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Primer Note

Development of polymorphic microsatellite markers for the Killarney Fern (*Vandenboschia speciosa*, Hymenophyllaceae)[1]

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- *Premise of the study:* We characterize 10 microsatellite loci in the endangered fern *Vandenboschia speciosa* (Hymenophyllaceae), enabling studies on the genetic population structure of this Macaronesian-European species using DNA hypervariable markers.
- *Methods and Results:* Ten primer sets were developed and tested on 47 individuals in a total of two Iberian populations of *V. speciosa*. The primers amplified di- and hexanucelotide repeats. The number of alleles ranged from two to eight, and the expected heterozygosity ranged from 0.107 to 0.807 among the populations analyzed.
- *Conclusions:* The 10 microsatellite markers developed will be useful in characterizing the genetic diversity of *V. speciosa* and understanding its population structure (including the possible structure between sporophyte and gametophyte phases) and biogeographic history, and will provide important genetic data for the conservation of this species.

Key words: endangered fern; Hymenophyllaceae; microsatellite markers; population genetics; *Vandenboschia speciosa*.

Vandenboschia speciosa (Willd.) G. Kunkel (Hymenophyllaceae) is a Macaronesian-European fern that has been considered one of the most vulnerable plant species in continental Europe, being protected under both the Bern Convention and the Habitats Directive (Anonymous, 1979, 1992). Both phases of its life cycle (sporophyte and gametophyte) are perennial and are capable of vegetative propagation, with extensive clonal development of either generation; therefore, independent sporophyte and gametophyte populations are usual throughout its distribution (Rumsey et al., 1998a). A differential geographic distribution exists between the two phases, with the gametophyte being more widely distributed than the sporophyte. Sporophyte populations are restricted to the European Atlantic coast and the Macaronesian Islands, in places considered shelters during the Quaternary glacial cycles, while gametophyte populations occur also in continental Northern Europe and Central Europe (Rumsey et al., 2005 [and references therein]). This property of *V. speciosa* raises questions about its biogeographic history (e.g., the refugial status of its populations, especially the Northern and Central European ones), as well as crucial implications for the conservation strategies of the species (e.g., how much genetic diversity do the gametophyte populations harbor in relation to the sporophyte ones?). These questions can be answered from a population genetics approach.

Earlier studies on the population genetics of *V. speciosa* have used allozymes as molecular markers and, although with low resolution, they have contributed to an understanding of the current distribution of *V. speciosa* (Rumsey et al., 1996, 1998b, 1999, 2005). Because *V. speciosa* is a tetraploid species (Manton, 1950; Manton et al., 1986), the allozyme banding patterns were coded as phenotypes in all the cited studies, thus decreasing further the resolution capacity of the allozymes. The aim of this paper is to develop a suite of microsatellite markers for *V. speciosa* to generate a highly informative evaluation of the genetic composition of its populations. To this end, 10 microsatellite markers were developed that will allow the genetic diversity and population structure of *V. speciosa* to be assessed throughout its distribution.

METHODS AND RESULTS

Genomic DNA was extracted from fresh fronds of *V. speciosa* from four samples of the population Canuto de Ojén Quesada (COQ), Cádiz Province, Andalusia, Spain (voucher deposited at the herbarium of the Universidad de Granada [GDA]: GDA 61589; georeference: 36.12794°N, 5.58523°W) using the NucleoSpin Plant Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). A final solution with 2 µg of total genomic DNA was created pooling ca. 400 ng of DNA from each sample. Microsatellite loci were isolated by

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GenoScreen (Lille, France) through 454 GS FLX Titanium pyrosequencing of enriched DNA libraries, following the methodology in Malausa et al. (2011). Genomic DNA was mechanically fragmented and probed for microsatellite repeats (AG, AC, AAC, AGG, ACG, AAG, ACAT, ATCT). Amplified and purified PCR products of the enriched fragments were used to construct GS FLX libraries following manufacturer's protocols (Roche Diagnostics, Penzberg, Germany) and sequenced on a GS FLX PicoTiterPlate (454 Life Sciences, a Roche Company, Branford, Connecticut, USA). The bioinformatics program QDD (Meglécz et al., 2010) was used to analyze the raw sequences, select the sequences with target microsatellites, and perform primer design. A total of 6484 sequences comprising microsatellite motifs were retrieved, and 158 primer pairs were designed for sequences longer than 100 bp with at least five-repeat microsatellite loci and tandem-repetition-free flanking regions.

For validation tests, 82 primer pairs were used for amplification in eight DNA samples of *V. speciosa* from two populations: COQ and Azketa Erreka (AZK), Gipuzkoa Province, Basque Country, Spain (voucher deposited at GDA: GDA 61588; georeference: 43.19458°N, 1.94015°W). Forward primers were synthesized with M13 tails (5′-CACGACGTTGTAAAACGAC-3′) preceding the 5′ end to facilitate cost-efficient fluorescent labeling of PCR products. DNA was isolated as above and PCR optimizations were performed by testing different magnesium chloride ($MgCl_2$) concentrations and by modifying the range of annealing temperatures. PCR reactions were performed in 10-µL reactions containing 10 ng of genomic DNA, 0.02 µM of the M13-labeled forward primer, 0.45 µM of each reverse primer and fluorolabeled M13 primer (5′-6FAM, 5′-HEX), 2–5 mM $MgCl_2$, 0.5 mM dNTPs, 0–0.5 M Betaine, 1 µL Biotools 10× Reaction Buffer, and 0.5 units of Biotools DNA polymerase (Biotools B&M Laboratories S.A., Madrid, Spain). Cycling parameters consisted of 3 min of denaturing at 94°C; followed by 35 cycles of 94°C for 1 min, 45–60°C for 1 min, and 72°C for 45 s; and a final extension at 72°C for 20 min. PCR products were analyzed with GeneScan 500 LIZ Size Standard (Applied Biosystems, Foster City, California, USA) on an ABI 3100-Avant Genetic Analyzer (Applied Biosystems) at the Centro de Instrumentación Científica at the Universidad de Granada (Granada, Spain). Microsatellite data were analyzed with GeneMarker version 1.51 (SoftGenetics, State College, Pennsylvania, USA). Of the 82 microsatellite markers tested, 47 gave a positive signal on electrophoretic gels; of these, 16 yielded an ambiguous allelic pattern, 21 were monomorphic, and 10 were polymorphic with easily interpretable electropherograms. The characteristics of the 10 polymorphic loci are shown in Table 1.

Genetic diversity of the selected loci was assessed by genotyping (as above) a total of 47 individuals of the two aforementioned populations (17 from AZK, 30 from COQ). Data were analyzed with the software GenoDive version 2.0b24 (Meirmans and Van Tienderen, 2004). In total, 41 unique multilocus genotypes were identified (AZK: 14, COQ: 27) by using the Assign Clones function and the Stepwise Mutation Model for calculating the interindividual distances in GenoDive. The distance threshold allowed between two individuals to be considered clones with the same multilocus genotype was 2 bp (selected after inspection of the distance histogram). The total clonal diversity (Meirmans and Van Tienderen, 2004) was 0.990 (AZK: 0.971, COQ: 0.991; intrapopulation average: 0.981). The average number of alleles per locus was 4.5 (AZK: 3.6, COQ: 2.9). Loci VS-CONS162, VS-HG1NJ, and VS-GT0D8-1 were monomorphic in COQ. Heterozygosity was only analyzed considering genets, on the basis of the unique multilocus genotypes identified, and applying the correction for unknown dosage of alleles as in GenoDive. The expected heterozygosity across all the populations analyzed was 0.523 (AZK: 0.523, COQ: 0.328; intrapopulation heterozygosity: 0.415). The values for allele number and heterozygosity for each locus and population are shown in Table 2.

CONCLUSIONS

The 10 microsatellite markers developed in this study will enable population genetics studies of *V. speciosa* throughout its distribution. These microsatellites will help to assess the clonality level in both mixed and independent sporophyte and gametophyte populations, the genetic diversity, and population and/or between-phase genetic structure. These population genetic data will be useful for improved management of populations of this endangered species. Moreover, future studies using these 10 microsatellite markers will allow questions about the biogeographic history of *V. speciosa* to be tested, such as the refugial status of its populations. Our results in this study show two sporophyte populations in the Iberian Peninsula—considered a glacial refuge (Bennett et al., 1991)—to be moderately diverse (AZK in the north being more diverse than COQ in the south), which contrasts with the lack of genetic diversity detected within Northern and Central European populations using allozyme markers (Rumsey et al., 1998b, 1999, 2005).

Table 1. Characteristics of 10 microsatellite loci developed in *Vandenboschia speciosa.*

Locus	Primer sequences (5′–3′)[a]	Repeat motif	Allele size range (bp)	Bet (M)	$MgCl_2$ (mM)	T_a (°C)	EMBL accession no.
VS-CA1-55	F: TTGGGAAGTCCCTCATGATT R: GCGGCTCTTGGAAGACAATA	$(AG)_{10}(GAAAT)_4$	240–258	0.5	5	55	LN864472
VS-CONS12	F: CAACCATAGCCACACCAACA R: TCAAGCAAACCAACAAACCA	$(CTACAT)_{11}$	233–247	0.5	3.75	52	LN864473
VS-CONS162	F: TTGATGTCATGCATCCAACC R: CATTCCCTCCTCATCTCACC	$(GA)_{10}$	107–111	0.5	3.75	50	LN864474
VS-GA76	F: GCATGCACACGAGCTTGTG R: ATGCATATAAATCAATAGAC	$(TC)_{14}(AC)_{14}$	99–115	0.5	2.75	50	LN864475
VS-GA87	F: GTGGGCATTACACATTCT R: GAGTCTCCTTGACATACC	$(TC)_{23}$	189–207	0.5	3.75	55	LN864476
VS-GT0D8-1	F: CAACCTCCCCCATTGTTTTA R: GGGTCCCCGACTGACTTTAT	$(AG)_8$	208–212	0.5	3.75	50	LN864477
VS-GXSPQ-2	F: TGAGTTGCTTGTGGATGAGG R: TTTCTTTTAGGGCCCTTTGG	$(AT)_6(GT)_{10}(GC)_4(GT)_3$	164–174	0.5	3.75	58	LN864478
VS-HG1NJ	F: AACAAAGTCAAATTTATGTCCCA R: TCATATTAATGCCATGCCCA	$(TC)_{10}$	145–147	0	2.5	50	LN864479
VS-HKPD7	F: ACATGATCTCACAACAGTCCTCT R: GCCCAATTTCCAGTTTCCTT	$(CT)_6$	207–209	0.5	3.75	50	LN864480
VS-HM60O-1	F: TCAAGCGATGATAGAGGGCTA R: TCCACCTTGATGACCTAATTGA	$(AC)_{10}$	213–227	0.5	2.5	45	LN864481

Note: Bet = optimal betaine concentration; EMBL = European Molecular Biology Laboratory; $MgCl_2$ = optimal magnesium chloride concentration; T_a = annealing temperature.

[a]Forward primer sequence and allele size range do not include the M13-tail sequence.

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TABLE 2. Summary genetic statistics for the two populations of *Vandenboschia speciosa* analyzed with the 10 newly developed microsatellites.[a]

	Azketa Erreka (n = 14)			Canuto de Ojén Quesada (n = 27)		
Locus	A	H_o	H_e	A	H_o	H_e
VS-CONS12	4	0.571	0.712	4	0.407	0.466
VS-CONS162	3	0.357	0.463	1	—	—
VS-GT0D8-1	3	0.429	0.415	1	—	—
VS-GXSPQ-2	4	0.429	0.553	2	0.111	0.107
VS-HG1NJ	2	0.429	0.423	1	—	—
VS-HKPD7	2	0.286	0.423	2	0.222	0.453
VS-HM60O-1	3	0.357	0.405	3	0.259	0.400
VS-CA1-55	6	0.857	0.807	6	0.333	0.586
VS-GA76	5	0.500	0.561	5	0.667	0.646
VS-GA87	4	0.286	0.267	4	0.593	0.620

Note: A = number of alleles sampled; H_e = expected heterozygosity; H_o = observed heterozygosity; n = number of individuals (genets) sampled.

[a]Locality and voucher information for the sampled populations: Azketa Erreka, Gipuzkoa Province, Basque Country, Spain (voucher: GDA 61588; georeference: 43.19458°N, 1.94015°W); Canuto de Ojén Quesada, Cádiz Province, Andalusia, Spain (voucher: GDA 61589; georeference: 36.12794°N, 5.58523°W).

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