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Conservation genetics of *Dichoropetalum schottii* (Apiaceae): is the legal protection of edge populations consistent with the genetic data?

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Dichoropetalum schottii is a species that lives at low to medium altitudes in the southern European mountain ranges, from the Balkans (N Greece) to the Pyrenees. Its legal protection status is not homogeneous along its distribution range, as only some of its edge populations, in the Pyrenees, are protected. Here, by means of allozyme electrophoresis, we examine the genetic variability of populations representing four different regions within its distribution area (Pyrenees, Maritime Alps, Karst Plateau in the SW Slovenia–NE Italy border region, and Pindus Mountains in Greece). The species as a whole exhibits relatively high levels of genetic diversity, partly due to the occurrence of several duplications among the loci surveyed. Genetic differentiation among populations and regions was low, which could be interpreted as the result of recent allopatric fragmentation. We conclude that the species as a whole is not threatened, and that the currently protected populations are, paradoxically, the least valuable from the genetic point of view.

Introduction

Recurrent discussion in conservation biology confronts local and global points of view on species protection and management (Gärdenfors 2001, Schmeller *et al*. 2008), an unavoidable consequence of the disagreement between the biogeography of the species and the distribution of management responsibilities, defined by political boundaries. Much effort has been made to develop multinational or multiregional management policies for the conservation of animal biodiversity (e.g., Delibes *et al*. 2000, Swenson *et al*. 2000, Donald *et al*. 2007). In contrast, boundary-based conservation policies for plants are often applied without scientific consideration of the value of these measures in a global context (Leppig & White 2006, Schmeller *et al*. 2008) and thus some species may be unnecessarily protected. For example, a species might be included in the protection list of a given territory because it is rare there, but that rarity may

be due to the fact that the protected populations are at the edge of the species' geographic range (Bunnell *et al*. 2004). Conservation of peripheral populations may be important, however, depending on the intraspecific variability, for example if they retain a substantial fraction of the genetic variability of the species (Millar & Libby 1991), if they represent an independent evolutionary or speciation lineage (Lesica & Allendorf 1995), or if they are ecologically significant (Millar & Libby 1991). Although ideally the maximum number of populations will be conserved, frequently the investment of resources needed for conservation of peripheral populations is not justified by their biological value. Relevant Genetic Units for Conservation (RGUC) (or similar measures) for a species is a science-based tool that can provide useful guidelines for management (Moritz 1994, Riddle & Hafner 1999, Caujapé-Castells & Pedrola-Monfort 2004, Pérez-Collazos *et al*. 2008).

The levels of genetic diversity in a plant species and its distribution within and among populations are the result of complex interactions between evolutionary factors (e.g., occurrence of bottlenecks and founder effects, range expansion or fragmentation, mode and timing of speciation) and life-history traits (e.g., ecological and demographic characteristics, mating system) (Gray 1996, Booy *et al*. 2000, Coates & Byrne 2005). For the European plant species, recent geological and climatic vicissitudes are generally considered to be the main factor in shaping not only the current population genetic structure but also their distributional patterns, due to the occurrence of major geomorphological events (Messinian salinity crisis, Cenozoic Mediterranean microplate movements, Alpine orogeny) but especially the extreme climatic oscillations that occurred during the Pleistocene, which produced dramatic vegetation changes in Europe (Hewitt 1999, Thompson 2005, Weiss & Ferrand 2007, Hu *et al*. 2009). Human activities, however, should also be regarded as an important element influencing the genetic structure of plant species on the European continent, as natural habitats have been greatly altered and fragmented (Silva *et al*. 2008). Landscape transformation has been especially severe in the southern region, the Mediterranean Basin, as humans have been present there for several thousands of years, establishing permanent settlements and developing intensive agricultural and livestock activities (Por 2003, Blondel *et al*. 2010).

Understanding the biogeographical processes underlying the present distribution of a taxon as well as the genetic diversity and relationships among populations and regions is extremely useful for evaluating the convenience of conserving the species in particular areas (Newton *et al*. 1999, Hu *et al*. 2009, Médail & Diadema 2009). An in-depth knowledge of the population genetic structure of a plant species allows us to identify those populations, or groups of populations, deserving conservation by virtue of their genetic wealth (high levels of allelic richness or heterozygosity) and/or distinctiveness (occurrence of exclusive alleles) (Petit *et al*. 1998, Caujapé-Castells & Pedrola-Monfort 2004, Li *et al*. 2005, Ávila-Díaz & Oyama 2007). One of the main goals of conservation genetics is the prioritization of populations for *in situ* conservation as well as the design of seed collection strategies, in order to preserve the maximum genetic variability at the lowest cost in economic, personal and infrastructural resources (Brown & Briggs 1991, Millar & Libby 1991, Ceska *et al*. 1997, Pérez-Collazos *et al*. 2008).

Dichoropetalum is a genus within the Apiaceae, composed of ca. 26 species. It is found mainly in Europe and western Asia, with a single species in North Africa, *D. munbyi* (Pimenov *et al*. 2007). The number of species decreases westwards: whereas there are up to 8 in the Caucasian–Iranian area, 11 (five of them endemic) in Anatolia and 8 in the Balkans, only 2 are found on the western side of the European continent (*D. carvifolia* and *D. schottii*; Pimenov *et al*. 2007). Of these two, *D. schottii* is found at low to moderate altitudes along the major mountain chains from Greece to the Iberian Peninsula (the Pindus, the Albanian ranges, the Dinarides, the Alps, the Apennines, and the Pyrenees). It is legally protected in Catalonia (Spain), from where a single population is known, in the Midi-Pyrenées and Languedoc-Roussillon (France). However, it is not protected or considered threatened in any other part of its range.

In the present study, allozyme electrophoresis was used to (i) study the genetic diversity of *D.*

schottii both at the population and species level; (ii) analyze the partitioning of genetic diversity within and between different geographic regions of its distribution range; and (iii) establish the conservation priorities for this species according to genetic data.

Material and methods

The plant studied

Dichoropetalum schottii is an hemicryptophyte of 30 to 120 cm in height, which develops rosettes of basal leaves and numerous floral stems. It is a diploid with 2n = 22 (Favarger 1959, Molero & Montserrat-Martí 1986). It lives mainly in rocky and mesic meadows, in forest clearings and roadsides between 100 and 1900 m a.s.l. (Strid & Tan 1986, Bolòs & Vigo 1990, Guillén & Laínz 2003; J. López-Pujol pers. obs.), and is widely distributed in the mountains of southern Europe (Strid & Tan 1986, Guillén & Laínz 2003, Pimenov *et al*. 2007). According to herbarium records and literature citations, it appears to be locally abundant but not widely spread in its westernmost range (the French Pyrenees and the Maritime Alps, where it forms large populations with no particular conservation concerns), in the center of its range (eastern Alps and Karst Plateau, which represents the northern tip of the Dinarides) and again in the Pindus in Greece, but seems to be less common in the Apennines and the central and southern Dinarides, from where abundant and/or precise occurrence data are generally lacking.

Dichoropetalum schottii is considered threatened and it is consequently protected in southern France (Anon. 1998, 2005) and in Spain (Molero *et al*. 2003, Anon. 2008). It is described as rare but not threatened in the current evaluation for the Greek Red Book (G. Kamari pers. comm.), and it is listed as LC (Least Concern) in Lombardy (Conti *et al*. 1997).

This species was originally described as *Peucedanum schottii*. However, recent studies involving molecular phylogeny (Spalik *et al*. 2004, Valiejo-Roman *et al*. 2006), as well as previous phytochemical studies (Hadaček *et al*. 1989, Reduron *et al*. 1997), divided the genus *Peucedanum* and assigned *P. schottii* to the genus *Holandrea*. More recently, Pimenov *et al*. (2007) gave nomenclatural priority to *Dichoropetalum* over *Holandrea*. All previously published direct and indirect evidence indicates that the *Dichoropetalum* species and clades studied up to date are monophyletic (Spalik *et al*. 2004, Doğan *et al*. 2010, Downie *et al*. 2010 and references therein).

Sampling

For the sampling, we prioritized the known peripheral populations of *D. schottii*, although samples from some populations within its core distribution area were also collected. Samples of the Pyrenean populations (MON, FDS, NDS, and SCG) were collected in June–July 2008 and those from the Maritime Alps (BRO and TEN), the Karst Plateau in the SW Slovenia–NE Italy border region (ZDM and DDP), and the Pindus Mountains in Greece (DIS) in June–July 2009 (Fig. 1 and Table 1). Fresh leaves of 25–36 individuals were collected from each population. In large populations, such as FDS, NDS, SCG, BRO and TEN, samples were collected at intervals along a linear transect. In smaller populations (MON, ZDM, DDP, and DIS), individuals were chosen at random from the entire population, making sure there was enough space (at least 1 m) among them to avoid collecting ramets from the same genet. All samples were placed in envelopes, transported to the laboratory and stored at 4 °C until extraction one day later.

Electrophoresis

Genetic diversity was assessed using standard methods for the starch gel electrophoresis of allozymes (Soltis & Soltis 1989). Leaf fragments were homogenized using an extraction buffer (0.05 M tris–citric acid, 0.1% cisteine–HCl, 0.1% ascorbic acid, 8% PVP-40 and 1 mM 2-mercaptoethanol). Extracts were absorbed onto 3 MM Whatman filter paper and analyzed immediately or stored at –80 °C until analysed. A total of 14 different enzymes were essayed on 11% starch gels, obtaining 8 interpretable loci (*Aco-1*,

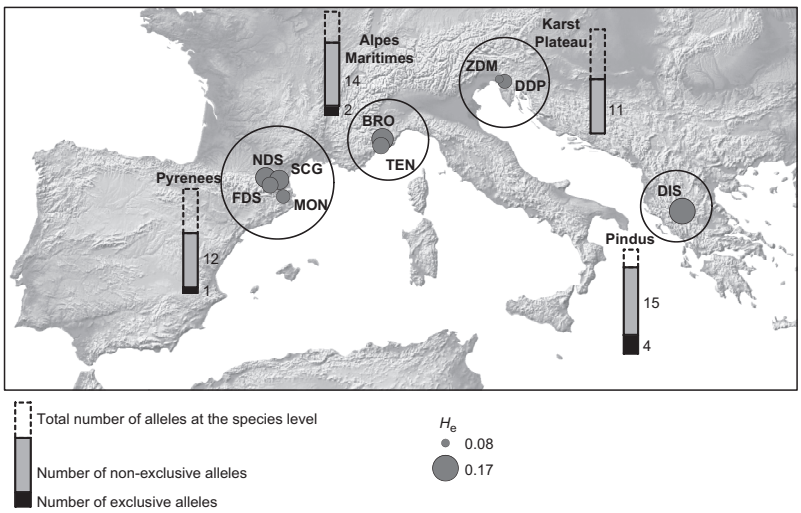


Fig. 1. Location of the studied populations of *Dichoropetalum schottii* in four geographical regions of the Mediterranean Basin, and results from their genetic diversity analyses. Sizes of the circles for each population are proportional to the expected panmictic heterozygosity (H_e). Bars indicate the total number of alleles and the number of exclusive alleles in each region in relation to the total number of alleles found for the species.

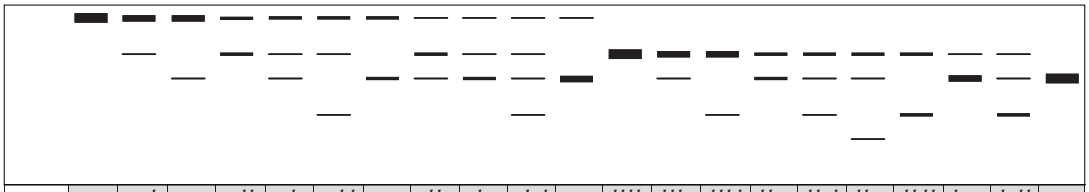
Aco-2, *Dia-1*, *Idh-1*, *Mdh-2*, *6Pgd-1*, *6Pgd-2* and *Pgm-2*). Diaphorase (DIA, EC 1.6.99.–) was resolved with a tris–citrate/lithium–borate buffer pH 8.2; isocitrate dehydrogenase (IDH, EC 1.1.1.42), phosphogluconate dehydrogenase (6PGD, EC 1.1.1.44), and malate dehydrogenase (MDH, EC 1.1.1.37) were resolved with a morpholine-citrate buffer pH 6.1; finally, phosphoglucomutase (PGM, EC 5.4.2.2) and aconitase (ACO, EC 4.2.1.3) were resolved with a histidine-citrate buffer pH 5.7. Staining procedures for all enzymes followed the method described by Wendel and Weeden (1989), with minor modifications.

Genetic analyses

Loci were numbered consecutively and alleles at each locus were labelled alphabetically beginning from the most anodal form. Banding patterns were interpreted according to standard

Table 1. Studied populations of *Dichoropetalum schottii*.

Population code	Location	Geographic coordinates	Altitude (m a.s.l.)	Sample size
MON	Santuari de la Mare de Déu del Mont (Girona Prov., Catalonia, Spain)	42°15′35.8′′N, 2°42′28.7′′E	1100	36
FDS	Fontanès-de-Sault (Aude Dept., France)	42°45′45.6′′N, 2°5′13.6′′E	900	30
NDS	Niort-de-Sault (Aude Dept., France)	42°48′6.9′′N, 1°59′45.1′′E	900	30
SCG	Sainte-Colombe-sur-Guette (Aude Dept., France)	42°45′26.2′′N, 2°13′51.1′′E	600	30
BRO	Col de Brouis (Alpes-Maritimes Dept., France)	43°59′43.4′′N, 7°23′41.7′′E	1400	32
TEN	Col de Tende (Alpes-Maritimes Dept., France)	44°8′17.9′′N, 7°34′0.5′′E	1300	30
ZDM	Zolla di Monrupino (Trieste Prov., Italy)	45°43′10.9′′N, 13°48′30.2′′E	400	35
DDP	Dolina di Perčadol (Trieste Prov., Italy)	45°44′24.1′′N, 13°43′19.1′′E	300	25
DIS	Distrato (Ioannina Prefecture, Greece)	40°2′54.4′′N, 21°0′31.2′′E	1000	36



	aaaa	*aaab*	*aaac*	*aabb*	*aabc*	*aabd*	*aacc*	*abbc*	*abcc*	*abcd*	*accc*	*bbbb*	*bbbc*	*bbbd*	*bbcc*	*bbcd*	*bbce*	*bbdd*	*bccc*	*bcdd*	*cccc*
MON[1]												2	11		6				14		3
FDS[1]													20		5				4		
NDS[1]													9		20						
SCG[1]													5		22						
BRO[2]		3	3	2			6					1	1		8				2		5
TEN[2]			2	2	4		8	1	1			1	2		8						1
ZDM[3]	2		1		2		11				2	2	2		6				5		2
DDP[3]		1			5		5		1			2	4		4				2		1
DIS[4]				1		1				1		4	8	2	6	3	1	3	1	1	
Total	**2**	**4**	**6**	**5**	**11**	**1**	**30**	**1**	**2**	**1**	**2**	**12**	**62**	**2**	**85**	**3**	**1**	**3**	**28**	**1**	**12**

Fig. 2. Banding patterns obtained for the locus *Pgm-2* in different populations of *Dichoropetalum schottii*. Above, the scheme indicates the observed phenotypes (the width of the band is proportional to its staining intensity). Below, the inferred genotypes and the number of individuals with a given genotype in each of the populations. Superscripts indicate the region: [1] Pyrenees, [2] Maritime Alps, [3] Karst Plateau and [4] Pindus.

principles (Gottlieb 1982, Soltis & Soltis 1989). Most loci appeared to be duplicated (*Aco-1*, *Aco-2*, *Idh-1*, *Mdh-2*, *6Pgd-2* and *Pgm-2*; Fig. 2), as inferred by the appearance of unbalanced heterozygous patterns (i.e. allele dosage effects; Tyler 1996, 2004). No evidence of duplication was found for the two remaining loci, although *Dia-1* was entirely monomorphic, and only three individuals (from a total of 284) had heterozygous *6Pgd-1* (balanced) phenotypes. It should be noted that these two loci could also be duplicated, as the banding patterns of individuals with the genotype AA (i.e. non-duplicated) are indistinguishable from those of individuals AAAA (duplicated). In the same way, the three heterozygous individuals at the *6Pgd-1* locus would be either AB or AABB.

To allow the statistical analyses, the *Dia-1* and the *6Pgd-1* loci were regarded as duplicated, and thus the species was treated as a tetraploid. The assumed error rate introduced in the analysis in case they are actually not duplicated is negligible, since *Dia-1* was monomorphic and only three individuals were heterozygous for the *6Pgd-1*. For the remaining duplicated loci, allele dosages observed on electrophoretic gels (that is, the different intensity of bands) allowed us to determine the allele copy number in heterozygote individuals, which was mandatory to further calculate the allele frequencies. The following statistics were computed: the number of multilocus genotypes (MLG); the percentages of polymorphic loci when the frequencies of the most common allele were < 0.95 and of < 0.99 (P_{95} and P_{99}, respectively); the mean number of alleles per locus (A); and the expected panmictic heterozygosity (H_e). Genetic relationships between the populations (and also between regions) were explored using Nei's genetic identity, I (Nei 1978), and an UPGMA tree was constructed on the basis of pairwise values of Nei *et al*.'s (1983) genetic distance. For the UPGMA, branch support was obtained by 1000 bootstrapping over loci. A principal coordinate analysis (PCoA) was performed using the Cavalli-Sforza chord distance (Cavalli-Sforza & Edwards 1967) among populations. All genetic parameters were calculated using the softwares SPAGeDi (Hardy & Vekemans 2002) and GENESTAT ver. 3.31 (Whitkus 1988), whereas the dendrogram was obtained with Populations ver. 1.2.30 (http://bioinformatics.org/~tryphon/populations/) and plotted with TreeView ver. 1.6 (Page 1996). PCoA was computed with Ginkgo ver. 1.7.0 (De Cáceres *et al*. 2007).

The geographical structure of genetic variation was estimated using an analysis of molecular variance (AMOVA) in Arlequin 2.0 (Schneider *et al*. 2000). Three different approaches were used: (i) no regional grouping, (ii) four geographically distinct regions (Pyrenees, Maritime Alps, Karst Plateau and Pindus), and (iii) three geographically distinct regions (grouping together the Maritime Alps and Karst Plateau, the central regions of the studied area).

Results

A total of 23 alleles in eight loci were scored for *D. schottii*. Interestingly, the richest population was that from the eastern extreme of its distribution range, the Pindus Mountains (DIS), which harbored almost all the alleles (19) detected at the species level. The poorest populations were DDP (ten alleles), located in the Karst Plateau, and MON (11 alleles), from the Spanish side of the Pyrenees (Table 2). Rare alleles (those present at frequencies below 0.05) occurred in almost all the populations with the exception of TEN and DDP. Only two populations had exclusive alleles, TEN (one allele) and DIS (four alleles). It should be noted, however, that all the exclusive alleles of the Greek population were present at low frequencies, and two of them were extremely rare (*Mdh-2c* and *Pgm-2e*, which occurred in just one individual; Table 2). At the regional level (Fig. 1), the Pyrenean populations had a total of 13 alleles (and one allele exclusive for the region), the Maritime Alps 16 alleles (two exclusive ones), the Karst Plateau 11 alleles (with no exclusive alleles), and the Pindus Mountains 19 alleles (four exclusive). The most common allele at each locus was the same for all the populations with the exception of *Pgm-2*, which was by far the most variable locus (with up to five alleles; Fig. 2). Only one locus was monomorphic across all the studied populations (*Dia-1*). MLG varied greatly among populations (from eight in SCG to 23 in DIS) and also among regions (from 14 in the Karst Plateau to 28 in the Maritime Alps; Table 3). A large part of MLG (ca. 75%) showed an unbalanced pattern of heterozygosity for at least one of the eight surveyed loci. Other evidence of polysomic inheritance for the duplicated loci was the occurrence of individuals having more than two alleles per locus: about 8.8% of all examined individuals possessed three or four alleles for at least one of the examined loci (with special relevance for the locus *Pgm-2*, for which up to eight genotypes of 3/4 alleles were found; Fig. 2).

The values of genetic diversity for *D. schottii* could be regarded as moderate (H_e = 0.122). The

Table 2. Allele frequencies for all loci in the nine studied populations of *Dichoropetalum schottii*. * indicates alleles restricted to a region, while ** indicates alleles exclusive to populations.

		Pyrenees				Maritime Alps		Karst Plateau		Pindus
Locus	Allele	MON	FDS	NDS	SCG	BRO	TEN	ZDM	DDP	DIS
Aco-1	a	0.000	0.000	0.000	0.000	0.016	0.000	0.000	0.000	0.067
	b	1.000	1.000	1.000	1.000	0.984	1.000	1.000	1.000	0.875
	c	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.058**
Aco-2	a	0.000	0.000	0.000	0.000	0.055	0.000	0.068	0.000	0.036
	b	1.000	1.000	1.000	1.000	0.945	1.000	0.902	1.000	0.936
	c	0.000	0.000	0.000	0.000	0.000	0.000	0.030	0.000	0.028
Dia-1	a	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Idh-1	a	0.007*	0.009*	0.028*	0.017*	0.000	0.000	0.000	0.000	0.000
	b	0.993	0.991	0.972	0.983	0.977	0.933	1.000	1.000	1.000
	c	0.000	0.000	0.000	0.000	0.023*	0.067*	0.000	0.000	0.000
Mdh-2	a	0.000	0.000	0.067	0.000	0.000	0.000	0.000	0.000	0.258
	b	1.000	1.000	0.933	1.000	1.000	1.000	1.000	1.000	0.727
	c	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.015**
6Pgd-1	a	0.000	0.000	0.000	0.000	0.000	0.083**	0.000	0.000	0.000
	b	1.000	1.000	1.000	1.000	1.000	0.917	1.000	1.000	1.000
6Pgd-2	a	0.000	0.087	0.000	0.034	0.032	0.000	0.000	0.000	0.000
	b	0.823	0.739	0.721	0.595	0.952	0.889	1.000	1.000	0.962
	c	0.177	0.174	0.279	0.371	0.016	0.111	0.000	0.000	0.038
Pgm-2	a	0.000	0.000	0.000	0.000	0.274	0.300	0.278	0.240	0.039
	b	0.465	0.638	0.578	0.546	0.258	0.308	0.236	0.370	0.609
	c	0.535	0.362	0.422	0.454	0.468	0.392	0.486	0.390	0.227
	d	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.117**
	e	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.008**

most variable population was, as expected, the easternmost population (DIS, $H_e = 0.175$), and the least variable were DDP ($H_e = 0.083$) and MON ($H_e = 0.101$) (Table 3), the latter being located at the other extreme of the species range (Fig. 1). At the regional level (Table 3), the Pindus Mountains were the richest in genetic diversity ($H_e = 0.175$) and the Karst Plateau the poorest ($H_e = 0.093$), whereas the Pyrenees and Maritime Alps had intermediate levels ($H_e = 0.120$ and $H_e = 0.129$, respectively). The genetic identities (I) between populations were very high (mean = 0.986 and range = 0.967–1.000). Despite these extremely low genetic distances between populations, three main geographical groups could clearly be distinguished in the UPGMA tree (Fig. 3): (i) the Pyrenean populations (MON, FDS, NDS and SCG), (ii) the populations from the Maritime Alps and the Karst Plateau (BRO, TEN, ZDM and DDP), and (iii) the Pindus population (DIS). The genetic identities among regions were also very high, two of them being genetically identical (Maritime Alps and Karst Plateau; Table 4). These three main geographical groups are also clearly identified in PCoA (Fig. 4). The variance explained by the first two axes was up to 90.2%.

AMOVA showed that most of the genetic variability of the species is found within populations (91.0% if no regional grouping is considered), and thus the genetic divergence between populations is relatively low (F_{ST} = 0.090; Table 5). When the populations were grouped within geographical regions (i.e. a third hierarchical level was introduced), the genetic variation assigned to differences among regions was low (9.5% and 8.5% when three or four regions were considered), but higher than that corresponding to differences among populations within regions (2.3% and 2.1% for three and four regions, respectively, Table 5).

Discussion

Levels of genetic diversity in *Dichoropetalum schottii*

One of the advantages of allozymes over other

Table 3. Summary of genetic variation for eight loci in the nine studied populations of *Dichoropetalum schottii*.

Population	MLG[1]/sample size[2]	P_{99}	P_{95}	A	H_e
Pyrenees					
MON	10/29	37.5	25.0	1.38	0.101
FDS	10/22	37.5	25.0	1.50	0.113
NDS	10/22	50.0	37.5	1.50	0.135
SCG	8/25	37.5	25.0	1.50	0.131
Population mean	–	40.6 ± 6.2	28.1 ± 6.2	1.47 ± 0.06	0.120 ± 0.016
Regional level[3]	25/98	50.0	25.0	1.63	0.122
Maritime Alps					
BRO	17/30	62.5	25.0	1.88	0.115
TEN	16/24	50.0	50.0	1.63	0.143
Population mean	–	56.3 ± 8.8	37.5 ± 17.7	1.76 ± 0.18	0.129 ± 0.020
Regional level[3]	28/54	75.0	25.0	2.00	0.129
Karst Plateau					
ZDM	12/24	25.0	25.0	1.50	0.102
DDP	9/25	12.5	12.5	1.25	0.083
Population mean	–	18.7 ± 8.8	18.7 ± 8.8	1.37 ± 0.18	0.093 ±0.013
Regional level[3]	14/49	25.0	25.0	1.50	0.095
Pindus					
DIS	23/25	62.5	50.0	2.38	0.175
All populations mean	–	41.7 ± 16.5	30.5 ± 12.7	1.61 ± 0.33	0.122 ± 0.027
Species level	69/226	87.5	25.0	2.87	0.133

[1] MLG = Number of multilocus genotypes; P_{99} = percentage of polymorphic loci (99% criterion); P_{95} = percentage of polymorphic loci (95% criterion); A = mean number of alleles per locus; H_e = expected panmictic heterozygosity. [2] Considering only those individuals with no missing data for any locus. [3] Pooling all populations within a region (e.g., Pyrenees).

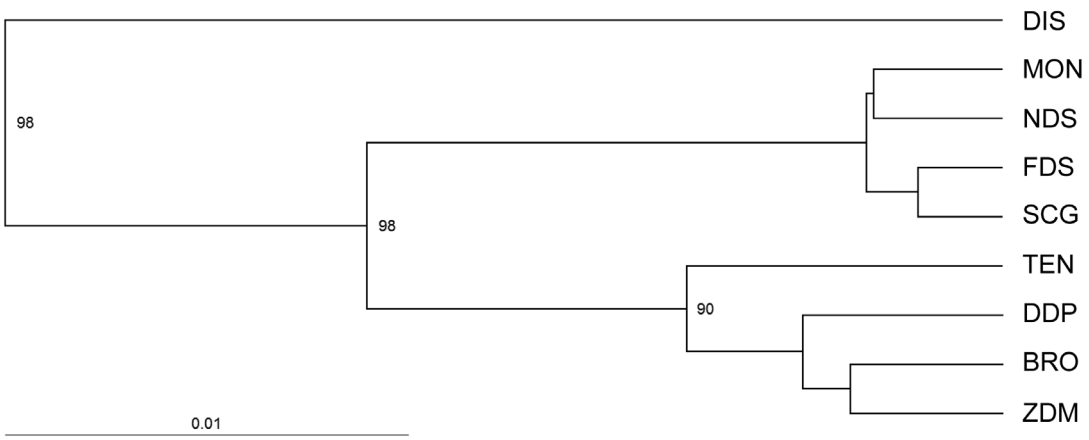


Fig. 3. UPGMA tree built from Nei *et al.*'s (1983) genetic distances among the studied populations of *Dichoropetalum schottii*. Only bootstrap values ≥ 90 are given.

molecular markers is the existence of a large body of literature (not yet available for other markers), which can aid in the analysis and interpretation of the data obtained (Parker *et al*. 1998, Lowe *et al*. 2004). Moreover, they are especially well suited for inter-specific comparisons because only a very small proportion of the thousands of plant enzymes (< 50) are routinely assayed by electrophoresis (Wendel & Weeden 1989, Berg & Hamrick 1997), which means that the same enzymes are used in almost all genetic studies. In *D. schottii*, the levels of genetic variability are clearly not low ($H_e = 0.122$), since they are slightly higher than those reported by Hamrick and Godt (1990, 1996) at the global level for both narrow ($H_e = 0.105$) and regional ($H_e = 0.118$) plant species, and also higher than those found by López-Pujol *et al*. (2009) for species native to the Mediterranean basin ($H_e = 0.109$).

The presence of a high number of duplicated loci may account, at least partially, for the relatively high levels of genetic diversity in *D. schottii*, as each duplicated locus may have up to four alleles, as in tetraploids with tetrasomic inheritance (Soltis & Rieseberg 1986, López-Pujol *et al*. 2004). Although the electrophoretic patterns observed for all the duplicated loci are indicative of polysomy, we must be cautious about this interpretation, as the inheritance model for the duplicated loci of *D. schottii* is completely unknown. The existence of duplications for some loci is not rare in diploid plants (Gottlieb 1982, Weeden & Wendel 1989, Wang *et al*. 2012), although cases of species with all (or almost all) their loci duplicated are virtually absent in the literature. However, in a study of the diploid grass *Melica ciliata*, Tyler (2004) reported a high number of duplicated enzymes and stated that this phenomenon "may be more common than reported in the literature". Due to intrinsic difficulties of dealing with duplicated loci data, loci that are duplicated or difficult to interpret may have been disregarded. In the case of *D. schottii*, most of the studied loci were apparently duplicated, whereas the remaining loci (*Dia-1* and *6Pgd-1*) showed patterns that may correspond both to duplicated or non-duplicated loci. Polyploidy was discarded on the basis of two independent chromosome counts of 2n = 22 (Favarger 1959, Molero & Montserrat-Martí 1986) and the stable chromosome number within the genus (Pimenov *et al*. 2007). These duplications may, instead, be the consequence of extensive chromosome segment duplication, although an ancient polyploidy within the genus

Table 4. Matrix of Nei's (1978) genetic identity between distinct geographic regions of *Dichoropetalum schottii*.

Region	Pyrenees	Maritime Alps	Karst Plateau
Pyrenees	–		
Maritime Alps	0.984	–	
Karst Plateau	0.981	1.000	–
Pindus	0.980	0.974	0.976

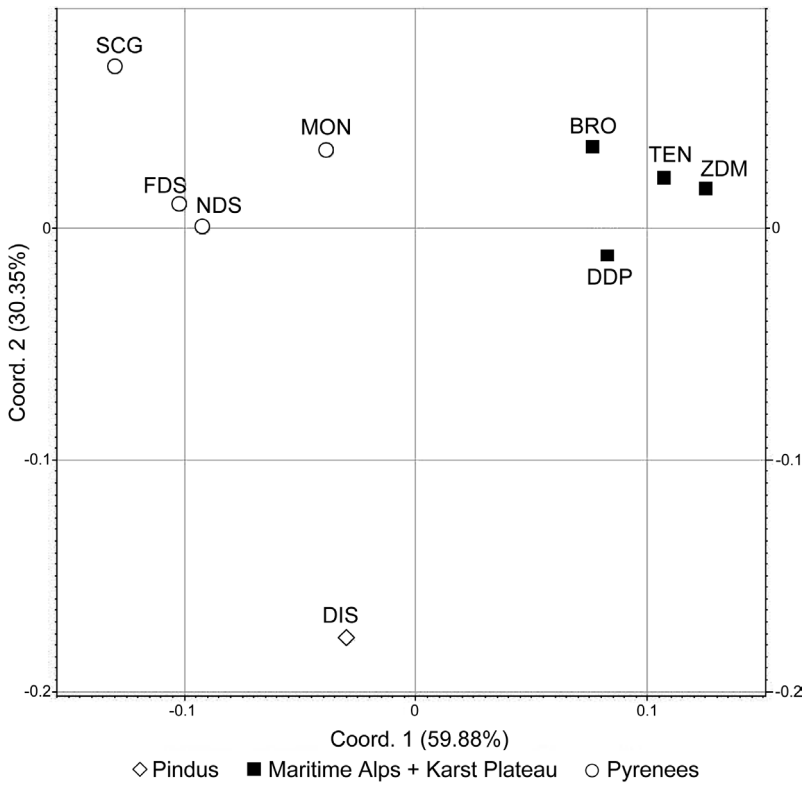


Fig. 4. PCoA of the nine studied populations of *Dichoropetalum schottii*. Percentages of the total variance explained by each axis are given in parentheses. Note that the populations are clustered in an analogous way to the UPGMA tree (Fig. 3).

cannot be ruled out (Gottlieb 1982, Weeden & Wendel 1989).

Population structure

The very high genetic similarity detected between the sampled isolated regions (Fig. 3 and Table 4) might indicate that the current population genetic structure of *D. schottii* is the result of recent allopatric fragmentation (i.e. vicariance as opposed to recent long-distance dispersal) as suggested for many species inhabiting the European mountains (Zhang *et al*. 2001, Comes & Kadereit 2003, Kropf *et al*. 2006, 2008, Schneeweiss & Schönswetter 2010, Zecca *et al*. 2011). Since habitats suitable for *D. schottii* (montane forests) may have shifted to lower altitudes during glacial periods (e.g., Zhang *et al*. 2001), populations of *D. schottii* probably had many opportunities for

Table 5. Analysis of molecular variance (AMOVA) of nine populations of *Dichoropetalum schottii* under three grouping hypotheses: no regional grouping, four geographical regions (Pyrenees, Maritime Alps, Karst Plateau, and Pindus) and three geographical regions (Pyrenees, Maritime Alps + Karst Plateau, and Pindus).

Grouping	Source of variation	Variance (%)	*F*-statistic	*p*
No regional grouping	Between populations	8.96	F_{ST} = 0.090	< 0.01
	Within populations	91.04		
Four regions	Between regions	8.54	F_{CT} = 0.065	< 0.01
	Between populations within regions	2.15	F_{SC} = 0.024	< 0.01
	Within populations	89.31	F_{ST} = 0.107	< 0.01
Three regions	Between regions	9.53	F_{CT} = 0.095	< 0.01
	Between populations within regions	2.28	F_{SC} = 0.025	< 0.01
	Within populations	88.19	F_{ST} = 0.118	< 0.01

contact. Because the glacial periods accounted for approximately 80% of the Quaternary (and the remaining 20% consisted of shorter interglacial periods; Birks & Willis 2008), one might hypothesize that the genetic flow between populations occurring in each of the long-lasting glacials could have maintained the genetic cohesion of this species. Following this reasoning, the putative contact through the flat Po Valley (which hosted montane vegetation during glacial periods; Estabrook 2001) may have permitted the maintenance of the genetic uniformity found between the southwestern tip of the Alps (Maritime Alps) and the Karst Plateau (which connect southeastern Alps with the Dinarides). In addition, the northern part of the Adriatic Sea (the gulf of Venice) was also passable during the glacial periods almost up to latitude 43°N because of marine regression (Lambeck & Purcell 2005), which would have facilitated genetic exchange with the populations of the Balkan Peninsula. With the onset of the present interglacial (the Holocene), populations would have retreat to highlands (i.e. putative interglacial refugia; Stewart *et al*. 2010), delineating the current species' genetic and distributional structure.

The higher genetic diversity detected in the Pindus region in Greece may have several reasons. Although some glaciers developed in the Pindus Mountains, these ranges were not as extensively glaciated as the Alps and the Pyrenees (Hughes *et al*. 2006). The whole Balkan Peninsula, and especially the Pindus Mountains, has been proposed as a major glacial refugium for many plant and animal lineages due to its relative environmental stability throughout the glacial/interglacial periods (Hewitt 1999, Tzedakis *et al*. 2002, Médail & Diadema 2009). Thus, plants were probably not forced to migrate such large latitudinal distances as in the Alps or in the Pyrenees, and thus much less dispersal bottlenecking and loss of allelic diversity is expected (Hewitt 1996, Tian *et al*. 2010). The Pindus population is the one with the highest number of alleles, and harbors up to four exclusive alleles, all of them at low frequencies, which may indicate that genetic drift was less severe than in the other, more westerly regions. Introgression could also have contributed to the higher diversity of the Greek population via introduction of new alleles. In the mountainous areas of NW Greece, at least four species of *Dichoropetalum* in addition to *D. schottii* occur (www.gbif.org). The distribution center of this genus is probably located nearby, in the Anatolian Peninsula (harboring up to 11 species), and the Balkans may represent a secondary center of speciation (with eight species occurring there).

In addition to support the recent allopatric fragmentation hypothesis, the occurrence of exclusive alleles, the high number of detected MLG, and the relatively high levels of genetic variation for both the Pyrenees and Maritime Alps (Tables 2 and 3) may also indicate that *D. schottii* would have persisted in multiple interglacial refugia throughout the Quaternary. This scenario of survival in multiple areas during the cold/warm stages of the Quaternary seems to be common to many widespread mountain species native to Europe; the varied topography of European mountain ranges allowed the plants to track the climate oscillations not only by latitudinal but also by altitudinal movements (e.g., Médail & Diadema 2009, Kramp *et al*. 2009, Hewitt 2011, Nieto-Feliner 2011, Zecca *et al*. 2011).

Local and/or contemporary factors may account for the particularly lower levels of diversity in the populations from the Karst Plateau (DDP and ZOL) despite the fact that they are also located in one of the suggested Mediterranean phylogeographical refugia (Médail & Diadema 2009). These populations are located at low altitudes (200–400 m), forming small, isolated populations in a highly human-affected area (Trieste Province). Genetically, they may represent a depauperate subset of the genetic diversity present in the Alps, as they are grouped together with the populations from the Maritime Alps.

Thus, we may conclude that the genetic structure of *D. schottii* is the result of Quaternary climatic oscillations, i.e. glacial genetic cohesion and post-glacial (Holocene) fragmentation. However, sampling more locations within the species' range and employing more markers for phylogeographic inference (e.g., cpDNA or AFLPs) are necessary for testing the allopatric fragmentation hypothesis and for getting better insight into the location and the significance of the species' Quaternary refugia. In addition, we should keep in mind that loci under balancing

selection are expected to show limited genetic differentiation among populations as compared with truly neutral loci; thus, the very high genetic similarity between populations and regions in *D. schottii* could be partly due to the lack of neutrality of allozyme systems (Karl & Avise 1992, Muirhead 2001).

Conservation implications

Dichoropetalum schottii is not globally threatened given its distribution range, the ecology and the conservation status of its populations. According to the IUCN (2001) criteria, it should be considered a species of Least Concern (LC). Nevertheless, it is protected in some parts of its distribution area owing to its local rarity (Anon. 1998, 2005, 2008). In species that are not imminently endangered, conservation should focus on guaranteeing evolutionary possibilities through the preservation of an adequate sample of their natural populations (Millar & Libby 1991, Neel & Cummings 2003). Among other factors (spatial isolation, ecological distinctiveness, etc.; Leppig & White 2006), two genetic features should be considered when selecting which populations to conserve: intrapopulation genetic diversity (richness and distinctiveness) and genetic differentiation among populations (Petit *et al*. 1998, Neel & Cummings 2003). The genetic data obtained here for *D. schottii* depict three relatively distinct genetic pools with exclusive alleles (i.e. the Pyrenees, the Alps plus Karst Plateau, and the Pindus). However, the genetic distances among them (and also among populations within each region) are small. On the other hand, almost all the exclusive alleles (either of a region or of a population) occur at very low frequencies (< 0.05). Several authors consider, when designing conservation strategies, that it is not necessary to preserve alleles whose frequencies are lower than 0.05, given that the effort to preserve them (they are likely to be lost in a few generations) is greater than the putative benefit for the evolution of the species (Brown & Briggs 1991, Lawrence *et al*. 1995, Lockwood *et al*. 2007).

The populations in the Pyrenees, which represent the western edge of the species range, are at present the only protected populations, both under the Spanish and French laws. The importance of edge or peripheral populations and the efforts devoted to their conservation have been widely discussed (*see* Eckert *et al*. 2008). Apparently, if only allozyme data are considered, conservation of *D. schottii* in this area should not be regarded as a priority since the genetic diversity of the Pyrenean populations is relatively low and the region as a whole harbors only one exclusive allele at a very low frequency. However, before additional molecular markers confirm low genetic originality (not necessarily all markers show the same patterns of variation), extreme caution should be applied because alternative threats could affect the Pyrenean populations. Actually, recent (autumn–winter 2012–2013) predation by domestic goats and a later wild-boar (*Sus scrofa*) perturbation considerably affected the MON population (J. Molero pers. obs.). Thus, non-genetic parameters could support protection of the Pyrenean populations at the western edge of the species range, and any change to conservation policy should be carefully evaluated. Additionally, it is advisable that the management of these populations is performed at a wide regional level, considering the French and Spanish populations together (that is, constituting a transboundary 'functional conservation unit') instead of developing border-based management policies.

In contrast, in the Pindus region, where *D. schottii* seems to harbor high levels of genetic diversity, it is not subject to any protection or conservation measures. There are numerous citations of the species in the area (Strid & Tan 1986; A. Strid pers. comm.) but most of them are quite old and were not confirmed by us during the fieldwork carried out in 2009. At present, the plant may not be as abundant as previously reported, since only few populations have recently been recorded (G. Kamari pers. comm.). However, metapopulation dynamics, which include local extinction and recolonization (Pannell 2003), may be occurring. It would be advisable, thus, to perform exhaustive surveys in order to estimate the current distribution of the species in the region, and subsequently to evaluate whether conservation measures are required. In any case, this region deserves special attention given that it retains most of the detected genetic

variability of the species: the population sampled from the Pindus contained up to 83% of the total allele richness (19 of the 23 alleles).

In the Alps, *D. schottii* has a relatively good conservation status, as there are numerous large populations. Those in the French Maritime Alps are currently being monitored by Conservatoire Botanique National de Porquerolles (CBNP), and show no decline (F. Boillot pers. comm.). Based on our field observations and herbarium records, the species should be considered not threatened overall in the Alps (or at least in the western part). Although the species is abundant in the Karst Plateau and neighbouring areas (such as in northern Velebit Mountain range; J. López-Pujol pers. obs.; M. Randić pers. comm.), human activities (urbanization) could threaten some of the populations as this area is densely populated and is experiencing a rapid economic development mainly associated with tourism. Knowledge of the distribution of *D. schottii* in other areas (central and southern Dinaric Alps, Apennines) is poor, and intensive fieldwork is required in order to confirm whether it is locally rare there or, alternatively, is more abundant than expected. With the present data, however, it is reasonable to believe that the Dinaric and Apennine populations would be genetically relatively similar to the already studied populations.

Dichoropetalum schottii represents a case of inconsistency between the legal status of a species and its current conservation and extinction probability. This kind of situation is relatively frequent, and one of the main causes is the local/short-term view of management and legal issues in comparison with the wide/long-term needs for an adequate plant conservation strategy. An example of this was studied by Bunnell *et al.* (2004) in the British Columbia, where most of the plants listed as 'endangered' in the region were in fact peripheral populations of species with a wide distribution area. Bunnell and colleagues claimed that these lists should include an analysis of the causes of rarity in the region in order to determine local conservation priorities. There are also examples of species whose peripheral populations deserve protection due to their ecological, evolutionary and economic significance, as described by Leppig and White (2006) for California.

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