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Short Communication

A new, highly effective primer pair to exclude algae when amplifying nuclear large ribosomal subunit (LSU) DNA from lichens

With *c.* 9500 sequences currently uploaded to GenBank (September 2014) for the class Lecanoromycetes alone, the nuclear large ribosomal subunit (LSU) is one of the top three loci used for amplification of fungal DNA for molecular phylogenetics of lichenized fungi. The availability of LSU sequences for a large number of species, and the effectiveness of this locus at resolving both higher- and lower-level relationships (Schoch *et al.* 2012), means that this is one of the most frequently targeted loci for barcoding following the internal transcribed spacer which is itself located immediately upstream of the LSU on the ribosomal operon. Because of these attributes, it was recently considered a top candidate for a universal fungal barcode (Schoch *et al.* 2012). The LSU is often referred to by its size in Svedberg units as the 25S, 26S or 28S ribosomal subunit in Eukaryotes although a wide range of sedimentation values have been measured in fungi (Taylor *et al.* 1967). LSU is essential for the translation of mRNA into polypeptides and is widely conserved across Eukaryotes. Nevertheless, large sections of the LSU accumulate phylogenetically informative polymorphisms.

Several primers have been used for over 20 years to target the most informative regions of LSU, especially in the 5' half (position: ~1–1500 bp), which usually exhibits greater variability (Porter & Golding 2012): LR0R, LR3R, LR5R, LIC24R [forward primers; R. Vilgalys, unpublished (http://sites.biology.duke.edu/fungi/mycolab/primers.htm); Miądlikowska & Lutzoni 2000] and LR3, LR5, LR6, LR7, LIC2044 (reverse primers; Vilgalys & Hester 1990; Kauff & Lutzoni 2002). In addition, Döring *et al.* (2000) published a set of ostensibly fungal-specific LSU primers developed from a set of Lecanoromycetes and tested against two accessions of *Trebouxia* photobionts, though these primers return shorter amplicons than, and have not been as widely used as, the common LSU primers mentioned above and on popular reference websites (e.g. http://lutzonilab.org/nuclear-ribosomal-dna).

The ever-increasing taxonomic range targeted in recent sequencing efforts has tested the specificity of popular primers in binding fungal genomic DNA co-extracted with previously unknown or obscure algal photobionts. This has led to frequent co-amplification of algal DNA or even selective amplification of only algal products using LSU primers intended for fungal targets. We suspect that this phenomenon is common though rarely mentioned in the

TABLE 1. *Names and sequences of primers used in amplification of nuLSU DNA in lichens; forw. = forward primer, rev. = reverse primer. Position is relative to the large ribosomal subunit gene of* Saccharomyces cerevisiae *(J01355).*

Name	Sequence (5' → 3')	Length [bases]	Position	Temp [°C]*
LRlecF (forw.)	CCTCAGTAACGGCGAG	16	81–96	58·5
LRlecR (rev.)	AGGCTTCGTCACGGAC	16	1417–1432	60·6

*Breslauer's nearest-neighbour method (Breslauer *et al.* 1986); temperatures obtained with other methods can deviate significantly from this estimate.

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                                                    25         35         45              80         90        100           660        670        680
1: 1VWS_A      Saccharomyces cerevisiae  GAGTACCCGC TGAACTTAAG CATATCAATA  GATTGCCTTA GTAACGGCGA GTGAAGCGGC  GCCGCCCGTCT TGAAACACGG ACCAAGGAG
2: JQ740011.1  Ramalina sp.              GGATACCCGC TGAACTTAAG CATATCAATA  GATTGCCTCA GTAACGGCGA GTGAAGCGGC  GCGACCCGTCT TGAAACACGG ACCAAGGAG
3: *           Lobaria pulmonaria        GGATACCCGC TGAACTTAAG CATATCAATA  GATTGCCTCA GTAACGGCGA GTGAAGCGGC  GCGACCCGTCT TGAAACACGG ACCAAGGAG
4: JQ740002.1  Umbilicaria tylorrhiza    GGATACCCGC TGAACTTAAG CATATCAATA  GATTGCCTCA GTAACGGCGA GTGAAGCGGC  GCGACCCGTCT TGAAACACGG ACCAAGGAG
5: **          Penicillium spp.          GGATACCCGC TGAACTTAAG CATATCAATA  GATTGCCCCA GTAACGGCGA GTGAAGCGGC  GCGA-CCGTCT TGAAACACGG ACCAAGGAG
6: ***         Trebouxia spp.            GAACACCCGC TGAACTTAAG CATATCAATA  GATTCCCTTA GTAGCGGCGA GCGAACCGGG  TTCGCCCGTCT TGAAACACGG ACCAAGGAG
7: HE610125.1  Stichococcus bacillaris   GACCACCCGC TGAACTTAAG CATATCAATA  GATTCCCCTA GTAACGGCGA GTGAACCGGG  ACTGCCCGTCT TGAAACACGG ACCAAGGAG
8: HE610126.1  Koliella longiseta        GACTACCCGC TGAACTTAAG CATATCAATA  GATTCCCCTA GTAACGGCGA GTGAACCGGG  TCTACCCGTCT TGAAACACGG ACCAAGGAG
                                         ____________ LR0R ____________  ___________ LRlecF ___________  ____________ LR3R ____________

                                                   980        990       1000            1415       1425       1435            1465       1475       1485
1: 1VWS_A      Saccharomyces cerevisiae  GTTCCTGCCG AAGTTTCCCT CAGGATAGCA  CGTGGAGGTC AGTGACGAAG CCTAGACCGT  CTAGTGCAGA TCTTGGTGGT AGTAGCAAAT
2: JQ740011.1  Ramalina sp.              GTTCCTGCCG AAGTTTCCCT CAGGATAGCA  CGTGGGGGTC AGTGACGAAG CCTTGGGAGT  CTAGTGCAGA TCTTGGTGGT AGTAGCAAAT
3: *           Lobaria pulmonaria        GTTTCAGCCG AAGTTTCCCT CAGGATAGCA  CGTGGGGGTC CGTGACGAAG CCTTGGGAGT  CCAGTGCAGA TCTTGGTGGT AGTAGCAAAT
4: JQ740002.1  Umbilicaria tylorrhiza    GTTCCGGCCG AAGTTTCCCT CAGGATAGCA  CGTGGGGGTC CGTGACGAAG CCTTGGGAGC  CTAGTGCAGA TCTTGGTGGT AGTAGCAAAT
5: **          Penicillium spp.          GTTCCTGCCG AAGTTTCCCT CAGGATAGCA  CGTGGGGGTC CGTGACGAAG CCTTGGGAGT  CTAGTGCAGA TCTTGGTGGT AGTAGCAAAT
6: ***         Trebouxia spp.            GTTCCCTCCG AAGTTTCCCC CAGGATAGCT  CGTGGGTGTC -GTGGGAAAG CCTGCGGCGT  CTGGTGCAGA TCTTGGTGGT AGTAGCAAAT
7: HE610125.1  Stichococcus bacillaris   GTTCCCTCCG AAGTTTCCCC CAGGATAGCT  CGTGGGTGTC -GTGGAGCAG CCTGCGGCGT  CTAGTGCAGA TCTTGGTGGT AGTAGCAAAT
8: HE610126.1  Koliella longiseta        GTTCCCTCCG AAGTTTCCCC CAGGATAGCT  CGTGGGTGTT -GTGACGCAG CCTGCGGCGT  CTAGTGCAGA TCTTGGTGGT AGTAGCAAAT
                                         ____________ LR5 _____________  ___________ LRlecR ___________  ____________ LR7 _____________
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For the following positions other sequences were used instead as there were no contiguous sequences available on GenBank for those taxa:

* for LR0R: **AY340549.1 *Lobaria pulmonaria***; for LRlecF, LR3R, LR5, LRlecR, and LR7: **AF183934.2 *Lobaria pulmonaria***

** for LR0R and LRlecF: **AB293968.1 *Penicillium janthinellum***; for LR3R, LR5, LRlecR, and LR7: **AF003355.1 *Penicillium aurantiogriseum***

*** for LR0R: **AM159503.1 *Trebouxia decolorans***; for LRlecF, LR3R, LR5, LRlecR, and LR7: **AY648107.1 *Trebouxia* sp.**

FIG. 1. Alignment regions of common Ascomycota and algae including binding sites (in bold) of six different LSU primers. NCBI accession numbers and genus names are denoted. The scale refers to the position on the large ribosomal subunit gene of *Saccharomyces cerevisiae* (J01355).

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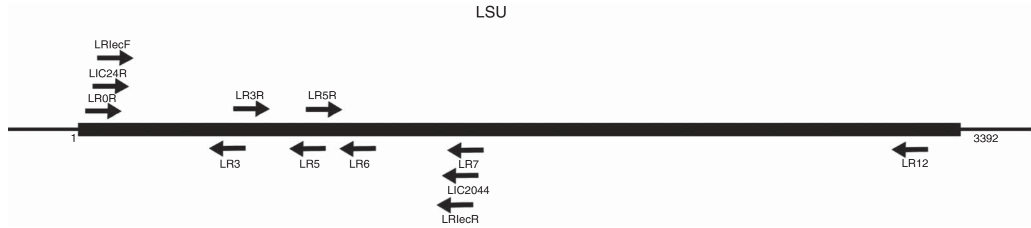

FIG. 2. LSU: schematic of primer binding sites. Numbered nucleotide positions refer to *Saccharomyces cerevisiae* nuLSU (J01355).

literature, as researchers probably develop more specific primers without mentioning the capture of algal sequences, or move on to use other loci. We know of unintentional amplification of algal products across a wide range of taxonomic groups in both the subclasses Lecanoromycetidae and Ostropomycetidae.

As part of an expanded study of the subclass Ostropomycetidae, we undertook a small study to find a primer combination that amplifies a large section of fungal LSU overlapping with the 3' end of most sequenced ITS fragments. In the present Short Communication we analyze LSU sequences over a cross-section of Lecanoromycetes to identify primer binding sites that would consistently discriminate against the alga and work for the core group of lichenized fungi.

We used sequence data from *c.* 180 species of Lecanoromycetes, as well as members of the classes Eurotiomycetes and Saccharomycetes, combined with algal LSU sequences from the *Trebouxiophyceae* to develop a set of LSU primers that both reliably amplify fungal, while excluding algal, LSU. This was achieved by searching for conserved regions across fungal taxa that differ in the algae. To avoid the potential formation of hair-pins and self-dimerization, the online tool OligoCalc was used to inspect primer candidates (Kibbe 2007). The primer pair candidates obtained were then subjected to a further analysis with the Multiple primer analyzer web tool to find optimal annealing temperatures and check for potential dimerization (http://www.thermoscientificbio.com/webtools/multipleprimer/).

Total DNA from voucher specimens was extracted using the QIAGEN DNeasy Plant Mini Kit Quick Start Protocol (Centrifugation Protocol), the E.Z.N.A. HP Plant DNA Mini Kit (Centrifugation Protocol for Fresh or Frozen Specimens), or the QIAGEN QIAamp DNA Investigator Kit (Protocol: Isolation of Total DNA from Tissues) for samples with little available material. For extraction we mainly used apothecia or soredia. Only in cases where the available apothecia or soredia were deemed insufficient did we use non-sorediate thallus material. The primer pair selected (Table 1) was then used for amplification of nuLSU of various test targets, with an optimal annealing temperature in the range of 56–58 °C determined by gradient PCR (Ta = 50–60 °C). Annealing temperatures below that range yielded unspecific PCR products in some cases. We used Illustra PuReTaq Ready-To-Go PCR Beads for amplification. The PCR conditions for amplification of LSU with the primers LRlecF and LRlecR were as follows: initial denaturation at 95 °C for 5 min; 35 cycles: denaturation at 95 °C for 1 min, annealing at 56 °C for 45 s, 1 min, or 1 min 15 s, elongation at 72 °C for 1 min, 1 min 30 s, or 2 min; final elongation at 72 °C for 7 min; storage at 4 °C until further use. We also performed PCRs with common LSU primers LR0R and LR7. The PCR conditions for these primers were: initial denaturation at 95 °C for 5 min; 35 cycles: denaturation at 95 °C for 1 min, annealing at 52 °C for 45 s, 1 min, or 1 min 15 s, elongation at 72 °C for 1 min, 1 min 30 s, or 2 min; final elongation at 72 °C for 7 min; storage at 4 °C until further use.

All PCRs were conducted on an Alpha Metrix Biotech G-STORM GS482 Thermal Cycler or, in some cases, on an AB GeneAmp PCR System 2700. We visualized amplified DNA fragments using ethidium bromide or Midori Green (NIPPON Genetics EUROPE) as the fluorescent dye under UV light. PCR products were purified using the Omega E.Z.N.A. Cycle Pure Kit Centrifugation Protocol, Agencourt AMPure XP Bead Cleanup, or the QIAGEN QIAquick PCR Purification Kit. Prior to the separate sequencing of double bands, each DNA band was excised and purified using the Omega E.Z.N.A. Gel Extraction Kit (Spin Protocol). Automated Sanger sequencing was performed on an ABI 3730xl by Microsynth (Switzerland). Subsequent nucleotide BLAST (Altschul *et al.* 1990; Johnson *et al.* 2008) was used for coarse orientation of taxonomic affiliations.

The optimal primer pair obtained (Table 1) includes a new forward primer (LRlecF) with two substitutions between Lecanoromycetes and common symbiotic algae such as *Trebouxia* or *Stichococcus*, as shown in Fig. 1. The new reverse primer (LRlecR) binding site (Figs 1 & 2) exhibits one indel and two to three

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TABLE 2. *Voucher information and GenBank accession numbers for LSUs obtained with the newly designed primers. For convenience, KS18 and KS33 were co-deposited with contiguous sequence regions obtained as separate products.*

Order/Family	Species	Country	Locality	Extract ID	GenBank Accession No	Voucher
Acarosporales						
Acarosporaceae						
	Acarospora glaucocarpa	Canada, British Columbia	ALCAN highway near Muncho Lake	T1320	KP794961	*Spribille* 29642 (GZU)
	A. schleicheri	USA, Arizona		SAR222	KP794963	*Sweat & Yansky* KGS1196 (UPS L-162697)
	Myriospora scabrida	Sweden, Harjedalen		SAR195	KP794966	*Westberg* 07-009g (LD)
	Pleopsidium chlorophanum	USA, Montana	Missoula Co., Finlay Lakes trail	T1321	KP794962	*Spribille* s. n., 09.2013 (GZU)
	Polysporina cyclocarpa	Sweden, Torne lappmark		SAR246	KP794967	*Westberg* P117 (S)
	Sarcogyne clavus	Sweden, Varmland		SAR220	KP794968	*Berglund* SAR220 (S)
	Timdalia intricata	Sweden, Harjedalen		SAR92	KP794969	*Westberg* SAR92 (LD)
Baeomycetales						
Trapeliaceae						
	Placopsis lambii	USA, Montana	Gallatin Co., S of Bozeman, Hyalite Canyon	KS72	KP794970	*Resl* 1152 (GZU)
	Trapelia coarctata	Austria, Carinthia	Hochrindl	KS18	KP794971	*Resl* 1149 (GZU)
	Trapeliopsis granulosa	USA, California	Yosemite National Park	KS33	KP794972	*Lendemer* 19688 (GZU)
Candelariales						
Candelariaceae						
	Candelaria concolor	Italy, Veneto		SAR78	KP794964	*Arup* L07018 (LD)
	C. pacifica	USA, California		SAR21	KP794965	*Westberg* 953 (LD holotype)
Lecanorales						
Mycoblastaceae						
	Violella fucata	Germany, Bavaria	Bayerischer Wald, Dreisesselberg	T462	KP794949	*Spribille* 32112 (GZU)
Ramalinaceae						
	Ramalina dilacerata	Russia, Khabarovskiy Krai	9 km SW of De Kastri along Bolshoi Somon River	T770	KP794953	*Spribille* 30671-B (GZU)
	R. geniculata	New Zealand, Auckland	Massey	T1003	KP794955	*Blanchon* 003715 (GZU)
Peltigerales						
Pannariaceae						
	Fuscopannaria laceratula	USA, Alaska	Glacier Bay National Park, Taylor Bay	T1188	KP794958	*Spribille* 39570 (GZU)
	Santessoniella grisea	USA, Alaska	6·3 km NW of Gustavus, Tower Road	T1214	KP794959	*Spribille* 38036 (GZU)
	Steineropsis alaskana	USA, Alaska	Glacier Bay National Park, Dundas Bay	T1187	KP794957	*Spribille* 38953 (GZU)

Table 2. *Continued*

Order/Family	Species	Country	Locality	Extract ID	GenBank Accession No	Voucher
Placynthiaceae						
	Placynthium tantaleum	USA, Montana	Flathead Co., Trail Creek	T1183	KP794956	*Spribille* s. n., 07.10.12 (GZU)
Pertusariales						
Megasporaceae						
	Aspicilia vagans	Russia, Altai Republic	Kosh-Agach	T1329	KP794973	*Resl* 1155 (GZU)
Ochrolechiaceae						
	Ochrolechia subplicans ssp. *hultenii*	USA, Alaska	Glacier Bay National Park, Excursion Ridge	T1300	KP794974	*Spribille* 38350 (GZU)
Pertusariaceae						
	Pertusaria pertusa	Bosnia-Herzegovina, Republika Srpska	Sutjeska National Park, Perucica forest	T1298	KP794960	*Bilovitz* 3636 (GZU)
Teloschistales						
Teloschistaceae						
	Caloplaca pyracea	USA, Montana	Lincoln Co., Lake Koocanusa, Rexford Bench	T592	KP794951	*Spribille* 21014 (GZU)
	C. tominii	USA, Montana	Lincoln Co., Lake Koocanusa, Rexford Bench	T635	KP794952	*Spribille* 21037 (GZU)
Physciaceae						
	Heterodermia speciosa	USA, Alaska	Klondike Gold Rush National Historical Park, Chilkoot Trail	T636	KP794975	*Spribille* 26300 (KLGO)
	Rinodina mniaraea var. *mniaraea*	USA, Idaho	Bonner Co., Mt. Roothaan	T803	KP794954	*Spribille* 15242 (GZU)
Umbilicariales						
Umbilicariaceae						
	Umbilicaria polyphylla	Austria, Styria	Zirbitzkogel, Großer Winterleitensee	T1324	KP794976	*Spribille* s. n., 2013 (GZU)
Incertae sedis						
Arthrorhaphidaceae						
	Arthrorhaphis alpina	USA, Alaska	Klondike Gold Rush National Historical Park, Chilkoot Trail	T563	KP794950	*Spribille* 26526 (KLGO)

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substitutions between Lecanoromycetes and algae. Based on the available sequence data (19.02.2015), we also expect the primers to discriminate against, for example, *Coccomyxa peltigerae* (FN597599), *Dictyochloropsis symbiontica* (EU734575) and *D. reticulata* (FJ792803). Using LRlecF and LRlecR we obtained single DNA bands instead of the double bands obtained for the same DNA extracts with, for example, LR0R and LR7 primers, with one band produced by fungal and the other by algal LSU fragments. Double bands have only been obtained in rare cases where untargeted, unannotated DNA was co-amplified at high primer concentrations (0.8 µM), as revealed by subsequent gel clean-up and sequencing. Sequencing revealed that the LSU of samples amplified with LRlecF and LRlecR was exclusively of fungal origin.

Our results indicate that the newly developed primers LRlecF and LRlecR are not only highly effective in excluding the photobiont partner in lichen symbioses from amplification of large ribosomal subunit DNA, but are also one of the most reliable primer pairs we have worked with for obtaining fungal LSU products across the whole of Lecanoromycetes. To date we have obtained clean fungal LSU products of ~1350–1850 bp length (depending on intron length) from representatives of 24 genera and 14 families in all five recognized subclasses of Lecanoromycetes after Miądlikowska *et al.* (2014): Lecanoromycetidae, Ostropomycetidae, Umbilicariomycetidae, 'Candelariomycetidae' and Acarosporomycetidae (Table 2). The co-amplification of DNA from co-occurring Ascomycota is still possible because of the broad affinity these primers exhibit to the phylum as a whole. The primers, however, exclude Basidiomycota such as *Tremella* in *in silico* tests as well as in actual PCRs conducted on co-extractions with *Violella fucata*.

Amplification of algal LSU with common primers is not random in our experience but instead specific to certain lichen genera and over time became predictable, amplifying especially algae of the genus *Pseudochlorella* and more rarely *Trebouxia*. We have no reason to believe that the phenomenon reflects anything more than minor homoplasies at primer binding sites in specific strains of *Trebouxia*. Most *Trebouxia* strains exhibit up to three substitutions at LR0R binding sites and are probably sufficiently discriminated by common LSU primers in most cases. This likely explains why co-amplification of 26S rDNA from *Trebouxia* photobionts has not been reported for most groups of lichens. However, co-amplification of algal DNA with standard LSU primers may be a more widespread problem in *Pseudochlorella* and other members of the *Prasiola* clade of *Trebouxiophyceae*, which are widespread in Ostropomycetidae. The primer pair we report here appears to offer a reliable way to obtain long fungal-specific products over a wide range of lichenized Lecanoromycetes, irrespective of photobiont.

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