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Mating System in *Blechnum spicant* and *Dryopteris affinis* ssp. *affinis* Correlates with Genetic Variability

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Abstract.—The monilophytes *Blechnum spicant* (L.) Sm. and *Dryopteris affinis* ssp. *affinis* (Lowe) Fraser-Jenkins show different reproductive strategies under *in vitro* conditions. While *B. spicant* exhibits asexual and sexual reproduction, with an antheridiogen system promoting outcrossing, *D. affinis* ssp. *affinis* reproduces only asexually through apogamy. Individuals of several populations of these species, collected in Principado de Asturias (Spain), were analyzed to test the influence of their mating system in the genetic variability displayed by each species. This study shows that the genetic diversity assessed in populations of each species collected *in situ* is in concordance with that expected according to their reproductive system previously observed *in vitro*. The genetic diversity found in *D. affinis* ssp. *affinis* was low and most of genetic variation can be attributed to differences among localities. This result indicates high fixation of the detected alleles within each locality, as expected for a clonal reproductive system. In the sexual species *B. spicant* the genetic diversity was higher. Our results confirm the importance of reproduction system in the genetic diversity present in populations of these fern species making essential to consider the definition and study of reproductive system and the associated consequences in the design of successful conservation actions.

Key Words.—AFLP, *Blechnun spicant*, clonal growth, *Dryopteris affinis*, sexual reproduction

The formation of a new fern sporophyte can be achieved through asexual or sexual pathways. During asexual reproduction, cells other than gametes develop a sporophyte through an asexual process without meiosis or fertilization called apogamy. The fern complex *Dryopteris affinis* (Lowe) Fraser-Jenkins includes diploid and triploid subspecies, all of them apogamic (Fraser-Jenkins, 1980). Apogamy has been reported to be a way of escaping hybrid sterility by alloploids (Fraser-Jenkins, 1980; Chao *et al.*, 2012). On the other hand, the mating system of the fern species *Blechnum spicant* (L.) Sm. implies the development of gametophytes that produce male and female gametes in the sexual organs, archegonia and antheridia, which form a sexual embryo through fertilization.

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Gametophytes are relatively easy to growth under *in vitro* controlled conditions (Fernández and Revilla, 2003) allowing a detailed study of their developmental process. Apogamous sporophyte formation during *in vitro* cultivation of *Dryopteris affinis* ssp. *affinis* has been described (Fernandez *et al.*, 1996; Menendez *et al.*, 2006). Gametophyte development has also been described under *in vitro* conditions for *Blechnum spicant* (Fernandez *et al.*, 1997).

Although self-fertilization is common in many fern species, both inbreeding and outcrossing schemes are possible. To promote outcrossing, some homosporous fern species present morphological barriers to prevent self-fertilization, such as asynchronous maturation time of gametophytes. Maturing female gametophytes produce antheridiogens that induce the younger, adjacent gametophytes to develop into male gametophytes both *in vivo* and *in vitro* culture conditions (Klekowski, 1969; Cousens, 1979; Fernández *et al.*, 1997; Menéndez *et al.*, 2006) promoting by this way genetic exchange. Ranker and Houston (2002) enquired whether gametophyte sexuality in the laboratory would be a good predictor of the mating system in nature, finding that *in vitro* culture gametophyte studies would lead to the same main conclusions than those performed on field collected ones. Although some species present mixed mating systems, mating strategies in fern species vary from mainly self-fertilization to obligate intergametophytic crossing, in a bimodal fashion (Soltis and Soltis, 1990, 1992). Recent results show that mixed mating systems in the same species are more frequent than previously reported, with high selfing capacity as a strategy for long dispersal and colonization (de Groot *et al.*, 2012).

The breeding system has been reported as an important determinant of genetic variation in plants (Hamrick and Godt, 1996). Outcrossers usually present higher polymorphic loci or number of genotypes per population than inbreeders (Holsinger, 2000). High genetic diversity has been observed in homosporous pteridophytes with high degree of outcrossing (Maki and Asada, 1998; Kang *et al.*, 2005). Genetic uniformity within populations was found in ferns whose gametophytes reproduce through intragametophytic selfing as *Asplenium csikii* (Vogel *et al.*, 1999). Also intragametophytic selfing has been reported as a trait that could be linked to invasiveness and successful colonization of new habitats (Lott *et al.*, 2003). Asexual reproduction, on the other hand, has been associated with low genetic variability by decreasing the effective population size in species such as *Ceratopteris pteridoides* (Chen *et al.*, 2010).

In this study, the genetic variability present in natural populations of two ferns representing asexual and sexual mating systems, *D. affinis* ssp. *affinis* and *B. spicant,* was assessed using AFLPs to test the repercussions of the reproductive biology on their genetic variability.

Materials and Methods

Sample collection and DNA extraction.—Two fern species characterized by different reproductive systems were selected for the present study, *Blechnum spicant* (L.) Sm. and *Dryopteris affinis* ssp. *affinis* (Lowe) Fraser-Jenkins. For

each species, plants growing in three separated localities were analyzed (Table 1). To avoid problems of repetition of samples due to the possible re-sampling of the same individual (i.e. rhizome mediated asexual reproduction), a safety distance was kept between each selected individual for sampling (Table 2). The number of samples used in the genetic fingerprinting analysis varied from four to ten plants per population (Table 1). Samples were individually stored at −80°C until DNA extraction.

Molecular analysis.—Total genomic DNA of each individual plant was extracted using a DNeasy Plant mini kit (Qiagen Ltd, Crawley, UK). AFLP analysis was performed according to Peredo *et al.* (2008). For each sample, AFLP analysis was performed using MseI and EcoRI digests. The pre-selective and selective amplifications were conducted using standard AFLP cycling parameters including a touch-down selective PCR. The primers used for the pre-selective amplification were EcoRI + A, and MseI + C, while for the selective amplification the primers used were *Eco* AGC/*Mse* CAT, AGC/CTT, AGA/CAT, AGA/CTT, AAC/CTT, ACC/CAT, and ACC/CTT. After PCR, samples were denatured by adding an equal volume of formamide buffer (98% formamide, 10 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol, pH 8.0) and heated for 3 min at 94 °C. Aliquots (6 ul) of each sample were loaded on 6% polyacrylamide gels (acrylamide/bisacrylamide; 19:1) containing 7.5 M urea and 1 x TBE, and then electrophoresed for 2 h. A standard silver staining protocol was used to reveal the bands: 20 min in fixing solution 1 (acetic acid 1% ethanol 10%), 3 min in fixing solution 2 (nitric acid 1.5%), 20 min in impregnate solution (silver nitrate 2 g/L), and developed in sodium carbonate (30 g/L) supplemented with 0.27 ml/L formaldehyde. The gel was rinsed in deionized water after each step.

Data analysis.—The presence/absence of AFLP bands was determined by visual inspection and scored as 1/0. Only the bands showing a clear and easily detectable signal were taken into account.

A first evaluation of the data was performed by scoring the number of detected loci per primer combination in each population and species. The percentage of monomorphic loci was evaluated for primer combination and population in all the analyzed samples with or without including singletons. A singleton is the presence of a locus alteration in a single individual, that is, the presence or absence of a band. The correction was applied to minimize the possible error due to scoring or other technique related mistakes. Additional precautions were taken to minimize the technical error such as a duplicate DNA extraction for each individual, which were treated as independent samples through all the molecular procedures to assess data repetitivity. All the data used in this study present less than one discrepancy per each 50 loci (over 95% repetitivity).

Estimates of genetic diversity within populations, including percentage of polymorphic loci, Shannon's index (Shannon and Weaver, 1949), and Nei's gene diversity assuming random mating (Nei, 1973), were calculated using the software POPGENE version 1.31 (Yeh and Boyle, 1997).

TABLE 1. List of sampled sites for *Blechnum spicant* (B) and *Dryopteris affinis ssp.affinis* (D) populations.

Species	Population	Sampling site	UTM coordinates (30T)	Lat/Lon Coordinates	Individuals (n)	Habitat	Date
Blechnum spicant	B 1	Nueva, river Nueva	0342868 4810718	43° 25′ 58.327″ N 4° 56′ 29.279″ W	10	Alder forest (*Alnus glutinosa*)	15/1/2009
	B 2	Novales river	0366566 4805620	43° 23′ 29.706″ N 4° 38′ 51.171″ W	10	Alder forest	
	B 3	Road to Purón	0362878 4806340	43° 23′ 50.639″ N 4° 41′ 35.684″ W	10	Shadow slope, heath	
Dryopteris affinis* ssp. *affinis	D 8	Nueva, river Nueva	0342852 4810789	43° 26′ 0.615″ N 4° 56′ 30.064″ W	4	Alder foresta	12/3/2009
	D 9	Gobiendes	0319177 4814827	43° 27′ 52.154″ N 5° 14′ 7.228″ W	4	Alder forest	
	D 10	Purón river, road to Purón	0362944 4805766	43° 23′ 32.083″ N 4° 41′ 32.233″ W	5	Alder forest	

TABLE 2. Distances (km) among different sampling sites of *Blechnum spicant* (B) and *Dryopteris affinis ssp.affinis* (D).

B	B1	B2	B3	D	D8	D9	D10
B1	-			**D8**	-		
B2	24.21	-		**D9**	24.48	-	
B3	20.43	3.8	-	**D10**	20.54	45.33	-

Data were analyzed with the program STRUCTURE (Pritchard et al, 2000), which employs a model-based Bayesian clustering procedure to determine the number of divergent groups (K) in a dataset. Ten independent runs were undertaken with K varying from 1 to 5. Each run was performed with 30,000 MCMC (Markov Chain Monte Carlo) repetitions following a burn-in of 30,000 iterations. The analysis used no prior information, assumed correlated allele frequencies and both admixture and no admixture. Posterior probabilities were calculated for each value of K, and the estimated ΔK (Evanno *et al.*, 2005) was used to choose the optimal K value. Following the analysis by STRUCTURE, data were also subjected to a standard analysis of molecular variance (AMOVA) using ARLEQUIN version 3.2 (Excoffier *et al.*, 2005) to assess the level of differentiation within and among different groupings of populations defined "a posteriori". A principal coordinate analysis (PCoA) was conducted on a similarity matrix using the Dice's index [$djk = 2M / (2M+N)$. where M is the number of matches and N is the total number of columns with presence in just one row] constructed from the primary binary dataset using the program PAST (version 1.54) (Hammer *et al.*, 2006). In addition, cluster analysis was performed on a matrix of distance values between individuals using paired group algorithm for binary data (presence/absence) with Dice distance as implemented in the PAST program (bootstrapped 1,000 times). To confirm the reliability of the groupings, trees were calculated for each primer combination and for the total detected loci.

RESULTS

Estimates of genetic diversity.—In the case of *Blechnum spicant*, 11 primer combinations of those tested were selected for the study based on their repetitivity. A total of 623 loci was detected (Table 3), and the number of loci per primer combination ranged from 35 (EAGAMCAT) to 85 (EACCMCTT) with a mean of 56.5±15.7. The mean percentage of monomorphic loci detected per primer combination in all the populations was 17.6% (ranging from 4.7% to 36.6%). To minimize the effect of possible artifacts that might lead to an overestimation of the polymorphisms, an additional calculation was done; the correction consisted in not considering singletons. The mean percentage of monomorphic sites increased to 26.32% (6.1–45.1%). The percentages were also calculated for each population: B1 39.17% (25.9–60.8%); B2 54.25% (26.4–79.2%) and B3 42.54% (11.6–69%).

TABLE 3. Genetic indexes, Nei's (HE) and Shannon's (I), and percentage of polymorphism (%P) calculated from AFLP analysis for *Blechnum spicant* (B1, B2, B3, total in grey) and *Dryopteris affinis* ssp *affinis* (D8, D9, D10, total in grey) using POPGENE version 1.31 (Yeh and Boyle 1997).

AFLP primers (EcoRI/ MseI)	*Blechnum spicant*												
		HE				*I*				*%P*			
	N loci	B1	B2	B3	B	B1	B2	B3	B	B1	B2	B3	B
AAC/CAT	72	0,18	0,10	0,10	0,19	0,28	0,16	0,16	0,30	54,17	29,17	30,56	63,89
AAC/CTT	53	0,19	0,07	0,13	0,25	0,28	0,11	0,20	0,37	49,06	20,75	37,74	69,81
AAC/TCC	72	0,19	0,23	0,22	0,27	0,30	0,36	0,34	0,42	59,72	73,61	70,83	94,44
ACC/CAT	42	0,23	0,21	0,21	0,25	0,36	0,33	0,33	0,40	73,81	71,43	71,43	88,10
ACC/CTT	85	0,24	0,21	0,21	0,26	0,37	0,32	0,33	0,40	72,94	65,88	70,59	91,76
ACG/CAT	51	0,13	0,08	0,14	0,16	0,20	0,12	0,22	0,27	41,18	27,45	50,98	72,55
ACT/CAT	43	0,17	0,09	0,27	0,21	0,27	0,13	0,40	0,35	58,14	27,91	76,74	88,37
AGA/CAT	35	0,16	0,13	0,14	0,19	0,24	0,18	0,21	0,29	48,57	28,57	37,14	60,00
AGA/CTT	63	0,18	0,09	0,21	0,24	0,29	0,14	0,31	0,37	58,73	30,16	57,14	84,13
AGA/TCC	65	0,23	0,24	0,22	0,34	0,35	0,36	0,33	0,51	67,69	72,31	67,69	95,38
AGG/CTT	42	0,22	0,08	0,13	0,18	0,34	0,13	0,20	0,29	69,05	28,57	40,48	71,43
TOTAL	623	0,20	0,15	0,18	0,24	0,30	0,22	0,28	0,37	60,03	45,75	56,50	81,38
	Dryopteris affinis ssp *affinis*												
	n loci	D8	D9	D10	D	D8	D9	D10	D	D8	D9	D10	D
AAG/CAT	107	0,15	0,05	0,01	0,15	0,22	0,07	0,01	0,25	34,58	11,21	1,87	56,07
AAG/CTT	85	0,31	0,02	0,01	0,20	0,44	0,03	0,01	0,32	68,24	4,71	2,35	74,12
AAC/CAT	70	0,05	0,11	0,05	0,21	0,07	0,15	0,08	0,31	11,43	24,29	12,86	57,14
AAC/CTT	44	0,05	0,17	0,06	0,16	0,08	0,25	0,09	0,25	13,64	38,64	15,91	54,55
AAC/TCC	50	0,00	0,15	0,05	0,22	0,00	0,22	0,07	0,33	0,00	34,00	12,00	66,00
ACC/CAT	62	0,06	0,01	0,04	0,14	0,08	0,01	0,06	0,21	12,90	1,61	11,29	38,71
ACC/CTT	63	0,01	0,00	0,00	0,05	0,01	0,00	0,00	0,07	1,59	0,00	0,00	12,70
ACC/TCC	33	0,03	0,00	0,01	0,11	0,04	0,00	0,02	0,16	6,06	0,00	3,03	27,27
AGA/CTT	71	0,21	0,03	0,00	0,20	0,30	0,04	0,01	0,32	46,48	7,04	1,41	71,83
AGC/TCC	71	0,21	0,01	0,03	0,18	0,30	0,02	0,04	0,29	49,30	2,82	7,04	66,20
TOTAL	656	0,13	0,05	0,02	0,16	0,18	0,07	0,04	0,26	28,66	11,43	6,10	54,73

For *Dryopteris affinis* ssp. *affinis*, 10 primers were selected using the same criterion detailed above. The total number of loci was 656, mean 65.6±20.97 ranging from 33 to 103. The mean percentage of monomophic loci detected in all the pooled populations was 46.4% (25.9 to 87.3%) and increased to 61.7% (42.1 to 88.9%) when singletons were not taken into account. The average percentages calculated for each *Dryopteris* population were: D8 75.3% (31.8–100%); D9 85.8% (61.4–100%), D10 92.5% (84.1–100%).

Two genetic indexes, Nei's (HE) and Shannon's (I), were calculated for each primer combination in each population and per species. Instead of calculating the average for the indexes, they were recalculated using total detected loci per primer combination and in total (Table 3). The total HE for *B. spicant* was 0.24 ranging from 0.16 (EACGMCAT) to 0.34 (EAGAMTCC), whereas in *D. affinis* ssp. *affinis* it was 0.16 (0.05–0.21). In the case of I, 0.37 (0.27–0.51) was

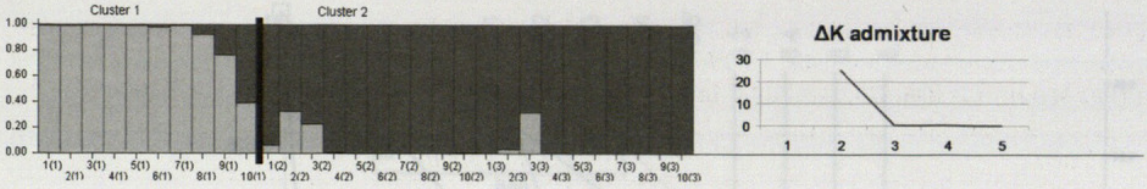

FIG. 1. Left: Bar plot of the proportion of an individual's of *Blechnum spicant* genome belonging to one or other cluster inferred by the STRUCTURE analysis. Cluster 1 is represented in green and includes individuals from B1 (Nueva) sampled site while cluster 2 is represented in red and includes individuals from sampled sites B2 (Novales) and B3 (Purón). Right: Plot of ΔK for each K value calculated as described in Evanno *et al.* (2005).

calculated for *Blechnum* and 0.26 (0.07–0.33) for *Dryopteris*. HE and I calculated for each population were: B1 0.20, 030; B2 0.15, 0.22; B3 0.18, 0.28; D8, 0.13, 0.18; D9 0.05, 0.07, and D10 0.02, 0.04 (consult Table 3 for a detailed list).

To test the existence of significant differences between the HE, I, and percentage of polymorphism (%P) values calculated for each species, the Mann-Whitney U test and Krustal-Wallis test were applied. Firstly, the suitability of the data to these tests was corroborated by checking whether the data fit to a normal distribution. To eliminate the possibility of variation due to other causes than the species as a source for the differences in the genetic variation (e.g. primer combinations chosen or population related variation) several tests were performed prior the comparison species to species. No significant differences were detected in any of the comparison within *D. affinis* ssp. *affinis* (data not shown), whereas for *B. spicant* statistically significant differences (%P $p = 0.03$) were found only in the percentage of polymorphism calculated for each primer combination. No significant differences were found using the same approach (Krustal-Wallis test) to the values calculated within each species.

The existence of differences between the values of heterozygosity (HE), Shannon index (I), and percentage of polymorphism (%P) in *D. affinis* ssp. *affinis* and *B. spicant* was tested using two-tailed Mann-Whitney U test. Significant differences were detected in each comparison: HE $p<0.0001$ (Monte Carlo $p<0.0001$); I $p<0.0001$ (Monte Carlo $p<0.0001$); and %P $p<0.0001$ (Monte Carlo $p<0.0001$).

Genetic structure in Blechnum spicant *and* Dryopteris affinis.—The Bayesian-clustering was applied to the datasets as implied in the STRUCTURE program assuming an admixture model, with the assumption of the possibility of mixed ancestry of the individuals. In *B. spicant*, Evanno's ΔK indicate the possibility of a $K=2$ grouping as the value for $\Delta K = 2$ was 25.4 and low values were calculated for $\Delta K > 2$ (<0.5) (Fig. 1). In each of the 10 replicates calculated for $K=2$ individuals from the locality B1 (Nueva) grouped in cluster 1 with a high probability (>0.904) while individuals from B2 (Novales) and B3 (Road to Puron) belonged to cluster 2 (probability over 0.928 and 0.958, respectively). This high degree of similarity among the individuals from populations B2 and B3 was also confirmed by the detected grouping in the

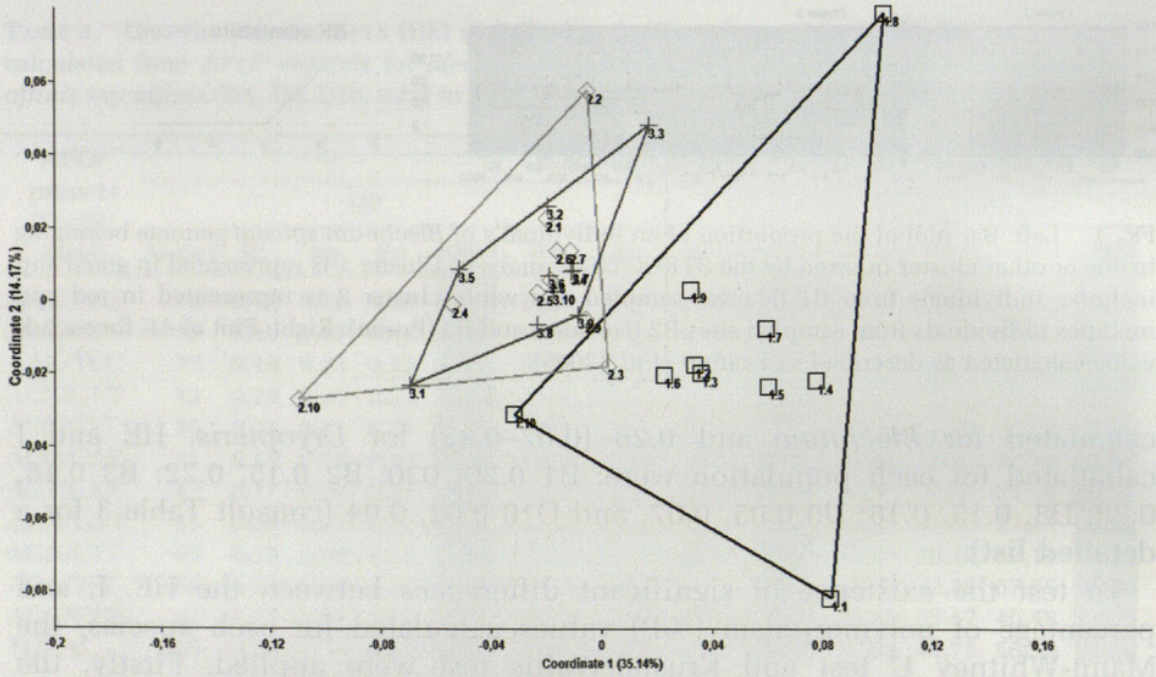

FIG. 2. Plot of principal coordinates 1 vs. 2 calculated using Dice's distance. Blue squares represent individuals from B1, green rhombus from B2, and red crosses from B3.

Principal Coordinates analysis (Fig. 2). Specific Fst values calculated for each sampled site were B1=0.26, B2=0.28 and B3=0.27. The *a posteriori* groupings of the sampling sites tested included an only group enclosing the three sampling sites, and two groups exploring the possible groupings (B1 vs B2 and B3; B1 and B2 vs B3; and B1 and B3 vs B2). No further statistical significance was found of population grouping detected by STRUCTURE (B1 vs B2 and B3) probably due to the amount of variation (over 60%) attributed to differences within populations.

For the *Dryopteris affinis* ssp. *affinis* populations, the Bayesian-clustering method employed in STRUCTURE failed to unequivocally demonstrate the existence of any clustering ($K > 1$) presents in the AFLP dataset probably due to the small sample size. While Evanno's ΔK might indicate the existence of two groups, as very low values were calculated for $K > 2$ (< 0.4), this result was not further confirmed by the probability of being assigned to each cluster calculated for each individual. While for individuals from populations D8 and D10 the probability of being assigned to cluster one or two was over 0.98, the individuals from D9 had similar probability of belonging to any of the clusters (aprox. 0.5).

This STRUCTURE result is in contradiction with the results by the AMOVA tests. The *a posteriori* groupings of the sampling sites tested included an only group enclosing the three sampling sites, and two groups (D8 vs D9 and D10; D8 and D10 vs D9; and D8 y D9 vs D10) indicated that there are differences among the sampling sites and there is no significant further grouping of the populations by geographical area indicating a high differentiation of each of them. Over a 60% of the variability is attributed to differences among

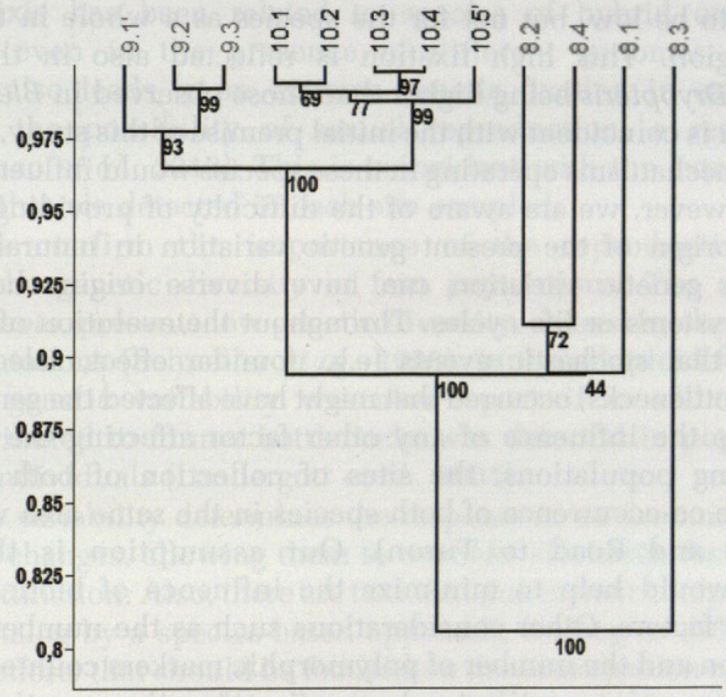

FIG. 3. Dendrogram calculated using Dice's distance as implied in PAST program representing the genetic relationships among *Dryopteris affinis* ssp. *affinis* individuals from the three sampling sites. Individuals from D8n are shown in green, D9 in pink, and D10 in blue. The bootstrap value is given in black in each node.

populations. The high Fst values also corroborate the high local fixation of variability (D8, 0.538; D9, 0.628; D10, 0.685). This high degree of similarity among the individuals from the same sampling site was confirmed by the high bootstrap support (>90%) of the different branches in the dendrogram calculated with PAST (Fig. 3) or the grouping in the PCoA (data not shown).

DISCUSSION

The genetic diversity calculated for the ferns *Blechnum spicant* and *Dryopteris affinis* ssp. *affinis* is in agreement with the reproductive mechanisms observed previously when cultured *in vitro* (Fernández and Revilla, 2003). Also, our results indicate the importance of small-scale sampling.

A relatively high percentage of polymorphism was estimated with AFLP for two species of ferns (81.38% for *B. spicant* and 54,73% for *D. affinis* ssp. *affinis*) in a small geographical area in the Principado de Asturias; no populations were found more than 45 km apart. However, when the distribution of this variation was analyzed in relation to the sampling sites, the polymorphism was drastically reduced to at least half and even one tenth in the case of *D. affinis* (28.66% (D8), 11.43% (D9) and 6.10% (D10). In the case of *B. spicant* a less notable effect was observed, with recalculated polymorphism ranging between 45.75% (B2) and 60.03% (B1). These changes in the percentage of polymorphism are explained by high genetic fixation, making the variation in each

sampling site to be low, but not for the species as a whole in this particular geographic region. This high fixation is reflected also in the Fst values calculated for *Dryopteris* being higher than those observed in *Blechnum*.

This fixation is coincident with the initial premise of this study, differences in reproduction mechanisms operating in these species would influence the genetic variability. However, we are aware of the difficulty of providing a conclusive probe of the origin of the present genetic variation in natural populations. Differences in genetic variation can have diverse origins beside specific reproduction systems or life cycles. Throughout the evolution of each species, it is possible that stochastic events (e.g., founder effect, selection of some genotypes or bottlenecks) occurred that might have affected the genetic diversity.

To minimize the influence of any other factor affecting the estimation of diversity among populations, the sites of collection of both species were selected by the co-occurrence of both species in the same area when possible (River Nueva and Road to Turon). Our assumption is that a shared environment would help to minimize the influence of biotic, abiotic, and anthropogenic factors. Other considerations such as the number of plants for each population and the number of polymorphic markers counted do not seem to produce any serious artifact when estimating the genetic intraspecific diversity in plants, as was shown by several studies using different dominant markers with this goal in gymnosperms and angiosperms (Nybom and Bartish, 2000). Finally, the variability could be caused by the experimental design (e.g. primer selection). Our analysis failed to detect any significant differences in the analyzed variables (HE, I and %P) that could be attributed to primer combinations used for each species or differences among populations within the same species. Our analysis, however, detected significant differences attributive to the two fern species in all estimates of genetic variability.

The different degree of genetic variation found in the natural populations of *B. spicant* and *D. affinis* ssp. *affinis* can be explained, then, to the differences in the reproductive biology of the species observed through *in vitro* culture. Traditionally, it has been maintained that most homosporous fern species are outbreeders or inbreeders (Soltis and Soltis, 1992) but more recent data suggest that the species could present a mix of reproductive strategies (de Groot *et al.*, 2012). Also, laboratory studies suggested that many fern species have mechanisms leading to the formation of functionally unisexual gametophytes that potentially could promote outcrossing. Because of the difficulty of working in *ex vitro* with gametophytes, not much work has been done to clarify whether outcrossing actually occurs in natural populations of ferns.

The sexual reproductive strategy of *B. spicant* is mediated by an antheridiogen system, which could favor intergametophytic reproduction (Schneller *et al.*, 1990), and enhance genetic variability. Soltis and Soltis (1990) estimated low levels of selfing in several populations of *B. spicant*, consistent with the statement that fern species with antheridiogen systems are supposed to be effective outcrossers. Our molecular results, even in small populations, also indicate a moderate genetic diversity. *D. affinis*, on the contrary, presented low genetic diversity and it has an apomictic reproductive

cycle. Apomixis has been related to species of hybrid origin, allowing reproduction even in the presence of different genomes. This asexual reproduction also leads to an extreme genetic fixation in each population and without the possibility of sexual reproduction, a reduced genetic variability (Chao *et al.*, 2012). This is coincident with the extreme reduction of variability that we detected in each site sampled.

Our results confirm the importance of the reproductive system in determining the genetic diversity in fern populations. The apomictic fern *Dryopteris remota*, presents low genetic diversity, caused by the accumulation of somatic mutations (Schneller *et al.*, 1998). In the alloploid fern *Dryopteris cristata*, low genetic variability was also reported. In addition, a further reduction attributed to former bottlenecks was detected in populations with less than 25 individuals (Landergott *et al.*, 2001).

The genetic variability determines the response to environmental short and medium-term changes, allowing them to overtake stochastic factors that could lead to local extinction. Also, there are taxa that due to their complexity could be difficult to catalog by a species-based approach, leading to difficulties in the conservation efforts that should be focused on the conservation of the processes that lead to the generation of the biodiversity (Ennos *et al.*, 2012). One of the species that could be favored by a comprehensive approach in conservation actions is *D. affinis*. The *Dryopteris affinis* complex has different ploidy levels and reticulate origin of the subspecies (Fraser-Jenkins, 1980). Because the lack of sexual reproduction, each population has extremely local fixation of its low genetic variability, possibly generated by somaclonal mutational events *in situ*, even among populations sited in a geographically close area, making highly risky the introduction into a certain locality of individuals from other areas, even if they are close. On the other hand, the conservation of *Blechnum spicant*, an outcrossing fern species, might need different actions. Even in the small populations analyzed here, a relatively high genetic diversity was assessed. In addition, the Bayesian-clustering of our molecular data detected two groups among our samples, one including the locality B1 (Nueva) and other B2 (Novales) and B3 (Purón). B2 and B3 are sited less than 4 km away, so it is not that surprising that some genetic exchange is present between these two localities. On the contrary, Nueva is over 20 km away. The high variability detected suggests a continuous genetic flow among locations that once properly identified could be stimulated to recover threatened populations.

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