawaiian genus *Brighamia* (Campanulaceae). *American Journal of Botany* **85**: 528–539.
- Godt MJW, Hamrick JL. 1998.** Low allozyme diversity in *Schalbea americana* (Scrophulariaceae), an endangered plant species. *Journal of Heredity* **89**: 89–93.
- Gottlieb LD. 1981.** Electrophoretic evidence and plant populations. In: Reinhold L, Harborne JB, Swain T, eds. *Progress in phytochemistry*. Oxford: Pergamon Press, 1–46.
- Grant V. 1971.** *Plant speciation*. New York: Columbia University Press.
- Hamrick JL, Godt MJW, Murawski DA, Loveles MD. 1991.** Correlation between species traits and allozyme diversity: implications for conservation biology. In: Falk DA, Holsinger KE, eds. *Genetics and conservation of rare plants*. New York: Oxford University Press, 75–86.
- Hartl DL. 1987.** *A primer of population genetics. 2nd edn*. Sunderland: Sinauer Associates.
- Hernández-Bermejo JE, Pujadas A, Clemente M. 1994.** Catálogo general de las especies de recomendada protección en Andalucía (endémicas, raras y amenazadas de extinción). In: Hernández-Bermejo JE, Clemente M., eds. *Protección de la Flora en Andalucía*. Sevilla: Agencia de Medio Ambiente, 43–66.
- Huber-Morath A. 1982.** *Phlomis*. In: Davis PH, ed. *Flora of Turkey*. Edinburgh: Edinburgh University Press, 102–126.
- Kimura M, Crow FJ. 1964.** The number of alleles that can be maintained in a finite population. *Genetics* **99**: 725–738.
- Levin DA, Kerster HW. 1974.** Gene flow in plants. *Evolutionary Biology* **7**: 139–220.
- Lewis PO, Zaykin D. 1997.** Genetic data analysis. Computer program for analysis of allelic data version 1.0. Free program distributed by the authors over the internet from GDA Home Page at <http://chee.unm.edu/gad/>.
- Marshall PR, Brown AHD. 1975.** Optimum sampling strategies in genetic conservation. In: Frankel OH, Whawkes JH, eds. *Crop genetic resources for today and tomorrow*. Cambridge: Cambridge University Press, 53–80.
- Mateu I. 1986.** Revisión del género *Phlomis* L. (Labiatae) en la Península Ibérica e Islas Baleares. *Acta Botanica Malacitana* **11**: 177–204.
- May B. 1998.** Starch gel electrophoresis of isozymes. In: Hoelzel AR, ed. *Molecular genetic analysis of populations. 2nd edn*. Oxford: IRC Press, Oxford University Press, 1–28.
- Nason JD, Ellstrand NC, Arnold ML. 1992.** Patterns of hybridization and introgression in populations of oaks, manzanitas, and irises. *American Journal of Botany* **79**: 101–111.

- Nei M. 1972. Genetic distances between populations. *American Naturalist* **106**: 283–292.
- Nickrent DL. 1986. Genetic polymorphism in the morphologically reduced dwarf mistletoes (*Arceutobium*, Viscaceae): an electrophoretic study. *American Journal of Botany* **73**: 1492–1502.
- Olmstead RG. 1990. Biological and historical factors influencing genetic diversity in the *Scutellaria angustifolia* complex (Labiatae). *Evolution* **44**: 54–70.
- Raspé O, Jacquemart AL, de Sloover J. 1998. Isozymes in *Sorbus aucuparia* (Rosaceae: Maloideae): genetic analysis and evolutionary significance of zymograms. *International Journal of Plant Sciences* **159**: 627–636.
- Rieseberg LH. 1997. Hybrid origin of plant species. *Annual Review of Ecology and Systematics* **28**: 359–389.
- Rieseberg LH, Ellstrand NC. 1993. What can molecular and morphological markers tell us about plant hybridization? *Critical Reviews in Plant Sciences* **12**: 213–241.
- Rieseberg LH, Wendel JF. 1993. Introgression and its consequences in plants. In: Harrison R, ed. *Hybrid zones and the evolutionary process*. New York: Oxford University Press, 70–109.
- Schemske DW, Husband BC, Ruckelhaus MH, Goodwillie C, Parker MI, Bishop JG. 1994. Evaluating approaches to the conservation of rare and endangered plants. *Ecology* **75**: 584–606.
- Silvestre S, Aparicio A. 1986. Un nuevo híbrido en el género *Phlomis* L.: *P.* × *margaritae* Aparicio and Silvestre. *Lagascalia* **14**: 100–102.
- Soltis DE, Haufler CH, Darrow DC, Gastony GJ. 1983. Starch gel electrophoresis of ferns: a compilation of grinding buffers, gel and electrode buffers, and staining schedules. *American Fern Journal* **73**: 9–27.
- Stebbins GL. 1959. The role of hybridization in evolution. *Proceedings of the American Philosophical Society* **103**: 231–251.
- Wendel JF, Weeden NF. 1989. Visualisation and interpretation of plant isozymes. In: Soltis DE, Soltis PS, eds. *Isozymes in plant biology*. London: Chapman & Hall, 5–45.
- Werth CR. 1985. Implementing a laboratory at a field station in Virginia. *Virginia Journal of Science* **36**: 53–76.
- Wolfe AD, Xiang Q-Y, Kephart SR. 1998. Diploid hybrid speciation in *Penstemon* (Scrophulariaceae). *Proceedings of the National Academy of Science of USA* **95**: 5112–5115.
- Wright S. 1951. The genetical structure of populations. *Annals of Eugenics* **15**: 323–354.