

Microsatellites reveal substantial among-population genetic differentiation and strong inbreeding in the relict fern *Dryopteris aemula*

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- **Background and Aims** A previous study detected no allozyme diversity in Iberian populations of the buckler-fern *Dryopteris aemula*. The use of a more sensitive marker, such as microsatellites, was thus needed to reveal the genetic diversity, breeding system and spatial genetic structure of this species in natural populations.
- **Methods** Eight microsatellite loci for *D. aemula* were developed and their cross-amplification with other ferns was tested. Five polymorphic loci were used to characterize the amount and distribution of genetic diversity of *D. aemula* in three populations from the Iberian Peninsula and one population from the Azores.
- **Key Results** Most microsatellite markers developed were transferable to taxa close to *D. aemula*. Overall genetic variation was low ($H_T = 0.447$), but was higher in the Azorean population than in the Iberian populations of this species. Among-population genetic differentiation was high ($F_{ST} = 0.520$). All loci strongly departed from Hardy–Weinberg equilibrium. In the population where genetic structure was studied, no spatial autocorrelation was found in any distance class.
- **Conclusions** The higher genetic diversity observed in the Azorean population studied suggested a possible refugium in this region from which mainland Europe has been recolonized after the Pleistocene glaciations. High among-population genetic differentiation indicated restricted gene flow (i.e. lack of spore exchange) across the highly fragmented area occupied by *D. aemula*. The deviations from Hardy–Weinberg equilibrium reflected strong inbreeding in *D. aemula*, a trait rarely observed in homosporous ferns. The absence of spatial genetic structure indicated effective spore dispersal over short distances. Additionally, the cross-amplification of some *D. aemula* microsatellites makes them suitable for use in other *Dryopteris* taxa.

Key words: Cross-amplification, *Dryopteris aemula*, ferns, inbreeding, genetic bottleneck, glacial refugium, microsatellites, population differentiation, spatial genetic structure.

INTRODUCTION

Microsatellites are tandem repeats of two to six nucleotides found in most nuclear genomes (Tautz, 1989; Chambers and MacAvoy, 2000). Since their discovery over three decades ago and with the rise of polymerase chain reaction (PCR) technology, microsatellite markers have progressively become the current molecular marker of choice for population genetic studies. Their popularity is based on their ubiquity across most organisms, their single-locus co-dominant inheritance, and their high variability resulting from high mutation rates (Tautz, 1989; Sunnucks, 2000; Selkoe and Toonen, 2006). Microsatellites are thus a valuable tool for detecting population genetic structure, evaluating genetic diversity, testing parentage relationships and interpreting recent population history (Zhang and Hewitt, 2003). The widespread use of microsatellites is, however, largely confined to vertebrates and seed plants; they remain largely unexploited in organisms such as lichens, algae, lycophytes and ferns.

Homosporous ferns have aroused the interest of researchers due to their capacity for long-distance dispersal and their exceptional life cycle. Their haploid spores germinate into potentially bisexual gametophytes, which are able to self-fertilize via intragametophytic selfing and to produce

completely homozygous sporophytes. Intragametophytic selfing enables homosporous ferns to found new populations from a single spore. Although these populations harbour little genetic diversity during their first generations, recurrent wind dispersal of spores over long distances may lead to additional gene flow among populations. Fern populations are therefore viewed as exhibiting low genetic differentiation, with most variation being intrapopulational (Soltis and Soltis, 1989), in agreement with expectations for long-lived plant species with potential for long-range gene movement (Hamrick and Godt, 1989). Most genetic studies on ferns have used allozyme electrophoresis (Ranker and Geiger, 2008). However, the low allelic diversity usually found at allozyme loci limits their applicability in population genetic studies as compared with microsatellites (Estoup *et al.*, 1998; Degen *et al.*, 1999), especially in the case of recently founded or bottlenecked populations (Quintanilla *et al.*, 2007). Recently, a few researchers have applied microsatellites to populations of ferns (Pryor *et al.*, 2001; Vitalis *et al.*, 2002; Woodhead *et al.*, 2005; Kang *et al.*, 2008) and demonstrated that these markers are useful for the characterization of genetic diversity and structure of populations of ferns.

Dryopteris aemula, a diploid buckler-fern, is considered to be a relict from the tropical flora that covered the

Mediterranean area during the Tertiary (Pichi-Sermolli, 1979). Its present distribution comprises oceanic refugial areas along the coasts of western Europe, some islands of the Macaronesian archipelagos, north-eastern Turkey and south-western Transcaucasia. It is listed as a vulnerable species in Spain because of the rarity of its habitat, i.e. deciduous, temperate oceanic forests at low altitude close to the sea (Bañares *et al.*, 2004). The production and response to antheridiogen, a pheromone released by female or hermaphrodite gametophytes promoting maleness in nearby asexual gametophytes, suggests that *D. aemula* may be mainly outbreeding (Jiménez *et al.*, 2008). Additionally, a previous survey found no genetic variation at 13 allozyme loci in three populations spanning the distribution area of *D. aemula* in the Iberian Peninsula (Jiménez *et al.*, 2009). Genetic bottlenecks and founder effects as the consequence of cycles of local extinction and recolonization from Macaronesian refugia during Pleistocene glaciations are a possible cause of the lack of allozyme diversity in the Iberian populations of this species. The use of more variable genetic markers is necessary to ascertain whether the lack of allozyme diversity in *D. aemula* is reflected in other genomic regions than the allozyme coding genes. The present study reports on eight newly developed nuclear microsatellite markers isolated from *D. aemula* and checks for their cross-amplification in other fern taxa including hybrids and polyploids incorporating the 'aemula' parental genome. Using five of these polymorphic microsatellite loci, the following hypotheses were then tested: (1) overall genetic variation is low, as indicated by allozymes, and higher in the Azores than in the Iberian Peninsula, because this archipelago acted as a refugium during glaciations; (2) among-population genetic differentiation is low due to long-distance gene flow via spore dispersal; (3) genotype frequencies within populations are in Hardy–Weinberg equilibrium, as a consequence of antheridiogen-mediated outcrossing; and (4) genetic variation within populations is not spatially structured because of effective spore dispersal and outcrossing.

MATERIALS AND METHODS

Sampling design

Four *Dryopteris aemula* (Ait.) Kuntze populations were sampled, three in the northern Iberian Peninsula (SAN, MUT and EUM), and a fourth in São Miguel Island, Azores (AZO, Table 1). Individuals were randomly sampled in all populations except in EUM, which was selected to study the spatial genetic structure within a population. Here, all individuals present along a 30 × 6-m transect were sampled, and the

position of each individual was mapped. One or two pinnules per individual were collected and directly dried in silica-gel until DNA extraction.

Microsatellite development and genotyping

DNA was extracted with the DNeasy 96 Plant Kit (Qiagen, Hilden, Germany). Genomic DNA of one *D. aemula* individual sampled from SAN was used by Ecogenics (Zurich, Switzerland) to make an enriched library from size-selected genomic DNA ligated into TspADshort/TspADlong-linker (Armour *et al.*, 1994) and enriched by magnetic bead selection with biotin-labelled (GT)₁₃, (CT)₁₃, (AAG)₁₀ and (TAC)₁₀ oligonucleotide repeats (Gautschi *et al.*, 2000a, b). Of 378 recombinant colonies screened, 59 gave positive signals after hybridization. Plasmids from these 59 positive clones were sequenced and primers were designed for 18 microsatellite inserts with PRIMER3 (Rozen and Skaletsky, 2000). Eight consistently amplifying microsatellite loci (Dae04, Dae05, Dae06, Dae07, Dae09-1, Dae09-2, Dae11 and Dae15; Table 2) were tested for cross-amplification in 13 *Dryopteris* species and hybrids of various ploidy levels (*D. oligodonta*, *D. oreades*, *D. affinis* subsp. *affinis*, *D. carthusiana*, *D. corleyi*, *D. crispifolia*, *D. dilatata*, *D. filix-mas*, *D. guanchica*, *D. × arecesiae*, *D. × asturiensis*, *D. × fraser-jenkinsii*, *D. × madalena*), three other Dryopteridaceae species (*Polystichum lonchitis*, *P. setiferum* and *P. aculeatum*) and two Woodsiaceae species (*Gymnocarpium dryopteris* and *Athyrium filix-femina*). Five of these loci (Dae06, Dae07, Dae09-2, Dae11 and Dae15) were finally used to genotype all *D. aemula* individuals sampled. As primer pair Dae09 seemed to amplify two loci, with two distinct amplification products consistently appearing 7–39 bp apart, the presence of an AG microsatellite in Dae09-2 was proven by sequencing of PCR products cut from 2% agarose gels using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

Forward primers of each primer pair were labelled with a fluorescent dye (6FAM, NED, PET or VIC; Applied Biosystems). Multiplexed PCR amplifications were performed with 20–40 ng of genomic DNA using the Multiplex PCR kit (Qiagen) in final reaction volumes of 10 µL. PCR profiles consisted of 15 min of initial denaturation at 95 °C, followed by 30 cycles of 94 °C for 30 s, 56–62 °C (depending on primer pair; Table 2) for 90 s and 72 °C for 60 s, and a final elongation step at 72 °C for 30 min. PCR products were separated on an ABI 3130 Avant Automated Sequencer (Applied Biosystems), and fragment sizes were determined with GENEMAPPER 3.7 (Applied Biosystems) using the internal size standards 400HD ROX™ for primer pair Dae15 and 500 LIZ® (Applied Biosystems) for all other primer pairs.

TABLE 1. Populations studied with five microsatellite loci in *Dryopteris aemula*

Acronym	Locality	Latitude (N)	Longitude (W)	Altitude (m a.s.l.)	Number of individuals
SAN	Spain, Asturias, Santianes del Agua	43°25'	5°20'	50	49
MUT	Spain, Gipuzkoa, Mutriku	43°18'	2°24'	90	50
EUM	Spain, A Coruña, Fragas do Eume	43°23'	8°00'	350	74
AZO	Portugal, Azores, São Miguel Island	37°48'	25°14'	950	56

TABLE 2. Characterization of eight microsatellites for *Dryopteris aemula*

Locus	GenBank accession number	Repeat motif	Size range (bp)	Primer sequences (5'–3')	T_a (°C)
Dae04	FJ455846	(AAG) ₁₆	111	F: GCCTCCTTGAAGGTCTACCC R: CCCCCGAAAAGAGTGTATG	56
Dae05	FJ554839	(CTT) ₈ (CTA) (CCT) (CTT) ₃	102–108	F: ATGGTCACCTTCGACCTTTG R: TAACCGACCATGAGTCCCTTG	59
Dae06	FJ455847	(TC) ₂₁	107–117	F: TGGCAAAAATAGAGAGAGGCAAC R: ATCAGGCGGCCAGTAGGAG	59
Dae07	FJ455848	(TG) ₂₂	77–101	F: GTACTACGCGGCCACAAG R: CGTGAGCTTCTGTGAAGAGC	59
Dae09-1	FJ455849	(AG) ₁₃	95–97	F: CTTTGCAGGCGGCATAGC R: AGTTGACAAGAATACTCGGATGC	56
Dae09-2	FJ455849	(AG) ₂₆	102–134	F: CTTTGCAGGCGGCATAGC R: AGTTGACAAGAATACTCGGATGC	56
Dae11	FJ455850	(AG) ₂₁	151–157	F: TCAACTCCTTGTAAGCCCAAG R: CCGTGGACGGTAATAAACTACTC	56
Dae15	FJ455851	(AG) ₂₇	106–140	F: TATGTTGGTCTGCGTGTGC R: AAACCATCAGTAGCCTCGACA	62

GenBank accession number, repeat motif, size range in base pairs, annealing temperature (T_a), and forward (F) and reverse primer (R) sequences are given for each locus.

Statistical analyses

Levels of genetic diversity were assessed based on the average number of alleles per locus (A) and, to allow comparison with allozyme studies, overall total heterozygosity (H_T). Genetic differentiation among populations was assessed with F_{ST} (Weir and Cockerham, 1984). Population structure was also studied by estimating R_{ST} , an analogue of F_{ST} developed for microsatellite loci mutating under the stepwise mutation model (Slatkin, 1995). The number of reproductively successful migrants per generation (Nm) was estimated using the private allele method (Barton and Slatkin, 1986). The observed heterozygosity (H_o), expected heterozygosity (H_e) under Hardy–Weinberg equilibrium, and Wright's (1943) fixation index $F = 1 - H_o/H_e$ were calculated for each locus in each population. Concordance of genotype frequencies with Hardy–Weinberg equilibrium was tested using chi-squared tests. Linkage disequilibrium was tested among all pairs of loci and its significance was determined after applying sequential Bonferroni correction (Rice, 1989). All statistics except H_T and R_{ST} were estimated with GENEPOP 4.0 (Rousset, 2008). H_T was calculated according to Nei (1973), and R_{ST} was estimated with FSTAT 2.9.3.2 (Goudet, 2002). The spatial autocorrelation of genotypes in population EUM was explored using Moran's I in SGS (1000 permutations; Degen et al., 2001) taking into consideration the four loci polymorphic in this population and ten distance classes of 3 m width between 0 and 30 m.

RESULTS

Microsatellite development and cross-taxa amplification

Of the 18 primer pairs tested in *D. aemula*, seven failed to amplify the target microsatellites and four amplified several fragments, whereas seven amplified products consistently amenable to interpretation. One of these primer pairs (Dae04) amplified monomorphic products, whereas the other six (Dae05, Dae06, Dae07, Dae09, Dae11 and Dae15) amplified polymorphic products (Table 2). Primer pair Dae09 amplified two microsatellite loci (Dae09-1 and Dae09-2), which

showed fragments varying in size according to an AG-microsatellite.

Transferability to other *Dryopteris* species and hybrids, especially those including an 'aemula' parental genome, was feasible for most microsatellites isolated from *D. aemula* (see Supplementary Data Table S1, available online). Transferability to *Polystichum* species was limited and there was no cross-amplification to *Gymnocarpium* or *Athyrium*. Locus Dae04, which was monomorphic in *D. aemula*, showed polymorphisms in other species.

Overall genetic variation

Total genetic variation considering all loci was $H_T = 0.447$. Overall, the Azorean population (AZO) showed a higher microsatellite variation than the three Iberian populations (EUM, MUT and SAN (Supplementary Data Table S2)). The mean number of alleles per locus (A) in AZO, EUM, MUT and SAN was 5.0, 2.6, 2.2 and 2.0, respectively, with an average of 3.0. In these populations, the number of private alleles was 13, one, three and two, respectively.

Among-population genetic structure

Genetic differentiation was high ($F_{ST} = 0.520$), and the value of R_{ST} (0.409) was lower than that of F_{ST} , although still high. In consequence, the estimated number of migrants per generation was low ($Nm = 0.25$).

Hardy–Weinberg equilibrium and linkage disequilibrium

All loci significantly departed from Hardy–Weinberg equilibrium in all populations, exhibiting a remarkable deficit of heterozygotes as shown by the positive fixation indexes (F), which ranged from 0.650 to 1.000 (Table 3) and had a value of 0.855 averaged over all loci and populations. Loci pairs Dae09-2/Dae11 and Dae11/Dae15 were in linkage disequilibrium ($P < 0.05$ after sequential Bonferroni correction) in

TABLE 3. Observed (H_o) and expected (H_e) heterozygosity as well as fixation index (F) for four *Dryopteris aemula* populations from the Iberian Peninsula (SAN, MUT, EUM) and Azores (AZO)

Population	Locus	H_o	H_e	F
SAN	Dae06	0	0	–
	Dae07	0	0	–
	Dae09-2	0.122	0.95	0.795***
	Dae11	0.061	0.414	0.853***
	Dae15	0.102	0.484	0.789***
MUT	Dae06	0	0	–
	Dae07	0	0.04	1.000***
	Dae09-2	0	0	–
	Dae11	0.02	0.134	0.851***
	Dae15	0.02	0.170	0.882***
EUM	Dae06	0.27	0.153	0.824***
	Dae07	0.014	0.040	0.650***
	Dae09-2	0	0	–
	Dae11	0	0.027	1.000***
	Dae15	0.054	0.581	0.907***
AZO	Dae06	0.071	0.385	0.816***
	Dae07	0.089	0.604	0.853***
	Dae09-2	0.054	0.444	0.878***
	Dae11	0.089	0.400	0.778***
	Dae15	0.018	0.336	0.946***

Asterisks indicate significant deviation from Hardy–Weinberg equilibrium; a dash indicates the locus is monomorphic within a population.

some but not all populations: loci Dae09-2 and Dae11 were linked in SAN, and loci Dae11 and Dae15 were linked in SAN and MUT.

Within-population genetic structure

In population EUM, all values of mean Moran's I per distance class fell within the confidence interval of the correlogram, indicating no significant spatial autocorrelation at any distance class. Hence, there was no spatial genetic structure within this population.

DISCUSSION

Microsatellite development and cross-taxa amplification

Only seven of 18 originally designed primer pairs for *D. aemula* consistently amplified interpretable products, a result comparable to the attrition rates reported by Squirrell *et al.* (2003) for primer optimization steps. Primer pair Dae09 amplified two putatively independent loci, Dae09-1 and Dae09-2, and it appeared that Dae09-2 was a duplicated locus. This locus duplication was probably a consequence of repeated polyploidization and gene silencing cycles in the evolution of genus *Dryopteris*, as reported formerly for many ferns (Nakazato *et al.*, 2008).

The microsatellite loci developed for *D. aemula* were largely transferable to other species and hybrids of the genus and, to some extent, to other Dryopteridaceae. Product amplifications were more successful in taxa which incorporated at least one 'aemula' parental genome, indicating that the flanking regions of these microsatellites were largely conserved. The microsatellites presented herein thus have the potential

to be successfully used in taxa that are phylogenetically close to *D. aemula*.

Overall genetic variation

In agreement with our first hypothesis, total genetic variation was low ($H_T = 0.447$). Even if the value of H_T is similar to those reported for ferns in allozyme studies (Gitzendanner and Soltis, 2000), microsatellites are likely to show much higher values due to their high allelic variability (Hedrick, 1999; Woodhead *et al.*, 2005). In the Iberian populations, the maximum number of microsatellite alleles per locus was four (Supplementary Data Table S2), which indicates an overall low genetic diversity as compared with other microsatellite-based studies on species with restricted and scattered distribution ranges (Salgueiro *et al.*, 2003; Mitrovski *et al.*, 2007; Kang *et al.*, 2008). Population AZO showed a maximum of seven microsatellite alleles per locus and was polymorphic for all five microsatellite loci studied, whereas populations SAN, MUT, and EUM were polymorphic for only a portion of them. In addition, the number and proportion of private alleles in population AZO was also larger than in the Iberian populations, and a large number of the alleles present in the Iberian populations were shared with population AZO (Supplementary Data Table S2). These results generally reinforce the hypothesis of a strong genetic bottleneck in the Iberian populations of *D. aemula* due to a recent founder effect, as suggested by the absence of allozyme diversity of this species in the Iberian Peninsula (Jiménez *et al.*, 2009). Rather than the persistence of *D. aemula* in Iberian refugia during Pleistocene glaciations (Hewitt, 1996; Quintanilla *et al.*, 2007), the higher genetic diversity of population AZO suggests that the Macaronesian archipelagos acted as a glacial refugium from which *D. aemula* spread and recolonized mainland Europe. This hypothesis is not only congruent with the higher genetic diversity of population AZO and with the fact that a high proportion of the alleles were shared among Azorean and Iberian populations, but also with the finding that the identity of the most frequent allele differed among Iberian populations for loci Dae09-2, Dae11 and Dae15, as expected under a scenario of repeated but independent colonization events (Schneller and Holderegger, 1996).

Among-population genetic structure

In marked disagreement with our second hypothesis, among-population genetic differentiation was very high, as shown by the values of both F_{ST} and R_{ST} (0.520 and 0.409, respectively). Population differentiation is intimately linked to the frequency and magnitude of gene flow among populations (Loveless and Hamrick, 1984), which, in ferns, takes place in the form of spore dispersal. Homosporous ferns have remarkable potential for long-distance dispersal and colonization, as illustrated by the fact that they are an important component of oceanic island floras (Muñoz *et al.*, 2004; Ranker and Geiger, 2008) and by their low among-population variation in both genetic and phenotypic markers (Sciarretta *et al.*, 2005; Schneller and Liebst, 2007). Given the high microsatellite differentiation among the four *D. aemula* populations studied, the corresponding number of migrants per

generation is low ($Nm = 0.25$). Theory predicts that, in the long run, a migration rate of 1.0 is generally needed to offset drift-mediated population differentiation (Spieth, 1974), and empirical studies suggest that even higher values of Nm are necessary to counteract drift (Lacy, 1987; Mills and Allendorf, 1996).

High population differentiation is well in agreement with the above presented colonization scenario of *D. aemula* on the Iberian Peninsula. However, other studies on ferns attributed high genetic population differentiation to the action of inbreeding. Substantial genetic differentiation among homo-spore fern populations has, for instance, been observed in *Alsophila spinulosa* (Su et al., 2004), which has functionally bisexual, self-fertilizing gametophytes, as well as in the polyploid taxa *Asplenium septentrionale*, *A. ruta-muraria* and *Polypodium vulgare* (Schneller and Holderegger, 1996), which are expected to self-fertilize due to polyploidy. Therefore, inbreeding (see below) might have contributed to high genetic population differentiation in *D. aemula*.

Hardy–Weinberg equilibrium and linkage disequilibrium

Our third hypothesis, that populations of *D. aemula* are in Hardy–Weinberg equilibrium, can be rejected. All loci departed from Hardy–Weinberg equilibrium in all populations and inbreeding coefficients were high, exhibiting heterozygote deficits. Several factors such as allelic dropout or null alleles can bias results obtained with microsatellites towards the inference of homozygous genotypes (Selkoe and Toonen, 2006). However, the substantial deviation from Hardy–Weinberg equilibrium found in *D. aemula* across loci and populations indicated rather that our results most likely point to inbreeding. In addition, the linkage disequilibrium detected may also be a consequence of successive generations of inbreeding, which lead to the fixation of different multilocus genotypes within populations (Loveless and Hamrick, 1984).

The marked tendency to inbreeding observed in natural populations of *D. aemula* contrasts with the high outbreeding rates displayed by most diploid ferns (Ranker and Geiger, 2008), and also with the substantial tendency to outcrossing shown by other diploid *Dryopteris* species such as *D. oreades* (Jiménez et al., 2008). The inbreeding coefficient detected in *D. aemula* ($F = 0.855$) approaches those found in *Botrychium* species, which have subterranean gametophytes that obligately self-fertilize via intragametophytic selfing (McCauley et al., 1985; Hauk and Haufler, 1999). Female gametophytes of *D. aemula* and other *Dryopteris* species produce antheridiogens that inhibit growth and favour maleness both within and among species (Jiménez et al., 2008). Most authors consider that antheridiogens promote outcrossing as they increase the probability of cross-fertilization between adjacent gametophytes by facilitating dioecy (e.g. Haig and Westoby, 1988; Hamilton and Lloyd, 1991). Reduction of intragametophytic selfing due to antheridiogens may confer a strong fitness advantage, given that zygotes produced by this breeding system are homozygous at all loci and, consequently, all deleterious recessive alleles are expressed. The present results are not consistent with this adaptive interpretation of antheridiogens, as *D. aemula* is mostly inbreeding. They are, instead, compatible with an alternative hypothesis of Willson

(1981), who proposed that antheridiogens are allelopathic substances secreted by females to stunt the growth of unrelated individuals. This interpretation, which considers antheridiogens as a mechanism for competition among gametophytes, holds for both outbreeding and inbreeding species.

Within-population genetic structure

In agreement with our fourth hypothesis, no significant spatial structure of genetic variation was observed in population EUM. However, this result conflicts with the results for highly inbred populations, which often present genetically structured populations (Loveless and Hamrick, 1984; Soltis et al., 1989). In ferns, within-population genetic structure has been found both in inbreeding (e.g. *Asplenium trichomanes* subsp. *quadrialeans*; Suter et al., 2000) and in outbreeding taxa (e.g. *Cheilanthes gracillima*; Soltis et al., 1989). Spore dispersal ability and other ecological factors may be more important than breeding system in determining the genetic structure of fern populations (Cousens et al., 1988; Soltis et al., 1989; Pryor et al., 2001). The lack of genetic structure in the studied *D. aemula* populations suggests that in its habitat, i.e. deciduous forest understorey, microsites suitable for spore germination and gametophyte development are randomly distributed in space, and that there is effective mixing of selfed genotypes by spore dispersal. Regardless, we cannot completely rule out the possibility that the absence of spatial genetic structure in population EUM is partly a consequence of using microsatellites, as high mutation rates can reduce the estimates of spatial structure in populations (Epperson, 2005).

CONCLUSIONS

Microsatellites revealed low levels of genetic diversity in *D. aemula*, as often observed in bottlenecked populations. Iberian populations harboured a lower number of alleles across loci than the Macaronesian population studied, which may be a consequence of post-glacial long-distance founder effects. Restricted gene flow among populations within the scattered distribution of *D. aemula* on the Iberian Peninsula maintained a strong genetic differentiation among populations, an effect reinforced by high inbreeding in this fern. Inbreeding is inferred from the substantial heterozygote deficits. This finding suggests that the ultimate role of the antheridiogen pheromone is not the promotion of outcrossing but competition avoidance via growth inhibition in nearby individuals. Homogeneity of habitats as well as effective spore dispersal over smaller distances caused, in spite of inbreeding, a lack of spatial genetic structure within a population of *D. aemula*.

The microsatellite loci reported here can potentially be used to design conservation strategies for the endangered *D. aemula*, to evaluate genetic diversity in other *Dryopteris* species or to clarify parental identities among diploid and polyploid members of this genus. Given the advantages of using microsatellites, we believe that this marker type will help in understanding the importance of evolutionary factors shaping population genetic variation and structure in ferns and other groups of organisms.

SUPPLEMENTARY INFORMATION

Supplementary data are available online at www.aob.oxfordjournals.org and consist of two tables. Table S1 shows the cross-amplification and fragment size of *D. aemula* microsatellites in 18 other fern taxa and hybrids. Table S2 shows the allele frequencies of five microsatellite loci in four *D. aemula* populations from the Iberian Peninsula and Azores.

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