

Population Genetic Structure and Hybridization Patterns in the Mediterranean Endemics *Phlomis lychnitis* and *P. crinita* (Lamiaceae)

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• **Background and Aims** The historical influence of gene flow and genetic drift after the last glacial phase of the Quaternary Period is reflected in current levels of genetic diversity and population structure of plant species. Moreover, hybridization after secondary contact might also affect population genetic diversity and structure. An assessment was made of the genetic variation and hybrid zone structure in Iberian populations of the Mediterranean *Phlomis lychnitis* and *P. crinita*, for which phylogenetic relationships are controversial, and hybridization and introgression are common.

• **Methods** Allozyme variation at 13 loci was analysed in 1723 individual plants sampled from 35 natural locations of *P. lychnitis*, *P. crinita* subsp. *malacitana* and *P. crinita* subsp. *crinita* in southern and eastern Spain. Standard genetic diversity parameters were calculated and patterns of genetic structure in each taxon were tested to fit the equilibrium between gene flow and genetic drift. Individual multilocus genotypes were subjected to Bayesian clustering analysis to estimate hybridization and introgression rates for both geographic regions.

• **Key Results** Contrasting patterns in the distribution of genetic variation among the three taxa were found. *Phlomis lychnitis* showed no significant inbreeding, low genetic differentiation among populations and no evidence of isolation by distance. *Phlomis crinita* subsp. *malacitana* and *P. crinita* subsp. *crinita* showed high levels of genetic structure consistent with a pattern of gene flow–drift equilibrium. Higher instances of hybridization and introgression were detected in locations from southern Spain compared with locations from eastern Spain, matching unimodal and bimodal hybrid zones, respectively.

• **Conclusions** High instances of historical gene flow, range expansion and altitudinal movement during the Quaternary Period, and lineage sorting can explain the diversity of patterns observed. The results suggest that *P. lychnitis* is the most differentiated lineage in the group; however, the relationship between the three taxa remains unclear.

Key words: Allozymes, glacial refuges, hybridization, Iberian Peninsula, introgression, isolation by distance, Lamiaceae, lineage sorting, gene flow–drift equilibrium, *Phlomis*.

INTRODUCTION

Historical processes play a major role as driving forces shaping the contemporary patterns of genetic architecture in natural populations (Comes and Kadereit, 1998; Taberlet *et al.*, 1998; Hewitt, 2001). Recent studies on patterns of genetic variation in plant species within areas that have historically acted as refuges for biodiversity, such as the peninsulas around the Mediterranean Basin, have revealed complex patterns that mostly result from the Quaternary Period (Gutiérrez Larena *et al.*, 2002; Olalde *et al.*, 2002; Bittkau and Comes, 2005). Although pinpointing the location of glacial refuges and migration routes is a challenging task, the scarce available data indicate that the mountain ranges of southern and eastern Spain (Betic mountains) acted as refuges for many forest plant species, when most lowlands on the Iberian Peninsula had sub-Saharan climatic conditions (Carrion, 2002). The climate changes that occurred during the Quaternary altered the altitudinal range of some species and resulted in a scattered mosaic of fragmented populations that are currently restricted to the massive Betic mountains (e.g. *Armeria* sp., Gutiérrez-Larena *et al.*, 2002; *Anthyllis montana*, Kropf *et al.*, 2002). Other plant species quickly

expanded their distribution range through horizontal migration from these refuges to the central and western Iberian Peninsula (e.g. *Pinus pinaster*, Salvador *et al.*, 2000; *Quercus ilex*, Lumaret *et al.*, 2002). However, in contrast to the knowledge about the recent plant recolonization of central and northern Europe (Hewitt, 2004), few studies have been conducted on plant species in glacial refuges except for forest trees (e.g. Petit *et al.*, 2003), and further knowledge is required to obtain a clearer picture of how historical factors have structured the current genetic variation of these species.

Furthermore, plant range expansion after the last glacial episode allowed the secondary contact of formerly isolated lineages, providing the opportunity for interspecific matings and the formation of hybrid zones (Hewitt, 2001). Hybrid zones might persist if pre-zygotic barriers against hybridization between divergent lineages are lacking or weakly developed, and/or if selection (either endogenous or exogenous) acting upon hybrid progeny is not remarkable. In this case, populations will usually consist of pure parental genotypes and a wide array of hybrids and backcrosses (i.e. a hybrid swarm; Harrison, 1993). In contrast, if nearly complete barriers against hybridization are developed and/or hybrids are overwhelmingly negatively selected, hybrid zones might be ephemeral and populations will comprise

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parental genotypes and a few hybrids. These two extremes have been referred to as 'unimodal' and 'bimodal' hybrid zones, respectively (Harrison and Bogdanowicz, 1997). Jiggins and Mallet (2000) have stated that this hybrid zone classification is not discrete and exists as a continuum from unimodality to bimodality towards the complete isolation of lineages on the way to speciation, although it might also take place in the opposite direction when two previously isolated lineages mate and merge to form a single lineage (Rhymer and Symberloff, 1996). Investigating the structure of hybrid zones might shed some light on the processes that maintain isolation between lineages. However, high rates of introgression between lineages also affect patterns of genetic diversity by increasing levels of polymorphism (Rieseberg and Wendel, 1993), and determining whether these polymorphisms are the result of hybridization or acquired through the divergence process is a complicated task.

Phlomis lychnitis L. (Iberian Peninsula and southern France) and *P. crinita* Cav. (southern and eastern Spain) are two species endemic to the Mediterranean region and constitute the morphological extremes of variation in a hybrid complex where hybridization and lineage sorting have played a major evolutionary role (Albaladejo *et al.*, 2005). An ecogeographic and morphometric analysis of the hybrid complex (Albaladejo *et al.*, 2004) demonstrated the existence of two allopatric sub-specific entities within *P. crinita* – *P. crinita* subsp. *malacitana* (Pau) Cabezudo, Nieto Caldera and Navarro (above 1000 m in the mountains of southern Spain) and *P. crinita* subsp. *crinita* (below 800 m in the mountains of eastern Spain). Intermediate morphotypes between each *P. crinita* sub-species and *P. lychnitis* are common in southern Spain but rare in eastern Spain. Karyologically these plants conform to a homoploid (diploid; $2n = 20$) hybrid complex; however, meiotic aberrations and pollen abnormalities (chromosome clumping, chromatinic bridges, occurrence of multivalents/univalents and polysporads) are common (Aparicio and Albaladejo, 2003). These data were interpreted as evidence for hybridization and introgression between *P. crinita* subsp. *malacitana* and *P. lychnitis* rather than between *P. crinita* subsp. *crinita* and *P. lychnitis*.

Furthermore, genetic support for hybridization and introgression in the hybrid complex was recently gained through a study of nuclear internal transcribed spacer (ITS) and non-coding plastid DNA sequence variation (Albaladejo *et al.*, 2005). This study showed that ITS sequence variation agrees with taxonomic delimitations, although the presence of nucleotide polymorphisms in the ITS sequences is the rule rather than the exception. In contrast, chloroplast DNA (cpDNA) variation follows a geographic trend through the sharing of haplotypes among taxa in different geographic areas (i.e. southern Spain vs. eastern Spain). The authors suggested localized instances of introgression, coupled with the sorting of polymorphisms among lineages through the divergence process, as possible causes for the incongruence between markers (Albaladejo *et al.*, 2005). However, the genetic relationships in this group remain controversial because of the low variability in nuclear and plastid DNA sequences that is inherently linked to the recent divergence of lineages.

In this study, allozyme variability was used to evaluate the relative historical influence of gene flow, genetic drift and hybridization on the genetic diversity and regional population structure of this Mediterranean endemic hybrid complex, and to deepen understanding of their biosystematics.

MATERIALS AND METHODS

Studied plants

All the taxa studied are common suffruticose chamaephytes growing in dry open places in disturbed helm-oak vegetation. The plants have vegetative spread and produce yellow to brown flowers, which are protandrous, nectariferous and visited by bumblebees (Brantjes, 1981). They have a self-compatible breeding system; however, insect visitation is required because of low fruit set through spontaneous selfing (R.G.A. and A.A., unpublished data). After fertilization, one (seldom two) nucule develops within the persistent calyx, supposedly as an adaptive mechanism to promote dispersal by wind (Aparicio, 1997).

Sampling and electrophoresis

To quantify genetic diversity and determine population structure of the three parental taxa, 35 locations were sampled covering the distribution range of the complex (Table 1 and Fig. 1). *Phlomis lychnitis* was collected from 23 populations (16 in southern Spain and seven in eastern Spain), *P. crinita* subsp. *malacitana* was collected from 16 populations and *P. crinita* subsp. *crinita* was collected from ten populations. To characterize hybrid zone structure in both geographic regions, morphologically intermediate plants – putative hybrids – that co-occurred with both parental types (i.e. mixed/sympatric zones) were sampled. Twelve such putative hybrid or contact zones were sampled across the studied area, nine in southern Spain (locations 1, 6, 8, 12, 13, 14, 15, 16 and 21; Table 1 and Fig. 1) and three in eastern Spain (locations 27, 32 and 35). The rarity of hybrid plants in eastern Spain limited the sampling. Individual plants were classified in the field as parental or hybrid phenotypes by their morphological traits, as detailed in Albaladejo *et al.* (2004). In all populations, plants were collected at least 2 m apart to avoid the sampling of different ramets from the same genet. Sampled leaves were refrigerated on ice until proteins were extracted by grinding small pieces of leaves in three drops of extraction buffer (Werth, 1985). Crude extracts were absorbed on chromatography paper wicks and stored at -80°C until they were electrophoresed.

Starch gel electrophoresis was conducted following the general methods of Weeden and Wendel (1989), which have been previously used in this genus by Aparicio *et al.* (2000). Electrophoreses were carried out in 9% starch (Sigma) gels containing 2.5% sucrose. Nine enzyme systems were used to resolve 13 loci, which provided consistent patterns of activity and reproducibility throughout the samples analysed. Malate dehydrogenase (MDH; EC 1.1.1.37), malic enzyme (ME; EC 1.1.1.40), phosphoglucisomerase (PGI; EC 5.3.1.9), phosphoglucumutase (PGM; EC 2.7.5.1), 6-phosphoglucuronate

TABLE 1. Location and population codes of the *Phlomis* taxa studied in southern and eastern Spain

Location	Taxon	Population code	<i>n</i>
Southern Spain			
1. Grazalema mountain range, 30S TF8676, 1000 m	<i>P. crinita</i> subsp. <i>malacitana</i>	MA1	30
	Hybrids		30
	<i>P. lychnitis</i>	LY1	30
2. Grazalema mountain range, 30S TF8874, 1300 m	<i>P. crinita</i> subsp. <i>malacitana</i>	MA2	30
3. Grazalema mountain range, 30S TF9167, 900 m	<i>P. lychnitis</i>	LY3	31
4. Ronda mountain range, 30S TF9763, 800 m	<i>P. lychnitis</i>	LY4	30
5. Ronda mountain range, 30S TF9868, 800 m	<i>P. lychnitis</i>	LY5	31
6. Ronda mountain range, 30S TF9766, 1100 m	<i>P. crinita</i> subsp. <i>malacitana</i>	MA6	33
	Hybrids		33
	<i>P. lychnitis</i>	LY6	33
7. Ronda mountain range, 30S UF1874, 1000 m	<i>P. lychnitis</i>	LY7	12
8. Ronda mountain range, 30S UF1360, 1000 m	<i>P. crinita</i> subsp. <i>malacitana</i>	MA8	35
	Hybrids		32
	<i>P. lychnitis</i>	LY8	35
9. Ronda mountain range, 30S UF1962, 1700 m	<i>P. crinita</i> subsp. <i>malacitana</i>	MA9	35
10. Ronda mountain range, 30S UF3372, 1000 m	<i>P. lychnitis</i>	LY10	35
11. Almijara mountain range, 30S VF1683, 1500 m	<i>P. crinita</i> subsp. <i>malacitana</i>	MA11	30
12. Almijara mountain range, 30S VF1587, 1100 m	<i>P. crinita</i> subsp. <i>malacitana</i>	MA12	30
	Hybrids		30
	<i>P. lychnitis</i>	LY12	25
13. Almijara mountain range, 30S VF2084, 1100 m	<i>P. crinita</i> subsp. <i>malacitana</i>	MA13	30
	Hybrids		30
	<i>P. lychnitis</i>	LY13	30
14. Sierra Nevada, 30S VF3488, 1300 m	<i>P. crinita</i> subsp. <i>malacitana</i>	MA14	31
	Hybrids		30
	<i>P. lychnitis</i>	LY14	30
15. Sierra Nevada, 30S VF3784, 1400 m	<i>P. crinita</i> subsp. <i>malacitana</i>	MA15	31
	Hybrids		30
	<i>P. lychnitis</i>	LY15	30
16. Sierra Nevada, 30S VF7688, 1400 m	<i>P. crinita</i> subsp. <i>malacitana</i>	MA16	32
	Hybrids		30
	<i>P. lychnitis</i>	LY16	30
17. Sierra Nevada, 30S VF7792, 1400 m	<i>P. crinita</i> subsp. <i>malacitana</i>	MA17	30
18. Sierra Nevada, 30S VG9611, 1800 m	<i>P. crinita</i> subsp. <i>malacitana</i>	MA18	32
19. Sierra Nevada, 30S VG9611, 1600 m	<i>P. lychnitis</i>	LY19	30
20. Lújar mountain range, 30S VF6577, 1600 m	<i>P. crinita</i> subsp. <i>malacitana</i>	MA20	30
	<i>P. lychnitis</i>	LY20	12
21. Gádor mountain range, 30S WF0985, 1600 m	<i>P. crinita</i> subsp. <i>malacitana</i>	MA21	32
	Hybrids		30
	<i>P. lychnitis</i>	LY21	30
22. Gádor mountain range, 30S WF1087, 1800 m	<i>P. crinita</i> subsp. <i>malacitana</i>	MA22	30

Continued

TABLE 1. Continued

Location	Taxon	Population code	<i>n</i>
Eastern Spain			
23. Revolcadores, 30S WH6515, 1600 m	<i>P. lychnitis</i>	LY23	30
24. El Pardal, 30S WH6360, 960 m	<i>P. lychnitis</i>	LY24	30
25. Elche de la Sierra, 30S WH8653, 500 m	<i>P. crinita</i> subsp. <i>crinita</i>	CR25	30
26. Los Belmontes, 30S WH6536, 800 m	<i>P. crinita</i> subsp. <i>crinita</i>	CR26	30
27. Crevillente mountain range, 30S XH8739, 800 m	<i>P. crinita</i> subsp. <i>crinita</i>	CR27	30
	Hybrids		1
	<i>P. lychnitis</i>	LY27	30
28. Ballones, 30S YH3391, 650 m	<i>P. lychnitis</i>	LY28	29
29. Tollos, 30S YH3696, 800 m	<i>P. crinita</i> subsp. <i>crinita</i>	CR29	30
30. Alcalá de la Jovada, 30S YH3697, 600 m	<i>P. crinita</i> subsp. <i>crinita</i>	CR30	26
	<i>P. lychnitis</i>	LY30	10
31. Bicorp, 30S XJ8330, 800 m	<i>P. crinita</i> subsp. <i>crinita</i>	CR31	30
32. Bicorp, 30S XJ8630, 600 m	<i>P. crinita</i> subsp. <i>crinita</i>	CR32	20
	Hybrids		2
	<i>P. lychnitis</i>	LY32	30
33. Quesa, 30S XJ9732, 260 m	<i>P. crinita</i> subsp. <i>crinita</i>	CR33	30
34. Barx, 30S YJ3221, 400 m	<i>P. crinita</i> subsp. <i>crinita</i>	CR34	30
35. Tous, 30S YJ0735, 200 m	<i>P. crinita</i> subsp. <i>crinita</i>	CR35	56
	Hybrids		2
	<i>P. lychnitis</i>	LY35	17

n is the sample size.

dehydrogenase (6-PGD; EC 1.1.1.44) and shikimate dehydrogenase (SKDH; EC 1.1.1.25) were resolved in a morpholine citrate buffer (Clayton and Tretiak, 1972), whereas isocitrate dehydrogenase (IDH; EC 1.1.1.42), colorimetric esterase (EST; EC 3.1.1) and aspartate aminotransferase (AAT; EC 2.6.1.1) were resolved in a lithium borate/Tris citrate buffer (Ashton and Branden, 1961). Staining recipes followed those previously reported by Soltis *et al.* (1983). Gels were run for 6–7 h at 40 and 50 mA for morpholine citrate and lithium borate/Tris citrate buffers, respectively. Loci and alleles within the gels were numbered and labelled, starting from the most anodally migrating form. Overall, 1723 allozyme multilocus genotypes were generated.

Genetic diversity and population structure in *P. lychnitis* and *P. crinita*

Allele frequencies were used to compute the percentage of polymorphic loci at the 99 % cut-off level (P_{99}), mean number of alleles per locus (A), expected heterozygosity (H_e) and observed heterozygosity (H_o), with the software GDA 1.1 (Lewis and Zaykin, 2001). Because differences in sampling intensity can bias comparisons for allelic

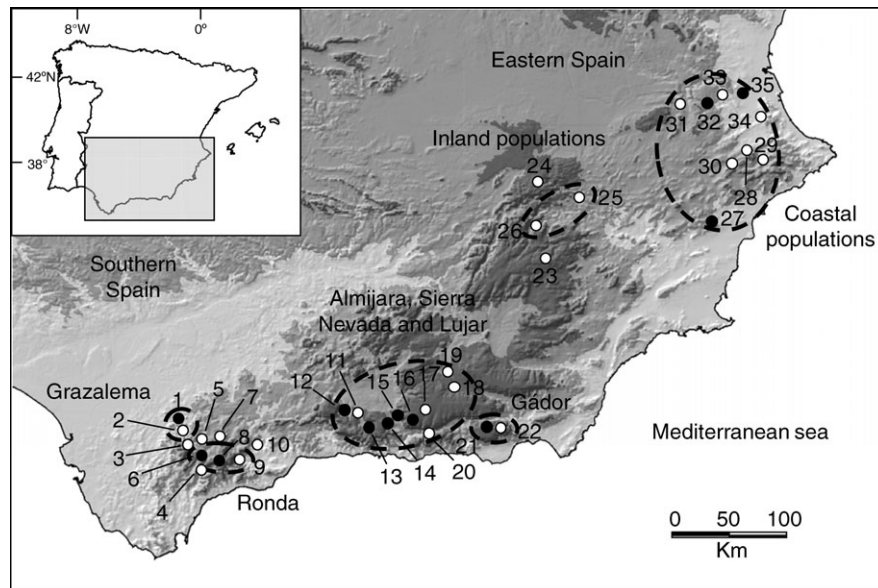


FIG. 1. The 35 locations in the Iberian Peninsula from which *Phlomis* individuals were sampled. Numbers correspond to those in Table 1: 1–22 southern Spain, 23–35 eastern Spain. Black circles correspond to the 12 hybrid or contact zones studied: nine in southern Spain and three in eastern Spain. Dashed lines represent the main clusters of *P. crinita* in the UPGMA phenogram.

richness, A was standardized to $A[g]$ on the basis of the smallest number of samples following a rarefaction method adapted for population genetic data by El Mousadik and Petit (1996). At the population level, g was set equal to 20 (the sample size for *P. lychnitis* at location 30 was ten individuals), and at the taxonomic level g was set equal to 500 as the standard sample size of gene copies (286 sampled plants, i.e. 572 gene copies, in *P. crinita* subsp. *crinita*). Allelic richness was computed with the software Rarefac (Petit *et al.*, 1998). The inbreeding coefficient (F_{IS} ; Weir and Cockerham, 1984) for each population was calculated with FSTATS 2.9.3 (Goudet, 2001), and significant heterozygote deficiency (HD) or excess (HE) tested by randomizing alleles among individuals.

At the taxonomic level, deviations from Hardy–Weinberg (HW) equilibrium and population structure were estimated by computing the inbreeding coefficient (F_{IS}) and the genetic differentiation (F_{ST}) unbiased estimators (Weir and Cockerham, 1984). Statistical significance was tested by constructing 95% confidence intervals by bootstrapping over loci (2000 replicates) with GDA 1.1 (Lewis and Zaykin, 2001).

Concordance with migration–drift equilibrium (‘isolation-by-distance’) in the three taxa was explored by plotting pairwise $F_{ST}/(1 - F_{ST})$ values (Rousset, 1997) against Euclidean geographic distances between all sample sites. Mantel test (1000 permutations) and reduced major axis (RMA) regression (Sokal and Rohlf, 1981) were conducted to assess the significance and strength of the relationship between genetic and geographic distances with the software IBD (Bohonak, 2002). Additionally, the absolute residuals of the regressions were saved and then correlated with the geographic distance (1000 permutations) to test whether the spread of the data increased

with geographic distance, following Hutchinson and Templeton (1999). For organisms with restricted dispersal ability, these authors stated that at gene flow–drift equilibrium, the scatter of points in a two-dimensional scatterplot should increase with the geographic distance because of the prominent role of genetic drift over larger distances.

Patterns of hybridization and introgression

The individual multilocus allozyme data in each of the 12 hybrid or contact zones ($n = 1020$ individuals) were subjected to the Bayesian clustering method implemented in the software Structure 2.0 (Pritchard *et al.*, 2000; Falush *et al.*, 2003) to determine and compare hybridization and introgression patterns between regions. Unlike previous methods that are based on maximum likelihood (e.g. Nason and Ellstrand, 1993), the algorithm behind Pritchard’s software does not need to specify the gene frequencies of the parental sources in advance and has been proven to work efficiently, even in complex hybrid swarms where parental frequencies could not be confidently estimated (e.g. Beaumont *et al.*, 2001). In this analysis, individuals were assigned to pre-defined K populations having homogeneous allele frequencies. First, it was checked whether the program was consistent in identifying the two expected parental populations (species in the present case) for each putative hybrid zone computing the *ad hoc* statistic ΔK described by Evanno *et al.* (2005). Briefly, for each hybrid zone, 20 simulations were run for each group, from $K = 1$ to $K = 5$, and then the statistic ΔK was computed, which detected the highest rate of change in the log-likelihood between successive K s (see Evanno *et al.* 2005 for a detailed graphic explanation about ΔK calculations). All simulations were run setting 10^5 iterations as the burn-in period, and 10^5 iterations for Markov chain

convergence. Preliminary runs using higher burn-in periods and Markov chain iterations did not produce different results. For each run, the admixture model was set, allele frequencies were allowed to be correlated and the previous classification of individuals based on morphological traits was not taken into account. Secondly, after estimating the most likely number of ‘genetic populations’ present in each hybrid zone ($K = 2$ in all cases; see Results), the individuals were classified as pure types (*P. lychnitis*, *P. crinita* subsp. *malacitana* and *P. crinita* subsp. *crinita*) or hybrids considering a somewhat relaxed criterion of 90 % posterior probability, which allowed the classification within pure types of some slightly introgressed individuals (see Valbuena-Carabaña *et al.*, 2007). Individuals with a probability between 40 and 60 % were considered hybrids, and individuals with a probability between 11 and 39 % or 61 and 89 % were considered introgressants.

Genetic differentiation and relationships among taxa

To address the genetic relationship among the taxa studied, genetic differentiation (F_{ST} ; Weir and Cockerham, 1984) and Nei’s (1972) genetic identities were computed. At the population level, genetic relationships were assessed by estimating pairwise genetic distances (Nei, 1972) and plotted through a UPGMA phenogram and multidimensional scaling (MDS) analyses conducted with Statistica 6.0 (StatSoft, 2001). To depict connections among genetically close populations, a minimum spanning tree (MST) was computed with Passage 1.1 (Rosenberg, 2004) and superimposed on the scatterplot of the samples against the first two MDS dimensions.

RESULTS

Overall, the allozyme analysis reflected limited variability. Eight of the 13 consistently resolved loci were polymorphic (*Pgi*, *Pgm*, *6-Pgd*, *Skdh-1*, *Skdh-2*, *Aat-1*, *Est-1* and *Est-2*), comprising 23 alleles. Three alleles were exclusive, i.e. occurring in some populations of only one taxon; however, all of these were present at low frequencies (<0.03) (Table 2). Additionally, 128 multilocus genotypes were detected; none of them was found to be unique, but 33 of them (25 % of the genotypes) were shared by only two individuals.

Population genetic diversity and structure in *P. lychnitis* and *P. crinita*

At the population and taxonomic level, *P. lychnitis* showed the highest diversity for all calculated parameters (Table 3). The two *P. crinita* sub-species displayed very similar values of diversity, except for heterozygosity. The expected heterozygosity across loci was somewhat higher than the observed heterozygosity in *P. lychnitis* and *P. crinita* subsp. *malacitana*. However, in *P. crinita* subsp. *crinita*, H_e was barely 2-fold higher than H_o (Table 3). Most analysed populations conformed to HW proportions. Four out of 23 populations of *P. lychnitis* showed a

TABLE 2. Allele frequencies, Weir and Cockerham’s (1984) unbiased estimators of inbreeding coefficient (F_{IS}) and population genetic differentiation (F_{ST}), and overall 95 % confidence intervals (95 % CI) for eight polymorphic loci in the studied taxa of *Phlomis*

	<i>P. lychnitis</i>	<i>P. crinita</i> subsp. <i>malacitana</i>	<i>P. crinita</i> subsp. <i>crinita</i>
<i>Pgi</i>			
a	0.000	0.000	0.025
b	0.009	0.972	0.973
c	0.964	0.028	0.000
d	0.027	0.000	0.002
F_{IS}	0.007	0.169	0.197
F_{ST}	0.102	0.019	0.072
<i>Pgm</i>			
a	0.034	0.061	0.005
b	0.249	0.705	0.810
c	0.683	0.178	0.184
d	0.018	0.023	0.002
e	0.017	0.033	0.000
F_{IS}	-0.293	0.050	0.122
F_{ST}	0.096	0.346	0.713
<i>6-Pgd</i>			
a	0.005	0.883	0.280
b	0.969	0.117	0.720
c	0.026	0.000	0.000
F_{IS}	0.015	-0.073	0.121
F_{ST}	0.090	0.254	0.423
<i>Skdh-1</i>			
a	0.018	0.000	0.000
b	0.982	1.000	1.000
F_{IS}	0.050	-	-
F_{ST}	0.026	-	-
<i>Skdh-2</i>			
a	0.969	0.978	1.000
b	0.031	0.022	0.000
F_{IS}	-0.133	0.010	-
F_{ST}	0.094	0.076	-
<i>Aat-1</i>			
a	0.005	0.000	0.038
b	0.995	1.000	0.963
F_{IS}	-	-	-0.137
F_{ST}	-	-	0.095
<i>Est-1</i>			
a	0.989	0.999	0.997
b	0.011	0.001	0.003
F_{IS}	-0.069	-	-
F_{ST}	0.065	-	-
<i>Est-2</i>			
a	0.073	0.022	0.000
b	0.537	0.038	0.825
c	0.390	0.940	0.175
F_{IS}	0.097	-0.140	0.253
F_{ST}	0.135	0.334	0.182
Overall			
F_{IS} (95 % CI)	-0.073 (-0.255 to 0.088)	0.003 (-0.087 to 0.070)	0.144 (0.023 to 0.224)
F_{ST} (95 % CI)	0.108 (0.078 to 0.126)	0.290 (0.152 to 0.336)	0.419 (0.146 to 0.603)

significant departure from HW equilibrium (two with heterozygote excess and deficit, respectively). In *P. crinita* subsp. *malacitana*, one population displayed a deficit and another one an excess of heterozygotes, while in *P. crinita* subsp. *crinita* two populations consistently showed a significant excess of homozygotes (Table 3). Accordingly, multilocus F_{IS} estimates over all populations were not significantly

TABLE 3. Population genetic statistics analysed for the studied populations of *Phlomis*; proportion of polymorphic loci (P_{99}), number of alleles per locus (A), allelic richness ($A[g]$), expected (H_e) and observed (H_o) heterozygosity, and inbreeding coefficient (F_{IS}). Population code as in Table 1

Taxon/population	n^\dagger	P_{99}	A	$A[g]^\ddagger$	H_e	H_o	F_{IS}
<i>P. lychnitis</i>							
LY1	30	38.5	1.692	1.460	0.103	0.113	-0.096
LY3	31	36.4	1.636	1.544	0.140	0.155	-0.113
LY4	30	15.4	1.231	1.197	0.073	0.082	-0.129
LY5	31	18.2	1.182	1.111	0.085	0.111	-0.714*
LY6	33	38.5	1.538	1.303	0.090	0.107	-0.189*
LY7	35	30.8	1.538	1.400	0.108	0.116	-0.075
LY8	35	38.5	1.615	1.459	0.115	0.119	-0.033
LY10	12	27.3	1.455	1.439	0.120	0.121	-0.011
LY12	25	30.8	1.538	1.384	0.092	0.074	0.199*
LY13	30	53.8	1.692	1.490	0.101	0.100	0.012
LY14	30	30.8	1.462	1.263	0.057	0.031	0.464*
LY15	30	30.8	1.538	1.396	0.093	0.105	-0.137
LY16	30	23.1	1.385	1.340	0.076	0.085	-0.109
LY19	30	38.5	1.615	1.276	0.071	0.074	-0.050
LY20	12	23.1	1.308	1.303	0.060	0.071	-0.175
LY21	30	23.1	1.308	1.264	0.055	0.064	-0.167
LY23	30	30.8	1.385	1.357	0.094	0.108	-0.153
LY24	30	38.5	1.462	1.365	0.104	0.118	-0.131
LY27	30	23.1	1.231	1.224	0.070	0.074	-0.069
LY28	29	30.8	1.462	1.356	0.091	0.093	-0.022
LY30	10	15.4	1.308	1.308	0.082	0.085	-0.037
LY32	30	15.4	1.231	1.222	0.081	0.087	-0.081
LY35	17	23.1	1.308	1.308	0.085	0.110	-0.319
Mean (\pm s.d.)	27.4 (7.2)	29.3 (9.5)	1.440 (0.157)	1.338 (0.103)	0.089 (0.021)	0.096 (0.026)	-0.093 (0.202)
<i>P. crinita</i> subsp. <i>malacitana</i>							
MA1	30	38.5	1.462	1.358	0.084	0.085	-0.007
MA2	30	27.3	1.364	1.307	0.079	0.091	-0.155
MA6	33	36.4	1.364	1.319	0.064	0.055	0.146
MA8	35	33.3	1.500	1.384	0.097	0.100	-0.036
MA9	35	38.5	1.538	1.499	0.128	0.145	-0.131*
MA11	30	23.1	1.231	1.143	0.026	0.018	0.305
MA12	30	23.1	1.385	1.234	0.032	0.033	-0.058
MA13	30	15.4	1.308	1.203	0.044	0.046	-0.046
MA14	31	7.70	1.154	1.131	0.042	0.017	0.588*
MA15	31	23.1	1.231	1.125	0.024	0.022	0.085
MA16	32	7.70	1.154	1.107	0.016	0.012	0.233
MA17	30	7.70	1.231	1.077	0.008	0.005	0.326
MA18	32	7.70	1.077	1.077	0.038	0.043	-0.151
MA20	30	38.5	1.385	1.214	0.039	0.033	0.139
MA21	32	23.1	1.231	1.182	0.052	0.055	-0.063
MA22	30	7.70	1.077	1.077	0.039	0.044	-0.128
Mean (\pm s.d.)	28.2 (9.3)	22.4 (12.1)	1.293 (0.145)	1.215 (0.126)	0.051 (0.039)	0.050 (0.055)	0.065 (0.211)
<i>P. crinita</i> subsp. <i>crinita</i>							
CR25	30	7.69	1.154	1.131	0.028	0.028	-0.013
CR26	30	15.4	1.154	1.100	0.021	0.008	0.631*
CR27	30	23.1	1.231	1.119	0.017	0.013	0.253
CR29	30	15.4	1.154	1.150	0.055	0.049	0.120
CR30	26	30.8	1.308	1.259	0.089	0.056	0.336*
CR31	30	23.1	1.231	1.221	0.063	0.059	0.065
CR32	20	15.4	1.154	1.115	0.043	0.038	0.112
CR33	30	38.5	1.385	1.316	0.105	0.092	0.120
CR34	30	38.5	1.385	1.269	0.069	0.069	-0.006
CR35	56	30.8	1.308	1.191	0.044	0.044	0.014
Mean (\pm s.d.)	31.2 (9.3)	23.9 (10.6)	1.246 (0.095)	1.187 (0.076)	0.053 (0.029)	0.046 (0.025)	0.163 (0.199)
Pooled at taxonomic level							
<i>P. lychnitis</i>	630	53.8	2.077 (1.188)	2.064 (1.187)	0.099 (0.185)	0.095 (0.180)	-
<i>P. crinita</i> subsp. <i>malacitana</i>	501	38.5	1.769 (1.166)	1.711 (1.107)	0.070 (0.137)	0.051 (0.091)	-
<i>P. crinita</i> subsp. <i>crinita</i>	312	38.5	1.692 (0.947)	1.672 (0.907)	0.088 (0.146)	0.046 (0.072)	-

Significant ($P < 0.05$) multilocus inbreeding coefficient within populations, assessed through randomization procedures, are marked with an asterisk.

† Sample size within populations.

‡ Expected allelic richness after rarefaction for 20 and 500 gene copies (g) as standard sample size at the population and taxonomic level, respectively.

different from zero in *P. lychnitis* ($F_{IS} = -0.073$) and *P. crinita* subsp. *malacitana* ($F_{IS} = 0.003$), but showed a significant excess of homozygotes ($F_{IS} = 0.144$) in *P. crinita* subsp. *crinita* (Table 2).

All three taxa showed a significant partitioning of the genetic variation among populations (Table 2). *Phlomis lychnitis* displayed a moderate F_{ST} value (0.108), whereas *P. crinita* subsp. *malacitana* and *P. crinita* subsp. *crinita* showed a markedly higher degree of genetic subdivision ($F_{ST} = 0.290$ and $F_{ST} = 0.419$, respectively). Mantel test correlation between geographic and genetic distance was not significant in *P. lychnitis* ($r = 0.126$, $P = 0.103$), with <2 % of the variation in genetic differentiation explained by geographic distance (Fig. 2). In contrast, correlation between genetic and geographic distances was significantly positive in *P. crinita* subsp. *malacitana* ($r = 0.531$, $P < 0.001$) and in *P. crinita* subsp. *crinita* ($r = 0.556$, $P = 0.011$), with geographic distance accounting for a similar proportion of the variance in both subspecies (28 % in *P. crinita* subsp. *malacitana* and 31 % in *P. crinita* subsp. *crinita*; Fig. 2). Correlation analysis of the absolute residuals of the regression showed a significant increase in the scatter of pairwise points with spatial distance ($r = 0.524$, $P = 0.001$ in *P. crinita* subsp. *malacitana* and $r = 0.405$, $P = 0.024$ in *P. crinita* subsp. *crinita*), suggesting the achievement of equilibrium between gene flow and drift among populations in these taxa.

Patterns of hybridization and introgression

The *ad hoc* approach used to infer the most likely number of populations consistently showed that $K = 2$ was the optimal number of clusters in all the hybrid or contact zones studied (see Table 4), which is consistent with the existence of two well-differentiated species. Based on the criterion of 90 % posterior probability of being a parental type, contrasting patterns of hybridization and introgression were evident between the areas in southern and eastern Spain (Fig. 3). In southern Spain, the number of potential hybrids and introgressed individuals between *P. lychnitis* and *P. crinita* subsp. *malacitana* ranged between 32 % (locations 13 and 14) and 79 % (location 8), with an average value of 48 % for the region. In southern Spain, introgression was apparently bidirectional because it occurred at similar rates towards both parental types. It is noteworthy that only 1.3 % (11/832) of the sampled individuals in these nine hybrid zones that were classified as hybrid phenotypes in the field were determined to be pure types by the allozyme analysis. In contrast, hybridization and introgression rates between *P. lychnitis* and *P. crinita* subsp. *crinita* in eastern Spain were extremely low because most individuals were assigned to parental types and only 4 % (7/188) displayed a multilocus genotype that could be considered hybrid or introgressant.

Genetic differentiation and genetic relationship among taxa

Nei's genetic identities over all localities among the three taxa ranged from 0.822 (for the pair *P. crinita* subsp. *malacitana*/*P. lychnitis*) to 0.922 (for the pair *P. crinita* subsp.

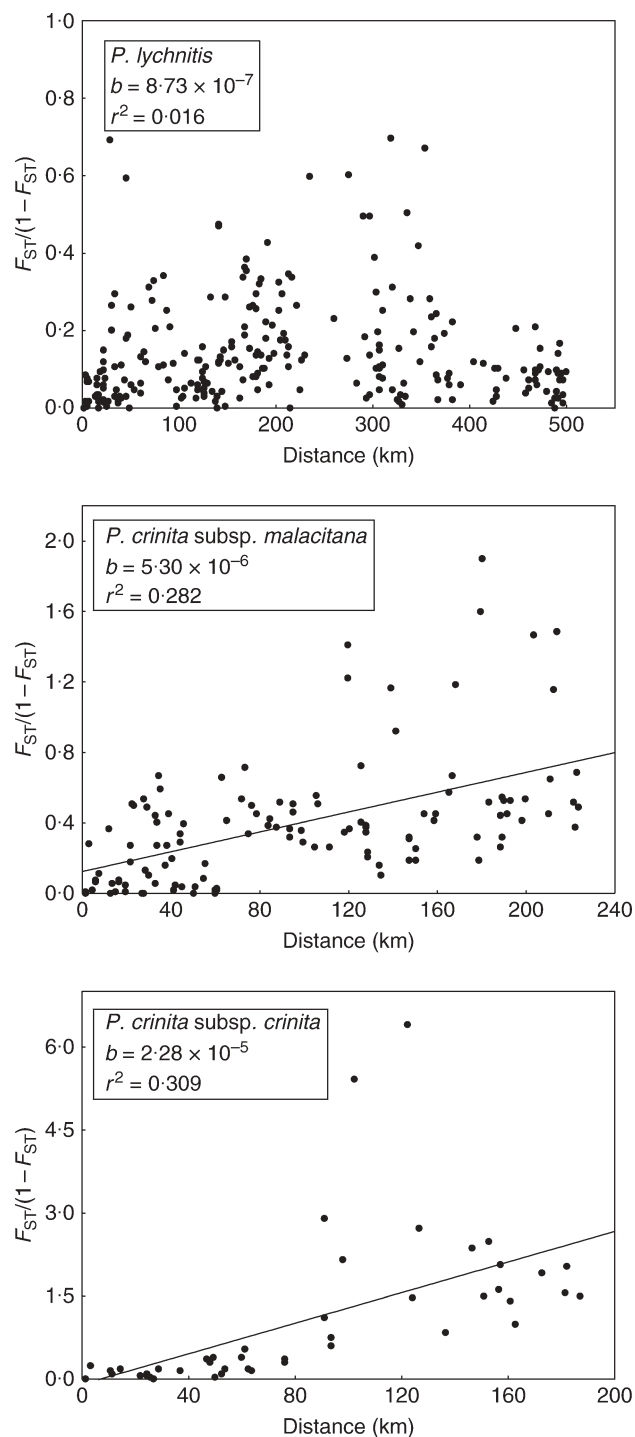


FIG. 2. Scatterplot of Rousset's (1997) genetic distance against geographic distance for the *Phlomis* taxa studied. The slope (b) and the explained variance (r^2) from reduced major axis (RMA) regressions are also shown for each taxon.

crinita/*P. crinita* subsp. *malacitana*). The genetic identity for *P. lychnitis*/*P. crinita* subsp. *crinita* was 0.887. Accordingly, F_{ST} values among taxa were very high: 0.652 for *P. crinita* subsp. *malacitana*/*P. lychnitis*, 0.520 for *P. crinita* subsp. *crinita*/*P. lychnitis* and 0.489 for *P. crinita* subsp. *malacitana*/subsp. *crinita*, indicating that

TABLE 4. Log-likelihood ($\ln K$) (\pm s.d.) computed by the software *Structure* (Pritchard et al. 2000) and ΔK values calculated following Evanno et al. (2005) for K groups (from $K = 1$ to $K = 5$) in 12 *Phlomis* hybrid zones located in southern and eastern Spain

K	$\ln K \pm$ s.d.	ΔK
Southern Spain		
Location 1		
$K = 1$	-733.81 ± 1.19	–
$K = 2$	-586.46 ± 1.09	129.72
$K = 3$	-580.17 ± 5.85	8.63
$K = 4$	-624.38 ± 34.73	1.49
$K = 5$	-649.62 ± 40.40	–
Location 6		
$K = 1$	-572.24 ± 0.65	–
$K = 2$	-477.92 ± 1.46	81.35
$K = 3$	-502.60 ± 21.10	2.04
$K = 4$	-538.10 ± 33.52	1.82
$K = 5$	-562.92 ± 27.00	–
Location 8		
$K = 1$	-638.73 ± 0.98	–
$K = 2$	-597.79 ± 2.67	28.42
$K = 3$	-632.85 ± 13.06	2.39
$K = 4$	-677.59 ± 40.23	2.50
$K = 5$	-729.79 ± 164.52	–
Location 12		
$K = 1$	-539.46 ± 0.55	–
$K = 2$	-412.75 ± 3.30	29.05
$K = 3$	-381.99 ± 2.58	17.45
$K = 4$	-396.20 ± 8.15	4.45
$K = 5$	-443.35 ± 15.21	–
Location 13		
$K = 1$	-628.72 ± 0.61	–
$K = 2$	-444.18 ± 0.34	480.05
$K = 3$	-424.91 ± 2.09	22.03
$K = 4$	-451.63 ± 22.27	1.44
$K = 5$	-470.41 ± 18.00	–
Location 14		
$K = 1$	-571.51 ± 0.70	–
$K = 2$	-326.95 ± 1.14	203.67
$K = 3$	-314.46 ± 1.18	6.59
$K = 4$	-306.98 ± 14.87	1.45
$K = 5$	-308.22 ± 11.68	–
Location 15		
$K = 1$	-561.80 ± 0.61	–
$K = 2$	-394.21 ± 1.18	107.79
$K = 3$	-353.43 ± 1.82	27.51
$K = 4$	-362.82 ± 5.74	6.14
$K = 5$	-406.08 ± 22.16	–
Location 16		
$K = 1$	-562.51 ± 0.41	–
$K = 2$	-334.41 ± 0.26	787.66
$K = 3$	-310.60 ± 2.83	19.23
$K = 4$	-341.16 ± 8.01	1.85
$K = 5$	-362.80 ± 11.00	–
Location 21		
$K = 1$	-512.19 ± 0.43	–
$K = 2$	-400.58 ± 0.89	86.10
$K = 3$	-365.60 ± 1.14	58.46
$K = 4$	-397.55 ± 11.94	2.44
$K = 5$	-406.46 ± 10.91	–
Eastern Spain		
Location 27		
$K = 1$	-315.47 ± 0.60	–
$K = 2$	-125.99 ± 0.12	1642.86
$K = 3$	-127.96 ± 5.49	2.50
$K = 4$	-133.16 ± 13.61	2.09
$K = 5$	-142.71 ± 11.07	–

Continued

TABLE 4. Continued

K	$\ln K \pm$ s.d.	ΔK
Location 32		
$K = 1$	-221.13 ± 0.44	–
$K = 2$	-151.71 ± 0.56	130.55
$K = 3$	-167.76 ± 4.00	2.40
$K = 4$	-170.15 ± 5.15	5.86
$K = 5$	-189.39 ± 82.24	–
Location 35		
$K = 1$	-399.13 ± 0.99	–
$K = 2$	-275.40 ± 0.80	141.30
$K = 3$	-264.72 ± 0.76	28.54
$K = 4$	-275.73 ± 1.67	5.94
$K = 5$	-293.78 ± 8.25	–

despite the low occurrence of exclusive alleles there were marked differences in allelic frequencies among the studied taxa.

The UPGMA phenogram grouped all populations of *P. lychnitis* together regardless of their geographic origin (Fig. 4). For instance, the population LY12 from southern Spain clustered together with populations LY28 and LY30 from eastern Spain, located >400 km away. In contrast, clustering of *P. crinita* subsp. *malacitana* populations was consistent with the mountain ranges of origin of the samples (population MA18 was the only exception). Populations of *P. crinita* subsp. *crinita* also grouped following a geographic pattern; however, they did not cluster together: populations CR25 and CR26 (inland populations in Fig. 1) clustered close to *P. lychnitis*, whereas populations CR27–CR35 (coastal populations) clustered close to *P. crinita* subsp. *malacitana*.

All the populations studied were consistently separated along the first two dimensions of the MDS scatterplot (Fig. 5). The first dimension clearly separated *P. lychnitis* and *P. crinita* subsp. *malacitana* populations, whereas the second dimension enhanced the separation of *P. crinita* subsp. *crinita* populations. The MST showed the role of *P. crinita* subsp. *crinita* connecting to *P. lychnitis* and *P. crinita* subsp. *malacitana* through coastal and inland populations, respectively.

DISCUSSION

Population genetic diversity and structure in P. lychnitis and P. crinita

Phlomis lychnitis populations consistently showed the highest levels of genetic diversity among the studied taxa. Over all loci, no significant inbreeding was apparent in this taxon, and most populations (83 %) did not depart from HW proportions, revealing the prevalence of outcrossed matings. This taxon had a moderate level of genetic differentiation among populations, and there was no evidence of isolation by distance, and, therefore, a lack of equilibrium between gene flow and drift. Accordingly, populations clustered together in the UPGMA irrespective of their geographic origin. This lack of equilibrium, together with the low degree of variance

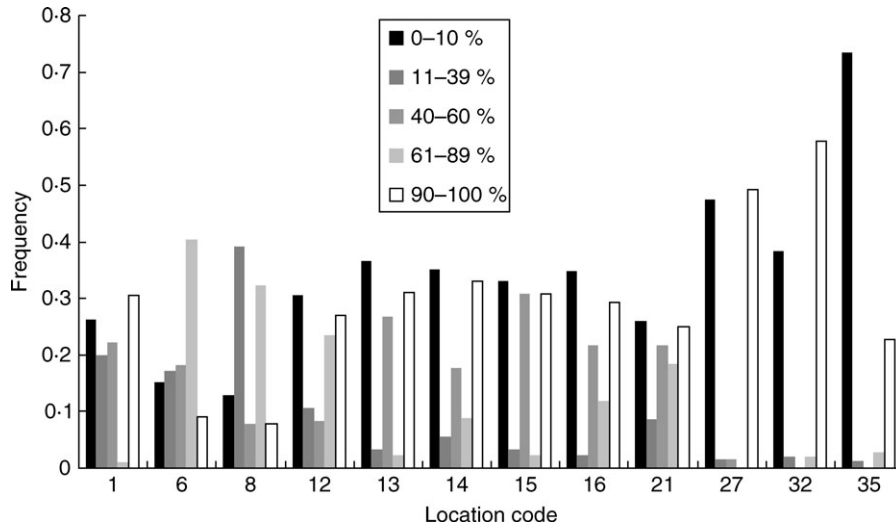


FIG. 3. Classes of posterior probabilities for belonging to the parental species *P. lychnitis*, derived from the software Structure (Pritchard *et al.* 2000), in 12 hybrid or contact zones across the *Phlomis* complex. The x-axis shows the identification code for each location (see Table 1 and Fig. 1). Zones between *P. crinita* subsp. *malacitana* and *P. lychnitis* in southern Spain are locations 1–21, and hybrid zones between *P. crinita* subsp. *crinita* and *P. lychnitis* in eastern Spain are locations 27–35.

of the pairwise points seen in the scatterplot (see Fig. 2), suggests high levels of historical gene flow among populations or a rapid range of expansion from a genetically homogenous ancestral source (Templeton *et al.*, 1995; Hutchison and Templeton, 1999). However, previous

results of cpDNA haplotype variation in the complex (including *P. lychnitis*) (Albaladejo *et al.*, 2005) have shown that a clear geographic trend exists (southern Spain vs. eastern Spain), which stresses that nuclear allozymes and plastid markers convey different historical information

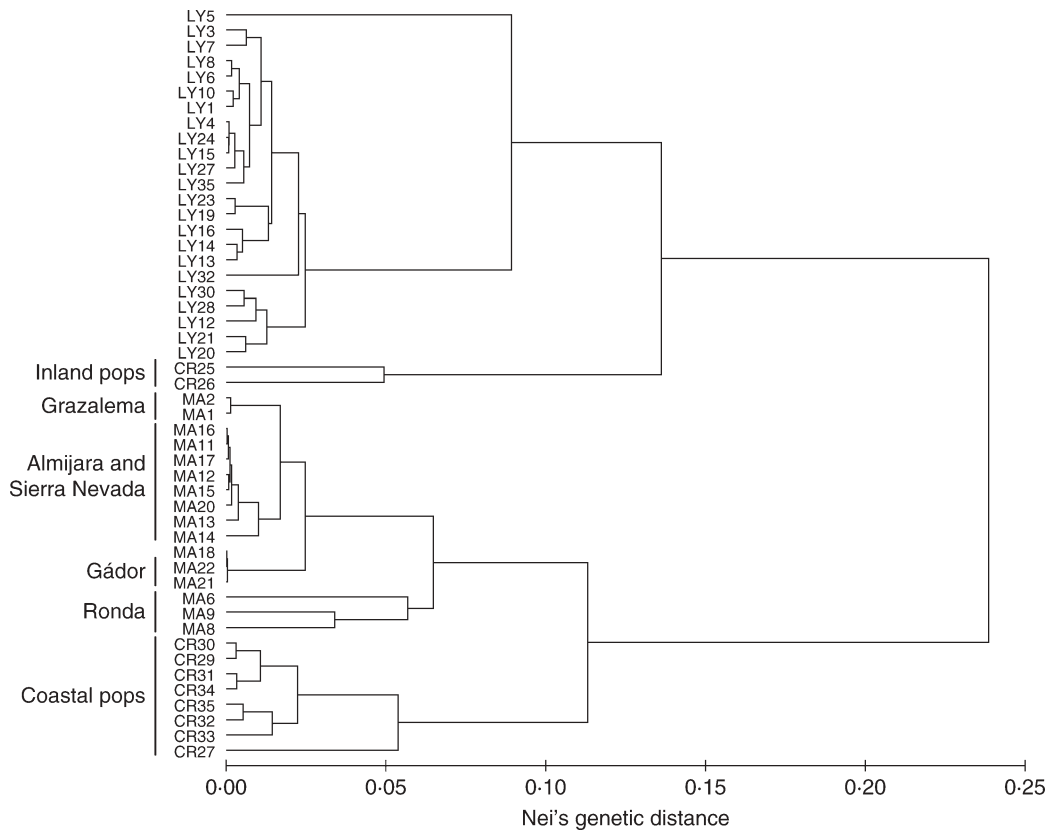


FIG. 4. UPGMA phenogram, based on Nei's genetic distance, for the populations of *Phlomis* studied. Population code is as in Table 1. The main clusters of *P. crinita* are mapped in Fig. 1.

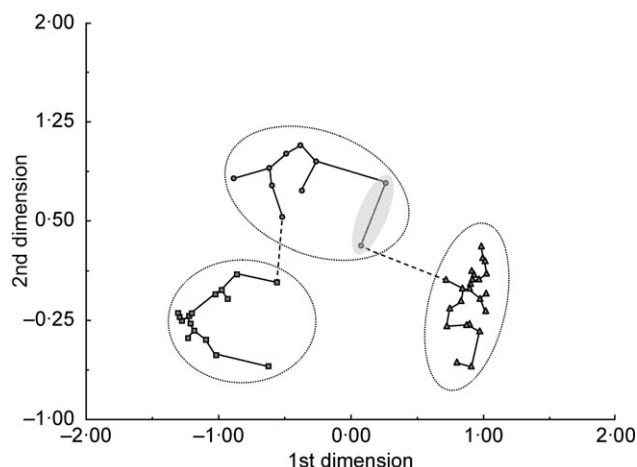


FIG. 5. Minimum spanning tree (MST) superimposed over the scatterplot of the first two dimensions extracted from multidimensional scaling (MDS) analysis. Symbols represent *Phlomis crinita* subsp. *malacitana* (squares), *P. crinita* subsp. *crinita* (circles) and *P. lychnitis* (triangles). Dashed lines connect different taxa and ellipses encompass populations of the same taxon. The shaded area in *P. crinita* subsp. *crinita* highlights the inland populations (see text).

in this species and argues against the existence of recurrent gene flow, at least via seeds, between regions. It is suggested that sorting of plastid lineages coupled with high levels of gene flow among populations within refuges and range expansion are not mutually exclusive explanations leading to the contrasting pattern of genetic vs. geographic distribution of nuclear and cpDNA variation observed in *P. lychnitis*. This taxon is not restricted to mountain environments and it seems likely that during the interglacial phases of the Quaternary Period the species expanded its distribution range connecting populations within refuges and colonizing new areas (e.g. Comes and Abbott, 1998). However, localized instances of introgression after secondary contact with *P. crinita* should also be taken into account to explain the sharing of haplotypes among taxa at some locations (see Albaladejo *et al.*, 2005).

The two subspecies of *P. crinita* showed similarly low levels of genetic diversity; however, slightly higher allelic richness was detected and heterozygosity was observed in *P. crinita* subsp. *malacitana* compared with *P. crinita* subsp. *crinita*. Higher rates of introgression in southern Spain compared with eastern Spain (see below) might allow the transfer of low-frequency allozyme alleles from *P. lychnitis* to the nuclear genome of *P. crinita* subsp. *malacitana* (e.g. *Pgi^c Est-2^b*), which may result in increased allelic diversity and heterozygosity (Rieseberg and Wendel, 1993).

As for *P. lychnitis*, F_{IS} values in *P. crinita* subsp. *malacitana* were consistent with a predominantly outcrossing mating system but, contrary to *P. lychnitis*, a significant strong genetic population structure ($F_{ST} = 0.290$) was evident in this taxon. Unlike these two taxa, *P. crinita* subsp. *crinita* showed a significant excess of homozygotes which, in the absence of selection or assortative mating and given the predominantly xenogamous mating system (R.G.A. and A.A., unpublished results), can be a consequence of strong local

genetic structure within some populations or natural variation in breeding systems. In fact, two out of the ten populations studied showed a consistent heterozygote deficit, which may greatly influence the overall pattern detected. Similar to *P. crinita* subsp. *malacitana*, high heterogeneity in allele frequencies among populations of *P. crinita* subsp. *crinita* was reflected by the markedly strong value of genetic differentiation ($F_{ST} = 0.419$), which was mainly because of strong genetic differences between the inland (CR25 and CR26) and the coastal set of populations (CR27–CR35).

The increase in pairwise $F_{ST}/(1 - F_{ST})$ values with geographic distance in both subspecies of *P. crinita* matched with an isolation-by-distance model. Furthermore, the spread of residuals also increased with geographic distance, indicating an equilibrium situation where, historically, nearby populations were connected by gene flow whereas genetic drift was more influential as spatial isolation increased (Hutchison and Templeton, 1999). Since the time (generations) to achieve this equilibrium is very long (sometimes even longer than the lifetime of a species; Whitlock and McCauley, 1999), it seems that populations of both sub-species of *P. crinita*, in contrast to *P. lychnitis*, might have remained relatively undisturbed during the last glacial periods, probably moving their distribution ranges across an altitudinal gradient, similarly to other Iberian endemic taxa (e.g. Gutiérrez-Larena *et al.*, 2002). Restriction to mountain habitats and the long-standing isolation, which should facilitate the divergence process within the complex, might explain why subspecies of *P. crinita* did not share cpDNA haplotypes (Albaladejo *et al.*, 2005).

Patterns of hybridization and introgression

Results of Bayesian clustering analysis based on multilocus allozyme genotypes underpin the existence of differential patterns of hybridization and introgression in the areas concerned in this study, southern Spain vs. eastern Spain, involving different taxa. Even considering a conservative criterion for detecting hybridization, high rates of hybridization and bidirectional introgression have been detected between *P. crinita* subsp. *malacitana* and *P. lychnitis* in southern Spain (average frequency = 48%), in accordance with the high morphological variability within populations (Albaladejo *et al.*, 2004) and the free flowing of chromosome rearrangements between taxa (Aparicio and Albaladejo, 2003). However, this average estimate of hybridization in populations in southern Spain is far higher than previously estimated based on morphometry alone (21.8%; Albaladejo *et al.*, 2004), which is probably because of the high occurrence (134/832) of slightly introgressed individuals having morphology that is indistinguishable from that of pure types. It is noted that multiallelic co-dominant DNA markers such as microsatellites would allow a more accurate classification of genotypic classes in hybrid or contact zones of *Phlomis* species.

In contrast, only a few hybrid genotypes (average frequency = 4%) were detected between *P. crinita* subsp. *crinita* and *P. lychnitis*, which agrees with previous estimates of hybridization based on morphology (2.1%;

Albaladejo *et al.*, 2004). Hybrid zones in southern Spain, with a predominance of hybrid genotypes in most populations, fitted to a unimodal structure (Harrison, 1993), whereas a bimodal structure was evident in populations from eastern Spain where most genotypes corresponded to pure types (Harrison and Bogdanowicz, 1997). Given the current distribution range of the studied *Phlomis* taxa, the scarcity of hybrid plants in eastern Spain reflects the existence of stronger barriers against hybridization between *P. crinita* subsp. *crinita* and *P. lychnitis*, rather than a lack of historical opportunities to hybridize. In a review dealing with the evolutionary consequences of hybridization, Jiggins and Mallet (2000) stated that bimodal hybrid zones, unlike unimodal hybrid zones, are invariably linked to strong assortative mating [e.g. flowering asynchrony (Cruzan and Arnold, 1993) and ethological isolation (Fulton and Hodges, 1999)] or assortative fertilization [e.g. interspecific pollen competition (Rieseberg *et al.*, 1998)], which avoids the formation of hybrid offspring. However, it does not exclude the existence of post-zygotic barriers because selection, endogenous (Barton and Hewitt, 1985) or exogenous (Arnold, 1997), against hybrids in the early stages of development might help discount hybrid plants from the populations, resulting in the overwhelming predominance of parental genotypes.

Genetic differentiation and relationships among taxa

Systematic and evolutionary relationships within the *P. lychnitis*/*P. crinita* complex are controversial because analyses of nuclear ribosomal DNA (nrDNA) and cpDNA sequences (Albaladejo *et al.*, 2005) suggest the existence of two sister lineages where lineage sorting and hybridization are central as evolutionary mechanisms. The MST superimposed over the MDS scatterplot and the UPGMA phenogram underlined the role of *P. crinita* subsp. *crinita* as a genetic link between *P. lychnitis* and *P. crinita* subsp. *malacitana*. Interestingly, the inland populations of *P. crinita* subsp. *crinita* (shaded populations in Fig. 5) were genetically closer to *P. lychnitis*, whereas the coastal set of *P. crinita* subsp. *crinita* populations were closer to *P. crinita* subsp. *malacitana*. These results were paralleled by nuclear ITS sequence variation where samples from the inland population CR25 (Elche de la Sierra) shared more parsimony-informative sites with the pure sequence of *P. lychnitis* (Albaladejo *et al.*, 2005).

This intermediate situation can be explained by the pattern of allelic additivity displayed by *P. crinita* subsp. *crinita* (see Table 2). As previously suggested for the patterns of nuclear and plastid DNA variation (Albaladejo *et al.*, 2005), sorting and gene transfer through introgression are likely to be responsible for the sharing of allozyme alleles among taxa during their evolutionary history.

Alternatively, an evolutionary scenario of homoploid hybrid speciation (Gross and Rieseberg, 2005) involving *P. crinita* subsp. *malacitana* and *P. lychnitis* could also be taken into account. It has been shown that these two taxa can hybridize easily, and species with a well-documented diploid hybrid origin (Gallez and Gottlieb, 1982; Wang *et al.*, 1990) are known to exhibit similar patterns of

allelic additivity. However, our data do not allow us to test whether or not homoploid hybrid speciation has occurred in this group of taxa.

Concluding remarks

The taxa involved in this study showed contrasting population genetic diversity, population structure and hybridization patterns despite their phylogenetic relatedness. It has been claimed that the climate changes that took place during the Quaternary Period in southern Europe have been of key importance in the evolution of these Mediterranean endemic *Phlomis* lineages, providing new information about how these changes shaped the genetic structure of plants within glacial refuges. With the present data, it could be envisaged that *P. lychnitis* was the ancestral lineage in the group; however, whether *P. crinita* subsp. *crinita* is a divergent taxon or the result of a homoploid hybrid speciation event is not possible to discern. Consequently, whether *P. crinita* subsp. *crinita* has quickly developed strong interspecific prezygotic barriers or whether *P. crinita* subsp. *malacitana* has suffered the breakdown of mating barriers through long-term contact with *P. lychnitis* remains unanswered and will require further research.

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