Allozyme Diversity in the Tetraploid Endemic *Thymus loscosii* (Lamiaceae)

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• *Background and Aims Thymus loscosii* (Lamiaceae) is a tetraploid perennial species endemic to the Ebro river basin (north-eastern Spain), which is included in the *National Catalogue of Endangered Species*. It is a tetraploid species ($2n = 54$), presumably an autotetraploid originated by the duplication of a $2n = 28$ genome and the subsequent loss of two chromosomes. Allozyme electrophoresis was conducted to survey the levels and distribution of genetic diversity and to test the previous autopolyploid hypothesis for its origin. In addition, both *in situ* and *ex situ* conservation measures are proposed.
• *Methods* Eight populations were sampled for analysis by standard methods of starch gel electrophoresis, and six putative enzymatic loci were resolved (five consistently and one only partially).
• *Key Results* Banding patterns exhibited no evidence of fixed heterozygosity and showed both balanced and unbalanced heterozygotes. In addition, most individuals showed a pattern consistent with the presence of three or four alleles at a single locus. High levels of genetic variability were found at population level (P = 85 %, A = 3·0, $H_e = 0·422$), in addition to a trend of an excess of heterozygotes.
• *Conclusions* Allozyme data support the hypothesis that *T. loscosii* is an autotetraploid, and the high number of alleles at some loci may be due to repeated polyploidization events. The high values of genetic variation found in this species agree with those expected for tetraploids. The excess of heterozygotes may be due to some barriers to inbreeding (e.g. occurrence of gynodioecy) and/or selection for heterozygosity.

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Key words: Allozyme electrophoresis, genetic diversity, conservation, endemic species, tetraploidy, Lamiaceae, *Thymus loscosii*.

INTRODUCTION

Polyploidy is considered a significant mode of species formation and an important source of evolution in higher plants (Stebbins, 1980). In fact, estimates of the frequency of polyploidy in angiosperms vary from 30 % to 80 % (Otto and Whitton, 2000; Soltis and Soltis, 2000). The success of polyploidy can be explained by several factors such as increased heterozygosity and genetic diversity, enzyme multiplicity and increased biochemical diversity (Levin, 1983; Soltis and Soltis, 2000) and their establishment and commonness in nature may be favoured by vegetative (asexual) multiplication and a perennial habit (Otto and Whitton, 2000; Soltis and Soltis, 2000). Moreover, polyploids have a broader ecological amplitude than the parental diploids and a better colonizing ability, which allows for an increased availability of new ecological niches (Ramsey and Schemske, 1998; Soltis and Soltis, 2000).

Two major types of polyploidy are recognized in nature, allopolyploidy and autopolyploidy (Stebbins, 1947; Crawford, 1989; Soltis and Soltis, 2000), although these two terms represent the ends of a spectrum of variation. Allopolyploidy is thought to be the result of an interspecific hybridization process and subsequent chromosome duplication, while autopolyploidy is probably the consequence of polyploidization of conspecific individuals, generally by

fusion of non-reduced gametes. Nevertheless, both allo- and autopolyploids may also be formed via a triploid bridge, in which there takes place a union of a reduced and an unreduced gamete (Ramsey and Schemske, 1998). Unfortunately, because allopolyploidy and autopolyploidy are often equated with interspecific and intraspecific polyploidy, respectively, classification of polyploids may also depend on species circumscription (Ramsey and Schemske, 1998).

Allopolyploids are characterized by disomic inheritance, bivalent formation at meiosis and fixed heterozygosity (non-segregating) due to the combination of two divergent parental genomes. In contrast, autopolyploids are expected to express polysomic inheritance (tetrasomic in autotetraploids) and may also show multivalent formation at meiosis. Two types of heterozygotes may be formed: balanced and unbalanced.

Thymus loscosii Willk. (Lamiaceae) is endemic to the Ebro river basin in north-eastern Spain. The classification of *Thymus* has long been considered difficult (Jalas, 1972; Morales, 1986), and *T. loscosii* has been included in three different sections, namely *Serpyllum* (Miller) Bentham, *Hyphodromi* (A. Kerner) Hálacsy and *Thymus*. The inclusion in the latter is the most widely recognized today (Greuter *et al.*, 1986; Morales, 1986). *Thymus loscosii* is a tetraploid ($2n = 54$; Morales, 1986), presumably an autotetraploid, which probably originated by the duplication of a $2n = 28$ genome and the subsequent loss of two

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TABLE 1. *Populations of* Thymus loscosii *studied*

Population code	Location	Population size	Sample size
ULL	Ulldemolins (Catalonia, Spain), 31TCF2075	~100	32
ALB	Albarca (Catalonia, Spain), 31TCF2075	300–500	33
LGR	La Granadella (Catalonia, Spain), 31TCF0380	~200	33
VAL	Vallclara (Catalonia, Spain), 31TCF3283	>500	32
MON	Montagut (Catalonia, Spain), 31TCF6885	~100	32
LPE	La Palma d'Ebre (Catalonia, Spain), 31TCF0172	50–100	30
CHI	Chiprana (Aragon, Spain), 30TYL3868	~100	32
ELC	Elciego (Basque Country, Spain), 30TWN3205	~100	33

Location of populations is detailed by UTM 1×1 km squares.
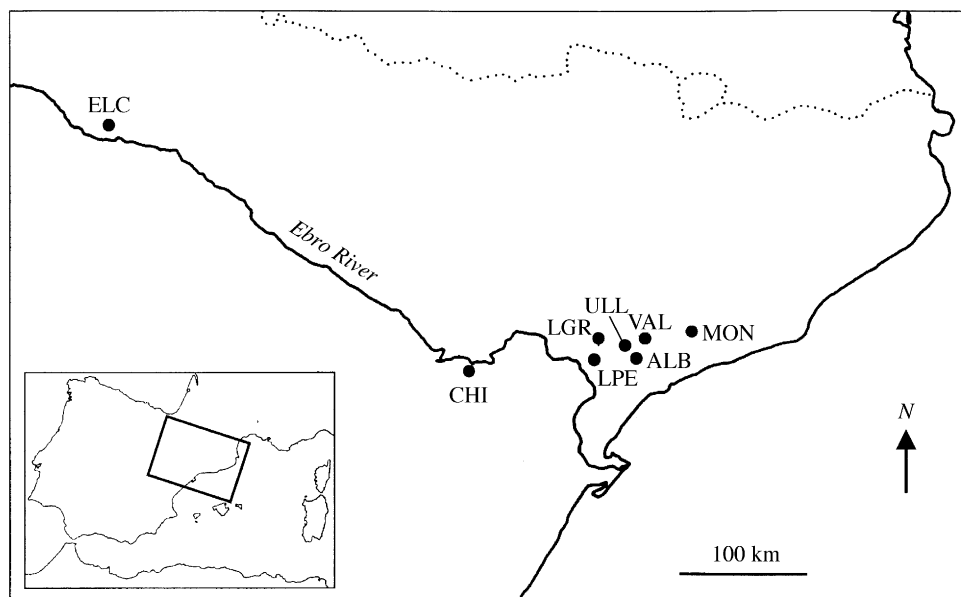

FIG. 1. Sampled populations of *Thymus loscosii*. ALB, Albarca, CHI, Chiprana, ELC, Elciego, LGR, La Granadella, LPE, La Palma d'Ebre, MON, Montagut, ULL, Ulldemolins, and VAL, Vallclara.

chromosomes, i.e. dysploidy ($2n = 4\times - 2$) (Morales, 1986). Morales (1986, 2002) has suggested a basic number in the genus *Thymus* of $x = 7$, which probably gave rise to the secondary basic numbers $x = 14$ and $x = 15$. Several studies report different ploidy levels in the same species, which indicates that polyploidization may occur frequently in this genus. Perhaps one of the most interesting cases is that of *Thymus herba-barona* Loisel., with $2n = 28$, 56 and 84 (Morales, 2002). In section *Thymus*, two ploidy levels (diploid and tetraploid) and five different numbers, $2n = 28$, 30, 54, 56 and 58, are found. According to Morales (1986), the tetraploid numbers (54, 56 and 58) have different origins: $2n = 56$ is probably derived from a duplication of a $2n = 28$ genome (i.e. autopolyploidy); $2n = 58$ may have originated from a hybridation of two taxa with $n = 14$ and $n = 15$ and a subsequent doubling of the chromosome number; and $2n = 54$ is probably derived from a $2n = 56$ plant which has lost two chromosomes (i.e. autopolyploidy followed by

dysploidy). The taxonomically closest species to *T. loscosii* is *T. zygis* Loefl. ex L. (Morales, 1986), which has three subspecies in the Iberian peninsula, two diploid (subsp. *zygis* and subsp. *gracilis*, both with $2n = 28$) and one probably autotetraploid (subsp. *sylvestris*, with $2n = 56$). Morales (1986) studied the meiosis of *T. carnosus* Boiss. ($2n = 56$), from the same section, and reported some tetravalents, which supports the hypothesis that the number $2n = 56$ is derived by autopolyploidy. Judging from its morphology, it is difficult to believe that *Thymus loscosii* should have arisen by hybridization and, accordingly, an allopolyploid origin of the species is not supported by morphological data (R. Morales, pers. comm.).

Thymus loscosii is a perennial woody plant, 9–10 (15) cm tall, with abundant stoloniferous branches. The inflorescences are composed of whorls of small, whitish, zygomorphic flowers, with seeds disposed in up to four nutlets of about 0·4 × 1·1 mm. The pollination in *T. loscosii* is

entomophilous, as in most *Thymus* species (Morales, 1986). The main pollinators are *Apis mellifera* and some species of *Bombylius*. It is gynodioecious, as for most species of *Thymus* (Morales, 1986; Manicacci *et al*., 1998). Despite being a self-compatible species, self-pollination is rare. The germination rate is low: 0 % reported by Morales (1986) and Sainz *et al*. (1996), 2 % by our group (Bosch *et al*., 2002). However, other researchers found higher rates under different temperature treatments, although with remarkable variation among populations (Albert *et al*., 2002). There is no active fruit dispersal; the nutlets are probably dispersed by gravity and/or surface water movements. *Thymus loscosii* might have a combined strategy of sexual and asexual reproduction, since vegetative propagation of stolons has been observed in the field. *Thymus loscosii* grows at 130–1010 m, in open sites in alkaline or saline loam substrates.

Populations of *T. loscosii* are located in several autonomous territories of Spain (Aragon, Castilla-León, Catalonia, Euskadi, La Rioja and Navarra), comprising 52 UTM 10 × 10 km squares (Blanché *et al*., 2000). The total number of individuals is probably greater than one million and the extent of occurrence is approx. 28 000 km². The species was included in the *Catálogo Nacional de Especies Amenazadas* (*National Catalogue of Endangered Species*) as endangered (Boletín Oficial del Estado, 1990), and at regional level is listed as rare in Aragon (Sainz *et al*., 1996), vulnerable in Navarra (Boletín Oficial de Navarra, 1997), and of special interest in Euskadi (Nekazal Ikerketa Eta Teknologia, 2002). However, the increasing number of new localities found in recent years has led to the exclusion of this species from the recent *Lista Roja de la Flora Vascular Española* (*Red List of the Vascular Spanish Flora*) (Aizpuru *et al*., 2000).

Allozyme electrophoresis was used to address the following issues: (*a*) whether there is evidence of autopolyploidy in *T. loscosii*, and (*b*) to describe the levels and distribution of genetic diversity. In addition, inferences are made about the status of conservation of this species together with some suggestions for strategies for its preservation.

MATERIALS AND METHODS

Sampling strategy

The study was focused in Catalonia (where six populations were sampled), but two additional populations were also sampled (one each from Aragon and Euskadi) to cover the entire geographic range of *Thymus loscosii* (Table 1 and Fig. 1). All populations were sampled in January–March 2001, and about 30 samples per population were collected. Sampling was conducted along a linear transect within each population and samples were collected about 50–100 cm apart to avoid collecting ramets from the same genet. Samples consisted of small fragments of branches which were placed into envelopes, transported to the laboratory, and stored at 4 °C until extraction 1 or 2 d later. Collection of samples was done carefully to minimize the potential damage to populations.

Electrophoresis

Genetic data were obtained through standard methods for starch gel electrophoresis of allozymes (Soltis *et al*., 1983; Wendel and Weeden, 1989). Leaves were detached from branches, and homogenized on refrigerated porcelain plates using a cold extraction buffer consisting of 0·011 M Tris–HCl, pH 7·6, 4 % sodium thioglycolate, 2 % polyethyleneglycol, and 8 % PVP-40 (polyvinyl-pyrrolidone). Analyses with an alternative extraction buffer (the composition is detailed in López-Pujol *et al*., 2001) were also performed but with poorer results. Extracts were absorbed onto 3 mm Whatman filter paper, and either analysed immediately or stored at –20 °C until analysis 1 or 2 d later. Using 12·5 % starch gels, 19 enzyme systems were assayed. 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; aconitate hydratase (ACO; EC 4.2.1.3) and phosphogluconate dehydrogenase (6-PGD; EC 1.1.1.44) were resolved on a morpholine buffer at pH 6·1 and phosphoglucomutase (PGM; EC 5.4.2.2) was resolved on histidine–citrate buffer 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 (Wendel and Weeden, 1989), but special attention was paid to phenotypes characteristic of tetraploids and to 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, i.e sister chromatids segregate into the same gamete, a phenomenon specific to autopolyploids and which is dependent on the amount of tetravalent formation and the proximity of the locus to the centromere (see Bever and Felber, 1992; Ronfort *et al*., 1998). It is not known whether double reduction takes place in *T. loscosii*, and it was assumed that only chromosomal segregation occurs. This consideration allows a more conservative data processing method to be used regarding the type of polyploid origin for this species (auto- or allopolyploid), since double reduction increases the production of homozygous gametes as compared with what is expected under random chromosomal segregation in diploids and allopolyploids (Ronfort *et al*., 1998). The mean fixation index (F) was also calculated for all variable loci in each population, to compare genotype proportions with those expected under the Hardy–Weinberg equilibrium. We used a chi-square (χ^2) test to evaluate deviations

Aat-1

aaaa	aaab	aabb	bbbb	bbbc	bbcc	bccc
N = 8	N = 1	N = 1	N = 234	N = 7	N = 3	N = 1

Dia-2

aaab	aabb	abbb	abbc	abcc	abcd	bbbb	bbbc	bbcc	bccc	bbcd	bccd	bcdd	bbbd	cccc
N = 1	N = 1	N = 5	N = 10	N = 1	N = 4	N = 31	N = 93	N = 32	N = 13	N = 44	N = 7	N = 1	N = 4	N = 7

6Pgd

aaab	aabb aabb	abbb	bbbc	bbcc	bccc
N = 8	N = 220	N = 14	N = 1	N = 2	N = 2

Pgi-2

aaaa	aaab	aabb	abbb	abbc	abcc	bbbb	bbbc	bbcc	bccc	bccd	bcdd
N = 3	N = 13	N = 30	N = 93	N = 25	N = 14	N = 19	N = 31	N = 11	N = 2	N = 2	N = 1

Pgm-2

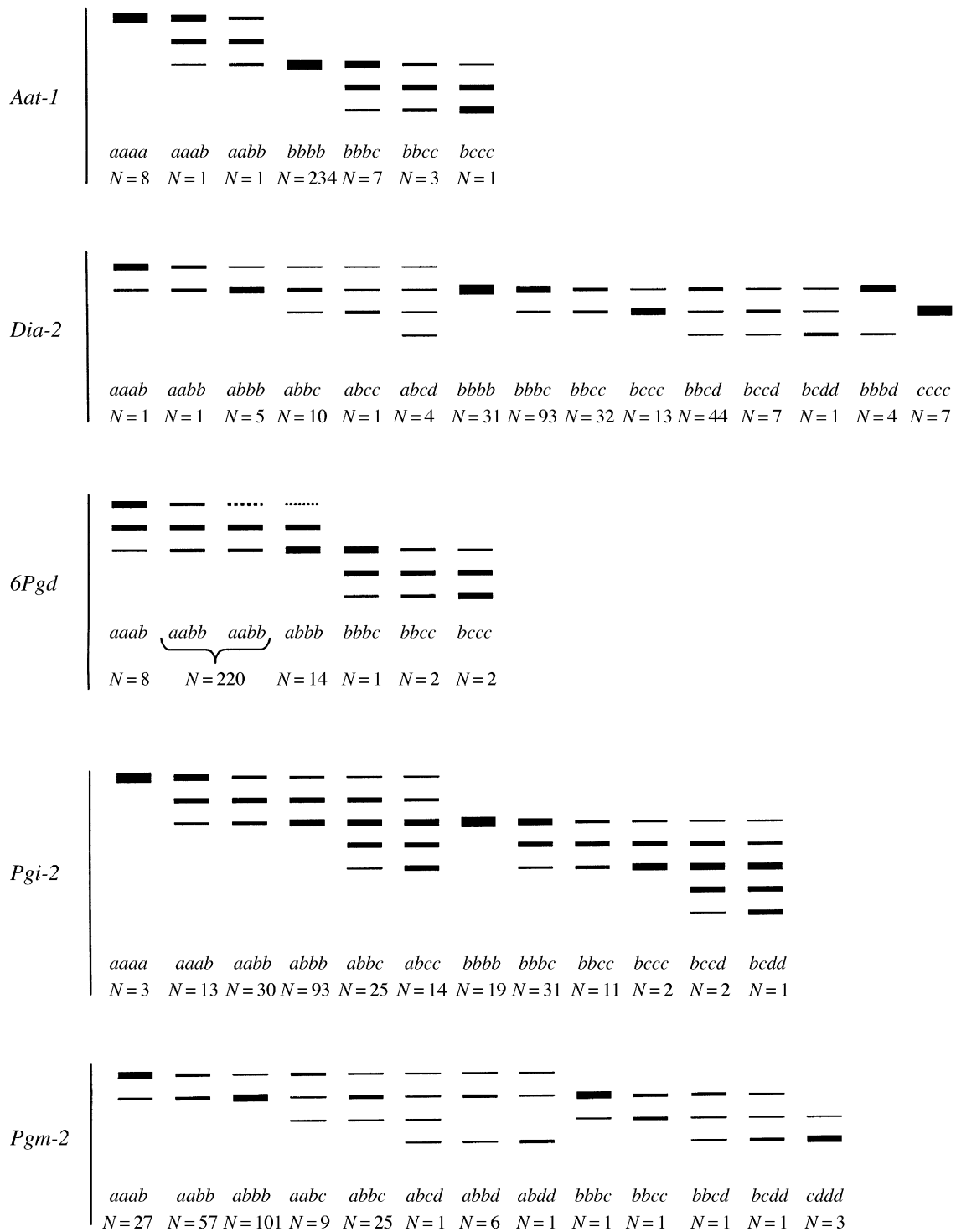| aaab	aabb	abbb	aabc	abbc	abcd	abbd	abdd	bbbc	bbcc	bbcd	bcdd	cddd
N = 27	N = 57	N = 101	N = 9	N = 25	N = 1	N = 6	N = 1	N = 1	N = 1	N = 1	N = 1	N = 3

FIG. 2. Schematic banding patterns obtained for the five resolved loci in *Thymus loscosii*. All the inferred genotypes and the number of individuals showing each genotype are given below each phenotype.

of F from zero. The partitioning of genetic diversity within and between populations was analysed using Nei's gene diversity statistics (Nei, 1973): 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 Wright's equation (Wright, 1951): $Nm = 1 - G_{ST}/4\ G_{ST}$. Nei's genetic identity (*I*) (Nei, 1978) was also calculated between pairs of populations. A program for analysis of

autotetraploid genotypic data, AUTOTET (Thrall and Young, 2000), was used for calculations of H_o, H_e and F. Employing the appropriate options for tetraploids, BIOSYS-1 (Swofford and Selander, 1989) was used for the calculation of P, while GeneStat version 3·31 (Whitkus, 1988) was used to calculate H_T, H_S, D_{ST} and G_{ST}. The mean number of alleles per locus (A) was calculated by hand because data was included for the partially resolved locus *Aco-1*.

RESULTS

Interpretation of enzyme banding patterns

There were considerable difficulties in resolving enzymes in *T. loscosii*, due to interference during the extraction from polyphenolic substances and volatile oils. Up to 100 preliminary experiments were needed to obtain acceptable resolution. Of the 19 enzymes assayed, five were satisfactorily resolved, each with one interpretable locus: *Aat-1*, *Dia-2*, *6Pgd*, *Pgi-2* and *Pgm-2* (Fig. 2). *Aco-1* was poorly stained on gels and allelic dosage in heterozygote phenotypes could not be determined, but the locus was used for calculation of A.

Banding patterns in AAT, 6-PGD and PGI were consistent with dimeric enzymes, and in ACO, DIA and PGM with monomeric enzymes. AAT displayed two regions of activity in *T. loscosii*, encoded by two independent putative loci: *Aat-1* and *Aat-2*. *Aat-1* was highly monomorphic, with the majority of individuals being homozygous for *Aat-1b* allele. Alleles *Aat-1a* and *Aat-1c*, although less frequent, were also present. The locus *Aat-2* was too faint to be interpretable, but appeared to be variable.

ACO presented two zones of activity, encoded by two different putative loci (*Aco-1* and *Aco-2*). *Aco-1* was too poorly stained to interpret the allelic dosage, but four different alleles were detected at this locus, *Aco-1b* and *Aco-1c* being the most common. Some analysed individuals showed patterns of three and four bands, which could be interpreted as possessing three and four different alleles for this enzyme. The locus *Aco-2* could not be interpreted due to the poor staining of the bands.

Two different regions of activity were identified for DIA, from which only the most cathodal was consistently interpreted (*Dia-2*). Four alleles were detected, with *Dia-2b* displaying the highest frequency. Single-banded homozygotes and several types of heterozygotes were observed at this locus. Heterozygous phenotypes were distinguished by the number of bands and/or the relative intensity of band staining. Thus, it was possible to observe two-banded heterozygotes, with balanced and unbalanced staining activity, three-banded phenotypes with unbalanced staining activity, and four-banded phenotypes with equally strong bands.

6-PGD exhibited a single zone of activity, encoded by only one putative locus (*6Pgd*). All studied individuals showed either a two-banded or a three-banded phenotype, which is not consistent with the dimeric structure of this enzyme. This pattern can be attributed to the inactivity of the dimer *aa* in some populations, since this band appeared in other populations but was poorly stained. Three-banded patterns are consistent with both balanced phenotypes (*aabb*), which were the majority of observed genotypes, and unbalanced heterozygotes (*aaab*, *abbb*), which were less frequent. In the Vallclara (VAL) population we also observed heterozygous phenotypes with the *6Pgdc* allele.

PGI showed two different regions of activity, encoded by two putative independent loci (*Pgi-1* and *Pgi-2*), of which only *Pgi-2* could be interpreted. Four different alleles were detected, *Pgi-2b* being the most common in all populations. Complex banding patterns with up to five bands were observed in some individuals, which are consistent with the expression of three different alleles. Five (not six) bands were observed due to the co-migration of a homodimeric band encoded by the 'intermediate' allele with the heterodimeric band formed between the 'fastest' and the 'slowest' allele. Both balanced and unbalanced phenotypes with three or five bands were observed, but homozygous phenotypes were also present.

PGM presented two zones of activity. Only the most cathodal region was interpretable, controlled by the putative locus *Pgm-2*. Analysed individuals showed four different alleles at this locus, *Pgm-2a* and *Pgm-2b* being the most common. Some phenotypes of three and four bands were found, which correspond to individuals possessing three and four different alleles at this locus.

Levels and distribution of genetic diversity

Among the six interpretable loci (including *Aco-1*) 22 alleles were detected, the frequencies of which are given in Table 2. The richest populations were ALB, VAL and ELC (all displaying 16 alleles); the poorest populations were ULL, LGR and LPE (all with 13 alleles). Only one population-specific allele was found in *T. loscosii*, at VAL (*6Pgd-c*). In contrast, rare alleles (alleles with a frequency of <0·05) were common to all populations, representing 8 % (LGR) to 31 % (ALB) of the total number of alleles. It was found that 37·2 % of all plants possessed three or four alleles for at least one of the loci examined, with a maximum percentage of 58 % at *Pgm-2* in the MON population. Genetic diversity was high in *T. loscosii*, with the mean values over all eight populations of P = 85 %, A = 3·0, and H_e = 0·422 (Table 3). The most variable population was LGR (H_e = 0·479), while the least diversity was found in CHI (H_e = 0·399); however, values of the standard deviation (Table 3) showed that differences between populations were not statistically significant.

Values of observed heterozygosity were higher than those of expected heterozygosity in all populations except LGR, the only population with a positive value of F (fixation index) (Table 3). At loci level, 15 F-values were in accordance with Hardy–Weinberg expectations ($P \geqslant$ 0·05), while 21 F-values differed significantly from zero ($P < 0.05$). Of these 21 values, four were positive and 17 were negative (Table 4). Positive values indicate deficiency of heterozygotes; negative values indicate excess.

Genetic diversity in *T. loscosii* was distributed mainly within populations (H_S = 0·429) rather than between them (D_{ST} = 0·015), i.e. genetic diversity attributable to

TABLE 2. *Allele frequencies for five loci in eight populations of* Thymus loscosii

Locus	Allele	ULL	ALB	LGR	VAL	MON	LPE	CHI	ELC
Aat-1	a	0·000	0·000	0·235	0·000	0·000	0·000	0·000	0·045
	b	**1·000**	**0·962**	**0·727**	**0·976**	**1·000**	**1·000**	**1·000**	**0·932**
	c	0·000	0·038	0·038	0·023	0·000	0·000	0·000	0·023
Dia-2	a	0·000	0·016	0·000	0·016	0·039	0·008	0·039	0·078
	b	**0·609**	**0·672**	**0·656**	**0·766**	0·297	**0·675**	**0·656**	**0·641**
	c	0·266	0·250	0·258	0·180	0·609	0·292	0·297	0·203
	d	0·125	0·062	0·086	0·039	0·055	0·025	0·008	0·078
6Pgd	a	**0·509**	**0·507**	0·485	0·427	0·492	0·475	0·500	0·476
	b	0·491	0·492	**0·515**	**0·484**	**0·508**	**0·525**	0·500	**0·523**
	c	0·000	0·000	0·000	0·089	0·000	0·000	0·000	0·000
Pgi-2	a	0·274	0·288	0·280	0·336	0·298	0·339	0·089	0·069
	b	**0·653**	**0·614**	**0·636**	**0·594**	**0·645**	**0·536**	**0·661**	**0·698**
	c	0·072	0·091	0·083	0·070	0·056	0·125	0·234	0·224
	d	0·000	0·007	0·000	0·000	0·000	0·000	0·016	0·009
Pgm-2	a	0·342	0·281	0·393	0·208	0·298	0·400	**0·577**	**0·537**
	b	**0·639**	**0·687**	**0·607**	**0·617**	**0·540**	**0·583**	0·388	0·361
	c	0·009	0·016	0·000	0·050	0·137	0·017	0·034	0·102
	d	0·009	0·016	0·000	0·125	0·024	0·000	0·000	0·000

The most frequent allele for each locus and population is indicated in bold.

TABLE 3. *Summary of genetic variation for five loci in eight populations of* Thymus loscosii

Population	N	P	A^*	H_o	H_e	F
ULL	30·2	80·0	2·83	0·491 (0·279)	0·402 (0·226)	−0·222
ALB	32·6	80·0	3·33	0·497 (0·249)	0·407 (0·189)	−0·221
LGR	29·0	100	2·83	0·469 (0·239)	0·479 (0·038)	0·022
VAL	31·4	80·0	3·17	0·439 (0·246)	0·418 (0·222)	−0·051
MON	31·4	80·0	3·00	0·470 (0·281)	0·425 (0·242)	−0·104
LPE	29·6	80·0	2·50	0·479 (0·070)	0·408 (0·232)	−0·175
CHI	31·2	80·0	3·00	0·452 (0·266)	0·399 (0·223)	−0·132
ELC	30·6	100	3·33	0·476 (0·246)	0·438 (0·178)	−0·086
Mean	30·7	85·0	3·00	0·472	0·422	−
s.d.		9·2	0·28	0·019	0·026	−

N, sample size; P, percentage of polymorphic loci; A, mean number of alleles per locus; H_o, observed heterozygosity; H_e, expected panmictic heterozygosity; F, mean fixation index.

Standard deviation in parentheses.

* Locus *Aco-1 is* included in its calculation.

interpopulation differentiation was low (G_{ST} = 0·033) (Table 5). Gene flow was high (Nm = 7·32), indicating a substantial interchange of genes between populations. Values for Nei's genetic identity (I) (Nei, 1978) were very high between pairs of populations (mean = 0·973, range 0·929–1·000; Table 6).

DISCUSSION

Autopolyploidy in Thymus loscosii

Morales (1986) hypothesized that *Thymus loscosii* is an autotetraploid. This hypothesis was based on chromosome numbers (as presented in the Introduction), and phylogenetic studies. Furthermore, according to R. Morales (pers. comm.) it may be inferred from its morphology that *T. loscosii* did not arise from hybridization of the potential diploid parentals, e.g. *T. zygis* or *T. vulgaris*.

To verify the hypothesis of an autopolyploid origin, extensive cytological (i.e. karyological meiotic analyses) and inheritance studies (allozyme progeny tests) would be needed. However, cytological analyses are technically extremely difficult in *Thymus*, due to the very small size of the chromosomes (cf. Morales, 1986), and inheritance studies would also be difficult due to low germination rates.

Allozyme data are extremely useful in distinguishing between autopolyploids and allopolyploids (Soltis and Rieseberg, 1986; Crawford, 1989). Tetrasomic inheritance in autotetraploids results in the formation of unbalanced as well as balanced heterozygotes in all possible combinations. In contrast, allotetraploids are expected to display fixed heterozygosity, at least at some loci at which loci on homoeologous chromosomes are fixed for different alleles. For example, when two alleles (*a* and *b*) are present at a locus in an autotetraploid, three types of heterozygotes can be produced, one balanced (*aabb*) and two unbalanced

TABLE 4. *Values of fixation index* (F) *for all polymorphic loci in eight populations of* Thymus loscosii

Locus	ULL	ALB	LGR	VAL	MON	LPE	CHI	ELC
Aat-1	–	0·238**	0·854***	0·204ns	–	–	–	0·492ns
Dia-2	−0·248**	−0·254***	−0·230**	0·013ns	0·057**	−0·199ns	0·077ns	−0·001ns
6Pgd	−0·299***	−0·324***	−0·314***	−0·084***	−0·301***	−0·303***	−0·303***	−0·305***
Pgi-2	−0·113*	−0·129*	0·078ns	−0·150ns	0·027ns	−0·043*	−0·065ns	0·007ns
Pgm-2	−0·223***	−0·256**	−0·148ns	0·015***	−0·192*	−0·179ns	−0·217ns	−0·179ns

Conformance to Hardy–Weinberg equilibrium was tested using chi-square analysis: ns, $P \geqslant 0.05$; * $P < 0.05$; **, $P < 0.01$;***, $P < 0.001$.

TABLE 5. *Gene diversity statistics (Nei, 1973) for five loci in eight populations of* Thymus loscosii

Locus	H_T	H_S	D_{ST}	G_{ST}
Aat-1	0·099	0·084	0·014	0·146
Dia-2	0·528	0·497	0·031	0·059
6Pgd	0·511	0·517	0·000	0·000
Pgi-2	0·531	0·520	0·010	0·019
Pgm-2	0·552	0·528	0·024	0·043
Mean	0·444	0·429	0·015	0·033
s.d.	0·193	0·193	0·012	0·056

H_T, Total genetic diversity; H_S, genetic diversity within populations; D_{ST}, genetic diversity between populations; G_{ST}, proportion of total genetic diversity between populations.

TABLE 6. *Matrix of Nei's genetic identity (Nei, 1978) between populations of* Thymus loscosii

Populations	ULL	ALB	LGR	VAL	MON	LPE	CHI	ELC
ULL	–							
ALB	1·000	–						
LGR	0·991	0·992	–					
VAL	0·994	1·000	0·976	–				
MON	0·968	0·957	0·938	0·932	–			
LPE	1·000	1·000	0·988	0·995	0·961	–		
CHI	0·978	0·971	0·967	0·953	0·941	0·985	–	
ELC	0·972	0·965	0·968	0·950	0·929	0·975	1·000	–

(*aaab* and *abbb*), which can be differentiated on zymograms by the intensity of band staining. Such a pattern would only be possible in an allopolyploid if the locus was heterozygous for *a* and *b* on both pairs of homoelogous chromosomes.

Allozyme data support the hypothesis that *T. loscosii* is an autotetraploid. No evidence of fixed heterozygosity was found in *T. loscosii*. Except for *6Pgd* and *Pgm-2*, all loci showed both homozygotes and heterozygotes (balanced and unbalanced). The *6Pgd* and the *Pgm-2* loci displayed only heterozygous phenotypes, but these were both balanced and unbalanced. Support for autopolyploidy was also given by genetic diversity data, as discussed below.

Levels of diversity in autopolyploids

The high levels of heterozygosity found in autotetraploids may also be a consequence of tetrasomic inheritance (Soltis and Soltis, 2000), because this mode of inheritance would reduce the effects of population bottlenecks and genetic drift. The literature provides us with several examples of higher levels of heterozygosity in autotetraploid plants than in their diploid counterparts: *Tolmiea menziesii* Torr. & Gray ($H_o = 0.237$ in tetraploid populations and $H_o = 0.070$ in diploid ones; Soltis and Soltis, 1989), *Heuchera micrantha* Dougl. ($H_o = 0.150$ in tetraploid and $H_o = 0.075$ in diploid; Ness *et al.*, 1989), *Heuchera grossulariifolia* Rydb. ($H_o = 0.159$ in tetraploid and $H_o = 0.058$ in diploid; Wolf *et al.*, 1990), *Dactylis glomerata* L. ($H_o = 0.43$ in tetraploid and $H_o = 0.17$ in diploid; Soltis and Soltis, 1993), *Rutidosis leptorrhinchoides* F. Muell. ($H_o = 0.34$ in tetraploid and $H_o = 0.22$ in diploid; Brown and Young, 2000), *Vaccinium oxycoccos* L. ($H_o = 0.213$ in tetraploid and $H_o = 0.067$ in diploid; Mahy *et al.*, 2000) and *Centaurea jacea* L. ($H_o = 0.54$ in tetraploid and $H_o = 0.29$ in diploid; Hardy and Vekemans, 2001), among others. Judging from allozyme banding patterns, no population in the examined material of *T. loscosii* appeared to be diploid. Accordingly, only its levels of heterozygosity ($H_o = 0.472$) can be compared, although cautiously, with the closely related diploid species *T. vulgaris* ($H_o = 0.295$; Tarayre and Thompson, 1997).

The low number of loci resolved in *T. loscosii* illustrates the problems raised when allozyme studies are addressed to polyploid, woody and aromatic species, which are traditionally considered to be difficult (P. Arús, pers. comm.). To exemplify this, only four loci were interpretable in the analysis of the population structure of the closely related *Thymus vulgaris* L. (Tarayre and Thompson, 1997).

Genetic diversity in Thymus loscosii

Tetraploidy allows for the presence of three or four alleles at a single locus since loci are duplicated (Soltis and Rieseberg, 1986; Soltis and Soltis, 1989; Mahy *et al.*, 2000). Compared with diploids, more enzyme variants and an increased biochemical diversity may contribute to the success of polyploids in nature (Levin, 1983). In *T. loscosii*, 37·2 % of all plants possessed three or four alleles for at least one of the examined loci, a value close to that obtained for an autotetraploid cytotype of *Tolmiea menziesii* (39 %; Soltis and Soltis, 1989), and higher than that reported for *Vaccinium oxycoccos* (12·1 %; Mahy *et al.*, 2000). It is possible that the high number of alleles at some loci may be due to repeated polyploidization events, as discussed, e.g. for the grass *Dactylis glomerata* (Lumaret, 1988).

TABLE 7. *Genetic diversity in* Thymus loscosii *and in other autotetraploid species*

Taxa	P	*A*	H_o	H_e	Reference
Aster kantoensis Kitam.	36·9	1·53	–	0·142	Maki *et al.*, 1996
Centaurea jacea L.	–	3·54	0·54	0·38	Hardy and Vekemans, 2001
Dactylis glomerata L.	80·0	2·36	0·43	–	Soltis and Soltis, 1993
Heuchera grossulariifolia Rydb.	31·0	1·55	0·159	–	Wolf *et al.*, 1990
Heuchera micrantha Dougl.	38·33	1·64	0·150	–	Ness *et al.*, 1989
Rutidosis leptorrhynchoides F. Muell.	98·0	3·2	0·34	0·36	Brown and Young, 2000
Swainsona recta A.T. Lee	–	4·3	0·24	0·42	Buza *et al.*, 2000
Thymus loscosii Willk.	85·0	3·0	0·472	0·422	This study
Tolmiea menziesii Torr. & Gray	40·8	1·5	0·237	–	Soltis and Soltis, 1989
Turnera ulmifolia var. *elegans* Urb.	65·3	2·03	0·42	0·27	Shore, 1991
Turnera ulmifolia var. *Intermedia* Urb.	20·1	1·20	0·07	0·04	Shore, 1991
Vaccinium oxycoccos L.	38·9	1·66	0·213	–	Mahy *et al.*, 2000

If a species has both diploid and tetraploid populations, values of diversity given here are only for tetraploid populations.
All values given here are population means.
P, Percentage of polymorphic loci; *A*, mean number of alleles per locus; H_o, observed heterozygosity; H_e, expected panmictic heterozygosity.

The expected tetrasomic inheritance leads to high levels of genetic diversity in autotetraploids, as described by values of *A*, P and heterozygosity (Soltis and Soltis, 1989). High values of genetic variation in *T. loscosii* are within the range of other autotetraploids (Table 7). As observed in the field, *T. loscosii* can undergo vegetative propagation through stolons, which can maintain genetic variation, once produced, within populations. This phenomenon has already been reported in other species, such as *Populus tremuloides* Michx. (Cheliak and Dancik, 1982), *Erythronium albidum* Nutt. and *E. propullans* A. Gray (Pleasants and Wendel, 1989), *Iris cristata* Aiton (Hannan and Orick, 2000) and *Delphinium montanum* DC. (Simon *et al.*, 2001).

In populations of autotetraploid species, equilibrium frequencies under random mating are reached after several generations, not in a single generation as for randomly mating diploid species (Bever and Felber, 1992). This can theoretically result in deviations from Hardy–Weinberg equilibrium (Mahy *et al.*, 2000). Negative values for the fixation index were found at most loci and most populations of *T. loscosii*, indicating an excess of heterozygotes compared with Hardy–Weinberg expectations. The *6Pgd* and the *Pgm-2* loci displayed only heterozygous phenotypes in all examined populations. A negative fixation index could be explained by: (*a*) selection against homozygote survival promoting high heterozygosity; (*b*) random stochastic events; (*c*) barriers to inbreeding; and (*d*) sampling error. The excess of heterozygotes detected in *T. loscosii* might be explained by some barriers to inbreeding, e.g. ocurrence of gynodioecy, together with a marked protandry and low rates of selfing (Bosch *et al.*, 2002). However, other reasons cannot be discarded, as for example selection for heterozygosity. Alternatively, vegetative propagation may have contributed to the maintenance of the heterozygote excess, but the extent to which vegetative reproduction occurs in *T. loscosii* needs further study.

Distribution patterns of genetic diversity within and among populations are primarily determined by genetic flow, genetic drift and natural selection. Only a minimal fraction of allozyme variation in *T. loscosii* (around 3 %) is due to differences between populations. This pattern is also expressed as a high value of gene flow (7·32), which is theoretically high enough to prevent divergence by genetic drift (Wright, 1951). However, judging from other species of *Thymus*, which are characterized by small pollen and seed dispersal distances (Tarayre and Thompson, 1997; Eriksson, 1998), gene flow should also be restricted in *T. loscosii*. Instead, the very high genetic identities found between populations and the high value of *Nm* may be explained by recent and rapid fragmentation from a wide, continuous area resulting in genetically similar populations. Chorological research in recent years has revealed the existence of many additional populations, which may indicate that *T. loscosii* formerly had a continuous distribution along the Ebro river basin. This area has been extensively replanted with forest during the last decades (Blanco *et al.*, 1997), and open sites at which the species could grow have been lost. In addition, this area is currently experiencing a fragmentation process caused by change of land use, e.g. the extensive plantation of new vineyards in the westernmost part of the distribution area (Nekazal Ikerketa Eta Teknologia, 2002, and field observations by J. López-Pujol). Fragmentation may result in genetic impoverishment (Barrett and Kohn, 1991; Ellstrand and Elam, 1993), but polyploids may be less sensitive since genetic diversity is maintained by genome duplications.

Conservation

The optimal measures for species preservation are those *in situ*. The conservation of a species in its habitat allows for the maintenance of interactions between the species and its ecosystem without detaining evolutionary processes (Falk and Holsinger, 1991). Protection of habitat is generally limited to the protection of selected populations of species. Using the formula proposed by Ceska *et al.* (1997), $1 - (G_{ST})^n$ (where *n* is the number of populations), conservation of only two populations allows us to preserve 99·9 % of genetic diversity found in *T. loscosii*, due to the high genetic

similarity between populations. Nevertheless, given the variation of G_{ST} among loci, this may be an underestimate of genetic diversity detected in this species. Moreover, when choosing the populations to preserve, other considerations must also be taken into account, for instance large populations and populations with more intermediate allele frequencies must be favoured in order to reduce the probability of loss of alleles (Martínez-Palacios *et al.*, 1999). For example, the combination of VAL and ALB populations contains 21 of the 22 detected alleles, and they are the largest sampled populations. However, if population LGR is also preserved (i.e. the three largest populations), then all alleles would be conserved. Collection of seeds for maintenance in germplasm banks as an *ex situ* strategy does not seem to be an appropriate alternative to conservation policy because of the low germination rates.

Apart from genetic criteria, two additional types of polymorphism may be considered: (1) chemical diversity and (2) the frequency of females within populations. Chemical diversity may play a role in adaptation to environment, as suggested in *T. vulgaris* (Thompson *et al.*, 1998) and, if this also applies to *T. loscosii*, all chemotypes must be conserved. At present, only a single population of *T. loscosii* has been surveyed for phytochemical diversity (Molero and Rovira, 1983).

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