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Development and characterization of fungal specific microsatellite markers in the lichen *Lobarina scrobiculata* (*Lobariaceae*, Ascomycota)

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Abstract: *Lobarina scrobiculata* (better known as *Lobaria scrobiculata*) is a widespread lichen, threatened and Red-Listed in various European countries. Microsatellite markers for the mycobiont of *L. scrobiculata* were developed in order to investigate its genetic diversity in the Iberian Peninsula and Europe and to design effective conservation strategies. A total of 7 polymorphic markers were isolated and characterized. These microsatellites were tested in natural populations found in the Iberian Peninsula. The number of observed alleles ranged from 3 to 8, and the Nei's unbiased gene diversity from 0·26 to 0·59. These microsatellite markers are the first to be developed for *L. scrobiculata* and they will be useful for population studies and for the assessment of the conservation status of this species.

Keywords: conservation status, cyanolichen, genetic diversity, *Lobaria scrobiculata*, SSR

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Introduction

In this study we focus on the foliose cyanolichen *Lobarina scrobiculata* (Scop.) Nyl. ex Cromb., previously included in the genus *Lobaria* but placed in *Lobarina* based on recent phylogenetic studies (Moncada *et al.* 2013). It is a widespread species, distributed in the Northern Hemisphere and in oceanic areas of Africa, Australia, New Zealand and South America (Nimis 1993; Smith *et al.* 2009). In the Iberian Peninsula, it is frequent in central Spain, common in oceanic areas of the north-west, and progressively rarer elsewhere (Burgaz & Martínez 1999). This species is preferentially epiphytic, occurring on deciduous trees and sometimes on mossy siliceous rocks. Although *L. scrobiculata* has been traditionally associated with old epiphytic communities (James *et al.* 1977; Burgaz *et al.* 1994), recent studies have shown that relatively young and/or slightly managed forests host more abundant populations of this species (Merinero *et al.* 2014). The most frequent mode of reproduction in *L. scrobiculata* is asexual via soredia, whereas sexual reproduction via apothecia is rather infrequent (Burgaz & Martínez 1999; Smith *et al.* 2009). Habitat destruction and air pollution have reduced the distribution of this species in Europe (Hallingbäck 1989; Nimis 1993; Smith *et al.* 2009; Pentecost & Richardson 2011). Consequently, it has been included in numerous European Red Lists under different threat categories (Sérusiaux 1989; Clerc *et al.* 1992; Randlane *et al.* 2008; Gärdenfors 2010). The conservation status of *L. scrobiculata* in the Iberian Peninsula is unknown, and although some studies have suggested classifying it as "endangered" or "vulnerable", threatened by forestry and agriculture (Burgaz *et al.* 1994; Martínez *et al.* 2003), we lack demographic and genetic data supporting these assertions.

Molecular studies contribute to an understanding of the population history and dynamics of lichen-forming fungi, providing information related to gene flow, dispersal

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and mating systems, which is essential for lichen conservation. Compared to other genetic markers, microsatellites show higher levels of variation, and they have been used for population studies in several lichen species (e.g. Otálora *et al.* 2011; Mansournia *et al.* 2012; Scheidegger *et al.* 2012; Werth *et al.* 2013). However, major drawbacks of microsatellite markers are that they might not work for other species and they may show less genetic variability than in those species for which they were originally developed (Werth 2010). Thus, new specific microsatellites and primers must be developed in order to work with new taxa. Here, we have developed and characterized new microsatellite markers for *L. scrobiculata* to assess its genetic diversity, connectivity and population structure across its distribution area in the Iberian Peninsula and Europe.

Methods

Total fungal genomic DNA of seven specimens of *L. scrobiculata* was isolated from different populations and mixed for library production. These populations are scattered across the Iberian Peninsula (Ávila, Ciudad Real, Gipuzkoa, Huelva, Palencia and Tarragona in Spain, and Coimbra in Portugal). The distances between populations range from 180 to 820 km. In these populations, *L. scrobiculata* presents mainly asexual propagules (i.e. soredia) and almost never reproduces sexually.

Before DNA extraction, the cyanobiont was manually removed from thalli under a dissecting microscope. Genomic DNA was extracted from the mycobiont tissue using DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. To test if the DNA extractions contained the cyanobiont, we performed PCR amplifications using the fungal nuITS ribosomal primers ITS1–ITS4 (White *et al.* 1990) and the cyanobacterial specific primers *rbcLX* CW-CX (Rudi *et al.* 1998). In these tests we used three samples in which DNA was extracted from complete lichen thalli, containing both myco- and cyanobiont DNA, as positive controls. Amplifications were performed in 20 µl volumes containing a reaction mixture of 6 µl of Multiplex PCR Master Mix (Qiagen), 1 µl of primer mixture (10 µM), 10 µl dH_2O and 3 µl of genomic DNA. Amplifications were carried out in a PTC-100 Peltier thermal cycler with the same PCR conditions as in Prieto *et al.* (2010) for the nuITS region, and with the following cycling conditions for the *rbcLX*: initial denaturation at 94°C for 15 min, followed by 3 cycles of 94°C for 30 s, 40°C for 30 s and 72°C for 2 min, 38 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 2 min, ending with an extension step at 72°C for 10 min. PCR products were stained with SYBR Green and visualized by electrophoresis through a 1% agarose gel.

The DNA libraries, highly enriched in microsatellite loci, were prepared using GS-FLX titanium pyrosequencing (Roche Diagnostics) at GenoScreen (Lille, France). The pooled DNA was used for creating GS-FLX libraries performed on the PCR product obtained as follows: fragmentation of genomic DNA, DNA enrichment in microsatellite loci and amplification with a High Fidelity Taq polymerase.

From the sequences obtained, 240 pairs of primers were validated bioinformatically with QDD software (Meglécz *et al.* 2010). Primers were designed for 24 microsatellite inserts using Primer3 (Rozen & Skaletsky 2000). We carried out a BLAST search of these primer candidates to exclude any match with cyanobacteria. These primers were tested to validate efficiency and specificity of PCR with the following conditions: PCR in a total volume of 25 µl, containing 2 µl of genomic DNA, 1 pmol of each primer, 1 U Taq DNA polymerase, 6 pmol dNTP and 37·5 pmol $MgCl_2$. PCR reactions were performed as follows: 10 min at 95°C, then 40 cycles of 30 s at 95°C, 30 s at 55°C, 1 min at 72°C, and a final extension of 10 min at 72°C.

Of the 18 successful pairs, 12 were selected for a polymorphic analysis at GenoScreen. Finally, we performed a broader polymorphism analysis using seven successful loci in four Iberian populations (80 thalli in total) from Ciudad Real (Robledo), Coimbra (Sicó), Navarra (Irati) and Palencia (Brañosera), 270–700 km apart from each other. Coordinates and further information on the localities can be found in Merinero *et al.* (2014). For this purpose, two multiplexed PCR reactions were carried out in a total volume of 10 µl containing 1 µl of genomic DNA, 2 µl of a primer mix of variable concentration, 5 µl of Multiplex PCR Master Mix (Qiagen) and 2 µl of distilled water (Table 1). PCR conditions were identical to those used for testing the markers (see above). The PCR products were electrophoresed on an ABI3130 capillary sequencer using the size marker GS400HD-ROX, and product sizes were estimated using Gene Mapper v5 (Applied Biosystems).

Results

Positive results were obtained in all samples with the nuITS fungal specific primers, whereas nothing was amplified using the cyanobacterial specific primers *rbcLX*, except for the three positive controls. Therefore, the cyanobiont was absent in our fungal extractions.

From the library production, out of the 35 471 raw data sequences obtained, 2594 sequences contained microsatellite motifs. Six of the 24 screened primer pairs did not

TABLE 1. *Microsatellite loci identified and screened for* Lobarina scrobiculata. *For each locus the following is provided: primer pairs (F: forward, R: reverse), repeat motif, fluorescent dye used, multiplex reaction, size of the original fragment (bp: base pairs), concentration of each primer (conc.) and GenBank accession number.*

Locus	Primer sequences (5'-3')	Motif	Dye	Multiplex	Size (bp)	Primer conc. (μM)	GenBank accession no.
LS03	F: ACCGATTGATACGCGTGC R: GCTCTTATCGGTGTGGGAAA	$(TG)_8$	FAM	1	131	0·08	KJ666764
LS07	F: TAAATACGCCACGACATGCT R: CCGTATTTTGCCTCGAAAGA	$(TTC)_{10}$	FAM	2	100	0·10	KJ666765
LS14	F: AGAAGGAGTAGGAGCAGGCA R: ATCACACCGTCGCTTCTCTT	$(AGG)_{11}$	TAMN	2	146	0·08	KJ666766
LS15	F: TGCGCATCTAAACATTCCAG R: TTGCGCGTTTTCTATAACCA	$(TG)_{12}$	HEX	1	104	0·10	KJ666767
LS18	F: AGAGGACGACGAGGAGAGTG R: ACACTCAATCAACCGTGCAA	$(GGA)_{12}$	TAMN	1	116	0·08	KJ666768
LS20	F: GAGTCTCGGAGGGAAGGAAG R: ATTTCAGGCATCAGCAGGAT	$(ATC)_{13}$	HEX	2	241	0·08	KJ666769
LS21	F: CTTTGTACGACGCAGATGTGA R: GATCCAATCATCCAACTCGATA	$(GAT)_{14}$	ROXN	2	190	0·08	KJ666770

TABLE 2. *Number of alleles (A) and Nei's gene diversity (He) in four populations of* Lobarina scrobiculata.

	Total			Robledo (Ciudad Real)			Brañosera (Palencia)			Sicó (Coimbra)			Irati (Navarra)		
Locus	Ns*	A	He	*n*†	A	He	*n*	A	He	*n*	A	He	*n*	A	He
LS3	79	4	0·54	19	3	0·46	20	3	0·66	20	4	0·53	20	3	0·52
LS07	77	6	0·58	19	4	0·54	20	2	0·49	20	5	0·64	18	4	0·66
LS14	77	4	0·52	17	2	0·46	20	3	0·53	20	4	0·41	20	4	0·69
LS15	79	4	0·26	19	2	0·10	20	2	0·08	20	3	0·27	20	4	0·58
LS18	79	5	0·57	19	4	0·59	20	3	0·57	20	4	0·60	20	4	0·54
LS20	75	3	0·59	19	3	0·65	20	3	0·67	20	3	0·66	16	3	0·40
LS21	76	8	0·54	19	3	0·62	20	4	0·68	20	3	0·49	17	4	0·39
Mean		4·86	0·52		3·00	0·49		2·86	0·53		3·71	0·51		3·71	0·54

*Number of successful amplifications; † = total number of samples analyzed per population.

produce visible PCR products. Among the 12 pairs selected for the polymorphic analysis, seven loci amplified successfully and were polymorphic. In the broader polymorphism analysis conducted on four Iberian populations, all primer pairs tested successfully for amplified unambiguous products.

The number of alleles and Nei's unbiased gene diversity were calculated using GenAlEx 6.501 (Peakall & Smouse 2006). The number of observed alleles ranged from 3 (locus LS20) to 8 (locus LS21) with a mean of 4·86, and the Nei's unbiased gene diversity ranged from 0·26 (locus LS15) to 0·59 (locus LS20) with a mean of 0·52 (Table 2). Over the four populations, the average gene diversities varied from 0·49 to 0·54 (Table 2).

These novel microsatellite markers are of paramount importance for genetic analyses and the conservation assessment of *L. scrobiculata*. We will investigate the population genetics of *L. scrobiculata* in the Iberian Peninsula and Europe, and will compare the fungal and cyanobacterial partners to determine if their genetic structures are coupled.

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