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Highly variable microsatellite markers for the fungal and algal symbionts of the lichen *Lobaria pulmonaria* and challenges in developing biont-specific molecular markers for fungal associations

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ABSTRACT

The availability of highly variable markers for the partners of a fungal symbiosis enables the integrated investigation of ecological and evolutionary processes at the symbiotic level. In this article we analyze the specificity of the first and to date only microsatellite markers that had been developed for an epiphytic lichen (*Lobaria pulmonaria*). We used DNA extracts from cultures of the fungal and of the green algal symbionts of *L. pulmonaria* as well as total DNA extracts from related *Lobaria* species associated with the same algal partner, and got evidence that five of the previously described microsatellite markers, proposed to be fungus-specific, are indeed alga-specific. Hence, highly variable microsatellite primer sets available for both, the algal and the fungal symbionts of *L. pulmonaria* are now at our hands, which allow us to investigate so far unexplored biological processes of lichen symbionts, such as codispersal and coevolution. In a broader sense, our work evaluates and discusses the challenges in developing biont-specific molecular markers for fungi forming close associations with other organisms.

Introduction

The estimation of genetic variation among individuals and within or among populations or species is the aim of much evolutionary and ecological research. Depending on mutation rates and patterns of inheritance of different molecular marker types (Parker *et al.* 1998; Zhang & Hewitt 2003), biological processes acting on different time scales can be detected (Bridge & Hawksworth 1998; Chenuil 2006).

For studying biological processes at the level of lichen populations, molecular markers with high variability are needed. In other organisms, corresponding markers of choice are microsatellites (Morgante & Olivieri 1993) or amplified fragment length polymorphisms (AFLPs; Vos *et al.* 1995). However, since anonymous DNA-fingerprinting methods such as AFLPs are only applicable if the genomes of symbionts can be separated, these methods are not applicable to field collected lichens where the genomes are together (Lohtander *et al.* 1998; Murtagh *et al.*

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1999). Within the lichen thallus, fungi, algae and/or cyanobacteria are living in close physical contact as myco- and photobionts. Their separation for later molecular analyses is a challenging task. Two possible routes are available to obtain pure DNA from each of the symbionts. First, one can separate them manually under a dissecting microscope or, second, one can cultivate them in axenic cultures (Grube 2005). Since hundreds of individuals have usually to be fingerprinted in population genetic analyses, it is unfeasible to separate the symbiotic partners of all samples before DNA extraction. The only reasonable alternative for studying population genetic processes is to use PCR-primers that are specific to either of the symbiotic partners. To date, there is only a limited number of fungus-specific primers available for population-genetic analyses in lichens (Zoller *et al.* 1999; Högberg *et al.* 2002; Printzen 2002; Printzen *et al.* 2003; Lindblom & Ekman 2006).

Walser *et al.* (2003) developed the first and to date only species-specific microsatellite markers (SSRs) for a lichen (*Lobaria pulmonaria* L. (Hoffm.)). The lichen *L. pulmonaria* contains a fungal partner and the green alga *Dictyochloropsis reticulata* (Tschermak-Woess) Tschermak-Woess and, less abundantly, the cyanobacterium *Nostoc* sp. as photobionts (Jordan 1973). The symbiotic nature of *L. pulmonaria* makes the isolation of fungus-specific microsatellites difficult, and Walser *et al.* (2003) manually separated algae and fungi before DNA extraction for microsatellite development. They subsequently excluded alga-specific loci by testing all developed markers with DNA from axenic cultures of the photobiont of *L. pulmonaria*.

In this study, we evaluate those microsatellite markers (Walser *et al.* 2003, 2004) by testing for their symbiont-specificity. We used DNA extracts from axenic cultures of the photobiont as well as of the mycobiont of *L. pulmonaria* and tested DNA purity with fungus- and alga-specific primers. When amplifying the 12 microsatellite loci from Walser *et al.* (2003, 2004) with genomic DNA extracted from these newly established cultures of the photobiont of *L. pulmonaria*, we got evidence that five of these markers were in fact amplifying the algal DNA. We then sequenced the three microsatellite loci *LPu16*, *LPu20* and *LPu27*, which had been redesigned (Walser *et al.* 2004) from the original primers (Walser *et al.* 2003) in order to verify whether the redesign might have caused a specificity shift from the fungus to the alga. Finally, we tested the identified fungus-specific and alga-specific microsatellite primers with three other *Lobaria* lichens having the same photobiont as *L. pulmonaria*. This study demonstrates and discusses the difficulties of developing species-specific molecular markers for fungi which form close associations and for which axenic isolation and cultivation is problematic. The alga- and fungus-specific microsatellite loci tested and discussed in this work are tools for studying so far unexplored evolutionary processes such as dispersal, gene flow or mutation in lichen-forming associations.

Materials and methods

Cultivation of and DNA extraction from the algal and the fungal symbionts of L. pulmonaria

Two axenic unialgal cultures of *Dictyochloropsis reticulata* were provided by Andreas Beck (Botanische Staatssammlung, Munich, Germany). The algae were isolated from two *Lobaria pulmonaria* specimens (algal culture collection of the Botanische Staatssammlung, Munich: AB06.006A2, Spain, in this study A1, and AB06.006B5, France, in this study A2). Algal cultures of *D. reticulata* isolated from *L. pulmonaria* var. *meridionalis* (Vain.) Zahlbr. (SAG 53.87) and from *Brigantiaea ferruginea* (Müll. Arg.) Kashiw. and Kurok. (CCHU 5616), as well as algal cultures of *D. splendida* Geitler (SAG 244.80), *D. symbiontica* Tschermak-Woess (SAG 46.85) and *D. irregularis* Nakano and Isagi (SAG 2036) were purchased from the Culture Collection of the University of Göttingen, Germany (SAG) and the Culture Collection of the Hiroshima University, Japan (CCHU). In order to obtain enough material for DNA isolation, all algal strains were inoculated on agar medium under sterile conditions. Cultivation took place under a diurnal light (12 h) and darkness (12 h) cycle with a constant temperature of 16 °C. The agar medium contained Bold's mineral solution (Ahmadjian 1967), 1.5 % agarose (AppliChem, Darmstadt, Germany), 0.5 % Bacto Peptone (BD, Franklin Lakes, NJ), 1 % Glucose (Sigma–Aldrich, St. Louis, MO) and 0.0715 % Hepes (Sigma–Aldrich).

To obtain pure DNA of the mycobiont, mycelia of two *L. pulmonaria* specimens (vouchers of the Herbarium of the Swiss Federal Research Institute WSL, Birmensdorf: 17061, Switzerland, in this study F1; 10161, Scotland, in this study F2) were cultivated according to Denison (2003) with some modifications. For each specimen a lobe of a recently collected thallus bearing fungal sporocarps (apothecia) was dried in silica gel for several days. Apothecia were then removed from the lobe and placed on filter paper wettened with sterilized water on the caps of inverted Petri dishes, which contained a sterile medium of 1 % alpha-cyclodextrin (Sigma–Aldrich) and 1.5 % corn meal agar (BD). Spores were discharged upward to the medium within 1–2 d and started to germinate after 3 d. After discharge of spores, filter papers with apothecia were removed and the caps of the Petri dishes replaced by new sterile ones. After 2 weeks of growth, axenic mycelia were transferred to new Petri dishes. Germination and growth took place at 16 °C in complete darkness during 3 m.

Algal cells (*ca* 20 mg for each algal culture) were disrupted in a mixer mill (MM300, Retsch, Haan, Germany), and DNA was extracted with the GenElute Plant Genomic DNA Miniprep Kit (Sigma–Aldrich) according to the manufacturer's instructions. The concentration of the isolated DNA was estimated by agarose gel electrophoresis.

For each fungal culture, *ca* 17 mg of the mycelia grown from hundreds of germinated spores were harvested under sterile conditions and pooled in an ice-cooled Eppendorf tube containing 30 μl of lysis buffer AP1 (Qiagen). The mycelia in the tube were frozen before disruption of cell material with a mixer mill (MM300, Retsch). DNA isolation with the DNeasy Plant Mini Kit (Qiagen, Venlo, The Netherlands) followed the manufacturer's instructions (Mini Protocol). The DNA concentration was estimated by agarose gel electrophoresis.

In addition, total genomic DNA, containing both fungal and algal DNA, was extracted from single lobes of several *Lobaria* specimens using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's recommendations. We extracted DNA from dried thallus material (*ca* 35–50 mg) of seven *L. pulmonaria* specimens and also from three other *Lobaria* lichens (*L. amplissima* (Scop.) Forss., *L. spathulata* (Inum.) Yoshim.

and *L. tuberculata* Yoshim.) which have the same photobiont as *L. pulmonaria* (Dal Grande 2007). The concentration of the isolated total genomic DNA was in the range of 10–25 ng/μl. For further description of the specimens used for DNA extraction see Supplementary Table 1.

Test for the purity of the DNA extracted from cultivated algal and fungal symbionts of L. pulmonaria

To ascertain that the DNA extracted from the algal (A1 and A2) and fungal (F1 and F2) cultures of *Lobaria. pulmonaria* symbionts were not contaminated by the partaking biont, we tested both DNA extracts with alga (rbcLa–rbcLb; F.D.G. *et al.*, unpubl. data; Supplementary Table 2) and fungus-specific (mrSSU1–mrSSU2R; Zoller *et al.* 1999; Supplementary Table 2) primer pairs. Additionally, we amplified the total genomic DNA of the lichen *L. pulmonaria* (T) with both primer pairs.

The primer pair rbcLa–rbcLb amplifies the large subunit of the ribulose-bisphosphate carboxylase gene (*rbcL*) and is specific for the chloroplast DNA of the photobiont of *L. pulmonaria*. The *rbcL* gene is absent in the fungus thus the rbcL primer pair being a molecular tool to test for algal contamination in pure fungal DNA of lichens associated with this algal species. In contrast, the primers mrSSU1–mrSSU2R, amplifying the mitochondrial small subunit (*mrSSU*), are specific for the mitochondrial DNA of lichen-forming ascomycetes. For PCR conditions see Supplementary Table 3 (PCR reaction mix) and Supplementary Table 4 (PCR cycling protocols). Amplification products were analyzed on 1.5 % Tris–Borate–ethylenediaminetetraacetic acid (EDTA)–agarose gels (Tris base: 89 mM, boric acid: 89 mM, EDTA: 2 mM), stained with GelRed (Biotium, Hayward, CA) and visualized with a Molecular Imager Gel Doc XR System (BioRad, Hercules, CA).

Amplification of L. pulmonaria *microsatellites with different DNA templates*

We tested the symbiont-specificity of the microsatellites developed for *Lobaria pulmonaria* with various DNA templates. The pure DNA of the myco- and photobiont of *L. pulmonaria*, total genomic DNA extracts from *L. pulmonaria* and from related *Lobaria* lichens and DNA from cultures of the photobiont of *L. pulmonaria* (CCHU 5616, SAG 53.87) were amplified with the 12 microsatellite primer pairs considered to be specific for the mycobiont of *L. pulmonaria* (Walser *et al.* 2003, 2004).

Additionally, we tested *Dictyochloropsis splendida*, *D. symbiontica* and *D. irregularis* for amplification with the same primers. The 12 microsatellite markers were amplified in three multiplex PCR reactions (multiplex 1: *LPu03*, *LPu09*, *LPu15*; multiplex 2: *LPu23*, *LPu24*, *LPu25*, *LPu28*; multiplex 3: *LPu16*, *LPu19*, *LPu20*, *LPu26*, *LPu27*; Supplementary Tables 3, 4). Fragment lengths were determined on a 3130 automated sequencer (Applied Biosystems) against an internal size standard (multiplex 1: ROX-500, multiplexes 2 and 3: LIZ-500; Applied Biosystems) and by using GeneMapper 3.7 (Applied Biosystems). For primer sequences and labelling see Supplementary Table 5.

The specificity of the microsatellite primers for *L. pulmonaria* was also assessed by amplifying the pure DNA of the myco- and photobiont as well as the total DNA extract (containing fungal and algal DNA) from *L. pulmonaria* with unlabelled microsatellite primers in single reactions. Amplification products were separated on 1.5 % TBE–agarose gels, stained with ethidium bromide and visualized with a Molecular Imager Gel Doc XR System (BioRad).

Sequencing of algal ITS nrDNA from the lichens L. pulmonaria, L. pulmonaria *var. meridionalis and* B. ferruginea *and locus comparison in the photobiont using original and redesigned primers*

Walser *et al.* (2003) tested the microsatellite markers developed for *L. pulmonaria* for specificity with DNA isolated from different axenic cultures of the alga *Dictyochloropsis reticulata*. Among other cultures, they used a culture of *D. reticulata* isolated from *Lobaria pulmonaria* var. *meridionalis* (SAG 53.87). To check if this photobiont belongs to the same algal strain as the photobiont isolated from *L. pulmonaria* (A2), we sequenced the ITS nrDNA of the photobionts of both lichens. Additionally, *D. reticulata* isolated from the lichen *Brigantiaea ferruginea* (CCHU 5616) was also sequenced.

Algal ITS nrDNA was amplified and sequenced using the forward primer nr-SSU-1780-5′ Algal (Piercey-Normore & DePriest 2001) in combination with the reverse primer A-ITS-R (F.D.G. *et al.*, unpubl. data; Supplementary Table 2). PCR products were purified with the MinElute PCR Purification Kit (Qiagen) following the manufacturer's recommendations, but DNA was eluted from membranes with the double volume of the elution buffer (EB). For sequencing, we used 2 μl of Big Dye Terminator v3.1 Cycle Sequencing mix (Applied Biosystems, Foster City, CA), about 20 ng of PCR product and 2 pmol primer. Cycle sequencing encompassed 26 cycles of 20 s at 96 °C, 5 s at 50 °C and 2 min at 60 °C. Cycle sequencing products were purified with Performa DTR Gel Filtration Cartridges (EdgeBio, Gaithersburg, MD), and sequences determined on a 3130 automated sequencer (Applied Biosystems) and analyzed with the Sequencing Analysis Software 3.4 (Applied Biosystems). Sequences were aligned using the BLASTN 2.2.20+ (http://blast.ncbi.nlm.nih.gov/Blast.cgi; Altschul *et al.* 1997).

Some of the original microsatellite primers for *L. pulmonaria* (Walser *et al.* 2003) were redesigned by Walser *et al.* (2004), namely *LPu16*, *LPu20* and *LPu27*. To test whether this redesign changed the biont specificity of the primers, we sequenced the amplification products of these microsatellite loci as generated by the original and the redesigned primers. We then compared the sequences generated from the total DNA of three *L. pulmonaria* specimens (vouchers of the Herbarium of the Swiss Federal Research Institute WSL, Birmensdorf: 8597, France; 8617, France; 491, Turkey) and the DNA of the photobiont isolated from *L. pulmonaria* (A1) and *L. pulmonaria* var. *meridionalis* (SAG 53.87), respectively. Sequencing was performed as given above for algal ITS nrDNA. Sequences were analyzed with DNADynamo (Bluetractor Software, Gwynedd, UK).

Results

Cultivation and DNA extraction of the algal and the fungal symbionts of L. pulmonaria

The algae isolated from different *Lobaria* lichens grew well under the selected cultivation conditions. The amount of the

DNA after extraction from about 20 mg of algal cells was *ca* 4 μg (20 ng/μl). In contrast, the fungal symbiont grew more slowly than the algal symbiont. After discharge and germination of hundreds of spores, it took 3 m of growth to obtain 160 ng (*ca* 0.8 ng/μl) of pure DNA, when pooling all the mycelia together.

Test for the purity of DNA extracted from cultivated algal and fungal symbionts of L. pulmonaria

The DNA extracted from the cultivated photobiont amplified with algal chloroplast-specific primers (rbcLa–rbcLb), but did not amplify with mtDNA primers for lichen-forming ascomycetes (mrSSU1–mrSSU2R). The fungal DNA showed no amplification with the algal chloroplast-specific primers but did so with the fungus-specific ones. This suggested purity of the DNA extracted from the cultures of the symbionts of *Lobaria pulmonaria*. DNA extracted from lichen thallus material contained algal and fungal DNA and thus amplified with both the alga- and the fungus-specific primers (Fig 1).

Amplification of L. pulmonaria *microsatellites with different DNA templates*

Using the pure DNA extracts of the algal and fungal symbionts of *Lobaria pulmonaria*, the primers for the microsatellite loci *LPu03*, *LPu09*, *LPu15*, *LPu23*, *LPu24*, *LPu25* and *LPu28* only amplified with pure fungal DNA, confirming the fungus-specific nature of these seven markers. In contrast, the microsatellite markers *LPu16*, *LPu19*, *LPu20*, *LPu26* and *LPu27* only amplified the pure algal DNA and showed no amplification with the fungal DNA. Total DNA extracted from a *L. pulmonaria* thallus amplified with all 12 microsatellite primer pairs (Fig 2). For a summary of the amplification results of *L. pulmonaria* with the different DNA templates consult Table 1 and Fig 2.

The total DNA extracts of related *Lobaria* lichens (*L. amplissima*, *L. spathulata* and *L. tuberculata*) having the same photobiont as *L. pulmonaria* amplified with the primer pairs *LPu16*, *LPu19*, *LPu20*, *LPu26* and *LPu27* (specific for the photobiont of *L. pulmonaria*). Their allele lengths matched with those found in *L. pulmonaria* and were in the same range as the allele lengths of the fungus-specific microsatellites. Cross-amplification with the primer pairs *LPu03*, *LPu09*, *LPu15*, *LPu23*, *LPu24*, *LPu25* and *LPu28* (specific for the mycobiont of *L. pulmonaria*) resulted in a non-homogeneous picture. None of the primers amplified in *L. amplissima*, three primers (*LPu15*, *LPu23*, *LPu28*) amplified in *L. spathulata* and five primers amplified in *L. tuberculata* (*LPu09*, *LPu15*, *LPu23*, *LPu24*, *LPu28*).

The DNA extract of *Dictyochloropsis reticulata* isolated from *L. pulmonaria* var. *meridionalis* (SAG 53.87) was successfully amplified with all alga-specific primer pairs, matching the allele sizes found in the photobiont of *L. pulmonaria*. There was no amplification with the fungus-specific primers. PCR amplification with all the primers completely failed for DNA extracted from *D. reticulata* isolated from *Brigantiaea ferruginea* (CCHU 5616).

DNA extracts of cultures of *D. splendida*, *D. symbiontica* and *D. irregularis* tested with the microsatellite primers of *L. pulmonaria* resulted in no amplification at all. Thus, there was no cross-species amplification for the alga-specific primers (*LPu16*, *LPu19*, *LPu20*, *LPu26* and *LPu27*) in these *Dictyochloropsis* species.

Sequencing of algal ITS nrDNA from the lichens L. pulmonaria, L. pulmonaria *var.* meridionalis *and* B. ferruginea *and locus comparison in the photobiont using original and redesigned primers*

The photobionts of *Lobaria pulmonaria* and *L. pulmonaria* var. *meridionalis* had almost identical 700 bp ITS nrDNA sequences (GenBank accession nos. FJ936173 and FJ936174; alignable for 100 % with a maximal identity of 99 %). On the other hand, the sequence of ITS nrDNA from the algal culture from *Brigantiaea ferruginea* (CCHU 5616, FJ936172) was clearly different from that of the photobiont of *L. pulmonaria* (alignable for only 60 % with a maximal identity of 95 %).

We sequenced the amplification products generated with the original microsatellite primers (Walser *et al.* 2003) and the redesigned microsatellite primers (*LPu16*, *LPu20*, *LPu27*; Walser *et al.* 2004) on DNA extracted from lichen thallus material and pure algal DNA. The flanking regions were identical and in agreement with the sequences published on GenBank (*LPu16*: AF513004, *LPu20*: AF513007, AF513008, *LPu27*:

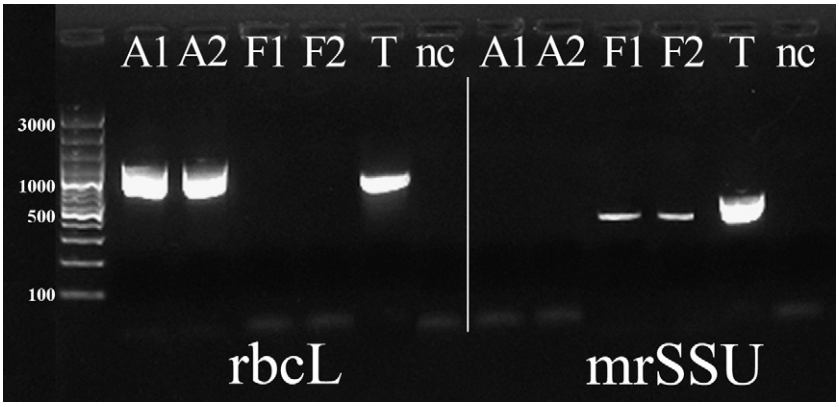

Fig 1 – Gel of PCR products obtained by amplifying DNA extracted from cultivated algal and fungal symbionts of *Lobaria pulmonaria* with alga- and fungus-specific markers. A1, A2: DNA extracted from cultures of the algal symbiont; F1, F2: DNA extracted from cultures of the fungal symbiont; T: total DNA extracted from a thallus of *L. pulmonaria*; rbcL: alga-specific markers; mrSSU: fungus-specific markers; nc: negative control.

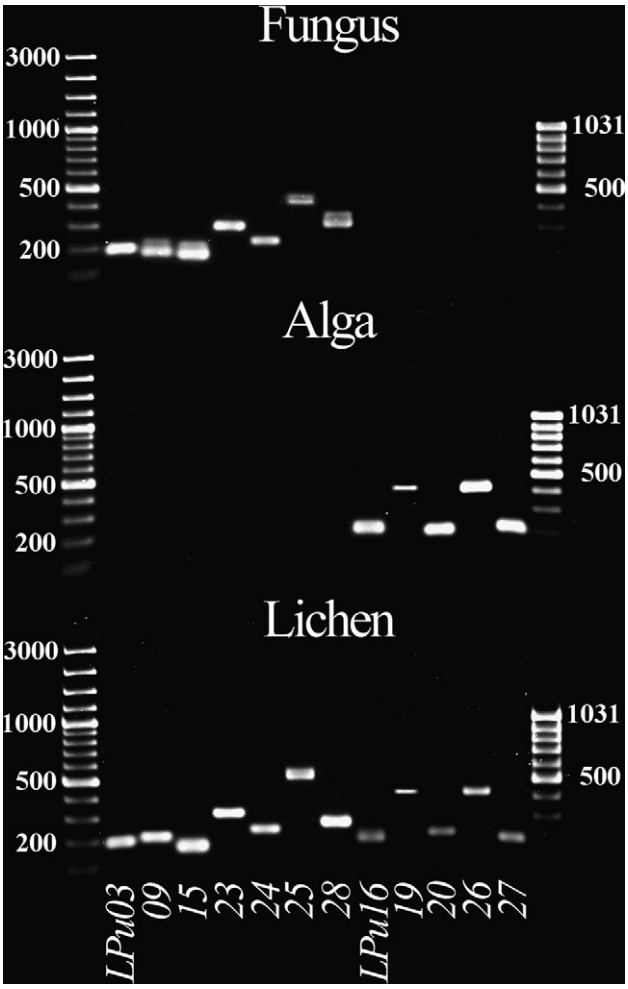

Fig 2 – Agarose gel electrophoresis of PCR products obtained by amplifying pure DNA of the algal and fungal symbionts of *Lobaria pulmonaria* as well as the total lichen DNA of *L. pulmonaria* with 12 microsatellite primers developed for *L. pulmonaria*. Fungus-specific microsatellites: *LPu03*, *LPu09*, *LPu15*, *LPu23*, *LPu24*, *LPu25*, *LPu28*; alga-specific microsatellites: *LPu16*, *LPu19*, *LPu20*, *LPu26*, *LPu27*. Note that pure fungal DNA was extracted from mycelia possibly having different multilocus genotypes, resulting in multiple bands for the more variable fungus-specific markers (*LPu09*, *LPu15*, *LPu25* and *LPu28*).

AF513013) when using both the original and the redesigned primers. The sequences generated with DNA extracted from total lichen thallus material and with pure algal DNA were identical (data not shown).

Discussion

Testing the symbiont-specificity of 12 microsatellites for L. pulmonaria

The conclusion that some of the microsatellite loci proposed to be fungus-specific by Walser *et al.* (2003) were indeed alga-specific was based on the following evidence. First, before testing microsatellite specificity, we tested the purity of the DNA extracted from the two cultivated symbionts of *Lobaria pulmonaria*. We thus performed PCR amplification with the DNA extracted from both cultivated symbionts with alga- and fungus-specific primers. For pure algal DNA, there was amplification with alga-specific primers only (rbcLa–rbcLb). For fungal DNA, only amplification with fungus-specific primers (mrSSU1–mrSSU2R) was observed. In this way, we verified that DNA extracts of algal and fungal cultures were not contaminated by the DNA of the other symbiont. We then showed that the microsatellite primers *LPu16*, *LPu19*, *LPu20*, *LPu26* and *LPu27* are specific for the algal symbiont, while the primers *LPu03*, *LPu09*, *LPu15*, *LPu23*, *LPu24*, *LPu25* and *LPu28* are specific for the fungal symbiont of *L. pulmonaria*.

Second, based on the identity of ITS nrDNA sequences of the photobionts, we confirmed that *L. amplissima*, *L. spathulata*, *L. tuberculata* and *L. pulmonaria* var. *meridionalis* had the same photobiont as *L. pulmonaria*. However, the photobiont isolated from *Brigantiaea ferruginea* did not show the same ITS nrDNA sequence as the photobiont of *L. pulmonaria*, indicating that it belonged to a different algal lineage. In addition, there was no amplification when testing the alga-specific microsatellite markers *LPu16*, *LPu19*, *LPu20*, *LPu26* and *LPu27* with the DNA isolated from the photobiont of *B. ferruginea*. The alga-specific primers *LPu16*, *LPu19*, *LPu20*, *LPu26* and *LPu27* amplified with total DNA of lichens having the same symbiotic alga as *L. pulmonaria* (*L. amplissima*, *L. spathulata*, *L. tuberculata* and *L. pulmonaria* var. *meridionalis*). In addition their allele lengths matched those found in *L. pulmonaria*. This provided us with another strong argument that the microsatellite primers *LPu16*, *LPu19*, *LPu20*, *LPu26* and *LPu27* are indeed alga-specific.

Third, only some of the fungus-specific primers *LPu03*, *LPu09*, *LPu15*, *LPu23*, *LPu24*, *LPu25* and *LPu28* cross-amplified when testing them with total DNA extracts from *L. amplissima*, *L. spathulata* and *L. tuberculata*. The number of primer pairs that amplify with a species parallels the taxonomic subdivision of the genus *Lobaria*. The three species *L. pulmonaria*, *L. spathulata* and *L. tuberculata* are closely related species belonging to the section *Lobaria*, whereas *L. amplissima* is a member of the section *Ricasolia* (Yoshimura 1971). The alga-specific primers (*LPu16*, *LPu19*, *LPu20*, *LPu26* and *LPu27*) did not amplify DNA extracts from cultures of *D. splendida*, *D. symbiontica* and *D. irregularis*.

Fourth, sequencing of the microsatellite loci *LPu16*, *LPu20* and *LPu27* with the original and the redesigned primers from Walser *et al.* (2003, 2004) verified the identity of the flanking regions. Hence, there was no specificity shift from fungus-specific to alga-specific amplification from the original to the new primers during redesign.

The above tests unequivocally prove that the microsatellite primers *LPu16*, *LPu19*, *LPu20*, *LPu26* and *LPu27* are specific for the algal symbiont of *L. pulmonaria*, while the primers *LPu03*, *LPu09*, *LPu15*, *LPu23*, *LPu24*, *LPu25* and *LPu28* are specific for its fungal symbiont. There are thus microsatellite primer sets available for both the fungal and the algal partners of *L. pulmonaria*.

Challenges in developing species-specific markers for fungi living in close association with other organisms

This study illustrates the challenges and risks of developing species-specific markers for fungal associations, where the

Table 1 – Amplification of different DNA templates with 12 microsatellite markers of *Lobaria pulmonaria*. +: PCR amplification; –: no PCR amplification; grey shading: specimens showing amplification with alga-specific microsatellites

DNA template	Microsatellite marker											
	Fungus-specific							Alga-specific				
	LPu03	*LPu09*	*LPu15*	*LPu23*	*LPu24*	*LPu25*	*LPu28*	*LPu16*	*LPu19*	*LPu20*	*LPu26*	*LPu27*
Lobaria pulmonaria												
Fungal symbiont of *L. pulmonaria* (F1)	+	+	+	+	+	+	+	–	–	–	–	–
Fungal symbiont of *L. pulmonaria* (F2)	+	+	+	+	+	+	+	–	–	–	–	–
Algal symbiont of *L. pulmonaria* (A1)	–	–	–	–	–	–	–	+	+	+	+	+
Algal symbiont of *L. pulmonaria* (A2)	–	–	–	–	–	–	–	+	+	+	+	+
L. pulmonaria (total lichen DNA, T)	+	+	+	+	+	+	+	+	+	+	+	+
Total DNA of lichens having the same alga as *L. pulmonaria*												
L. amplissima	–	–	–	–	–	–	–	+	+	+	+	+
L. spathulata	–	–	+	+	–	–	+	+	+	+	+	+
L. tuberculata	–	+	+	+	+	–	+	+	+	+	+	+
Pure algal DNA												
Dictyochloropsis reticulata (SAG 53.87)	–	–	–	–	–	–	–	+	+	+	+	+
Alga isolated from *Brigantiaea ferruginea* (CCHU 5616)	–	–	–	–	–	–	–	–	–	–	–	–
D. splendida	–	–	–	–	–	–	–	–	–	–	–	–
D. symbiontica	–	–	–	–	–	–	–	–	–	–	–	–
D. irregularis	–	–	–	–	–	–	–	–	–	–	–	–

axenic cultivation of the fungus living in close association with other organisms is problematic (e.g. for lichen-forming, mycorrhizal or parasitic fungi). Separation of the associated organisms for subsequent DNA extractions is the key step. A minor contamination with the DNA of the non-target organism can lead to erroneous primer development and, hence, erroneous data sets. For the lichen *Lobaria pulmonaria*, it is extremely difficult to manually separate the algal from the fungal symbionts. Even in fruit bodies (apothecia) of *L. pulmonaria*, algal cells are found. Since Walser *et al.* (2003) developed their microsatellite primers from DNA of apothecia, the presence of algae could explain why five out of 12 microsatellite loci are situated on the algal and not on the fungal genome. Furthermore, if we consider that microsatellite loci seem to be less abundant in fungi than in other organisms (Dutech *et al.* 2007), the chance of picking algal DNA during the enrichment is substantial in the development of fungus-specific microsatellites.

Due to the fact that Walser *et al.* (2003) had no pure DNA of the mycobiont of *L. pulmonaria* at their hand, they could only test the fungus-specific nature of the isolated microsatellites with pure DNA of the photobiont of *L. pulmonaria*. Amongst others they tested the microsatellite markers developed for the fungal part of the symbiosis with the photobiont extracted from *L. pulmonaria* var. *meridionalis* (SAG 53.87). There was no amplification with this DNA extract. Perhaps there were PCR inhibitors present in the reaction such as secondary compounds or polysaccharides. This result led Walser *et al.* (2003) to the wrong conclusion that all microsatellites were fungus-specific. However, in this study, we showed that *L. pulmonaria* var. *meridionalis* has the same photobiont as *L. pulmonaria* and that there is indeed PCR amplification with DNA from the very same culture of the photobiont with the microsatellite primers *LPu16*, *LPu19*, *LPu20*, *LPu26* and *LPu27*, once adequate PCR conditions are met. There was no amplification with the *Dictyochloropsis reticulata* culture from *Brigantiaea ferruginea* (CCHU 5616) when amplified with *LPu16*, *LPu19*, *LPu20*, *LPu26* and *LPu27* because, as shown in this study, this culture did not belong to the same algal lineage as the photobiont of *L. pulmonaria*. Therefore, even referring to some established reference cultures of a biont can be misleading. In fact, its phylogenetic position might be uncertain and in need of a revision, as it is in our case with the culture of *D. reticulata* from *B. ferruginea* (CCHU 5616).

Our findings showed that a very careful evaluation is necessary when developing species-specific molecular primers such as microsatellites for fungi living in close association with other organisms.

Over the last few years, several studies (Walser *et al.* 2004, 2005; Werth *et al.* 2006, 2007) have been carried out based on the assumption that the microsatellites isolated for *L. pulmonaria* by Walser *et al.* (2003, 2004) are fungus-specific. However, we showed here that the microsatellites *LPu16*, *LPu19*, *LPu20*, *LPu26* and *LPu27* are in fact alga-specific. Preliminary analyses showed that population pairwise genetic differentiation measurements substantially changed when estimating F_{ST} values based only on fungus- or alga-specific microsatellite data sets (data not shown). However, there was no clear trend in the rate and direction of change. This implies that the population genetic work carried out so far with the microsatellite primers for *L. pulmonaria* should be reanalyzed and critically revised. The present study also provides microsatellite primer sets for both the fungal and algal symbionts of a lichen symbiosis (Dal Grande *et al.* 2010). These microsatellite sets represent very effective tool for studying many unexplored evolutionary processes in lichens, such as codispersal, vertical *versus* horizontal transfer of symbionts or algal sharing within fungal communities. They thus enable us to shed more light on evolutionary patterns and processes at different spatial scales.

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Supplementary material

Supplementary data associated with this article can be found in online version at doi:10.1016/j.funbio.2010.04.003.

REFERENCES

Ahmadjian V, 1967. *The Lichen Symbiosis*. Blaisdell, Waltham, Mass.

Altschul SF, Madden TL, Schaffer AA, Zhang JH, Zhang Z, Miller W, Lipman DJ, 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**: 3389–3402.

Bridge PD, Hawksworth DL, 1998. What molecular biology has to tell us at the species level in lichenized fungi. *Lichenologist* **30**: 307–320.

Chenuil A, 2006. Choosing the right molecular genetic markers for studying biodiversity: from molecular evolution to practical aspects. *Genetica* **127**: 101–120.

Dal Grande F, 2007. Photobiont guild and phylogeography of *Dictyochloropsis* sp., the photobiont of the lichen *Lobaria pulmonaria* (L.) Hoffm., and a re-evaluation of its phylogeny. Master-thesis, University of Trieste, Trieste, Italy.

Dal Grande F, Widmer I, Beck A, Scheidegger C, 2010. Microsatellite markers for *Dictyochloropsis reticulata* (Trebouxiophyceae), the symbiotic alga of the lichen *Lobaria pulmonaria* (L.). *Conservation Genetics* **11**: 1147–1149.

Denison WC, 2003. Apothecia and ascospores of *Lobaria oregana* and *Lobaria pulmonaria* investigated. *Mycologia* **95**: 513–518.

Dutech C, Enjalbert J, Fournier E, Delmotte F, Barres B, Carlier J, Tharreau D, Giraud T, 2007. Challenges of microsatellite isolation in fungi. *Fungal Genetics and Biology* **44**: 933–949.

Grube M, 2005. Nucleic acid isolation from ecological samples – fungal associations, lichens. *Methods in Enzymology* **395**: 48–57.

Högberg N, Kroken S, Thor G, Taylor JW, 2002. Reproductive mode and genetic variation suggest a North American origin of European *Letharia vulpina*. *Molecular Ecology* **11**: 1191–1196.

Jordan WP, 1973. The genus *Lobaria* in North America north of Mexico. *Bryologist* **76**: 225–251.

Lindblom L, Ekman S, 2006. Genetic variation and population differentiation in the lichen-forming ascomycete *Xanthoria parietina* on the island Storfosna, central Norway. *Molecular Ecology* **15**: 1545–1559.

Lohtander K, Källersjö M, Tehler A, 1998. Dispersal strategies in *Roccellina capensis* (Arthoniales). *Lichenologist* **30**: 341–350.

Morgante M, Olivieri AM, 1993. PCR-amplified microsatellites as markers in plant genetics. *The Plant Journal* **3**: 175–182.

Murtagh GJ, Dyer PS, McClure PC, Crittenden PD, 1999. Use of randomly amplified polymorphic DNA markers as a tool to study variation in lichen-forming fungi. *Lichenologist* **31**: 257–268.

Parker PG, Snow AA, Schug MD, Booton GC, Fuerst PA, 1998. What molecules can tell us about populations: choosing and using a molecular marker. *Ecology* **79**: 361–382.

Piercey-Normore MD, DePriest PT, 2001. Algal switching among lichen symbioses. *American Journal of Botany* **88**: 1490–1498.

Printzen C, 2002. Fungal specific primers for PCR-amplification of mitochondrial LSU in lichens. *Molecular Ecology Notes* **2**: 130–132.

Printzen C, Ekman S, Tønsberg T, 2003. Phylogeography of *Cavernularia hultenii*: evidence of slow genetic drift in a widely disjunct lichen. *Molecular Ecology* **12**: 1473–1486.

Vos P, Hogers R, Bleeker M, Reijans M, Vandelee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M, 1995. AFLP – a new technique for DNA-fingerprinting. *Nucleic Acids Research* **23**: 4407–4414.

Walser JC, Sperisen C, Soliva M, Scheidegger C, 2003. Fungus-specific microsatellite primers of lichens: application for the assessment of genetic variation on different spatial scales in *Lobaria pulmonaria*. *Fungal Genetics and Biology* **40**: 72–82.

Walser JC, Gugerli F, Holderegger R, Kuonen D, Scheidegger C, 2004. Recombination and clonal propagation in different populations of the lichen *Lobaria pulmonaria*. *Heredity* **93**: 322–329.

Walser JC, Holderegger R, Gugerli F, Hoebee SE, Scheidegger C, 2005. Microsatellites reveal regional population differentiation and isolation in *Lobaria pulmonaria*, an epiphytic lichen. *Molecular Ecology* **14**: 457–467.

Werth S, Wagner HH, Holderegger R, Kalwij JM, Scheidegger C, 2006. Effect of disturbances on the genetic diversity of an old-forest associated lichen. *Molecular Ecology* **15**: 911–921.

Werth S, Gugerli F, Holderegger R, Wagner HH, Csencsics D, Scheidegger C, 2007. Landscape-level gene flow in *Lobaria pulmonaria*, an epiphytic lichen. *Molecular Ecology* **16**: 2807–2815.

Yoshimura I, 1971. The genus *Lobaria* of Eastern Asia. *Journal of the Hattori Botanical Laboratory* **34**: 231–331.

Zhang DX, Hewitt GM, 2003. Nuclear DNA analyses in genetic studies of populations: practice, problems and prospects. *Molecular Ecology* **12**: 563–584.

Zoller S, Scheidegger C, Sperisen C, 1999. PCR primers for the amplification of mitochondrial small subunit ribosomal DNA of lichen-forming ascomycetes. *Lichenologist* **31**: 511–516.