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Methodological Advances

Assessing intraspecific diversity in a lichen-forming fungus and its green algal symbiont: Evaluation of eight molecular markers

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ABSTRACT

To facilitate marker selection in sequence-based studies on genetic diversity and symbiont selectivity in lichens we conducted a comparison of eight molecular markers in the lichen-forming fungus *Lasallia pustulata* and its trebouxioid photobiont. We compared mtSSU rDNA, mtLSU rDNA, *MCM7*, *TSR1* (mycobiont) and nrITS rDNA, *COX2*, psbJ-L intergenic spacer, *rbcL* (photobiont) of 45 individuals from European populations of *L. pustulata*. Mycobiont and photobiont loci had congruent phylogenetic signals. Based on the results of this study we recommend the use of *MCM7* and *TSR1* (mycobiont), and nrITS rDNA and *COX2* (photobiont). In this specific study system we found no sequence variability in the mycobiont loci *EF1*, nrITS rDNA, *RPB1*, and *RPB2*, which we sequenced for a subset of individuals. We had limited success amplifying *GPD* (mycobiont), actin and chloroplast LSU rDNA (photobiont), however, we do not rule out that these loci could be valuable markers in other species.

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Introduction

Understanding the phylogeography and population biology of symbiotic organisms is of special interest because symbiosis may be an important mechanism of adaptation. The choice and identity of symbiotic partners can influence the distribution and ecological tolerance of a species. This has been shown, for example, in corals and their photosymbionts (Howells *et al.* 2012), plants and endophytic fungi (Rodriguez *et al.* 2008; Redman *et al.* 2011), or plants and mycorrhizal fungi (Rosendahl 2008; Johnson *et al.* 2010). Recent studies suggest that lichen-forming fungi select their green algal photobionts according to habitat (Muggia *et al.* 2008; Fernandez-Mendoza *et al.* 2011; Peksa & Škaloud 2011; Vargas Castillo & Beck 2012), and that in some species multiple algal genotypes with different ecological tolerances are present in a single lichen individual (Casano *et al.* 2011). These findings support the idea that photobiont selection may influence the lichen's ability to respond to environmental change, and to occupy diverse ecological niches (Piercey-Normore 2006).

Genetic diversity in lichens has been assessed at the species or subspecies level. Some studies focus only on the mycobiont (e.g. Leavitt *et al.* 2011; Spribille *et al.* 2011; and studies summarized by Werth 2010), only the photobiont (e.g. Werth & Sork 2008), or both symbiotic partners (e.g. Yahr *et al.* 2006; Fernandez-Mendoza *et al.* 2011; Vargas Castillo & Beck 2012). Most of these studies are based on molecular sequence markers that can be generated from metagenomic DNA extracts using symbiont specific primers (Gardes & Bruns 1993; Zoller *et al.* 1999; Kroken & Taylor 2000; Printzen 2002; Schmitt *et al.* 2009). While sequence markers have been successfully employed to analyze symbiont diversity, selectivity and evolution, genomic markers, such as single sequence repeats (microsatellites), are probably most promising for high-resolution population studies in lichen-forming fungi (Werth 2010; Dal Grande *et al.* 2012). However, the development of such markers typically requires axenic cultivation of the mycobiont, which is often not feasible. To date, microsatellite markers have only been published for *Lobaria pulmonaria* and its photobiont *Dictyochloropsis reticulata* (Trebouxiophyceae) (Walser *et al.* 2003; Dal Grande *et al.* 2010; Widmer *et al.* 2010), *Parmotrema tinctorum* and its photobiont (Mansournia *et al.* 2012), for the mycobionts of *Peltigera dolichorhiza* (Magain *et al.* 2010) and *Buellia frigida* (Jones *et al.* 2012), and for *Trebouxia decolorans*, the photobiont found in *Xanthoria parietina* and *Anaptychia ciliaris* (Dal Grande *et al.* 2013). Thus, sequence-based approaches to understanding the phylogeography and population structure of lichens are an important method in lichenology. Furthermore, suitable sequence markers are necessary for the analysis of co-evolution and symbiont selectivity in the lichen symbiosis. Some mycobiont species are highly selective, forming lichens only with certain photobiont species or lineages, whereas others may utilize a great variety of photobiont lineages (e.g. Blaha *et al.* 2006; Piercey-Normore 2006; Nelsen & Gargas 2009). On the other hand, a single photobiont species can associate with different species of lichen-forming fungi (e.g. Beck *et al.* 2002). For such studies it is important to evaluate the available locus markers in order to maximize the retrieved number of fungal and algal haplotypes, as well as the number of unique symbiotic pairs.

A number of ribosomal, protein coding, and intron markers have been used to study mycobiont and photobiont diversity at the species level or below (e.g. Škaloud & Peksa 2010; Werth & Sork 2010; Fernandez-Mendoza *et al.* 2011; Leavitt *et al.* 2011; Spribille *et al.* 2011). However, an overall comparison of the performance of molecular loci at the population level using a single lichen species is missing. Here, we compared the variability of eight loci (four mycobiont, four photobiont) using 45 specimens from the European populations of *Lasallia pustulata* and its green algal photobiont (*Trebouxia* sp.). For a subset of individuals we tested additional loci. We chose a fungus with trebouxioid photobiont, because unicellular green algae of the genus *Trebouxia* are the most common photobionts in lichens (Tschermak-Woess 1988; Friedl & Büdel 1996). They can be found in more than 60 % of the described taxa, approximately 9 000 species (Ahmadjian 1993; Friedl & Büdel 1996). Thus, *Trebouxia* is the most common and widely distributed terrestrial algal genus in the world (Ahmadjian 2004). Genetic variability and phylogenetic relationships of Trebouxiophyceae are still poorly understood, although this taxon makes up a significant portion of terrestrial algal diversity (Škaloud & Peksa 2010; Leliaert *et al.* 2012). Our comparative study helps to facilitate marker selection for studies in biogeography, population biology, and co-evolution/selectivity of lichens with trebouxioid photobionts.

Methods

Taxon sampling and molecular methods

We selected 45 thalli from 30 populations sampled across the species' Europe-wide range (Table 1). We extracted total genomic DNA using the CTAB method (Cubero & Crespo 2002). PCR reactions (25 μl) contained 0.65 U Ex Taq polymerase (TaKaRa BIO INC.), 1× buffer, 0.2 mM dNTP mixture, 0.5–1.0 μM of each primer, 2–50 ng DNA template, and H_2O. Primers used for PCR and cycle sequencing are referenced in Table 2. We used the following PCR cycling conditions to amplify the photobiont loci nrITS rDNA, psbJ-L, *COX2*, *rbcL*, and the mycobiont loci mtSSU rDNA, mtLSU rDNA, *MCM7*, *TSR1*: initial denaturation 95 °C for 4 min, followed by 38 cycles of 95 °C for 30 s, 50 °C for 40 s, 72 °C for 1 min, and final elongation 72 °C for 5 min. It was our experience that the type of Taq we used (and even the brand of PCR machine) had a higher influence on amplification success than varying the annealing temperature. Methodological details on the amplification of additional loci that we tested for a subset of individuals (photobiont actin, 23S rDNA; mycobiont *GPD*, *EF1*, nrITS, *RPB1*, *RPB2*) are provided in Table S1. For some loci we designed new primers that are specific for *L. pustulata* or its photobiont (Table 2, Table S1). Amplification products were separated on 1 % agarose gels. If single bands were present we diluted the PCR products and added 5–12 ng of the amplicon to 10 μl sequencing reactions. If multiple bands were present we extracted fragments of the expected size using the peqGOLD Gel Extraction Kit (PEQLAB Biotechnologie GmbH). We

Table 1 – Material used in the current study and GenBank accession numbers. Herbarium acronyms follow Thiers (2012)

# Of individual	Source	Photobiont				Mycobiont			
		nrITS rDNA	COX2	psbJ-L	*rbcL*	*MCM7*	*TSR1*	mtLSU rDNA	mtSSU rDNA
A0102	Austria, Styria, hiking path to Sporiroa-Ofen, 760 m, lat. 46.926469, long. 15.178028, leg. Sadowska-Deś, 27.07.2011 (FR)	JX474323	JX474233	JX844291	JX474278	JX474413	JX474458	JX474503	JX474368
E0101	Estonia, Lääne-Virumaa Lahemaa Rahvuspark, Vihula vald, lat. 59.5156104, long. 25.9356541, leg. Jüriado, 31.07.2008 (TU)	JX474324	JX474234	JX844292	JX474279	JX474414	JX474459	JX474504	JX474369
G0116	Germany, Hesse, Eppstein, 342 m, lat. 50.13949, long. 8.40439, leg. Sadowska-Deś, Nuñez Zapata, Schmitt, 26.05.2011 (FR)	JX474325	JX474235	JX844293	JX474280	JX474415	JX474460	JX474505	JX474370
G0126	Germany, Hesse, Eppstein, 342 m, lat. 50.13949, long. 8.40439, leg. Sadowska-Deś, Nuñez Zapata, Schmitt, 26.05.2011 (FR)	JX474326	JX474236	JX844294	JX474281	JX474416	JX474461	JX474506	JX474371
G0136	Germany, Hesse, Eppstein, 342 m, lat. 50.13949, long. 8.40439, leg. Sadowska-Deś, Nuñez Zapata, Schmitt, 26.05.2011 (FR)	JX474327	JX474237	JX844295	JX474282	JX474417	JX474462	JX474507	JX474372
G0305	Germany, Saarland, Lohfelden, Elsenfeld, 400 m, lat. 49.59647222, long. 7.00676389, leg. Sadowska-Deś, John, Nuñez Zapata, Schmitt, 17.06.2011 (FR)	JX474328	JX474238	JX844296	JX474283	JX474418	JX474463	JX474508	JX474373
G0507	Germany, Saarland, Orscholz, 300 m, lat. 49.500876, long. 6.542163, leg. Sadowska-Deś, John, Nuñez Zapata, Schmitt, 17.06.2011 (FR)	JX474329	JX474239	JX844297	JX474284	JX474419	JX474464	JX474509	JX474374
G0703	Germany, Thuringia, Bad Blankenburg, Ingoklippefelsen, 309 m, lat. 50.664914, long. 11.246982, leg. Sadowska-Deś, 25.06.2011 (FR)	JX474330	JX474240	JX844298	JX474285	JX474420	JX474465	JX474510	JX474375
G0910	Germany, Saxony-Anhalt, Bodetal, Rosstrappe, 403 m, lat. 51.735064, long. 11.246982, leg. Sadowska-Deś, 13.08.2011 (FR)	JX474331	JX474241	JX844299	JX474286	JX474421	JX474466	JX474511	JX474376
H0102	Hungary, Baranya district, Kővágószőlős, Mecsek Mts, Jakab-hegy, Zsongor-kő, 450 m, lat. 46.09083333, long. 18.1330555, leg. Farkas, Lőkös, 30.10.2010 (HB Farkas-268)	JX474332	JX474242	JX844300	JX474287	JX474422	JX474467	JX474512	JX474377
H0301	Hungary, Baranya district, Kővágószőlős, Mecsek Mts, Jakab-hegy, Babas-szerkovek, 410 m, lat. 46.09194444, long. 18.12916667, leg. Farkas, Lőkös, 30.10.2010 (HB Farkas-270)	JX474333	JX474243	JX844301	JX474288	JX474423	JX474468	JX474513	JX474378
H0302	Hungary, Baranya district, Kővágószőlős, Mecsek Mts, Jakab-hegy, Babas-szerkovek, 410 m, lat. 46.09194444, long. 18.12916667, leg. Farkas, Lőkös, 07.04. 2011 (HB Farkas-311)	JX474334	JX474244	JX844302	JX474289	JX474424	JX474469	JX474514	JX474379
H0502	Hungary, Pest District, Kamence, Borzsony Mts, Nagy-Mána, 695 m, lat. 46.09083333, long. 18.13305556, leg. Farkas, Lőkös, Molnár, 07.05.2011 (HB Farkas-346)	JX474335	JX474245	JX844303	JX474290	JX474425	JX474470	JX474515	JX474380
N0101	Norway, Aust-Agder, Lilesand: Hellekilen, 1 m, lat. 58.698777, long. 8.088226, leg. Klepsland, Jon, 07.06.2009, L164675 (O)	JX474336	JX474246	JX844304	JX474291	JX474426	JX474471	JX474516	JX474381
N0201	Norway, Hedmark, Ringsaker: Brøttum sag, 125 m, lat. 60.062922, long. 10.737061, 11.05.2008, L159236 (O)	JX474337	JX474247	JX844305	JX474292	JX474427	JX474472	JX474517	JX474382
N0203	Norway, Hedmark, Ringsaker: Brøttum sag, 125 m, lat. 60.910672, long. 10.737061, leg. Breili, Anders, 11.05.2008, L159236 (O)	JX474338	JX474248	JX844306	JX474293	JX474428	JX474473	JX474518	JX474383
N0301	Norway, Akershus, Ski: Nord Bjørke, 110 m, lat. 60.062922, long. 11.375427, leg. Breili, Anders, 18.06.2006, L150858 (O)	JX474339	JX474249	JX844307	JX474294	JX474429	JX474474	JX474519	JX474384
N0701	Norway, Oppland, Vågå: Russvassbue, 1 185 m, lat. 61.11926, long. 10.466137, leg. Haugan, 24.06.2008, L160450 (O)	JX474340	JX474250	JX844308	JX474295	JX474430	JX474475	JX474520	JX474385

(*continued on next page*)

Table 1 – (*continued*)

# Of individual	Source	Photobiont				Mycobiont			
		nrITS rDNA	*COX2*	psbJ-L	*rbcL*	*MCM7*	*TSR1*	mtLSU rDNA	mtSSU rDNA
N0801	Norway, Oppland, Vågå: Trollhø, 1 300 m, lat. 61.752031, long. 9.050303, leg. Breili, Anders, 25.03.2005, L142521 (O)	JX474341	JX474251	JX844309	JX474296	JX474431	JX474476	JX474521	JX474386
N0901	Norway, Rogland, Vindafjord: Saltvika, 1 m, lat. 59.301234, long. 5.985718, leg. Jordal, 02.09.2008, L155335 (O)	JX474342	JX474252	JX844310	JX474297	JX474432	JX474477	JX474522	JX474387
N1001	Norway, Sogn Og Fjordane, Edi: Hamnest, 2 m, lat. 61.665375, long. 6.315307, leg. Breili, Anders, 08.04.2009, L159494 (O)	JX474343	JX474253	JX844311	JX474298	JX474433	JX474478	JX474523	JX474388
N1101	Norway, Vestfold, Larvik, Stavern, 20 m, lat. 58.983333, long. 10.03333, leg. Rui, Timdal, 02.04.2011, L169175 (O)	JX474344	JX474254	JX844312	JX474299	JX474434	JX474479	JX474524	JX474389
N1104	Norway, Vestfold, Larvik, Stavern, 20 m, lat. 58.983333, long. 10.03333, leg. Rui, Timdal, 02.04.2011, L169175 (O)	JX474345	JX474255	JX844313	JX474300	JX474435	JX474480	JX474525	JX474390
P0101	Poland, Lower Silesia, Sobieszów, Zbójeckie Skały, 600 m, lat. 51.0244444, long. 15.904444, leg. Sadowska-Deś, 23.06.2011 (FR)	JX474346	JX474256	JX844314	JX474301	JX474436	JX474481	JX474526	JX474391
P0103	Poland, Lower Silesia, Sobieszów, Zbójeckie Skały, 600 m, lat. 51.0244444, long. 15.904444, leg. Sadowska-Deś, 23.06.2011 (FR)	JX474347	JX474257	JX844315	JX474302	JX474437	JX474482	JX474527	JX474392
P0109	Poland, Lower Silesia, Sobieszów, Zbójeckie Skały, 600 m, lat. 51.0244444, long. 15.904444, leg. Sadowska-Deś, 23.06.2011 (FR)	JX474348	JX474258	JX844316	JX474303	JX474438	JX474483	JX474528	JX474393
P0206	Poland, Lower Silesia, Sobieszów, Chojnik Hill, 530 m, lat. 50.83333, long. 15.63333, leg. Sadowska-Deś, 23.06.2011 (FR)	JX474349	JX474259	JX844317	JX474304	JX474439	JX474484	JX474529	JX474394
P0309	Poland, Lower Silesia, Sobieszów, Chojnik Castle, 530 m, lat. 50.833725, long. 15.644181, leg. Sadowska-Deś, 29.08.2011 (FR)	JX474350	JX474260	JX844318	JX474305	JX474440	JX474485	JX474530	JX474395
P0401	Poland, Lower Silesia, Izery Mts, Bobrowe Skały, 699 m, lat. 50.866666, long. 15.58333, leg. Sadowska-Deś, 25.08.2011 (FR)	JX474351	JX474261	JX844319	JX474306	JX474441	JX474486	JX474531	JX474396
P0501	Poland, Lower Silesia, Ostrzyca Proboszczowicka, Kaczawskie Mts, 501 m, lat. 51.14888889, long. 15.96944444, leg. Sadowska-Deś, 02.09.2011 (FR)	JX474352	JX474262	JX844320	JX474307	JX474442	JX474487	JX474532	JX474397
P0502	Poland, Lower Silesia, Ostrzyca Proboszczowicka, Kaczawskie Mts, 501 m, lat. 51.14888889, long. 15.96944444, leg. Sadowska-Deś, 02.09.2011 (FR)	JX474353	JX474263	JX844321	JX474308	JX474443	JX474488	JX474533	JX474398
P0503	Poland, Lower Silesia, Ostrzyca Proboszczowicka, Kaczawskie Mts, 501 m, lat. 51.14888889, long. 15.96944444, leg. Sadowska-Deś, 02.09.2011 (FR)	JX474354	JX474264	JX844322	JX474309	JX474444	JX474489	JX474534	JX474399
P0601	Poland, Lower Silesia, Rudawy Janowickie, Bolczów Castle, 561 m, lat. 51.03388889, long. 16.08972222, leg. Sadowska-Deś, 30.09.2011 (FR)	JX474355	JX474265	JX844323	JX474310	JX474445	JX474490	JX474535	JX474400
P0602	Poland, Lower Silesia, Rudawy Janowickie, Bolczów Castle, 561 m, lat. 51.03388889, long. 16.08972222, leg. Sadowska-Deś, 30.09.2011 (FR)	JX474356	JX474266	JX844324	JX474311	JX474446	JX474491	JX474536	JX474401

Table 1 – (*continued*)

# Of individual	Source	Photobiont				Mycobiont			
		nrITS rDNA	COX2	psbJ-L	*rbcL*	*MCM7*	*TSR1*	mtLSU rDNA	mtSSU rDNA
P0605	Poland, Lower Silesia, Rudawy Janowickie, Bolczów Castle, 561 m, lat. 51.03388889, long. 16.08972222, leg. Sadowska-Deś, 30.09.2011 (FR)	JX474357	JX474267	JX844325	JX474312	JX474447	JX474492	JX474537	JX474402
P0701	Poland, Lower Silesia, Jelenia Góra, 383 m, lat. 50.873319, long. 15.761103, leg. J. and Z. Sadowscy, 12.09.2011 (FR)	JX474358	JX474268	JX844326	JX474313	JX474448	JX474493	JX474538	JX474403
P0716	Poland, Lower Silesia, Jelenia Góra, 383 m, lat. 50.873319, long. 15.761103, leg. J. and Z. Sadowscy, 12.09.2011 (FR)	JX474359	JX474269	JX844327	JX474314	JX474449	JX474494	JX474539	JX474404
S0101	Spain, Almeria, Garganta de Chilla, 1 700 m, lat. 40.2325, long. 5.30722222, leg. Vivas Rebuelta, 11.07.2010 (FR)	JX474360	JX474270	JX844328	JX474315	JX474450	JX474495	JX474540	JX474405
S0401	Spain, Almeria, Garganta de Chilla, 1 000 m, lat. 40.206666, long. −5.29472222, leg. Vivas Rebuelta, 11.07.2010 (FR)	JX474361	JX474271	JX844329	JX474316	JX474451	JX474496	JX474541	JX474406
S0405	Spain, Almeria, Garganta de Chilla, 1 000 m, lat. 40.206666, long. −5.29472222, leg. Vivas Rebuelta, 11.07.2010 (FR)	JX474362	JX474272	JX844330	JX474317	JX474452	JX474497	JX474542	JX474407
W0105	Slovakia, Tribeč Mts, Zobor Mts: Nitra, 546 m, lat. 48.337255, long. 18.10623, leg. Guttova, 27.07.2011 (FR)	JX474363	JX474273	JX844331	JX474318	JX474453	JX474498	JX474543	JX474408
W0131	Slovakia, Tribeč Mts, Zobor Mts: Nitra, 546 m, lat. 48.337255, long. 18.10623, leg. Guttova, 27.07.2011 (FR)	JX474364	JX474274	JX844332	JX474319	JX474454	JX474499	JX474544	JX474409
W0133	Slovakia, Tribeč Mts, Zobor Mts: Nitra, 546 m, lat. 48.337255, long. 18.10623, leg. Guttova, 27.07.2011 (FR)	JX474365	JX474275	JX844333	JX474320	JX474455	JX474500	JX474545	JX474410
X0121	Portugal, Algarve, Serra de Monchique, 890 m, lat. 37.317139, long. −8.590722, leg. Divakar, Agudo, Ruibal, 19.05.2011 (FR)	JX474366	JX474276	JX844334	JX474321	JX474456	JX474501	JX474546	JX474411
Y0103	Serbia, Pčinja District, Surdulica, Vardenik Mts, 1 640 m, lat. 42.647805, long. 22.261555, leg. Lőkös, 24.06.2011 (HB Farkas-446)	JX474367	JX474277	JX844335	JX474322	JX474457	JX474502	JX474547	JX474412

sequenced the amplicons using Big Dye 3.1 chemistry (Applied Biosystems). Cycle sequencing was executed with the following program: initial denaturation for 1 min at 95 °C, followed by 30 cycles of 96 °C for 10 s, 50 °C for 10 s, 60 °C for 2 min. Sequenced products were precipitated and loaded on an ABI PRISMTM 3730 DNA Analyzer (Applied Biosystems). We assembled partial sequences using Geneious v5.4 (Drummond *et al.* 2011) and edited conflicts manually.

Sequence analyses and networks

We aligned sequences of each locus individually using MUSCLE (Edgar 2004) implemented in Geneious. We analyzed the alignments using RAxML (Stamatakis 2006). The maximum likelihood (ML) analyses were performed assuming the general time reversible model of nucleotide substitution with six rate categories including estimation of invariant sites and assuming a discrete gamma distribution (GTR + I + G) for the psbJ-L dataset and GTR + G for all other datasets. These models were determined as best fitting models using the program MrModeltest v2 (Nylander 2004). ML bootstrapping was performed based on 1 000 replicates. We examined the ML trees for the presence of supported conflicts (individuals group in a clade supported by >75 % ML bootstrap support in one dataset, but in a different supported clade in another dataset). We determined DNA polymorphisms and haplotype numbers with DnaSP v5 (Librado & Rozas 2009). For each locus we measured: the number of polymorphic sites to assess locus variability; the number of parsimony informative positions to assess possible use of the locus as a phylogenetic marker; the number of gaps to indicate the potential for ambiguous alignment of the locus; and nucleotide diversity, average differences between individuals, the number of haplotypes, and haplotype diversity to estimate utility of the locus as an intraspecific marker. Gaps were treated as missing data. We constructed median joining haplotype networks using Network 4.6 (Bandelt *et al.* 1999).

Results

We generated 45 sequences of each of the following loci: photobiont nrITS rDNA, psbJ-L intergenic spacer, *COX2*, *rbcL*, and mycobiont mtSSU rDNA, mtLSU rDNA, *MCM7*, *TSR1*.

Table 2 – Loci and primers used in the current study

Locus	Genome	Primer name, orientation	Primer sequence (5′–3′)	Reference
Photobiont				
nrITS rDNA	Nuclear	nrITS1T (f)	GGAAGGATCATTGAATCTATCGT	Kroken & Taylor (2000)
		nrITS4T (r)	GGTTCGCTCGCCGCTACTA	
		nrITSaJOFOR2 (f)	TGAATCTATCGTGCAMACACC	This study
		nrITSaJOREV2 (r)	GCCGCTACTAAGGGAATCCT	
COX2	Mitochondrial	Cox2-P2fw-5′ (f)	GGCATGAAAGCATGGTTAGC	Fernandez-Mendoza *et al.* (2011)
		Cox2-P2rv-3′ (r)	TCTGGATGTTAGCAAGAACTTTGT	
psbJ-L	Chloroplast	psbF (f)	GTWGTWCCAGTATTRGACAT	Werth & Sork (2008)
		psbR (r)	AACCRAATCCANAYAAACAA	
rbcL	Chloroplast	a-ch-rbcL-203 (f)	GAATCWTCWACWGGWACTTGGACWAC	Nelsen *et al.* (2011)
		a-ch-rbcL-991 (r)	CCTTCTARTTTACCWACAAC	
Mycobiont				
MCM7	Nuclear	MCM7-709 (f)	ACIMGIGTITCVGAYGTHAARCC	Schmitt *et al.* (2009)
		MCM7-1348 (r)	GAYTTDGCIACICCIGGRTCWCCCAT	
		MCM7FOR2 (f)	AGGTGAACGCTTACACATGC	This study
		MCM7REV2 (r)	CGGGAGCTATGGATCTTGAG	
TSR1	Nuclear	TSR1-1453F (f)	GARTTCCCIGAYGARATYGARCT	Schmitt *et al.* (2009)
		TSR1-2308R (r)	CTTRAARTAICCRTGIGTICC	
		TSR1LAPUFOR (f)	ACTACAAAGGCGCAAAGAGC	This study
		TSR1LAPUREV (r)	TGAACCAGTTGACGTCTTCG	
mtLSU rDNA	Mitochondrial	mtLSU3A (f)	GCTGGTTTTCTGCGAAACCTATATAAG	Printzen (2002)
		mtLSU4A (r)	GTTAGTTTGCCGAGTTCCTTAATG	
mtSSU rDNA	Mitochondrial	mrSSU1 (f)	AGCAGTGAGGGATATTGGTC	Zoller *et al.* (1999), Zhou & Stanosz (2001)
		MSU7 (r)	GTCGAGTTACAGACTACAATCC	

Detailed information on specimens and corresponding GenBank accession numbers is given in Table 1. We also generated 12 sequences of chloroplast 23S rDNA (GenBank accession numbers JX474221–JX474232).

A comparison of alignment features and variability among photobiont loci (nrITS rDNA, psbJ-L, *COX2*, *rbcL*) and mycobiont loci (mtSSU rDNA, mtLSU rDNA, *MCM7*, *TSR1*) is presented in Table 3. The ML 75 % bootstrap support method for testing datasets for incongruence indicated no supported conflicts among photobiont or mycobiont alignments. This suggests that the tested loci have similar evolutionary histories. ML trees are presented in Fig S1. The median joining networks of the four photobiont loci are also largely congruent (Fig 1).

Variability of the photobiont loci was higher than that of the mycobiont loci (Table 3). Nuclear ITS rDNA of the photobiont showed the highest number of variable sites (71) and haplotypes (13), followed by the mitochondrial loci psbJ-L intergenic spacer and *COX2*. The psbJ-L intergenic spacer is non-coding and highly variable, thus causing ambiguities/gaps in the alignment. *RbcL* was the least variable photobiont locus (Table 3). Among the mycobiont loci, the nuclear protein-coding gene *MCM7* possessed the highest number of polymorphic sites, average differences between individuals,

Table 3 – Comparison of variability among four photobiont and four mycobiont loci. The alignment included 45 sequences from the European populations of *Lasallia pustulata*

Symbiont	Photobiont				Mycobiont			
Locus	nrITS rDNA	*COX2*	psbJ-L	*rbcL*	*MCM7*	*TSR1*	mtLSU rDNA	mtSSU rDNA
Alignment length	507	419	614	441	370	485	728	706
Polymorphic sites [*N*] (%)	71 (14.0 %)	44 (10.5 %)	44 (7.2 %)	5 (1.1 %)	6 (1.6 %)	5 (1.0 %)	2 (0.3 %)	1 (0.1 %)
Parsimony informative positions [*N*]	69	44	43	5	6	5	2	1
Gaps [*N*]	5	0	331	0	0	0	1	4
Nucleotide diversity[a]	0.051	0.045	0.075	0.005	0.0029	0.002	0.0008	0.0003
Ø differences between individuals[a]	26.24	18.6	26.7	2.04	1.07	0.97	0.58	0.24
Haplotypes [*N*]	13	5	10	4	3	3	3	2
Haplotype diversity	0.81	0.66	0.7	0.61	0.47	0.24	0.52	0.24

a Based on pairwise comparisons.

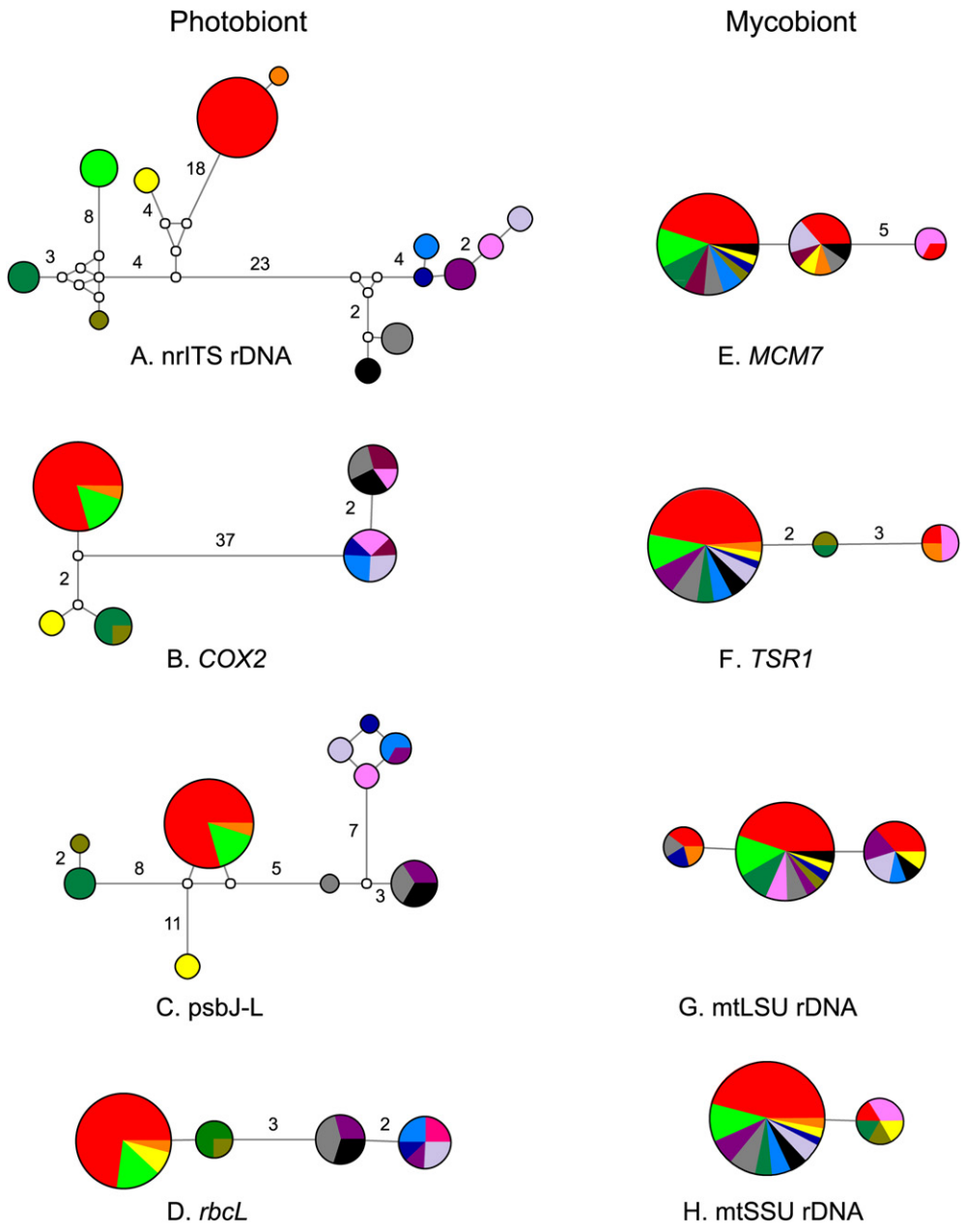

Fig 1 – Median joining haplotype networks. (A–D) photobiont, (E–H) mycobiont. (A) nrITS rDNA, (B) *COX2*, (C) psbJ-L intergenic spacer, (D) *rbcL*, (E) *MCM7*, (F) *TSR1*, (G) mtLSU rDNA, (H) mtSSU rDNA. Colored circles indicate haplotypes; the size of circles is relative to the number of individuals carrying the haplotype. Empty circles indicate missing haplotypes. Individuals are color-coded based on the nrITS rDNA photobiont haplotype they carry. Numbers indicate mutational steps > 1.

and the highest nucleotide diversity. It also had the shortest alignment. The second nuclear, protein-coding locus (*TSR1*) was only marginally less polymorphic than *MCM7* (Table 3). Despite much longer alignments, the mitochondrial ribosomal loci yielded very little variability (1–2 polymorphic sites). However, the little variation observed among mycobiont loci was largely congruent. For example, the largest haplotype group in the nuclear *TSR1* and in the mitochondrial mtSSU rDNA consisted of the same individuals (Fig 1F, H).

For a subset of individuals we tested additional loci (Table S1). The mycobiont loci *EF1*, nrITS rDNA, *RPB1*, and *RPB2* showed no sequence variability in *L. pustulata*. Despite PCR optimization we had very limited success in amplifying/sequencing the mycobiont locus *GPD* and the photobiont locus actin in the present study system (methodological details and experimental outcomes are provided in Table S1). We were able to generate 12 sequences of chloroplast LSU rDNA (23S rDNA). This marker was recently recommended for diversity assessment in the genus *Trebouxia* (del Campo *et al.* 2009, 2010). The new 23S rDNA sequences contained no introns. We compared 23S rDNA and nrITS rDNA variability of these 12 individuals, and found nrITS rDNA to be more variable (alignment length excluding sites with gaps 23S rDNA: 1 072, nrITS rDNA: 503; polymorphic sites 23S rDNA: 20 (1.9 %), nrITS:

64 (12.7 %); nucleotide diversity 23S rDNA: 0.00594, nrITS: 0.03915; number of haplotypes 23S rDNA: 6, nrITS rDNA: 9).

Discussion

It is difficult to find mycobiont loci with sufficient variability at the population level. Intraspecific variability has been reported for nuclear ITS rDNA in some lichen-forming fungi (e.g. Printzen *et al.* 2003; Lindblom & Ekman 2006; Del Prado *et al.* 2011; Núñez-Zapata *et al.* 2011; Wirtz *et al.* 2012), and also in a number of non-lichenized fungi (e.g. Nilsson *et al.* 2008; Bonito *et al.* 2010; Kovács *et al.* 2011). However, a factor complicating the use of nrITS rDNA as a population marker in fungi is the potential presence of intragenomic variation (e.g. Simon & Weiss 2008). Furthermore, species boundaries in fungi are often not well understood and different ITS types may indicate the presence of morphologically cryptic species complexes (Del Prado *et al.* 2011; Núñez-Zapata *et al.* 2011; Wirtz *et al.* 2008, 2012). Overall, nrITS rDNA is conserved enough in the majority of fungal species to serve as a species identifier, rather than a population marker (Schoch *et al.* 2012). Our finding of lack of sequence variability in the nrITS rDNA of *L. pustulata* is consistent with this observation. A second locus from the nuclear ribosomal cistron, IGS rDNA, has also been employed at the subspecies level, sometimes showing slightly lower (Lindblom & Ekman 2006), sometimes slightly higher variability than nrITS rDNA (Printzen *et al.* 2003; Leavitt *et al.* 2011; Wirtz *et al.* 2012).

Protein-coding genes, such as *GPD*, *MCM7*, *RPB1*, *RPB2*, *TSR1 or TUB* may be alternatives to ribosomal loci for population studies of fungi (Buschbom & Mueller 2006; Fernandez-Mendoza *et al.* 2011; Leavitt *et al.* 2011; Spribille *et al.* 2011; Wirtz *et al.* 2012). Paralogs, sometimes present in these genes, are typically too divergent to cause problems in studies at the species level or below (Buschbom & Mueller 2006). *RPB1* and *RPB2* had very little or no variability (Buschbom & Mueller 2006; Wirtz *et al.* 2012; this study), while *MCM7* showed an acceptable level of polymorphism in the present and other studies (Leavitt *et al.* 2011; Spribille *et al.* 2011). *TSR1*, which was tested at the population level for the first time in the present study, performed very similarly to *MCM7*. For both markers (*MCM7* and *TSR1*) it is best to generate a few sequences using universal ascomycete primers (Schmitt *et al.* 2009) and then design more specific primers for the target group, as PCR and sequencing can be problematic using universal primers for protein-coding genes (Schoch *et al.* 2012). In the present study we had very limited success sequencing *GPD* (see Table S1), a marker that was successfully used to analyze population dynamics in *Cetraria aculeata* (Fernandez-Mendoza *et al.* 2011). This suggests that utility of certain loci may depend on the species studied.

Markers covering the entire genome, such as short sequence repeats (microsatellites), or single nucleotide polymorphisms (SNPs), are likely to be more powerful in resolving population structure in lichen mycobionts. However, the development of such markers typically requires axenic cultivation of fungal partners, which is often time consuming due to the slow growth rates of isolated mycobionts (Widmer *et al.* 2010). As more genome sequences of lichen-forming fungi and their photobionts become available, we expect that bioinformatics approaches will become important tools for separating the genomes of the symbiotic partners. Genomic data generated with next generation sequencing technology will be a valuable resource for marker development in population studies in non-model organisms (Davey *et al.* 2011).

Photobionts can be highly diverse, and it has been shown repeatedly that multiple photobiont lineages can form associations with a single fungus (e.g. Blaha *et al.* 2006; Piercey-Normore 2006; Nelsen & Gargas 2009; Bačkor *et al.* 2010). In this study, too, the trebouxioid photobiont showed considerable variation, indicating that multiple genetic lineages of algae form symbioses with *L. pustulata*. Three out of four tested photobiont loci showed substantial variability. The nrITS rDNA and *COX2* median joining networks indicate that there are at least two major photobiont haplotype groups separated from each other by 23/37 steps (Fig 1A, B). These highly divergent genotype groups probably belong to different *Trebouxia* species. Nuclear ITS rDNA and *COX2* are therefore most useful to assess the phylogenetic range of possible trebouxioid symbiotic partners in a lichen mycobiont, i.e. they are suitable markers for studies of selectivity. Our study shows that phylogenetic signal in all tested photobiont loci is congruent, with nrITS rDNA providing the highest resolution. Thus, nrITS rDNA, *COX2*, *rbcL*, and psbJ-L are probably all useful markers for multi-locus phylogenetic studies at different taxonomic levels within the genetically diverse genus *Trebouxia*. Whether these loci are sufficiently polymorphic to assess population structure at the level of individuals remains to be tested. Since trebouxioid photobionts of lichens are considered mostly clonal organisms, care needs to be taken not to underestimate their potential genetic diversity by relying on a single marker type (Arnaud-Haond *et al.* 2005). However, the loci analyzed here are likely to be very useful to define genetic lineages in *Trebouxia*, for which more powerful, high-resolution markers can then be developed (Dal Grande *et al.* 2013).

Additional loci have been used successfully in other studies of lichen photobiont diversity, for example actin (Fernandez-Mendoza *et al.* 2011), or chloroplast LSU (del Campo *et al.* 2010), demonstrating their value as phylogenetic or population markers. In the present study we confirm that nrITS rDNA appears to be more suitable for analyzing genetic variability at the intraspecific level, whereas chloroplast LSU seems to be adequate at the interspecific level in the genus *Trebouxia* (del Campo *et al.* 2010). The chloroplast LSU sequences generated in the present study belong to the same general group of species analyzed by del Campo *et al.* (2010). In BLAST searches they were most similar to *Trebouxia simplex* FJ804756: N1104, P0206, W0133 (identities 1 070 out of 1 072 bp), P0103, P0309, W0133 (identities 1 069 out of 1 072), W0105, P0701, P0101, X0121, G0910 (identities 1 066 out of 1 072), or to *Trebouxia brindabellae* FJ804757: G0507 (identities 1 071 out of 1 072). The sequence deviations between the new sequences and the BLAST hits may be an indication of unexplored cryptic diversity in *Trebouxia*. The fact that we had only limited success in generating chloroplast LSU and actin sequences (Table S1) indicates that – in analogy to the mycobiont loci – not all photobiont markers appear to be equally suitable for all species/lineages.

Understanding the genetic variability of lichens may provide insights into basic ecological phenomena, such as local adaptation (Peksa & Škaloud 2011; Vargas Castillo & Beck 2012), symbiont specificity (Nelsen & Gargas 2008; Dal Grande *et al.* 2012), and cryptic speciation (Leavitt *et al.* 2011). We have yet much to learn about the local and global distribution of genetic diversity in populations of lichen-forming fungi and their photobionts, selectivity of the symbiotic partners, and the reaction of lichens to anthropogenic change. While a number of appropriate markers are available to assess photobiont selectivity in lichenized fungi with trebouxioid green algae, it will be our challenge to develop powerful markers for assessing variability within and among populations of both symbionts.

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Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funeco.2012.12.001.

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