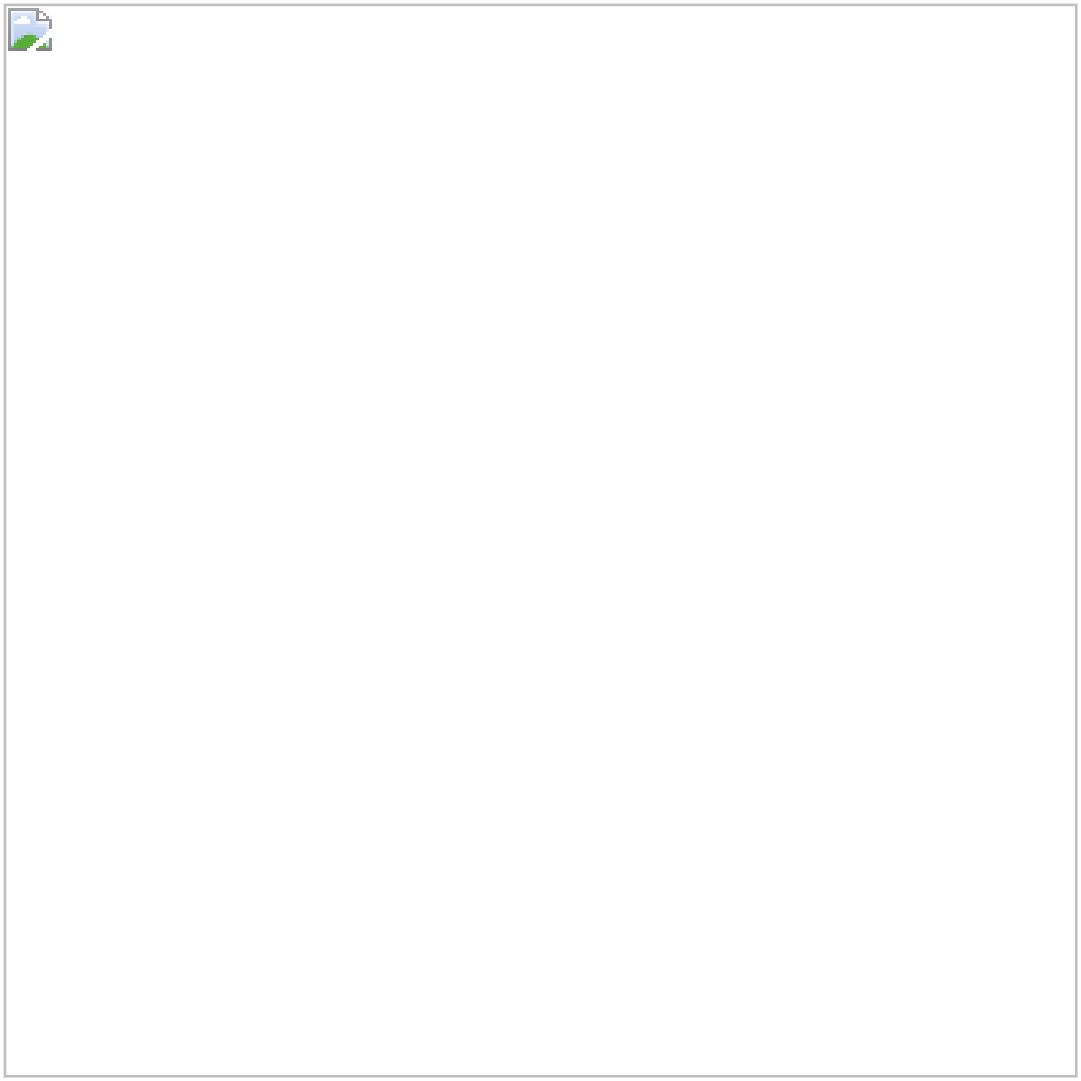

Characterization of Fungus-Specific Microsatellite Markers in the Lichen-Forming Fungus Parmelina carporrhizans (Parmeliaceae)

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Primer Note

Characterization of fungus-specific microsatellite markers in the lichen-forming fungus *Parmelina carporrhizans* (Parmeliaceae)[1]

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- *Premise of the study:* Microsatellite loci were developed to study the lichen-forming fungus *Parmelina* (Parmeliaceae) in different habitats of western Europe and the Mediterranean for baseline studies to understand the effects of climate change on its distribution.
- *Methods and Results:* We cultured *P. carporrhizans* from ascospores for genomic sequencing with Illumina HiSeq. We successfully developed 11 polymorphic microsatellite markers and associated primer sets and assessed them with 30 individuals from two of the Canary Islands. The average number of alleles per locus was 8.8. Nei's unbiased gene diversity of these loci ranged from 0.53 to 0.91 in the tested populations. Amplification in two closely related species (*P. tiliacea*, *P. cryptotiliacea*) yielded only limited success.
- *Conclusions:* The new microsatellite markers will allow the study of genetic diversity and population structure in *P. carporrhizans*. We propose eight markers to combine in two multiplex reactions for further studies on a larger set of populations.

Key words: Ascomycota; lichen-forming fungi; microsatellites; multiplex; *Parmelina carporrhizans*; population genetics.

Parmelina carporrhizans (Taylor) Poelt & Vêzda (Parmeliaceae) is a sexually reproducing foliose lichen species that has long been considered synonymous with the morphologically similar *P. quercina* (Willd.) Hale. Thus, the geographic distribution and degree of conservation of both species are poorly known (Argüello et al., 2007; Clerc and Truong, 2008). These two species are largely allopatric but they occasionally overlap, being apparently parapatric depending on the climatic conditions. Hence they possibly may be used as indicators of climate change. *Parmelina carporrhizans* has an Atlantic-Mediterranean distribution in Europe. It is abundant in the central-western Iberian Peninsula in the humid supra- and mesomediterranean level on deciduous *Quercus* L. vegetation (Argüello et al., 2007; Nuñez-Zapata, 2013). The species also occurs across open forest and in isolated trees above the Canarian monteverde forest in central Macaronesia from 800 to 1500 m and is locally common on Gran Canaria. Further, *P. carporrhizans* is listed as "vulnerable" on the Red Lists of England and Wales (Church et al., 1996; Woods, 2010). Despite these conservation concerns, our knowledge of the population genetics of this species is currently limited.

We developed 11 microsatellite markers for high-resolution population studies in *P. carporrhizans* to provide a better understanding of its genetic diversity, gene flow, and population structure. The enhanced knowledge will allow us to implement an informed conservation plan and investigate potential impacts of climate change on this narrowly distributed species. In addition, we also investigate whether this set of high-resolution microsatellite markers can be applied to other closely related species in the genus *Parmelina* Hale.

METHODS AND RESULTS

We isolated the mycobiont of *P. carporrhizans* from ascospores of two thalli (deposited in the herbarium of the Universidad Complutense de Madrid [MAF], Madrid, Spain: MAF-Lich 19191 and MAF-Lich 19192) collected in Cuevas del Valle, Spain (40°18′28.4″N, 5°00′39.0″W), in October 2012, following the inverted Petri dish method (Ahmadjian, 1993). We germinated spores in Basal Bold Medium (Deason and Bold, 1960), and after two weeks these were transferred to corn meal agar (CMA) and malt yeast (Honegger et al., 2004), where the cultures were grown for four months.

Prior to DNA extraction, we removed secondary metabolites with acetone, and then crushed the samples with pestles in liquid nitrogen and extracted genomic DNA with the DNeasy Plant Kit (QIAGEN, Redwood City, California, USA) according to the manufacturer's instructions.

To confirm the identity of the mycobiont cultures, we amplified the internal transcribed spacer (ITS) region of the nuclear rDNA from the axenic cultured tissues. Genomic DNA (10–25 ng) was used for PCR amplifications. Primers, PCR, and cycle sequencing conditions were the same as described previously

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TABLE 1. Overview of the microsatellite loci and associated primer sets successfully developed for *Parmelina carporrhizans* and deposited in the National Center for Biotechnology Information (NCBI) database.

Locus	Primer sequences (5′–3′)	Repeat motif	Dye	T_a (°C)	Allele size range (bp)[a]	GenBank accession no.
Pcar1	F: *CATCAAATCATCCGCTACCA	$(AC)_{18}$	FAM	57	124–147	KM875582
	R: GGGGAGGTGAGGAGAACAA					
Pcar2	F: *TCACCATGTGGTAGGGTAGC	$(GTA)_{15}$	NED	57	206–265	KM875583
	R: CTGTATCGAACAAGGCATCG					
Pcar3	F: *TGACCCTGTGACCTCTTGC	$(AAT)_{17}$	PET	57	109–249	KM875584
	R: GCCTCGGGTCCATACAGAT					
Pcar4	F: *AGGAGGGGGTGAAAAAGAGA	$(AAGAG)_{16}$	VIC	57	280–318	KM875585
	R: GCTGGTCTTTGCACTCATCA					
Pcar5	F: *GATGCGTATAGCGGTGCAT	$(AG)_{18}$	FAM	57	227–309	KM875586
	R: TTCTGTGGGATGTATTGCAGA					
Pcar6	F: *GCATTGCATGAGGCTGAAC	$(CTT)_{15}$	NED	57	203–270	KM875587
	R: TGCAGTGGCAATCAATGTG					
Pcar7	F: *CTGGGGTGGTGATTGTGAG	$(AAG)_{19}$	PET	57	120–223	KM875588
	R: GCAAGCAGAAAGCAGCAAC					
Pcar8	F: *GCTTGAATTGGAGGGAAGC	$(GAT)_{20}$	VIC	57	372–474	KM875589
	R: GAGGCGTGTATGCCTTAACC					
Pcar9	F: *GAAACTCCCACCACCGTTC	$(AG)_{16}$	FAM	57	89–165	KM875590
	R: AAGCATTTTGGTGCATTGG					
Pcar10	F: *GCCCTCCAATGAAGGAGTC	$(AC)_{16}$	FAM	57	341–390	KM875591
	R: CCTTGGCTGGGATAAGCAT					
Pcar11	F: *CGATAGCGGAGGATTTTCAG	$(ACTC)_{17}$	FAM	57	250–371	KM875592
	R: GTCTGCGTCGCCTCTAATTC					

Note: T_a = annealing temperature.

[a] Size range indicates allele size based on two populations collected in the Canary Islands (see Appendix 1).

* M13 tail: TGTAAAACGACGGCCAGT.

(Argüello et al., 2007). Sequencing was conducted on an ABI 3730 DNA analyzer (Applied Biosystems, Foster City, California, USA) at Centro de Genómica y Proteómica del Parque Científico de Madrid. The identity of the sequences and specimens were confirmed using the MegaBLAST search function in GenBank. ITS sequences were deposited in GenBank (accession numbers KM357892 and KM357893).

From the extracted DNA, approximately 0.5 µg of genomic DNA was used to construct an Illumina library using the Nextera XT multiplex paired-end kit (Illumina, San Diego, California, USA). The library was paired-end sequenced using an Illumina HiSeq 2000 with 100 cycles (version 3 chemistry). Standard Illumina protocols (http://www.illumina.com/) were used to generate the library. Sequencing was carried out at the Stab Vida Laboratory (Madan Parque, Caparica, Portugal). Illumina reads were assembled to contigs using the "De novo assembly" option of the CLC Genomics Workbench version 6.0.4 (CLC bio, Aarhus, Denmark). A total of 38,115,484 reads with an average length of 69.06 bases and a total of 2,632,336,717 bases were recovered. De novo assembly produced 31,035 contigs (N50 = 3615 bp) with an average of approximately 73× coverage, which totaled 36.2 Mbp of genome data.

All the contigs were screened for microsatellites using MSATCOMMANDER 1.0.8 (Faircloth, 2008), accepting di-, tri-, tetra-, penta-, and hexanucleotide repeats of ≥15. We found 63 contigs containing microsatellite sequences with 15 to 20 repeats (29 dinucleotides, 24 trinucleotides, 7 tetranucleotides, 2 pentanucleotides, and 1 hexanucleotide). From these contigs, we designed short primers of 19–21 bp in length with the program Primer3 using default parameters (Rozen and Skaletsky, 2000), expecting some transferability within the genus as reported in other lichen mycobionts (Jones et al., 2012; Devkota et al., 2014). We excluded contigs with short flanking regions, as well as repeated motifs on the flanking region, and selected primer pairs with amplicons between 100 and 400 bp. Finally, an M13 tag (5′-TGTAAAACGACGGCCAGT-3′) was appended to forward primers for subsequent amplification.

Microsatellite PCRs were performed in a 10-µL reaction volume containing ~0.5–5 ng of genomic DNA, 1× Type-it Multiplex Master Mix (QIAGEN, Hilden, Germany), 0.15 µM of reverse primer, 0.01 µM of M13-tailed forward primer, and 0.15 µM of dyer–M13-labeled primer (Schuelke, 2000). PCRs were carried out with an initial 5-min denaturation at 94°C; followed by 35 cycles of 94°C for 30 s, 57°C for 45 s, and 72°C for 45 s; and a final extension of 72°C for 30 min.

We tested the 24 primer pairs with seven accessions of *P. carporrhizans* from different areas of its distribution range and one accession of *P. tiliacea* (Hoffm.) Hale (MAF-Lich 17252); see Appendix 1 for specific localities. Out of these 24 primers, only 12 pairs successfully amplified all of the *P. carporrhizans* samples, and four pairs amplified in *P. tiliacea*. We then tested this subset of 12 primer pairs for variability with 30 samples of *P. carporrhizans* from Gran Canaria and Tenerife (MAF-Lich numbers 19123–19152; Appendix 1), as well as one accession each of *P. tiliacea* and *P. cryptotiliacea* Crespo & Núñez-Zapata (MAF-Lich 19403 and MAF-Lich 19402, respectively). Eight of these primer pairs (Pcar1–Pcar8) amplified all *P. carporrhizans* samples, while the other three (Pcar9–Pcar11) had 3.3–10% missing data. Four of these primer pairs (Pcar3, Pcar5, Pcar7, Pcar9) amplified in *P. tiliacea* and none amplified in *P. cryptotiliacea*. We deposited these 11 primer sequences in GenBank (Table 1); other primer pairs were excluded due to their low amplification rate (<60%). Our limited cross-species amplification results suggest that it may be possible to use some of these markers in other species of the *P. carporrhizans* clade (Nuñez-Zapata, 2013).

Polymorphism within the eight microsatellite loci that amplified across all *P. carporrhizans* samples was determined by counting the number of alleles and calculating Nei's unbiased haploid diversity (Table 2) using GenAlEx version 6.41 (Peakall and Smouse, 2006). The number of alleles ranged from four to 14, and the average unbiased diversity was 0.76, a relatively high number for

TABLE 2. Number of alleles (A) and Nei's unbiased genetic diversity (H_e) of the eight polymorphic microsatellite loci that were amplified with 100% success across 30 samples from the Canary Islands.

	Total		Gran Canaria (n = 20)		Tenerife (n = 10)	
Locus	A	H_e	A	H_e	A	H_e
Pcar1	6	0.55	4	0.56	4	0.53
Pcar2	4	0.64	4	0.73	2	0.56
Pcar3	14	0.89	11	0.87	6	0.91
Pcar4	8	0.82	7	0.78	6	0.87
Pcar5	9	0.78	6	0.68	7	0.87
Pcar6	9	0.78	8	0.87	4	0.71
Pcar7	12	0.89	9	0.90	6	0.89
Pcar8	9	0.75	8	0.89	3	0.60
Average	8.88	0.76	7.13	0.79	4.75	0.74

just 30 individuals from a small geographic area. No identical multilocus genotypes were found among the samples as is expected for a sexually reproducing lichen-forming fungus.

CONCLUSIONS

We developed 11 polymorphic fungus-specific microsatellite markers to facilitate studies of population genetics in *P. carporrhizans*. Eight of the 11 microsatellite primer pairs are being used to analyze *P. carporrhizans* populations. The results from future population genetic studies will help inform us on population responses to global changes, clarify the mechanisms of speciation, as well as define populations of this narrowly distributed species for conservation purposes.

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APPENDIX 1. Voucher information for specimens of *Parmelina carporrhizans*, *P. cryptotiliacea*, and *P. tiliacea* used in this study. All the specimens are deposited in the Lichen section of MAF herbarium, Faculty of Pharmacy, Universidad Complutense de Madrid, Madrid, Spain (MAF-Lich).[a]

Voucher no.	Species	Locality	Substrate[b]	Geographic coordinates	Elevation (m)	Collectors	Collection date
16476	*P. carporrhizans*	Canakkale (Tr)	*Quercus* sp.	40°06′N 26°55′E	400	A. Crespo, P. K. Divakar & M. Candan	15/06/2007
17252	*P. tiliacea*	Tenerife	Rock	28°07′14″N 16°40′19″W	982	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	19/06/2009
19191	*P. carporrhizans*	Avila (Es)	*Castanea sativa*	40°18′28″N 05°00′39″W	1007	A. Crespo, D. Alors & C. Ruibal	11/10/2012
19404	*P. carporrhizans*	Tenerife	*Castanea sativa*	28°27′11″N 16°24′55″W	894	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19405	*P. carporrhizans*	Gran Canaria	*Pinus radiata*	28°01′50″N 15°37′12″W	1420	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	19/06/2009
19406	*P. carporrhizans*	Gran Canaria	*Prunus* sp.	28°00′01″N 15°32′29″W	954	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	17/06/2009
19407	*P. carporrhizans*	Tetouan (Ma)	Unidentified dead tree	35°20′43″N 05°22′20″W	687	D. Alors & C. G. Boluda	22/10/2013
19408	*P. carporrhizans*	Gran Canaria	*Ulmus* sp.	28°01′29″N 15°35′15″W	1305	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	18/06/2009
19402	*P. cryptotiliacea*	Agadir (Ma)	*Quercus ilex*	30°38′51″N 09°40′34″W	711	D. Alors & C. G. Boluda	23/10/2013
19403	*P. tiliacea*	Azilal (Ma)	*Quercus ilex*	33°25′40″N 05°11′26″W	1439	D. Alors & C. G. Boluda	20/10/2013
19123	*P. carporrhizans*	Gran Canaria	*Pinus radiata*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19124	*P. carporrhizans*	Gran Canaria	*Pinus radiata*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19125	*P. carporrhizans*	Gran Canaria	*Pinus radiata*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19126	*P. carporrhizans*	Gran Canaria	*Pinus radiata*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19127	*P. carporrhizans*	Gran Canaria	*Pinus radiata*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19128	*P. carporrhizans*	Gran Canaria	*Pinus radiata*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19129	*P. carporrhizans*	Gran Canaria	*Pinus radiata*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009

Appendix 1. Continued.

Voucher no.	Species	Locality	Substrate[b]	Geographic coordinates	Elevation (m)	Collectors	Collection date
19130	*P. carporrhizans*	Gran Canaria	*Pinus radiata*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19131	*P. carporrhizans*	Gran Canaria	*Pinus radiata*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19132	*P. carporrhizans*	Gran Canaria	*Pinus radiata*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19133	*P. carporrhizans*	Gran Canaria	*Castanea sativa*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19134	*P. carporrhizans*	Gran Canaria	*Castanea sativa*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19135	*P. carporrhizans*	Gran Canaria	*Castanea sativa*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19136	*P. carporrhizans*	Gran Canaria	*Castanea sativa*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19137	*P. carporrhizans*	Gran Canaria	*Castanea sativa*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19138	*P. carporrhizans*	Gran Canaria	*Castanea sativa*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19139	*P. carporrhizans*	Gran Canaria	*Castanea sativa*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19140	*P. carporrhizans*	Gran Canaria	*Castanea sativa*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19141	*P. carporrhizans*	Gran Canaria	*Castanea sativa*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19142	*P. carporrhizans*	Gran Canaria	*Castanea sativa*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19143	*P. carporrhizans*	Gran Canaria	*Castanea sativa*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19144	*P. carporrhizans*	Tenerife	*Castanea sativa*	28°27′11″N 16°24′55″W	894	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	23/06/2009
19145	*P. carporrhizans*	Tenerife	*Castanea sativa*	28°27′11″N 16°24′55″W	894	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	23/06/2009
19146	*P. carporrhizans*	Tenerife	*Castanea sativa*	28°27′11″N 16°24′55″W	894	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	23/06/2009

Appendix 1. Continued.

Voucher no.	Species	Locality	Substrate[b]	Geographic coordinates	Elevation (m)	Collectors	Collection date
19147	*P. carporrhizans*	Tenerife	*Castanea sativa*	28°27′11″N 16°24′55″W	894	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	23/06/2009
19148	*P. carporrhizans*	Tenerife	*Castanea sativa*	28°27′11″N 16°24′55″W	894	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	23/06/2009
19149	*P. carporrhizans*	Tenerife	*Castanea sativa*	28°27′11″N 16°24′55″W	894	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	23/06/2009
19150	*P. carporrhizans*	Tenerife	*Castanea sativa*	28°27′11″N 16°24′55″W	894	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	23/06/2009
19151	*P. carporrhizans*	Tenerife	*Castanea sativa*	28°27′11″N 16°24′55″W	894	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	23/06/2009
19152	*P. carporrhizans*	Tenerife	*Castanea sativa*	28°27′11″N 16°24′55″W	894	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	23/06/2009

Note: Tr = Turkey; Es = Spain; Ma = Morocco.

[a] The first eight samples were tested against all 24 microsatellite primer pairs. The last 32 samples were tested against a subset of 12 microsatellite primer pairs (see Methods and Results).

[b] Scientific authorities for substrate species: *Castanea sativa* Mill., *Pinus radiata* D. Don, *Prunus* L., *Quercus* L., *Quercus ilex* L., *Ulmus* L.