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Low genetic diversity and allozymic evidence for autopolyploidy in the tetraploid Pyrenean endemic larkspur *Delphinium montanum* (Ranunculaceae)

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Allozyme electrophoresis was conducted to survey the levels and distribution of genetic diversity in the tetraploid perennial larkspur *Delphinium montanum* (Ranunculaceae), which is endemic to the eastern Pyrenees of Spain and France and considered to be endangered. Seven populations were sampled, resolving 14 putative enzymatic loci belonging to eight enzymes. Banding patterns stained in gels revealed several enzymatic duplications attributable to autotetraploidy, such as the presence of both balanced and unbalanced heterozygotes and the lack of fixed heterozygosity. However, variability in *D. montanum* ($P = 23.8\%$, $A = 1.48$, and $H_e = 0.082$) was lower than that expected for an autotetraploid species. This, in addition to the scarcity of loci showing three or four different alleles, could indicate that this species has suffered genetic erosion through population bottlenecks, or, alternatively, that it is undergoing diploidization. *Botanical Journal of the Linnean Society*, 2007, **155**, 211–222.

ADDITIONAL KEYWORDS: diploidization – heterozygosity – isozyme electrophoresis.

INTRODUCTION

Polyploidy is a significant mode of speciation and a force in the evolution of higher plants (Stebbins, 1980; Otto & Whitton, 2000). Although it is widespread, estimates of its frequency in angiosperms have varied widely: for example, 30–35% (Stebbins, 1950), 47% (Grant, 1981), and 70–80% (Lewis, 1980; Masterson, 1994). Recent genomic studies have revealed that most plants possess genomes with a considerable gene redundancy, which may arise from several rounds of polyploidization or gene duplications (Soltis, Soltis & Tate, 2004). Repeated cycles of genome duplication, followed by extensive diploidization, have led to many polyploids being functional diploids (Soltis *et al*., 2004). The success of polyploidy is a result of several genetic factors, such as increased heterozygosity, genetic diversity, and enzyme multiplicity (that is the capacity to produce new heterodimeric proteins; Thompson & Lumaret, 1992; Soltis & Soltis, 2000; Soltis *et al*., 2004).

Two major types of polyploidy are recognized in nature, allopolyploidy and autopolyploidy (Stebbins, 1947, 1950; Grant, 1981; Crawford, 1989; Soltis & Soltis, 2000), although these two terms represent the ends of a spectrum of variation (Grant, 1981; Thompson & Lumaret, 1992; Soltis *et al*., 2004). Three major types of criteria have been used to distinguish between them: cytological, genetic, and taxonomic (Soltis *et al*., 2004). Following Stebbins (1947, 1950) and Jackson (1982), allopolyploids are characterized by their disomic inheritance, bivalent formation at meiosis, and nonsegregating, fixed heterozygosity, as a result of the combination of two divergent parental genomes within them. By contrast, autopolyploids are

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expected to express polysomic inheritance (tetrasomic in autotetraploids) and may also show multivalent formation at meiosis. However, these rules are often broken and, although autopolyploids may exhibit disomic inheritance, allopolyploids rarely exhibit multivalent formation (Otto & Whitton, 2000; Soltis *et al*., 2004).

Alternatively, Kihara & Ono (1926) and Lewis (1980) described allopolyploidy and autopolyploidy as interspecific and intraspecific polyploidy, respectively. Allopolyploids are the result of a hybridization process between different species, with subsequent chromosome duplication, whereas autopolyploidy is probably the consequence of crosses between conspecific individuals (within or between populations), generally by fusion of nonreduced gametes. Nevertheless, both allo- and autopolyploids may also be formed via a triploid bridge, that is the union of a reduced and an unreduced gamete to produce a triploid (Ramsey & Schemske, 1998; Husband, 2004). This classification of polyploids, however, depends on species' taxonomic circumscription, a troublesome issue, especially for related taxa (Ramsey & Schemske, 1998).

Allopolyploids have been suggested to be much more common than autopolyploids (Stebbins, 1950; Grant, 1981). However, autopolyploids are now recognized to be more frequent than traditionally believed (Thompson & Lumaret, 1992; Ramsey & Schemske, 1998; Soltis *et al*., 2004). Autopolyploids were considered to be maladaptive (Soltis & Rieseberg, 1986) because of their presumably slower development and reduced fertility as a result of meiotic irregularities (Ramsey & Schemske, 1998). Permanent hybridity in allopolyploids as a result of fixed heterozygosity, which confers 'intrinsic advantages' (heterosis and homeostasis) and which is lacking in autopolyploids, has been suggested as another reason for the differences in frequency of the two types of polyploid (Ramsey & Schemske, 1998). Nevertheless, tetrasomic ratios provide autopolyploids with levels of heterozygosity higher than those in their diploid parents, and the possibility of the occurrence of three or four alleles at a single locus, which may explain their success in nature as a strategy of rapid speciation and evolution.

Delphinium montanum DC. (Ranunculaceae) is a perennial larkspur endemic to the eastern Pyrenees, on both the Spanish and French sides. It is placed in series *Montana* B. Pawl., with *D. dubium*, endemic to the Alps, and *D. oxysepalum*, restricted to the Carpathians (eastern Europe). All three species are tetraploids ($2n = 4x = 32$; Blanché, 1991), probably having differentiated during the Quaternary from a common ancestor (Pawlowsky, 1970). *D. montanum* is a forb up to 70 cm in height, with blue flowers showing a spur which contains nectar. Its pollination is entomophilous and the breeding system is basically outcrossing (Simon *et al*., 2001). It grows in rocky places, such as mobile or fixed screes, over calcareous substrates, at altitudes between 1600 and 2400 m. Currently, this species is restricted to seven locations of the ten originally described, four on the French side of the Pyrenees and three within Spain. The total population size is about 6000–6500 mature individuals (Table 1). According to the World Conservation Union (IUCN) recommendations for estimating the distribution area of the species (IUCN, 2001), the extent of occurrence is about 827.5 km^2 and the occupancy area is less than 20 km^2. The species has been listed as VU ('vulnerable') in the *Red List of the Vascular Spanish Flora* (Aizpuru *et al.*, 2000), and will also be included in the second volume of the *Red Book of the French Endangered Flora* (see Olivier, Galland & Maurin, 1995).

In this work, allozyme electrophoresis was used to obtain insights into the polyploid typology of *D. montanum* (i.e. autopolyploid or allopolyploid), and to describe the levels and distribution of its genetic diversity. The status of conservation of this species was also revised and some strategies are suggested for its preservation.

MATERIAL AND METHODS

Sampling strategy

All seven extant populations of *D. montanum* were sampled (Table 1, Fig. 1). Because the aerial part of the plant is present for only a short period (from June to September), and plants can be distinguished from *Aconitum* spp. only during flowering, samples were collected in three different years: the two populations from Vall d'Eina (EI1 and EI2) and the population from Els Cortils (COR) were sampled in July 2002, those from Bastanist (BAS) and Coma de l'Orri (ORR) were sampled in July 2003, and those from Noedes (NOE) and Cambra d'Ase (CAS) were sampled in July 2004. Sampling was conducted along a linear transect within each population, and samples were collected at least 50–100 cm apart to avoid collecting ramets from the same genet. Small fragments of leaves were placed in envelopes and stored at 4 °C prior to extraction 1 or 2 days later.

Electrophoresis

Genetic data were obtained by standard methods of starch gel electrophoresis of allozymes (Soltis *et al*., 1983). Leaves were homogenized on refrigerated porcelain plates using a cold extraction buffer consisting of 0.05 M tris-citric acid, 0.1% cysteine·HCl, 0.1% ascorbic acid, 8% polyvinylpyrrolidone (PVP-40), and 1 mM 2-mercaptoethanol. Extracts were absorbed

Table 1. Extant and studied populations of *Delphinium montanum*

Population code	Location†	Elevation (a.s.l.) (m)	Population size and year of last record	Sample size	Habitat protection (and date of establishment)
BAS	Spain, Bastanist, 31TCG98	2200	~ 2500 (2003)	72	Cadí-Moixeró Natural Park (1983)
COR	Spain, Els Cortils, 31TCG98	2200	~ 1000 (2002)	76	Cadí-Moixeró Natural Park (1983)
ORR	Spain, Coma de l'Orri, 31TDG39	1850	~ 500 (2003)	74	PEIN* 'Capçaleres del Ter i del Freser'
EI1	France, Vall d'Eina (Lower), 31TDG29	1900	~ 1500 (2002)	50	Vallée d'Eyne Nature Reserve (1993)
EI2	France, Vall d'Eina (Upper), 31TDG29	2100	~ 80 (2002)	24	Vallée d'Eyne Nature Reserve (1993)
CAS	France, Cambra d'Ase, 31DH20	2400	~ 200 (2004)	70	Unprotected
NOE	France, Noedes, 31TDH31	1750	~ 700 (2004)	72	Nohèdes Nature Reserve (1986)
–	Spain, Pedraforca, 31TCG97	1950	25 (1984); no individuals found in 2000 and 2002 surveys	–	Cadí-Moixeró Natural Park (1983)
–	France, Portella de Mantet, 31TDG49	2410	Not found since 1886	–	Mantet Nature Reserve (1984)
–	Spain, Cava, 31TCG88	2050	Not found since 1985 (200 individuals)	–	Cadí-Moixeró Natural Park (1983)

*PEIN is an acronym which stands for 'Pla d'Espais d'Interès Natural' (Spots of Natural Interest) created by the Autonomous Government of Catalonia (Generalitat de Catalunya).
†Location of populations is detailed by UTM 10×10-km squares.

onto Whatman 3MM filter paper, either to be analysed immediately or stored at –80 °C for long-term conservation until electrophoresis. Using 11% starch gels, eight enzyme systems were resolved. Aspartate aminotransferase (AAT, EC 2.6.1.1), diaphorase (DIA, EC 1.6.99), and phosphoglucoisomerase (PGI, EC 5.3.1.9) were satisfactorily resolved on a tris-citrate–lithium-borate buffer at pH 8.2; acid phosphatase (ACP, EC 3.1.3.2), isocitrate dehydrogenase (IDH, EC 1.1.1.42), and phosphogluconate dehydrogenase (6-PGD, EC 1.1.1.44) were resolved on a morpholine buffer at pH 6.1; finally, malate dehydrogenase (MDH, EC 1.1.1.37) and phosphoglucomutase (PGM, EC 5.4.2.2) were resolved on histidine–citrate buffer at pH 5.7.

Genetic analyses

Loci were numbered consecutively and alleles at each locus were labelled alphabetically, beginning from the most anodal form. Isozyme phenotypes were interpreted genetically according to standard principles, but special attention was paid to characteristic phenotypes of tetraploids and their interpretation (Gottlieb, 1981). To describe the levels of genetic diversity, the following statistics were calculated: P, the percentage of polymorphic loci at which the most common allele had a frequency of < 0.95; A, the mean number of alleles per locus; H_o, the observed heterozygosity; and H_e, the expected panmictic heterozygosity. In autotetraploids, two types of H_e can be computed: $H_e(Ce)$, the expected heterozygosity assuming random chromosomal segregation; and $H_e(Cd)$, the expected heterozygosity assuming some level of chromatid segregation. Chromatid segregation is produced if 'double reduction' takes place, that is, when sister chromatids segregate into the same gamete, a phenomenon that is specific to autopolyploids and is dependent on the amount of quadrivalent formation and the proximity of the locus to the centromere (see Bever & Felber, 1992; Ronfort *et al*., 1998). A study of the meiotic behaviour (Blanché, 1991) has suggested that double reduction does not take place in *D. montanum*, and therefore it is assumed that only chromosomal segregation occurs. This consideration allowed us to perform a more conservative data processing with regard to the type of polyploid condition in this species (auto- or allopolyploid), as double reduction increases the production of homozygous gametes compared with that expected under random chromosomal segregation in diploids and allopolyploids (Ronfort *et al*., 1998). We also calculated the mean fixation index (F) for all variable loci in each population, in order to compare the genotype proportions with those expected under Hardy–Weinberg equilibrium. The chi-squared (χ^2) test was used to evaluate deviations

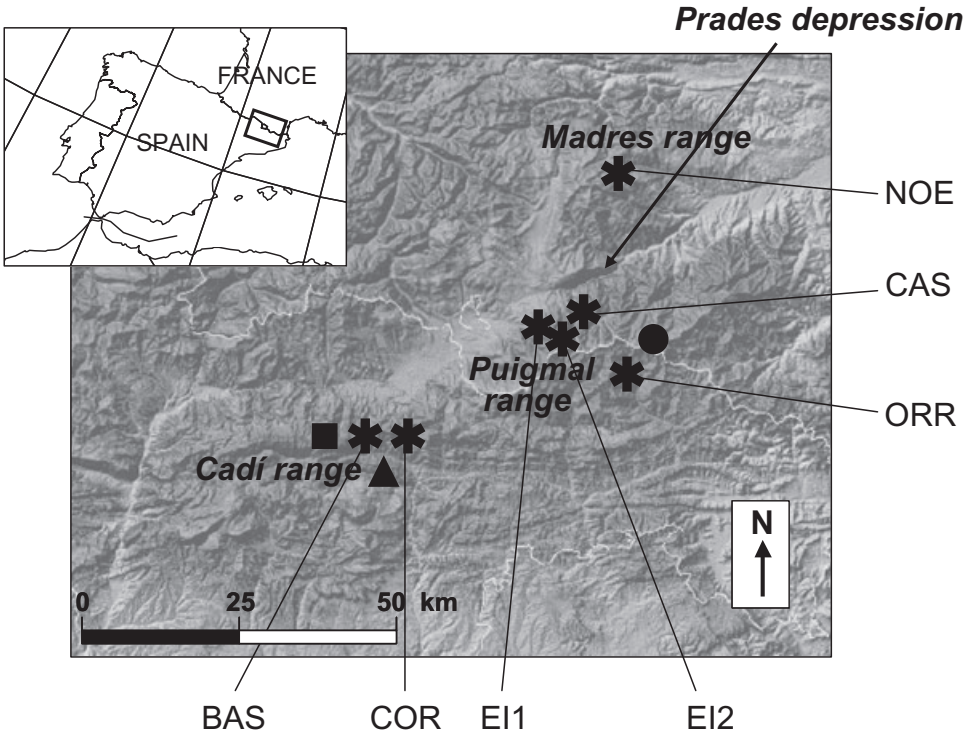

Figure 1. Sampled populations of *Delphinium montanum*. BAS, Bastanist; CAS, Cambra d'Ase; COR, Els Cortils; EI1, Vall d'Eina (Lower); EI2, Vall d'Eina (Upper); NOE, Noedes; ORR, Coma de l'Orri. The triangle, circle, and square correspond to extinct populations (Pedraforca, Portella de Mantet, and Cava, respectively).

of F from zero. The partitioning of genetic diversity within and between populations was analysed using Nei's gene diversity statistics (Nei, 1973): the total genetic diversity (H_T), mean genetic diversity within populations (H_S), genetic diversity between populations (D_{ST}), and proportion of total genetic diversity between populations (G_{ST}) were calculated for all populations. Gene flow (Nm) was estimated by the equation: $Nm = 1 - G_{ST}/4G_{ST}$ (Wright, 1951). Nei's genetic identity (I) was calculated between pairs of populations (Nei, 1978), and was used to cluster them into a dendrogram following the unweighted pair group method with averaging (UPGMA). A program for the analysis of autotetraploid genotypic data, AUTOTET (Thrall & Young, 2000), was used for the calculation of A, H_o, H_e, and F. The choice of the appropriate options for tetraploids, BIOSYS-1 (Swofford & Selander, 1989), was used for the calculation of P, and GeneStat version 3.31 (Whitkus, 1988) was used to calculate H_T, H_S, D_{ST}, and G_{ST}.

RESULTS

Interpretation of enzyme banding patterns

The satisfactorily resolved enzymes displayed 14 putative loci: *Aat-1*, *Aat-2*, *Acp-2*, *Dia*, *Idh-1*, *Mdh-1*, *Mdh-2*, *6Pgd-1*, *6Pgd-2*, *6Pgd-3*, *6Pgd-4*, *Pgi-2*, *Pgm-1*, and *Pgm-2*. Seven of these loci (*Aat-2*, *Mdh-2*, *6Pgd-1*, *6Pgd-2*, *6Pgd-4*, *Pgi-2*, and *Pgm-1*) were monomorphic across populations, showing a single band in the phenotype of all examined individuals (homozygote genotypes). By contrast, the seven remaining loci were polymorphic, with some exhibiting a wide array of bands (Fig. 2). Banding patterns in AAT, ACP, IDH, MDH, and 6-PGD were consistent with dimeric enzymes and, in DIA and PGM, with monomeric enzymes. All banding patterns found for the interpretable loci are described briefly below.

AAT displayed two regions of activity in *D. montanum*, encoded by two independent putative loci, *Aat-1* and *Aat-2*, the latter being monomorphic. *Aat-1* showed three different alleles. Although homozygotes corresponded only to the two fastest alleles (*Aat-1a* and *Aat-1b*), combinations of heterozygous phenotypes amongst the three alleles could be found. At this locus, several types of heterozygote were observed, which were distinguished by the number of bands and/or the relative intensity of band staining (Fig. 3). Thus, we were able to observe three-banded heterozygotes (genotypes which are combinations of two alleles), with either balanced (same allelic dosage) or unbalanced (different allelic dosage) staining activity.

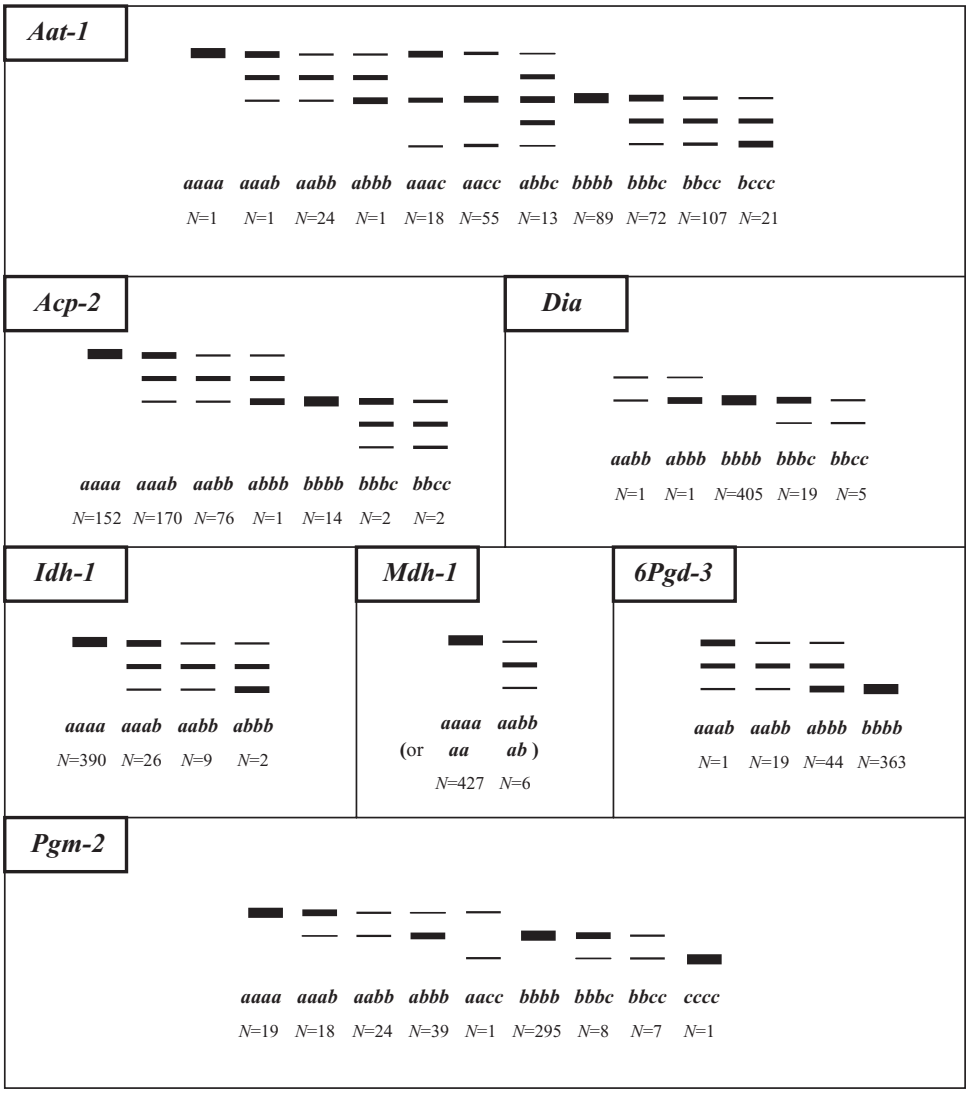

Figure 2. Schematic banding patterns obtained for polymorphic loci in *Delphinium montanum*. All the inferred genotypes and the number of individuals showing each genotype are given below each genotype.

Although most of these heterozygotes comprised *Aat-1b* and *c* alleles, some consisted of combinations between *a* and *b* alleles or even *a* and *c*. Furthermore, some individuals showed a five-banded phenotype with balanced staining activity, which could be interpreted as having three different alleles for this enzyme (genotype *abbc*).

ACP presented two zones of activity, encoded by two different putative loci (*Acp-1* and *Acp-2*). *Acp-1* was stained too poorly to be interpretable. *Acp-2* exhibited three different alleles, although the slowest (*Acp-2c*) was at a very low frequency (only four individuals from the 417 analysed; see Fig. 2). Both single-banded homozygotes and several types of heterozygote (both balanced and unbalanced) were observed, the genotypes *aaaa*, *aaab*, and *aabb* being the most common.

A single zone of activity was detected for DIA, encoded by one putative locus (*Dia*). Most of the individuals ($N = 405$; see Fig. 2) showed a single-banded phenotype (interpreted as the *bbbb* genotype). Nevertheless, some plants exhibited two-banded phenotypes, both balanced and unbalanced heterozygotes, as a result of the monomeric structure of this enzyme. The presence of the fastest allele (*Dia-a*) was very rare (shown by only two individuals).

Two regions of activity were identified for IDH, from which only the most anodal was consistently interpreted (*Idh-1*). Just two alleles were detected, but the slower of these (*Idh-1b*) appeared only rarely (shared by 37 individuals of the 427 analysed; see Fig. 2). The most common inferred genotype was the homozygote *aaaa* ($N = 390$). Although at low frequency, two kinds of

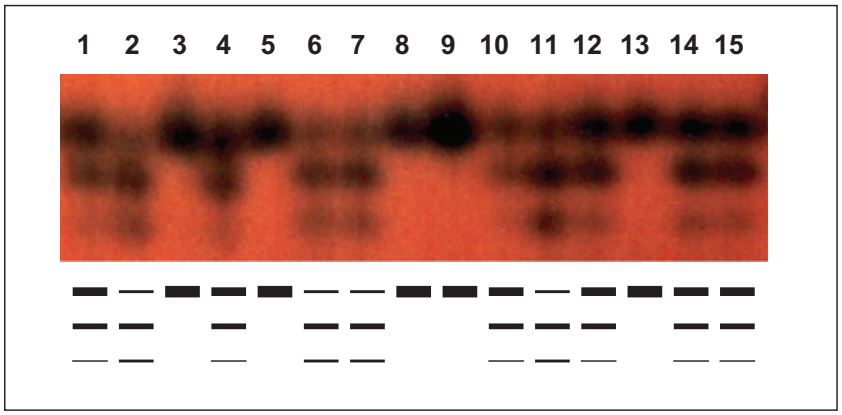

Figure 3. Banding patterns obtained for *Aat-1* in the COR population of *Delphinium montanum*. Three types of genotype may be inferred: homozygotes *bbbb* (individuals 3, 5, 8, 9, and 13), balanced heterozygotes *bbcc* (individuals 2, 6, 7, and 11), and unbalanced heterozygotes *bbbc* (individuals 1, 4, 10, 12, 14, and 15).

three-banded heterozygote were found, unbalanced (*aaab* and *abbb*) and balanced (*aabb*).

MDH showed at least three zones of activity, although only the most anodal was interpretable, controlled by the putative locus *Mdh-1*. Although most of the analysed individuals were homozygotes for the fastest allele (*Mdh-1a*), a few showed a three-banded balanced phenotype (interpreted as either *aabb*, i.e. with a tetraploid load, or, alternatively, as *ab*, i.e. diploidized).

6-PGD exhibited up to five regions of activity, four of which could be interpreted. The two most anodal loci (*6Pgd-1* and *6Pgd-2*) and the *6Pgd-4* locus showed a single-banded phenotype for all the analysed individuals, interpreted as the *aaaa* genotype. The *6Pgd-3* locus showed two different alleles, with the slower of these (*6Pgd-3b*) being more common (Fig. 2). Both balanced (*aabb*) and unbalanced (*aaab* and *abbb*) heterozygous genotypes were observed, but homozygotes were found only for the *6Pgd-3b* allele. The most cathodal isozyme (*6Pgd-5*) was too faint to be interpretable, but below the *6Pgd-4* locus was an additional region of enzymatic activity, which might be interpreted as an intergenic heterodimer between *6Pgd-4* and *6Pgd-5*.

PGM presented two zones of activity. Only the more cathodal region was interpretable, controlled by the putative locus *Pgm-2*. The analysed individuals showed three different alleles at this locus, *Pgm-2b* being the most common. Several types of two-banded heterozygote (as this enzyme behaves as a monomer), either balanced and unbalanced, were revealed in the starch gels, even between the fastest and the slowest allele (e.g. *aacc*). Although homozygotes with all the alleles were found, the most common was the genotype *bbbb* ($N = 295$).

Levels and distribution of genetic diversity

Amongst the 14 interpretable loci, 25 alleles were detected. The allelic frequencies of the polymorphic loci are given in Table 2. The richest population in terms of the number of alleles was COR, displaying 23 alleles; the poorest was BAS, with 19 alleles observed. The COR population showed two private (population-specific) alleles (*Acp-2c* and *Dia-a*), whereas EI2 harboured one private allele (*Mdh-1b*). Rare alleles (those with a frequency of less than 0.050) were present in all populations, with a maximum of six in the COR population. All the population-specific alleles were rare, with the exception of *Mdh-1b* in population EI2. Furthermore, some populations harboured extremely rare alleles, that is those with frequencies of less than 0.010, such as COR (*Dia-c*), ORR (*Aat-1a*, *Dia-c*), CAS (*Idh-1b*, *6Pgd-3a*), and NOE (*Pgm-2a*). All the populations shared the same allele as the most frequent across loci, with the exception of *Aat-1*, where ORR and NOE each exhibited a different allele (*Aat-1c* and *Aat-1a*, respectively). By contrast, 13 of the 402 plants (3.2%) examined for the *Aat-1* locus had three alleles; no additional loci exhibited more than two alleles in any individual (see Fig. 2).

Genetic diversity was moderate to low in *D. montanum*, with mean values over all seven populations of $P = 24.5\%$, $A = 1.49$, and $H_e = 0.088$ (Table 3). The most variable population was CAS ($H_e = 0.107$), and the least diverse was BAS ($H_e = 0.065$); however, the standard deviations (Table 3) showed that the differences between populations were not statistically significant. Values of H_o were lower than those of H_e in all populations, except ORR and NOE, which showed a negative value of F (Table 3). At the locus level, of

Table 2. Allele frequencies for polymorphic loci in seven populations of *Delphinium montanum*. The most frequent allele for each locus and population is given in italic

Locus	Allele	BAS	COR	ORR	EI1	EI2	CAS	NOE
Aat-1	*a*	0.102	0.048	0.004	0.080	0.011	0.066	*0.564*
	b	*0.828*	*0.747*	0.448	*0.675*	*0.728*	*0.575*	0.000
	c	0.070	0.205	*0.548*	0.245	0.261	0.358	0.436
Acp-2	*a*	*0.875*	*0.589*	*0.761*	*0.831*	*0.833*	*0.631*	*0.865*
	b	0.125	0.389	0.239	0.169	0.167	0.369	0.135
	c	0.000	0.022	0.000	0.000	0.000	0.000	0.000
Dia	*a*	0.000	0.010	0.000	0.000	0.000	0.000	0.000
	b	*0.972*	*0.987*	*0.996*	*0.990*	*0.979*	*0.968*	*0.979*
	c	0.028	0.003	0.004	0.010	0.021	0.032	0.021
Idh-1	*a*	*1.000*	*0.964*	*0.864*	*1.000*	*1.000*	*0.996*	*1.000*
	b	0.000	0.036	0.136	0.000	0.000	0.004	0.000
Mdh-1	*a*	*1.000*	*1.000*	*1.000*	*1.000*	*0.875*	*1.000*	*1.000*
	b	0.000	0.000	0.000	0.000	0.125	0.000	0.000
6Pgd-3	*a*	0.000	0.039	0.101	0.057	0.033	0.007	0.103
	b	*1.000*	*0.961*	*0.899*	*0.943*	*0.967*	*0.993*	*0.897*
Pgm-2	*a*	0.212	0.236	0.045	0.077	0.205	0.197	0.007
	b	*0.788*	*0.764*	*0.955*	*0.923*	*0.795*	*0.739*	*0.955*
	c	0.000	0.000	0.000	0.000	0.000	0.064	0.038

BAS, Bastanist; CAS, Cambra d'Ase; COR, Els Cortils; EI1, Vall d'Eina (Lower); EI2, Vall d'Eina (Upper); NOE, Noedes; ORR, Coma de l'Orri.

Table 3. Summary of genetic variation for 14 loci in seven populations of *Delphinium montanum*

Population	*P*	*A*	H_o	H_e	*F*
BAS	21.4	1.36	0.050 (0.092)	0.065 (0.122)	0.232 (0.075)
COR	21.4	1.64	0.076 (0.130)	0.102 (0.176)	0.253 (0.094)
ORR	28.6	1.50	0.104 (0.191)	0.098 (0.162)	–0.056 (0.063)
EI1	28.6	1.43	0.071 (0.140)	0.073 (0.142)	0.027 (0.013)
EI2	28.6	1.50	0.083 (0.140)	0.095 (0.145)	0.121 (0.074)
CAS	21.4	1.57	0.095 (0.201)	0.107 (0.199)	0.107 (0.135)
NOE	21.4	1.43	0.081 (0.175)	0.074 (0.142)	–0.087 (0.068)
Mean	24.5	1.49	0.080	0.088	0.085
Standard deviation	3.8	0.09	0.017	0.017	0.132

BAS, Bastanist; CAS, Cambra d'Ase; COR, Els Cortils; EI1, Vall d'Eina (Lower); EI2, Vall d'Eina (Upper); NOE, Noedes; ORR, Coma de l'Orri. *A*, mean number of alleles per locus; *F*, mean fixation index; H_o, observed heterozygosity; H_e, expected panmictic heterozygosity; *P*, percentage of polymorphic loci. Standard deviation in parentheses.

the 38 valid tests, the χ^2 test showed that 22 *F* values were in accordance with Hardy–Weinberg expectations ($P = 0.05$), and 16 *F* values differed significantly from zero ($P < 0.05$). Of these 16 values, 12 were positive and four were negative (data not shown). Positive values indicate a deficiency of heterozygotes; negative values indicate an excess.

The genetic diversity in *D. montanum* was distributed mainly within populations ($H_S = 0.089$) rather than between them ($D_{ST} = 0.013$); nevertheless, a significant fraction of the genetic variability was attributable to differences between populations ($G_{ST} = 0.131$; data not shown), that is the populations are quite divergent. The number of migrants per generation was only moderate ($Nm = 1.66$), from which we can infer that gene flow is quite limited between populations. The UPGMA dendrogram (Fig. 4) showed a clear separation of the NOE population from the remainder, which were clustered together. The most distant populations geographi-

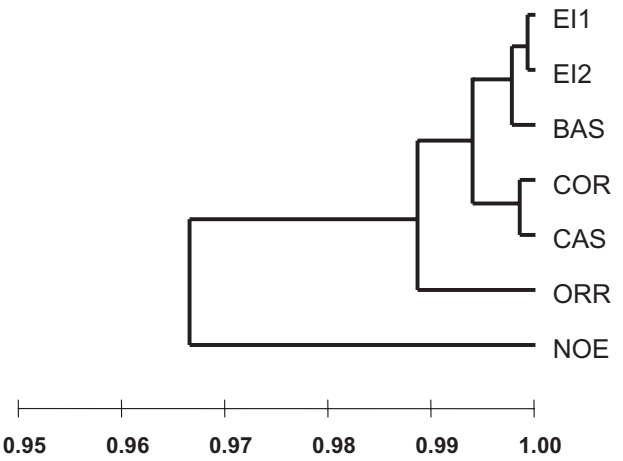

Figure 4. Dendrogram resulting from analysis by unweighted pair group method with averaging (UPGMA) of seven populations of *Delphinium montanum* based on pairwise values of Nei's (1978) genetic identity (standard deviation, 0.474%; cophenetic correlation, 0.933).

cally (NOE and BAS, which are separated by 60 km) were the most genetically distant ($I_{\text{NOE-BAS}} = 0.957$), and the closest pair (EI1–EI2; 0.8 km apart) were the most genetically similar ($I_{\text{EI1-EI2}} = 0.999$; data not shown).

DISCUSSION

Autopolyploidy and genetic diversity in *D. montanum*

Allozyme data are extremely useful in distinguishing between autopolyploids and allopolyploids (Soltis & Rieseberg, 1986; Crawford, 1989; López-Pujol *et al*., 2004). Tetrasomic inheritance in autotetraploids results in the formation of unbalanced as well as balanced heterozygotes in all possible combinations, because alleles at a given locus on the homologous chromosomes segregate at random. By contrast, allotetraploids are expected to display fixed heterozygosity, as alleles at a given locus on homologous chromosomes segregate independently (Ramsey & Schemske, 2002). No evidence of fixed heterozygosity was found in any of the loci examined in *D. montanum*. In the seven polymorphic loci, all but one showed both balanced and unbalanced heterozygotes (see Fig. 2). Thus, allozyme data support an autotetraploid condition in *D. montanum*.

Tetraploidy allows for up to four different alleles at a single locus (Soltis & Rieseberg, 1986; Soltis & Soltis, 1989; Mahy *et al*., 2000). In autopolyploids, segregation at a given locus can involve as many alleles as there are homologous chromosomes (Ramsey & Schemske, 2002), that is up to four in autotetraploids. Although only 13 (about 3%) of the electrophoretically analysed individuals possessed three alleles in *D. montanum* (all of those sharing the genotype *abbc* at the *Aat-1* locus), this provides further support for the hypothesis that this species is an autopolyploid. Nevertheless, these figures are much lower than those in other studied autotetraploid species. For example, in *Thymus loscosii*, 37.2% of all plants exhibited three or four alleles at at least one of the examined loci (López-Pujol *et al*., 2004), and the autotetraploid cytotypes of *Tolmiea menziesii* and *Vaccinium oxycoccos* had 39% (Soltis & Soltis, 1989) and 12.1% (Mahy *et al*., 2000), respectively, of individuals with three or four alleles. The large number of alleles at some loci have been attributed to repeated polyploidization events, as discussed, for example, in the grass *Dactylis glomerata* (Lumaret, 1988).

Autotetraploids are usually characterized by high levels of heterozygosity, which may also be a consequence of tetrasomic inheritance (Soltis & Soltis, 2000). The literature provides several examples of high levels of heterozygosity in autotetraploid plants (mean $H_o = 0.279$; see Table 4) that are substantially greater than in their diploid counterparts (Soltis & Soltis, 1989; Mahy *et al*., 2000; Hardy & Vekemans, 2001; see Table 4). Data from the literature indicate that values of heterozygosity are relatively low for *D. montanum* ($H_o = 0.075$). None of the examined populations of Pyrenean larkspur appeared to be diploid, as deduced from their allozyme banding patterns, and all the reported chromosome counts are tetraploid in this species (Blanché, 1991). Therefore, we can only cautiously compare the data with some diploid taxa of *Delphinium* (see Table 4).

Tetrasomic inheritance leads to high levels of genetic diversity in autotetraploids, as described by values of A, P, and heterozygosity (Soltis & Soltis, 1989). The mean diversity values for autotetraploids are very high ($P = 50.74\%$, $A = 2.23$, and $H_e = 0.264$; Table 4), significantly greater than for diploid species (for example, Hamrick & Godt, 1989; Gitzendanner & Soltis, 2000). Values of variability in *D. montanum* ($P = 24.5\%$, $A = 1.49$, and $H_e = 0.088$) are lower than the average for autotetraploid species, only comparable with those of *Turnera ulmifolia* var. *intermedia* (Shore, 1991), and even lower than those found for the surveyed diploid perennial larkspurs (mean values: $P = 33.3\%$, $A = 1.52$, and $H_e = 0.118$; see Table 4).

These relatively low levels of diversity and heterozygosity, coupled with the low incidence of three or four different alleles at surveyed loci, may be the result of several causes. It could be suggested that *D. montanum* is currently undergoing diploidization, i.e. accumulating not only structural differences in homologous chromosomes (producing a further diver-

Table 4. Allozyme diversity in *Delphinium montanum* compared with other autotetraploid taxa and other *Delphinium* taxa. If a species has both diploid and tetraploid populations, the values of diversity given here are only for tetraploid populations except for H_o

Taxon	*P*	*A*	H_o	H_e	Reference
Autotetraploid taxa					
Aster kantoensis	36.9	1.53	–	0.142	Maki, Masuda & Inoue (1996)
Centaurea jacea	–	3.54	0.540 (0.29*)	0.38	Hardy & Vekemans (2001)
Dactylis glomerata	80.0	2.36	0.430 (0.17*)	–	Soltis & Soltis (1993)
Delphinium montanum	24.5	1.49	0.080	0.088	This study
Heuchera grossulariifolia	31.0	1.55	0.159 (0.058*)	–	Wolf, Soltis & Soltis (1990)
Heuchera micrantha	38.33	1.64	0.150 (0.075*)	–	Ness, Soltis & Soltis (1989)
Rutidosis leptorrhynchoides	98.0	3.20	0.340 (0.22*)	0.36	Brown & Young (2000)
Swainsona recta	–	4.30	0.240	0.42	Buza, Young & Thrall (2000)
Thymus loscosii	85.0	3.00	0.472	0.422	López-Pujol *et al*. (2004)
Tolmiea menziesii	40.8	1.50	0.237 (0.070*)	–	Soltis & Soltis (1989)
Turnera ulmifolia var. *elegans*	65.3	2.03	0.420	0.27	Shore (1991)
Turnera ulmifolia var. *intermedia*	20.1	1.20	0.07 (0.11*)	0.04	Shore (1991)
Vaccinium oxycoccos	38.9	1.66	0.213 (0.067*)	–	Mahy *et al.* (2000)
Mean of tetraploid taxa	50.74	2.23	0.279	0.264	
Other *Delphinium* taxa					
D. bolosii	28.9	1.40	0.107	0.104	Orellana *et al*. (in press)
D. decorum	40.9	1.90	0.143	0.161	Koontz, Soltis & Brunsfeld (2001)
D. fissum ssp. *fissum*	17.8	1.30	0.053	0.061	Orellana *et al*. (in press)
D. fissum ssp. *sordidum*	4.0	1.10	0.013	0.012	Orellana *et al*. (in press)
D. luteum†	69.2	1.80	0.184	0.211	Koontz *et al*. (2001)
D. mansanetianum	6.7	1.20	0.013	0.013	Orellana *et al*. (in press)
D. nudicaule	65.0	2.00	0.191	0.295	Koontz *et al*. (2001)
D. pentagynum ssp. *formenteranum*	40.7	1.60	0.125	0.180	López-Pujol *et al*. (2003)
D. variegatum ssp. *kinkiense* and ssp. *thornei*‡	24.5	1.28	0.070	0.074	Dodd & Helenurm (2002)
D. variegatum ssp. *variegatum*	33.6	1.52	0.041	0.064	Dodd & Helenurm (2002)
D. viridescens	35.3	1.60	0.103	0.119	Richter, Soltis & Soltis (1994)
Mean of *Delphinium*	33.3	1.52	0.095	0.118	

A, mean number of alleles per locus; H_o, observed heterozygosity; H_e, expected panmictic heterozygosity; *P*, percentage of polymorphic loci. All values given here are population means.
*Values of H_o in diploid populations.
†Only the wild population was considered.
‡The two subspecies are combined in the analysis.

gence) but also genetic factors that enforce preferential pairing in polyploids (Wolfe, 2001; Ramsey & Schemske, 2002), resulting in a loss of duplicate expression in the case of autotetraploids. Allozymes can be very useful in detecting the loss of duplicate expression, because the diploidized loci will not exhibit unbalanced banding patterns. However, unbalanced heterozygotes are lacking only from *Mdh-1* in *D. montanum*. Rather, the absence of unbalanced heterozygotes at this locus may be the result of sampling error, given the low frequency of the *b* allele (only six individuals from the 433 analysed harboured this allele). Although some cytological (karyotypes show that chromosome pairs are quite distinct, quadrivalents are absent at meiosis) and morphological (pollen size, stomata size and density, and stamen number are not higher than in some diploid counterparts) data suggest that the plant has suffered a significant process of diploidization in its genome (Blanché, 1991; Bosch, 1999), to verify the hypothesis of an autopolyploid origin and further diploidization, extensive cytological (i.e. additional karyological and meiotic analyses) and inheritance (allozyme progeny tests) studies are needed.

Alternatively, the period of time spent from the formation of this 'palaeotetraploid' in the Tertiary to today may have contributed to the extensive occurrence of genetic drift and allelic erosion within populations, processes enhanced if the different populations of *D. montanum* have experienced episodes of

demographic bottlenecks. Moreover, the genome doubling could have occurred during a time of low diversity in the ancestor. These reasons together might explain the near absence of loci with three or four different alleles, the monomorphism of seven of the 14 surveyed loci, and the overall low genetic diversity found. Indeed, the genetic variability values of *D. montanum* are mostly lower than those for other surveyed autopolyploids (Table 4), most of them of recent origin.

Population genetic structure and conservation of *D. montanum*

As explained above, population history may be a more feasible factor contributing to the relatively low levels of genetic variability found in *D. montanum*, rather than the hypothesized diploidization. There is a significant fraction of rare alleles, and even extremely rare ones (see 'Results'), which may easily be lost by genetic drift. Genetic erosion of populations could lead to a decrease in the frequency of some alleles and their elimination, or nearly so. The very ancient establishment of the population (probably before the Pleistocene glaciations) may have promoted this erosion. The presence of some population-specific alleles may also be caused by this fact (that is, they still persist in some populations, but have disappeared in others), although these alleles may also have a more recent origin through the accumulation of mutations. The long history of populations (from the Quaternary) has been demonstrated to be a crucial factor in modelling the genetic structure of populations in the related species *Aconitum lycoctonum* (Utelli, Roy & Baltisberger, 1999); this may also be the case for *D. montanum*.

The moderate to low levels of inbreeding detected within populations (mean $F = 0.089$) present a picture of current conservation stability of extant populations. Most populations have a size (see Table 1) apparently sufficient to counteract the negative effects of inbreeding. Nevertheless, populations are genetically divergent ($G_{ST} = 0.135$), probably as a consequence of their geographical isolation (the mean inter-population distance is about 27 km), which limits to a great extent any possibility of migration. Although a direct estimation of gene flow has not been carried out, we can suggest that the indirect value of *Nm* (1.66) found reflects historical rather than current levels of genetic exchange in *D. montanum*.

The separation of NOE from the rest of the populations, showed by the UPGMA dendrogram, may be the result of the higher level of genetic isolation suffered by this population. All the populations, with the exception of NOE, are located within the mountainous axis formed by the Cadí and Puigmal ranges (Fig. 1). The NOE population is located in the Madres range, segregated from the rest by the Prada-de-Conflent depression. The six southern populations are clustered together because they have had more likelihood of genetic interchange between them, whereas NOE has remained isolated, probably from the last glacial maximum, allowing an independent evolution, i.e. constituting an 'evolutionarily significant unit' (ESU) (Moritz, 1994).

Fortunately, all but one of the *D. montanum* populations are located within the boundaries of an area of legal habitat protection, even those considered currently extinct (Table 1). Nevertheless, these belong to different administrations (French and Spanish) and the degree of protection is uneven. For example, the ORRI population is located in a Spot of Natural Interest (PEIN; see Table 1), which has a level of protection much lower than a Nature Reserve or a Natural Park, whereas the CAS population has no degree of protection and is situated very close to a ski station. The NOE population deserves special protection because of its genetic singularity. This locality is a Nature Reserve and a Natura 2000 site, and can be considered a 'hotspot' of rare plants because of the presence of other endangered species, such as *Hormathophylla pyrenaica*, *Ligularia sibirica*, and *Dracocephalum austriacum* (the three species are listed in Annex I of the Convention of Bern, in Annexes II and IV of the Habitats Directive, and in Annex I of the French Red Book, where it also appears as VU), although the latter may be currently extinct from the Pyrenees (Aymerich & Sáez, 2001).

Although the size of the extant populations seems sufficient to ensure an appropriate capability to respond to stochastic events, at least three classical localities seem to have been lost (one in the central scree of Pedraforca Mountain, another in Portella de Mantet, and the last over Cava; see Table 1). Therefore, close monitoring of extant populations is necessary to detect demographic fluctuations that have been widely reported for this species (total population size reached almost 10 000 individuals in recent years; Aymerich & Sáez, 2001), or other incidences, to ensure their long-term viability. One of these incidences may be the predation by several types of mountain-dwelling ungulates, such as Pyrenean chamois (*Rupicapra rupicapra pyrenaica*) and argali (*Ovis ammon*) (Aymerich, 2003). Predation by ungulates may represent a loss of over 95% of the seed set in some populations (Simon *et al.*, 2001; Aymerich, 2003), and our recent field observations (July 2004) have revealed a massive predation of the reproductive structures of *D. montanum* in the NOE population. Therefore, the monitoring of populations, regulation of ungulate density, and even the installation of physical barriers against ungulates may be necessary for the plant's survival.

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