Barcoding lichen-forming fungi using 454 pyrosequencing is challenged by artifactual and biological sequence variation¹

Kristiina Mark, Carolina Cornejo, Christine Keller, Daniela Flück, and Christoph Scheidegger

Abstract: Although lichens (lichen-forming fungi) play an important role in the ecological integrity of many vulnerable landscapes, only a minority of lichen-forming fungi have been barcoded out of the currently accepted ~18 000 species. Regular Sanger sequencing can be problematic when analyzing lichens since saprophytic, endophytic, and parasitic fungi live intimately admixed, resulting in low-quality sequencing reads. Here, high-throughput, long-read 454 pyrosequencing in a GS FLX+ System was tested to barcode the fungal partner of 100 epiphytic lichen species from Switzerland using fungal-specific primers when amplifying the full internal transcribed spacer region (ITS). The present study shows the potential of DNA barcoding using pyrosequencing, in that the expected lichen fungus was successfully sequenced for all samples except one. Alignment solutions such as BLAST were found to be largely adequate for the generated long reads. In addition, the NCBI nucleotide database—currently the most complete database for lichen-forming fungi—can be used as a reference database when identifying common species, since the majority of analyzed lichens were identified correctly to the species or at least to the genus level. However, several issues were encountered, including a high sequencing error rate, multiple ITS versions in a genome (incomplete concerted evolution), and in some samples the presence of mixed lichen-forming fungi (possible lichen chimeras).

Key words: 454 pyrosequencing, DNA barcoding, intragenomic variation, internal transcribed spacer, lichenized fungi.

Résumé : Bien que les lichens (champignons lichénisés) jouent un rôle important dans l'intégrité écologique de plusieurs environnements vulnérables, seule une minorité de champignons lichénisés ont été examinés au moyen de codes à barres sur un total de ~18 000 espèces reconnues. Le séquençage Sanger standard peut s'avérer problématique lorsqu'on analyse les lichens en raison de la présence de champignons saprophytes, endophytes et parasitiques qui y sont intimement associés, ce qui produit des lectures de faible qualité. Dans ce travail, un pyroséquençage 454 à haut débit produisant de longues séquences sur un système GS FLX+ a été testé pour réaliser un codage à barres de la composante fongique de 100 espèces de lichens épiphytes provenant de la Suisse. Des amorces spécifiques des champignons ont été employées pour amplifier l'espaceur interne transcrit (ITS) au complet. Ce travail montre le potentiel des codes à barres réalisés au moyen du pyroséquençage, car les champignons lichénisés attendus ont été séquencés avec succès pour tous les échantillons sauf un. Les outils d'alignement comme BLAST se sont avérés largement adéquats pour l'analyse des longues séquences. De plus, la base de données NCBI – de loin la plus complète pour les champignons lichénisés – peut servir de base de référence pour l'identification des espèces communes, puisque la majorité des lichens analysés ont été correctement identifiés à l'espèce ou à tout le moins au genre. Cependant, plusieurs problèmes ont été rencontrés, incluant un taux d'erreurs élevé, de multiples versions de l'ITS au sein d'un génome (évolution concertée incomplète), et la présence dans certains échantillons de plus d'un champignon lichénisé (possiblement dû à des lichens chimériques). [Traduit par la Rédaction]

Mots-clés : pyroséquençage 454, codage à barres de l'ADN, variation intragénomique, espaceur interne transcrit, champignons lichénisés.

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Introduction

Lichens are intimate and long-term symbiotic associations consisting of a heterotrophic fungal partner—also called the mycobiont—and photosynthetic algae or cyanobacteria—also called the photobiont (Nash 2008). In a systematic context, lichens are named after the fungal partner, and according to most recent estimates, about 18% of all fungal species are lichen-forming (Feuerer and Hawksworth 2007). While lichens include many bio-indicators for monitoring environmental quality—including air pollution and ecological integrity of forest landscapes (Nimis et al. 2002)—accurate identification of lichenized fungal species remains challenging (Lumbsch and Leavitt 2011). DNA-based specimen identification to a species level is useful in a system of well-circumscribed taxa and a high-quality reference database (Seifert 2009; Begerow et al. 2010). Recently, the internal transcribed spacer region (ITS) of the nuclear ribosomal RNA cistron was proposed as the primary fungal barcode marker (Schoch et al. 2012). However, only a minority of lichen-forming fungi have been barcoded from the estimated 17 500 species (Feuerer and Hawksworth 2007). For example, the largest and most typical order of lichen-forming fungi (Lecanorales) consists of about 5700 species, but only about 29% of them have publicly available ITS sequences in sequence databases such as the National Institute of Health's (NIH) genetic sequence database, GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>; accessed 28 September 2015), the Barcode of Life Data System (BOLD; www.boldsystems.org; Ratnasingham and Hebert 2007), and UNITE (<https://unite.ut.ee/>; accessed 24 November 2015; Abarenkov et al. 2010).

Barcoding lichens using Sanger sequencing has been successful in some groups of lichens (Kelly et al. 2011; Divakar et al. 2016), especially with foliose and fruticose lichens; however, in crustose lichens—that constitute by far the vast majority of lichenized species (Bergamini et al. 2005)—it often proves quite challenging (Flück 2012). Sampling difficulties occur where other very similar lichen species live mixed or close by, and many saprophytic, endophytic, and parasitic fungi also live intimately admixed with the lichen mycobiont, making the application of Sanger sequencing insufficient in many cases (Flück 2012; Orock et al. 2012). A limited number of studies are known to have successfully applied pyrosequencing to recover the identity of a lichen, when Sanger sequencing failed to produce usable results (Hodkinson and Lendemer 2013; Lücking et al. 2014a).

Recent advancements in pyrosequencing methods now allow the amplification of fragments up to 1000 base pairs (bp) in the GS FLX+ system of Roche/454 pyrosequencing. However, 454 pyrosequencing is notorious for its high indel (short insertions and deletions) error rate in homopolymeric regions (three or more identical nucleotides) and carry-forward-incomplete-extension (CAFIE) errors (Margulies et al. 2005; Huse et al. 2007; Gilles et al. 2011; Lücking et al.

2014b). In addition to artifactual sequence variation, biological sequence variation—such as intragenomic and (or) intramycelial (i.e., allelic heterozygosity) variation of this multi-copy gene—may be possible (Wörheide et al. 2004; Simon and Weiß 2008; Lindner et al. 2013).

The aim of our study was to test whether lichen mycobiont can be successfully identified to a single species using DNA barcoding through pyrosequencing the ITS marker of 100 species of both foliose–fruticose and crustose lichens, using fungal-specific markers in the high-throughput 454 sequencing in the GS FLX+ system, while elucidating and quantifying artifactual and biological sequence variation.

Materials and methods

Taxon sampling and morphology-based identification

One hundred lichen specimens, including 52 crustose species and 48 macrolichens (foliose and fruticose thalli), were collected from different locations in Switzerland in 2011 and 2014. The full list of specimens with voucher information is given in Table 1. Additional specimen data—such as the collection GPS coordinates, substrate, specimen photographs, the primary ITS barcodes—are available through a public BOLD dataset DOI: dx.doi.org/10.5883/DS-LICODE. All project data can also be accessed through the PlutoF cloud database under project “Barcoding Swiss lichens using 454 pyrosequencing” (<https://plutof.ut.ee/#/study/view/31890>). Specimen identifications of the collected material were based on morphological and chemical characters, following the nomenclature of Clerc and Truong (2012). Lichen chemistry was examined with standardized thin layer chromatography (TLC) using solvent systems A, B, and C (Culberson and Ammann 1979, White and James 1985). Sorediate *Bacidia* and *Lecanora* species without apothecia, samples #6, #7, and #42, could not be identified to the species level and were left as *Bacidina arnoldiana* aggregate (aggr.) and *Lecanora strobilina* aggr., respectively. Specimen LC-088, determined as *Lecidella* sp., includes an undescribed chemotype for *Lecidella* (arthothelin, 2,4-dichloronorlichexanthone), and BC-47-1 *Lecanora* sp. morphologically resembles *Haematomma ochroleucum* or *Biatora flavopunctata*, with atranorin, usnic acid, and zeorin identified as secondary substances. Specimens with poorly expressed diagnostic characters were re-checked in light of barcoding data, and the determination of five specimens was corrected (LC-023 *Lecanora* cf. *umbrina* ⇒ *Lecania* cf. *cyrtella*, BC-027-1 *Haematomma* aff. *ochroleucum* or *Biatora flavopunctata* ⇒ *Lecanora* sp., BC-155-5 *Lecidea nylanderii* ⇒ *Loxospora elatina*, BC-161-5 *Lecanora farinaria/expersa* ⇒ *Loxospora elatina*, KM-03-02 *Usnea florida* ⇒ *Usnea intermedia*). All specimens are stored at the WSL Swiss Federal Research Institute at –20 °C.

Molecular methods

About 3–5 mg of visually uncontaminated lichen thallus of each specimen was sampled for molecular analyses. In sampling, vegetative thallus was preferred, but in some apotheciate crustose species with no visible

Table 1. Studied species with specimen voucher info, the primary barcode length for the sequenced ITS region, the position and length of an intron in the barcode (if present), and the primary barcode accession numbers in the NCBI and BOLD databases.

No.	Target taxon	Collection data (site, date, ID)	Barcode length (bp)	Group I intron length (bp) and position	GenBank No.	BOLD No.
1	<i>Alectoria sarmentosa</i> (Ach.) Ach.	CH-Bern; 06.02.2014; LC-064	579		KX132948	LIFU039-16
2	<i>Anaptychia crinalis</i> (Schleich. ex Schaer.) Vězda	CH-Graubünden; 18.02.2014; LC-071	1067	512 (18–322; 332–538)	KX132954	LIFU045-16
3	<i>Arthrosporum populum</i> A. Massal.	CH-Valais; 06.02.2014; LC-046	586		KX132986	LIFU078-16
4	<i>Bacidia rubella</i> (Hoffm.) A. Massal.	CH-Valais; 06.02.2014; LC-041	774	216 (26–241)	KX132984	LIFU076-16
5	<i>Bacidia vermifera</i> (Nyl.) Th. Fr.	CH-Bern; 04.07.2012; RE-515-19	578		KX132992	LIFU084-16
6	<i>Bacidina arnoldiana</i> aggr.*	CH-Lucerne; 30.03.2011; BC-038-1	583		KX132958	LIFU049-16
7	<i>Bacidina arnoldiana</i> aggr.*	CH-Zurich; 15.07.2011; BC-132-1	795	213 (26–238)	KX132972	LIFU063-16
8	<i>Bryoria capillaris</i> (Ach.) Brodo & D. Hawksw.	CH-Bern; 06.02.2014; LC-058	803	223 (26–248)	KX132945	LIFU036-16
9	<i>Buellia arborea</i> Coppins & Tønsberg	CH-Obwalden; 13.08.2011; BC-154-2	584		KX132975	LIFU066-16
10	<i>Bunodophoron melanocarpum</i> (Sw.) Wedin	CH-Bern; 06.02.2014; LC-065	584		KX132949	LIFU040-16
11	<i>Cetrelia monachorum</i> (Zahlbr.) W.L. Culb. & C.F. Culb.	CH-Lucerne; 22.02.2014; KM-03-07	577		KX132924	LIFU015-16
12	<i>Chaenotheca cf. stemonea</i> (Ach.) Müll. Arg.	CH-Neuchâtel; 13.05.2011; BC-087-3	571		KX133006	LIFU098-16
13	<i>Cladonia chlorophaea</i> (Flörke ex Sommerf.) Spreng.	CH-Zurich; 01.02.2014; KM-01-8	872	227 (26–252)	KX132914	LIFU005-16
14	<i>Cladonia coniocraea</i> (Flörke) Spreng.	CH-Bern; 06.02.2014; LC-068	868	226 (26–251)	KX132951	LIFU042-16
15	<i>Cladonia digitata</i> (L.) Hoffm.	CH-Bern; 06.02.2014; LC-067	650		KX132950	LIFU041-16
16	<i>Cladonia squamosa</i> (Scop.) Hoffm.	CH-Bern; 06.02.2014; LC-069	873	227 (26–252)	KX132952	LIFU043-16
17	<i>Evernia divaricata</i> (L.) Ach.	CH-Bern; 06.02.2014; LC-057	585		KX132944	LIFU035-16
18	<i>Evernia prunastri</i> (L.) Ach.	CH-Graubünden; 18.02.2014; LC-070	578		KX132953	LIFU044-16
19	<i>Fellhanera bouteillei</i> (Desm.) Vězda	CH-Lucerne; 03.2014; LC-118	609		KX132990	LIFU082-16
20	<i>Flavoparmelia caperata</i> (L.) Hale	CH-Zurich; 15.02.2014; KM-02-02	1176	594 (26–619)	KX132916	LIFU007-16
21	<i>Frutidella pullata</i> (Norman) Schmall	CH-Bern; 17.05.2011; BC-115-2	828	232 (26–257)	KX132970	LIFU061-16
22	<i>Fuscidea arboricola</i> Coppins & Tønsberg	CH-Vaud; 10.03.2007; BC-185-5	n.a.		KX132962	LIFU053-16
23	<i>Fuscidea pusilla</i> Tønsberg	CH-St. Gallen; 20.04.2011; BC-063-2	542			LIFU072-16 ^a
24	<i>Hyperphyscia adglutinata</i> (Flörke) H. Mayrhofer & Poelt	CH-St. Gallen; 11.04.2011; BC-055-3	795	228 (26–253)	KX132959	LIFU050-16
25	<i>Hypocenomyce scalaris</i> (Ach. ex Lilj.) M. Choisy	CH-Valais; 06.02.2014; LC-016	564		KX132982	LIFU074-16
26	<i>Hypogymnia farinacea</i> Zopf	CH-Bern; 06.02.2014; LC-062	580		KX132947	LIFU038-16
27	<i>Hypogymnia physodes</i> (L.) Nyl.	CH-Valais; 06.02.2014; LC-039	810	228 (26–253)	KX132937	LIFU028-16
28	<i>Hypogymnia tubulosa</i> (Schaer.) Hav.	CH-Jura; 01.03.2014; LC-107	797	220 (26–245)	KX132956	LIFU047-16
29	<i>Hypotrachyna laevigata</i> (Sm.) Hale	CH-Lucerne; 22.02.2014; KM-03-05	582		KX132922	LIFU013-16
30	<i>Lecania cf. cyrtella</i> (Ach.) Th. Fr.	CH-Valais; 06.02.2014; LC-023	801	223 (26–248)	KX132983	LIFU075-16
31	<i>Lecanora albella</i> (Pers.) Ach.	CH-Bern; 17.05.2011; BC-118-1	590		KX133002	LIFU094-16
32	<i>Lecanora allophana f. sorediata</i> Nyl.	CH-Valais; 07.05.2011; BC-72-3	813	223 (26–248)	KX133001	LIFU093-16
33	<i>Lecanora argentata/subrugosa</i>	CH-Bern; 17.05.2011; BC-118-2	598		KX133003	LIFU095-16
34	<i>Lecanora carpinea</i> (L.) Vain.	CH-Valais; 16.04.2011; BC-58-3	840	248 (26–273)	KX132999	LIFU091-16
35	<i>Lecanora horiza</i> (Ach.) Röhl.	CH-St. Gallen; 11.04.2011; BC-54-3	591		KX132998	LIFU090-16
36	<i>Lecanora impudens</i> Degel.	CH-St. Gallen; 11.04.2011; BC-49-1	813	223 (26–248)	KX132996	LIFU088-16
37	<i>Lecanora muralis</i> (Schreb.) Rabenh.	CH-Valais; 06.02.2014; LC-045	590		KX132985	LIFU077-16
38	<i>Lecanora praesistens</i> Nyl.	CH-Bern; 20.05.2013; MG-076-49	833	231 (26–256)	KX132991	LIFU083-16

Table 1 (continued).

No.	Target taxon	Collection data (site, date, ID)	Barcode length (bp)	Group I intron length (bp) and position	GenBank No.	BOLD No.
39	<i>Lecanora pulicaris</i> (Pers.) Ach.	CH-Jura; 01.03.2014; LC-101	831	226 (26–251)	KX132989	LIFU081-16
40	<i>Lecanora saligna</i> (Schrader) Zahlbr.	CH-Glarus; 11.04.2011; BC-47-1	597		KX132995	LIFU087-16
41	<i>Lecanora</i> sp.*	CH-Bern; 22.05.2011; BC-027-1	796	206 (26–231)	KX133005	LIFU097-16
42	<i>Lecanora strobilina</i> aggr.*	CH-Glarus; 11.04.2011; BC-52-1	590		KX132997	LIFU089-16
43	<i>Lecanora subcarpineae</i> Szatala	CH-Bern; 01.03.2014; LC-090	595		KX132988	LIFU080-16
44	<i>Lecanora varia</i> (Hoffm.) Ach.	CH-Zurich; 16.04.2011; BC-60–5	808	218 (26–243)	KX133000	LIFU092-16
45	<i>Lecidella albida</i> Hafellner	CH-St. Gallen; 20.04.2011; BC-067-3	590		KX132987	LIFU079-16
46	<i>Lecidella</i> cf. <i>elaeochroma</i> (Ach.) M. Choisy	CH-Graubünden; 18.02.2014; LC-072	592		KX132966	LIFU057-16
47	<i>Lecidella</i> cf. <i>leprothalla</i> (Zahlbr.) Knoph & Leuckert	CH-Neuchâtel; 13.05.2011; BC-077-2	599		KX132965	LIFU056-16
48	<i>Lecidella flavosorediata</i> (Vězda) Hertel & Leuckert	CH-Valais; 08.05.2011; BC-074-14	599		KX132994	LIFU086-16
49	<i>Lecidella flavosorediata</i> (Vězda) Hertel & Leuckert	CH-Bern; 01.03.2014; LC-091	597		KX132960	LIFU051-16
50	<i>Lecidella scabra</i> (Taylor) Hertel & Leuckert	CH-St. Gallen; 11.04.2011; BC-055-4	595		KX132993	LIFU085-16
51	<i>Lecidella</i> sp.*	CH-Bern; 01.03.2014; LC-088	596		KX132978	LIFU069-16
52	<i>Lepraria elobata</i> Tønsberg	CH-Obwalden; 13.08.2011; BC-158-2	604		KX132979	LIFU070-16
53	<i>Lepraria jackii</i> Tønsberg	CH-Obwalden; 13.08.2011; BC-156-3	601		KX132974	LIFU065-16
54	<i>Lepraria lobificans</i> Nyl.	CH-Zurich; 18.07.2011; BC-133-3	564		KX132961	LIFU052-16
55	<i>Lepraria rigidula</i> (B. de Lesd.) Tønsberg	CH-Valais; 16.04.2011; BC-056-2	598		KX132973	LIFU064-16
56	<i>Lepraria vouauxii</i> (Hue) R.C. Harris	CH-Zurich; 18.07.2011; BC-133-2	601		KX132927	LIFU018-16
57	<i>Letharia vulpina</i> (L.) Hue	CH-Valais; 06.02.2014; LC-006	581		KX132976	LIFU067-16
58	<i>Loxospora elatina</i> (Ach.) A. Massal.	CH-Obwalden; 13.08.2011; BC-155-5	564		KX133004	LIFU096-16
59	<i>Loxospora elatina</i> (Ach.) A. Massal.	CH-Obwalden; 19.08.2011; BC-161-5	564		KX132969	LIFU060-16
60	<i>Megalaria pulverea</i> (Borrer) Hafellner & E. Schreiner	CH-Bern; 17.05.2011; BC-113-3	569		KX132913	LIFU004-16
61	<i>Melanelixia fuliginosa</i> subsp. <i>glabratula</i> (Lamy) J.R. Laundon	CH-Zurich; 01.02.2014; KM-01-6	807	229 (26–254)	KX132939	LIFU030-16
62	<i>Melanelixia glabra</i> (Schaer.) O. Blanco, A. Crespo, Divakar, Essl., D. Hawksw. & Lumbsch	CH-Valais; 06.02.2014; LC-043	580		KX132938	LIFU029-16
63	<i>Melanelixia subargentifera</i> (Nyl.) O. Blanco, A. Crespo, Divakar, Essl., D. Hawksw. & Lumbsch	CH-Valais; 06.02.2014; LC-042	582		KX132935	LIFU026-16
64	<i>Melanohalea exasperata</i> (De Not) O. Blanco, A. Crespo, Divakar, Essl., D. Hawksw. & Lumbsch	CH-Valais; 06.02.2014; LC-028	801	220 (26–245)	KX132943	LIFU034-16
65	<i>Menegazzia terebrata</i> (Hoffm.) A. Massal.	CH-Bern; 06.02.2014; LC-054	583		KX132981	LIFU073-16
66	<i>Micarea cinerea</i> (Schaer.) Hedl.	CH-Lucerne; 22.02.2014; KM-03-03	571		KX132957	LIFU048-16
67	<i>Micarea xanthonica</i> Coppins & Tønsberg	CH-St. Gallen; 15.03.2011; BC-015-6	755	192 (18–209)	KX132971	LIFU062-16
68	<i>Mycoblastus sanguinarius</i> (L.) Norman	CH-Bern; 17.05.2011; BC-119-4	596		KX132946	LIFU037-16
69	<i>Parmelia saxatilis</i> (L.) Ach.	CH-Bern; 06.02.2014; LC-060	583		KX132926	LIFU017-16
70	<i>Parmelia sulcata</i> Taylor	CH-Valais; 06.02.2014; LC-004	593		KX132912	LIFU003-16
71	<i>Parmelina tiliacea</i> (Hoffm.) Hale	CH-Zurich; 01.02.2014; KM-01-4A	578		KX132918	LIFU009-16
72	<i>Parmeliopsis ambigua</i> (Wulfen) Nyl.	CH-Valais; 06.02.2014; LC-002	581		KX132923	LIFU014-16

Table 1 (concluded).

No.	Target taxon	Collection data (site, date, ID)	Barcode length (bp)	Group I intron length (bp) and position	GenBank No.	BOLD No.
73	<i>Parmotrema crinitum</i> (Ach.) M. Choisy	CH-Lucerne; 22.02.2014; KM-03-06	609		KX132915	LIFU006-16
74	<i>Parmotrema perlatus</i> (Huds.) M. Choisy	CH-Zurich; 14.02.2014; KM-02-01	610		KX132941	LIFU032-16
75	<i>Phaeophyscia ciliata</i> (Hoffm.) Moberg	CH-Valais; 06.02.2014; LC-050	998	418 (18–224; 233–443)	KX132942	LIFU033-16
76	<i>Phaeophyscia orbicularis</i> (Neck.) Moberg	CH-Valais; 06.02.2014; LC-051	992	418 (18–224; 233–443)	KX132940	LIFU031-16
77	<i>Phaeophyscia poeltii</i> (Frey) Clauzade & Cl. Roux	CH-Valais; 06.02.2014; LC-049	801	221 (26–246)	KX132963	LIFU054-16
78	<i>Phlyctis argena</i> (Spreng.) Flot.	CH-St. Gallen; 20.04.2011; BC-065-1	924	351 (18–368)	KX132910	LIFU001-16
79	<i>Physcia adscendens</i> (Fr.) H. Olivier	CH-Zurich; 01.02.2014; KM-01-2B	784	222 (26–247)	KX132934	LIFU025-16
80	<i>Physcia aipolia</i> (Ehrh. ex Humb.) Fürnr.	CH-Valais; 06.02.2014; LC-026	783	216 (26–241)	KX132933	LIFU024-16
81	<i>Physconia distorta</i> (With.) J.R. Laundon	CH-Valais; 06.02.2014; LC-021	972	402 (18–217; 226–427)	KX132967	LIFU058-16
82	<i>Placynthiella dasaea</i> (Stirt.) Tønsberg	CH-Neuchâtel; 13.05.2011; BC-084-1	900	335 (26–360)	KX132911	LIFU002-16
83	<i>Pleurosticta acetabulum</i> (Neck.) Elix & Lumbsch	CH-Zurich; 01.02.2014; KM-01-3A	577		KX132925	LIFU016-16
84	<i>Pseudevernia furfuracea</i> (L.) Zopf	CH-Valais; 06.02.2014; LC-001	574		KX132917	LIFU008-16
85	<i>Punctelia jeckeri</i> (Roum.) Kalb	CH-Zurich; 15.02.2014; KM-02-03	901	336 (26–361)	KX132977	LIFU068-16
86	<i>Pycnora sorophora</i> (Vain.) Hafellner	CH-Obwalden; 13.08.2011; BC-156-1	565		KX132955	LIFU046-16
87	<i>Ramalina pollinaria</i> (Westr.) Ach.	CH-Jura; 01.03.2014; LC-093	770	215 (26–240)	KX133007	LIFU099-16
88	<i>Scoliciosporum umbrinum</i> (Ach.) Arnold	CH-Valais; 06.02.2014; LC-024	598		KX133008	LIFU100-16
89	<i>Scoliciosporum umbrinum</i> (Ach.) Arnold	CH-Bern; 16.05.2013; MG-114-3C	598		KX132936	LIFU027-16
90	<i>Tuckermannopsis chlorophylla</i> (Willd.) Hale	CH-Valais; 06.02.2014; LC-032	560		KX132929	LIFU020-16
91	<i>Usnea barbata</i> (L.) Weber ex F.H. Wigg.	CH-Valais; 06.02.2014; LC-011	578		KX132932	LIFU023-16
92	<i>Usnea barbata</i> (L.) Weber ex F.H. Wigg.	CH-Valais; 06.02.2014; LC-019	578		KX132921	LIFU012-16
93	<i>Usnea ceratina</i> Ach.	CH-Lucerne; 22.02.2014; KM-03-04	578		KX132919	LIFU010-16
94	<i>Usnea intermedia</i> (A. Massal.) Jatta	CH-Lucerne; 22.02.2014; KM-03-01	578		KX132920	LIFU011-16
95	<i>Usnea intermedia</i> (A. Massal.) Jatta	CH-Lucerne; 22.02.2014; KM-03-02	578		KX132930	LIFU021-16
96	<i>Usnea lapponica</i> Vain.	CH-Valais; 06.02.2014; LC-013	579		KX132928	LIFU019-16
97	<i>Usnea substerilis</i> Motyka	CH-Valais; 06.02.2014; LC-009	577		KX132968	LIFU059-16
98	<i>Violella fucata</i> (Stirt.) T. Sprib.	CH-Bern; 17.05.2011; BC-109-4	834	235 (26–260)	KX132980	LIFU071-16
99	<i>Violella fucata</i> (Stirt.) T. Sprib.	CH-Obwalden; 13.08.2011; BC-159-4	592		KX132931	LIFU022-16
100	<i>Vulpicida pinastri</i> (Scop.) J.-E. Mattsson & M.J. Lai	CH-Valais; 06.02.2014; LC-015	792	216 (26–241)	KX132948	LIFU039-16

Note: The morphology and chemistry of the undescribed taxa marked with an asterisk (*) are discussed in text.

^aNo sequence, specimen data only.

vegetative thallus, 1–2 apothecia were used for DNA extraction. Frozen lichen samples were lyophilized and disrupted with a stainless steel bead in a Retsch MM2000 mill (Düsseldorf, Germany) for 2 min at 30 Hz. The full genomic DNA was extracted using the Qiagen DNEasy Plant Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's Plant Tissue Mini Protocol and diluted in 50 μ L elution buffer. A two-step polymerase chain reaction (PCR) approach was used for unidirectional amplification sequencing. First, the full ITS was amplified via PCR by using short fungal-specific primers, ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990). Each PCR reaction (25 μ L) consisted of 2 $\times$ KAPA HiFi HotStart ReadyMix PCR mix (KAPA Biosystems), 5 μ mol/L of each of the forward and reverse primer, and 1 μ L of template DNA. Thermal cycle conditions included the initial denaturation at 95 °C for 2 min; 35 cycles of denaturation for 20 s at 98 °C, primer annealing for 15 s at 57 °C, and extension for 20 s at 72 °C; and final extension for 3 min at 72 °C. The PCR products were visualized on 1.5% agarose gel and purified via Exo-SAP (Fermentas) treatment. In the second PCR step, the products were re-amplified with full-length fusion primers designed by Microsynth AG (Balgach, Switzerland). The fusion primers contained the GS FLX Titanium A- and B-adapters of the kit Lib-L (Roche Diagnostics), the multiplex identifier (MID) sequence, four base library key sequence (TCAG), and the same sample-specific primers used in the first step. The MID tags were used only on the forward, A-adaptor for unidirectional sequencing. Amplifications were conducted in 50 μ L reaction volumes containing 39 μ L of mastermix with reagents 5 $\times$ KAPA HiFi Buffer, 10 mmol/L KAPA dNTP Mix, 2U/100 μ L KAPA HiFi HotStart DNA Polymerase (KAPA HiFi HotStart PCR Kit, KAPA Biosystems), 4 μ mol/L of each of the forward and reverse primer, and 1 μ L of template DNA. The cycle conditions were as follows: initial denaturation at 95 °C for 3 min; 12 cycles of denaturation at 98 °C for 20 s, primer annealing at 56 °C for 30 s, and elongation at 72 °C for 30 s; and a final elongation step at 72 °C for 5 min. The aliquots were then purified via AMPure Beads XP (Beckman Coulter, Brea, Calif., USA). Concentrations of purified PCR products were quantified by fluorometry using the Quant-iTTM PicoGreen[®] dsDNA Assay Kit (Molecular Probes, Eugene, Oreg., USA). Probes were pooled in four equimolar pools (25 each), purified in 1.5% preparative gel, and run on 4/16 of a sequencing plate using Titanium FLX+ reagents on a GS Roche Sequencer (454 technology, Roche Diagnostics). Pre-sequencing steps (starting from the second PCR), sequencing, raw data processing using #3 pipeline preconfigured by Roche for long amplicons, and sorting the resulting reads into samples based on their MID's (demultiplexing) were all carried out by Microsynth AG.

Data processing and analyses

We followed a traditional and rather conservative approach when sorting and clustering sequences. Reads

with a non-matching forward primer, that were shorter than 500 bp, included more than two ambiguous sites, and (or) with a mean quality score lower than 25 were disregarded using the programs Cutadapt v1.7 (Martin 2011) and PRINSEQ-lite v0.20.4 (Schmieder and Edwards 2011). The quality-sorted reads were screened for chimeras using the uchime_ref command in the sequence analysis tool USEARCH v8.0.1623 (Edgar et al. 2011), searching against the UNITE/INSDC reference database of fungal ITS sequences (Nilsson et al. 2015) available at <http://unite.ut.ee/repository.php> (accessed 26 July 2015). The sequence data were not denoised, and singleton sequences were included to better understand the artifactual and biological sequence variation. The remaining reads for each sample were sorted by length, then clustered with USEARCH at a 95% similarity threshold using the centroids function in the UCLUST algorithm (Edgar 2010). This approach assumes that the longest read of a cluster is the most appropriate and assures that the longest fragment of the full ITS marker will be used for downstream analyses. The less stringent clustering was opted for our data to compensate for a high sequence error rate in the 454 system and to better reflect biological units, based on preliminary analyses where the suggested 97% threshold (see Blaailid et al. 2013) was applied. The resulting centroid sequences were compared against the NCBI nucleotide database (NCBI Resource Coordinators 2013) using the blastn algorithm to obtain their initial taxonomic affiliations.

Even though the Roche GS FLX+ system is capable of sequencing fragments up to 1000-bp long, the average read length remains between 500 and 600 bp (Erguner et al. 2015). The quality of the barcodes becomes an issue towards the end of the read as the base-call quality drops, and the further away from the start, the more ambiguous the base calling becomes (Fig. 1). The full ITS region in fungi has an average length of 500 bp in ascomycetes (Porter and Golding 2011). Based on the available sequences of the studied fungi, the barcoded ITS region is usually more than 550-bp long, and depending on the presence and length of the group I intron, it can be more than 1000-bp long. Consequently, ITS barcodes of more than 500–600 bp get fewer full-length target reads with low-quality sequence ends. Furthermore, various sequencing and PCR errors, such as homopolymer indel errors, CAFIE errors, and chimeras, are frequent and not always detected using the quality scores (reviewed in, for example, Margulies et al. 2005; Huse et al. 2007; Gilles et al. 2011; Lücking et al. 2014b).

To obtain a more reliable species reference sequence, the reads of the target taxon that were concordant with the quality parameters specified above were aligned using the MAFFT v7.017 automatic algorithm (Katoh and Standley 2013) in the Geneious v7.1.6 platform (Kearse et al. 2012). To gather the relevant sequences from a pool, we used the cluster identifications from a previous step. Sequences within the target species (the expected myco-

Fig. 1. General overview of pyrosequencing reads, with (A) number of reads per sample, (B) average read length, and (C) read quality distribution over read length. [Colour online.]

biont of the observed and determined lichen species) cluster(s)—identified as the taxon and (or) closely related taxon (for closely related species groups) using the GenBank BLAST function—were gathered and aligned together with reference sequences from GenBank. Clear outliers in the alignment were removed, the primer binding sites were trimmed, and the remaining region—including the end of ribosomal RNA gene 18S, ITS1, 5.8S rRNA gene, ITS2, the beginning of the 28S rRNA gene, and in some species, also the group I intron at the end of the 18S gene—was designated as the barcode region for the species. Nucleotide diversity was estimated for alignments with removed reference sequences using DnaSP v5.10.1 (Librado and Rozas 2009). The consensus sequence of this region was assigned as the barcode for this

species. The barcode and the representative centroid sequence—the longest read of the biggest cluster of the taxon—were aligned using the same alignment function in MAFFT, to estimate sequence similarity and the quantity of indels and nucleotide differences. Using the consensus sequence approach allowed us to ignore much of the random noise within sequences, but it is dependent on the accuracy of a given alignment. Alternatively to MAFFT, we applied the PaPaRa alignment (Berger and Stamatakis 2011) to better account for erroneous insertions. Finally, however, PaPaRa alignments were not used due to multiple reasons, discussed and exemplified in the supplementary data, File S1².

We tested the identification of the generated barcodes using the NCBI nucleotide database, currently the most

²Supplementary data are available with the article through the journal Web site at <http://nrcresearchpress.com/doi/suppl/10.1139/gen-2015-0189>.

complete database for lichen-forming fungi. The barcodes were compared against the database using the megaBLAST function in GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>; accessed 11 January 2015; Madden 2002). To evaluate the success of specimen identification using DNA barcoding we only counted the identity of the best match in GenBank BLAST, ignoring the sequence similarity since no single meaningful threshold can be applied over the species set, and for many of the taxa, too little or no information is known for accurate threshold estimation. We compared the morphological species determination with the best match (= with the highest bit score) from the barcode sequence megaBLAST search and recorded for each specimen if the identification via DNA barcoding resulted in concordant results at the species or the genus level (= correct species / = correct genus but different species or species not present in the database), divergent (= different genus and species), or neutral results (= unidentified or uncultured fungus). In cases where the hypothesized identity differed from the GenBank match, or the similarity between the GenBank sequences and our sequence was less than 97% (following Blaaidid et al. 2013), a phylogeny-based identification was used. To achieve this, available closely related representative ITS sequences of the same genus or species group (depending on the known information of the species phylogeny) were aligned with the barcode using the program MAFFT v7 (Katoh and Standley 2013), where the G-INS-i alignment algorithm (Katoh and Toh 2008) with 1PAM / K = 2 scoring matrix was set. The alignments of ITS sequences were analyzed using a maximum likelihood (ML) criterion in the program RAXML v7.3.1 (Stamatakis 2006) where the evolutionary model was set to GTRGAMMA, and node support was assessed using 1000 “fastbootstrap” replicates (Stamatakis et al. 2008).

Where distinct nucleotide variation—possibly representing intragenomic variation of ITS sequences or a mixture of individuals of the same or closely related species—was noted, the target species alignments were studied carefully, and different ITS version frequencies were counted. In cases where (a) less-dominant version(s) of ITS was/were higher in frequency than 10% of the target reads, consensus barcode(s) for the less-dominant versions were also generated. The most frequent barcode version was assigned as the primary barcode for the specimen. The different barcode versions were aligned using the MAFFT v7.017 automatic algorithm (Katoh and Standley 2013), and sequence variation, nucleotide diversity, and distances (p-distances, with indels treated as missing data with pairwise deletion) were estimated using DnaSP v5.10.1 (Librado and Rozas 2009) and MEGA5 (Tamura et al. 2011). More distant barcodes of a sample—possibly resulting from a mixture of closely related species—were aligned with closely related representative ITS sequences (for the species list and references see Table S1²), and phylogenetic trees were constructed us-

ing the ML approach executed with RAXML v7.3.1 as described above. In some samples, unalignable, up to 10-bp-long regions were detected. Since these regions were usually within or after GC-rich areas, we suspected a problematic PCR accompanied by CAFIE errors, rather than genuine mutations (Dutton et al. 1993). A known issue for GC-rich islands is knot formations in the RNA fold. The RNA secondary structure for such problematic regions was estimated using RNAstructure web servers (available at: <http://rna.urmc.rochester.edu/RNAstructureWeb>; accessed 18 November 2015) utilizing the default parameters (Bellaousov et al. 2013).

Results

The sequencing of 100 lichens resulted in 128 449 reads. The number of reads per sample varied between 207 and 5473, with an average of 1285. A general overview of sequencing reads is given in Fig. 1. Through quality sorting and chimera checking, on average 21% of the reads were removed, varying from 9% to 57% among samples. Clustering of reads at 95% resulted in an average of 88 clusters per sample (minimum – mean ± standard deviation – maximum; 5 – 88 ± 57 – 231). Despite conservative quality sorting, high nucleotide variation per sample was present in the data. While a high proportion of variation resulted from contaminant fungi within samples, significant nucleotide variation within the target taxon was also present (Table 2). The mean number of clusters per target species was 8 (0 – 8 ± 7 – 32), and the average nucleotide diversity within a target was 0.01128 ± 0.01862 . The pairwise identity between the consensus sequence (barcode) and centroid sequence of the biggest cluster was on average 98.5%, with 9.65 as the mean number of differences per sequence pair. Comparing centroid sequences with consensus barcodes revealed a mean difference of 1.46%. About 90% of the differences were insertions (69%) and deletions (21%) that were especially prominent towards the end of the sequences, while the rest were nucleotide substitutions or CAFIE errors.

From 100 samples, we were able to recover the fungal ITS sequence of the lichen mycobiont for all sequenced species except one. *Fuscidea arboricola* sequences were not recovered in its sequence pool (#22). Full barcodes, including the end of 18S rRNA, ITS1, 5.8S rRNA, ITS2, and the beginning 28S rRNA, were generated for all species, except for the barcode of *Anaptychia crinalis*, which remained incomplete, with a partly missing ITS1 sequence due to two long group I introns towards the end of 18S rDNA (altogether 512 bp). To recover the end of the barcode, chimeric sequences without the introns were used. Introns at the end of 18S rDNA were identified in 37 target taxa (Table 1). The length of the intron varied from 60 to 594 bp. The primary barcode sequences have been deposited in the NCBI and BOLD databases under the accession codes as indicated in Table 1. All project data—specimen voucher information, photographs, the complete

Table 2. Pyrosequencing results with nucleotide variation in the studied samples and comparison of consensus barcodes with representative sequencing reads for each sample.

No.	Species	Reads	Clusters ^a	Reads of target taxon (%)	Target clusters ^a	Nucleotide diversity π	Comparison between consensus barcode and representative centroid		
							Pairwise identity (%)	No of base differences (%)	TS/TV/Ind ^b
1	<i>Alectoria sarmentosa</i>	582	28	463 (79.6)	1	0.00385	97.2	14 (2.4)	1/3/10
2	<i>Anaptychia crinalis</i>	342	70	52 (15.2)	3	0	98.6	9 (1.4)	0/0/9
3	<i>Arthrosporum populorum</i>	2937	78	2499 (85.1)	24	0.00433	98.0	12 (2.0)	0/0/12
4	<i>Bacidia rubella</i>	2194	38	2167 (98.8)	24	0.00643	98.6	11 (1.4)	0/0/11
5	<i>Bacidia vermifera</i>	4052	195	1813 (44.7)	24	0.00865	97.6	14 (2.4)	0/0/14
6	<i>Bacidina arnoldiana</i> aggr.	1715	231	116 (6.8)	8	0.03161	98.6	8 (1.4)	0/1/7
7	<i>Bacidina arnoldiana</i> aggr.	1406	185	100 (7.1)	4	0.01275	99.0	8 (1.0)	0/0/8
8	<i>Bryoria capillaris</i>	636	45	424 (66.7)	4	0.00811	98.5	12 (0.5)	2/2/8
9	<i>Buellia arborea</i>	891	98	281 (31.5)	8	0.00902	97.6	14 (2.4)	0/0/14
10	<i>Bunodophoron melanocarpum</i>	128	19	82 (64.1)	3	0.02226	98.0	12 (2.0)	0/0/12
11	<i>Cetrelia monachorum</i>	1903	93	1644 (86.4)	32	0.00487	97.6	14 (2.4)	0/1/13
12	<i>Chaenotheca</i> cf. <i>stemonea</i>	542	94	156 (28.8)	8	0.08548	99.3	4 (0.7)	2/0/2
13	<i>Cladonia chlorophaea</i>	1159	131	485 (41.8)	1	0.00099	98.9	10 (1.1)	0/0/10
14	<i>Cladonia coniocraea</i>	425	52	241 (56.7)	1	0.00274	98.9	10 (1.2)	0/0/10
15	<i>Cladonia digitata</i>	718	69	582 (81.1)	6	0.00195	97.5	16 (2.5)	0/2/14
16	<i>Cladonia squamosa</i>	291	82	159 (54.6)	1	0.0022	98.9	10 (1.1)	1/0/9
17	<i>Evernia divaricata</i>	472	48	366 (77.5)	5	0.0014	99.2	5 (0.8)	0/0/5
18	<i>Evernia prunastri</i>	382	55	112 (29.3)	8	0.00118	99.0	6 (1.0)	0/0/6
19	<i>Fellhanera bouteillei</i>	422	78	159 (37.7)	12	0.02398	99.7	2 (0.3)	0/0/2
20	<i>Flavoparmelia caperata</i>	553	109	42 (7.6)	9	0.00442	97.9	19 (2.1)	0/1/18
21	<i>Frutidella pullata</i>	841	161	31 (3.7)	3	0.00283	98.9	9 (1.1)	1/0/8
22	<i>Fuscidea arboricola</i>	1369	180	0	0	—	—	—	—/—/—
23	<i>Fuscidea pusilla</i>	1568	165	411 (26.2)	3	0.00409	99.6	2 (0.4)	1/0/1
24	<i>Hyperphyscia adglutinata</i>	1433	218	198 (13.8)	5	0.00389	99.5	4 (0.5)	0/0/4
25	<i>Hypocenyomyce scalaris</i>	1446	34	1349 (93.3)	8	0.00467	98.8	7 (1.2)	1/2/4
26	<i>Hypogymnia farinacea</i>	752	33	621 (82.6)	4	0.00097	97.0	18 (3.0)	0/1/17
27	<i>Hypogymnia physodes</i>	806	57	615 (76.3)	2	0.00352	97.7	19 (2.3)	2/1/16
28	<i>Hypogymnia tubulosa</i>	716	43	532 (74.4)	2	0.00219	98.0	16 (2.0)	0/0/16
29	<i>Hypotrachyna laevigata</i>	1137	92	867 (76.3)	16	0.00145	99.1	5 (0.9)	0/0/5
30	<i>Lecania</i> cf. <i>cyrtella</i>	728	78	413 (56.7)	7	0.02063	98.0	16 (2.0)	0/0/16
31	<i>Lecanora albella</i>	267	11	12 (4.5)	3	0.11882	77.7	118 (19.9)*	26/47/45
32	<i>Lecanora allophana</i> f. <i>sorediata</i>	1717	19	1397 (81.4)	3	0.00643	99.5	4 (0.5)	0/0/4
33	<i>Lecanora argentata/subrugosa</i>	625	24	553 (88.5)	13	0.01542	98.8	7 (1.2)	0/0/7
34	<i>Lecanora carpinea</i>	224	6	187 (83.5)	2	0.02157	99.5	4 (0.5)	0/0/4
35	<i>Lecanora horiza</i>	963	9	963 (100)	9	0.0019	98.5	9 (1.5)	1/0/8
36	<i>Lecanora impudens</i>	221	14	186 (84.2)	7	0.00799	99.4	5 (0.6)	1/1/3
37	<i>Lecanora muralis</i>	1231	97	737 (59.9)	22	0.00641	99.3	4 (0.7)	0/0/4
38	<i>Lecanora praesistens</i>	773	19	127 (16.4)	1	0.00532	99.1	7 (0.9)	0/0/7

Table 2 (continued).

No.	Species	Reads	Clusters ^a	Reads of target taxon (%)	Target clusters ^a	Nucleotide diversity π	Comparison between consensus barcode and representative centroid		
							Pairwise identity (%)	No of base differences (%)	TS/TV/Ind ^b
39	<i>Lecanora pulicaris</i>	379	10	347 (91.6)	2	0.01427	99.0	8 (1.0)	0/0/8
40	<i>Lecanora saligna</i>	298	29	209 (70.1)	12	0.00692	99.0	6 (1.0)	0/0/6
41	<i>Lecanora</i> sp.	868	124	104 (12)	4	0.03276	99.0	7 (1.0)	3/0/4
42	<i>Lecanora strobilina</i> aggr.	269	11	247 (91.9)	3	0.01246	98.5	9 (1.5)	0/0/9
43	<i>Lecanora subcarpineae</i>	1551	14	1390 (89.6)	7	0.00754	98.0	12 (2.0)	2/5/5
44	<i>Lecanora varia</i>	430	5	404 (94)	2	0.00386	98.9	9 (1.1)	2/0/7
45	<i>Lecidella albida</i>	1096	163	247 (22.5)	4	0.01403	97.7	14 (2.3)	0/1/13
46	<i>Lecidella</i> cf. <i>elaeochroma</i>	1215	58	1152 (72.7)	8	0.00403	99.2	5 (0.8)	1/0/4
47	<i>Lecidella</i> cf. <i>leprothalla</i>	1585	133	254 (20.9)	5	0.00127	99.3	4 (0.7)	3/1/0
48	<i>Lecidella flavosorediata</i>	634	59	644 (69.1)	4	0.0462	98.7	8 (1.3)	0/0/8
49	<i>Lecidella flavosorediata</i>	932	76	214 (33.8)	2	0.00199	99.3	4 (0.7)	0/0/4
50	<i>Lecidella scabra</i>	2186	185	337 (15.4)	13	0.00193	97.7	14 (2.3)	2/0/12
51	<i>Lecidella</i> sp.	331	39	160 (48.3)	14	0.01223	99.7	2 (0.3)	0/0/2
52	<i>Lepraria elobata</i>	1134	194	197 (17.4)	22	0.0049	98.2	11 (1.8)	1/0/10
53	<i>Lepraria jackii</i>	960	166	161 (16.8)	18	0.00537	99.2	5 (0.8)	0/0/5
54	<i>Lepraria lobificans</i>	1021	212	349 (34.2)	16	0.00497	99.3	4 (0.7)	1/0/3
55	<i>Lepraria rigidula</i>	704	184	144 (20.5)	13	0.01333	99.2	5 (0.8)	0/0/5
56	<i>Lepraria vouauxii</i>	744	90	312 (41.9)	7	0.00822	98.8	7 (1.2)	0/0/7
57	<i>Letharia vulpina</i>	621	19	574 (92.4)	7	0.0011	98.1	11 (1.9)	0/0/11
58	<i>Loxospora elatina</i>	722	90	291 (40.3)	13	0.01002	98.9	6 (1.1)	0/0/6
59	<i>Loxospora elatina</i>	3077	60	2713 (88.2)	23	0.00785	99.1	5 (0.9)	0/0/5
60	<i>Megalaria pulvereae</i>	1516	198	290 (19.1)	9	0.01208	99.6	2 (0.4)	0/0/2
61	<i>Melanelixia fuliginosa</i> subsp. <i>glabrata</i>	975	163	294 (30.2)	4	0.00344	97.7	19 (2.3)	0/0/19
62	<i>Melanelixia glabra</i>	890	96	476 (53.5)	10	0.00457	98.3	10 (1.7)	0/0/10
63	<i>Melanelixia subargentifera</i>	698	58	478 (68.5)	3	0.00111	99.3	4 (0.7)	0/0/4
64	<i>Melanohalea exasperata</i>	891	85	330 (37)	3	0.00361	99.0	8 (1.0)	1/0/7
65	<i>Menegazzia terebrata</i>	926	94	672 (72.6)	20	0.00207	97.1	17 (2.9)	1/6/10
66	<i>Micarea cinerea</i>	422	32	341 (80.8)	4	0.00999	99.1	5 (0.9)	0/0/5
67	<i>Micarea xanthonica</i>	711	87	373 (52.5)	7	0.00728	99.3	5 (0.7)	0/0/5
68	<i>Mycoblastus sanguinarius</i>	824	102	222 (26.9)	3	0.00246	98.8	6 (1.0)	0/0/6
69	<i>Parmelia saxatilis</i>	362	58	241 (66.6)	1	0.00159	96.9	18 (3.1)	0/3/15
70	<i>Parmelia sulcata</i>	753	143	400 (53.1)	21	0.00604	98.0	11 (1.8)	0/0/11
71	<i>Parmelina tiliacea</i>	1391	133	1031 (74.1)	18	0.00203	99.0	6 (1.0)	1/0/5
72	<i>Parmeliopsis ambigua</i>	1401	89	878 (62.7)	7	0.00294	98.1	11 (2.1)	1/0/10
73	<i>Parmotrema crinitum</i>	1284	83	1146 (89.3)	15	0.0023	98.4	10 (1.6)	0/1/9
74	<i>Parmotrema perlatum</i>	1981	112	1234 (62.3)	17	0.00143	98.2	11 (1.8)	0/0/11
75	<i>Phaeophyscia ciliata</i>	365	86	90 (24.7)	3	0.00115	98.2	17 (1.8)	0/0/17
76	<i>Phaeophyscia orbicularis</i>	407	92	83 (20.4)	2	0.01161	99.4	5 (0.6)	0/0/5
77	<i>Phaeophyscia poeltii</i>	756	71	423 (56)	3	0.00104	97.9	17 (2.1)	0/0/17

Table 2 (concluded).

No.	Species	Reads	Clusters ^a	Reads of target taxon (%)	Target clusters ^a	Nucleotide diversity π	Comparison between consensus barcode and representative centroid		
							Pairwise identity (%)	No of base differences (%)	TS/TV/Ind ^b
78	<i>Phlyctis argena</i>	1078	127	246 (22.8)	3	0	99.1	8 (0.9)	0/0/8
79	<i>Physcia adscendens</i>	962	111	474 (49.3)	29	0.04417	98.5	12 (1.5)	0/0/12
80	<i>Physcia aipolia</i>	612	73	377 (61.6)	4	0.002	98.6	11 (1.4)	0/0/11
81	<i>Physconia distorta</i>	1081	182	183 (16.9)	3	0.04149	98.2	17 (1.8)	0/0/17
82	<i>Placynthiella dasaea</i>	1089	133	179 (16.4)	5	0.00495	99.1	8 (0.9)	1/0/7
83	<i>Pleurosticta acetabulum</i>	585	134	20 (3.4)	14	0.05287	99.1	5 (0.9)	1/2/2
84	<i>Pseudevernia furfuracea</i>	1017	27	879 (86.4)	9	0.0017	95.7	25 (4.3)*	9/7/9
85	<i>Punctelia jeckeri</i>	499	79	107 (21.4)	2	0.07591	99.4	5 (0.6)	0/0/5
86	<i>Pycnora sorophora</i>	984	112	351 (35.7)	8	0.01008	98.9	6 (1.1)	0/0/6
87	<i>Ramalina pollinaria</i>	705	22	654 (92.8)	9	0.00525	98.5	12 (1.5)	1/0/11
88	<i>Scoliosporum umbrinum</i>	1244	101	8 (1.4)	5	0.05371	99.5	4 (0.7)	0/1/3
89	<i>Scoliosporum umbrinum</i>	592	201	148 (11.9)	3	0.01479	99.0	6 (1.0)	0/0/6
90	<i>Tuckermannopsis chlorophylla</i>	347	72	146 (42.1)	5	0.0035	97.6	14 (2.4)	0/0/14
91	<i>Usnea barbata</i>	1498	39	1269 (84.7)	10	0.00258	97.8	13 (2.2)	0/1/12
92	<i>Usnea barbata</i>	2202	51	2101 (95.4)	11	0.00209	96.8	19 (3.2)	0/4/15
93	<i>Usnea ceratina</i>	2627	69	2518 (95.9)	14	0.00213	97.0	18 (3.0)	0/1/17
94	<i>Usnea intermedia</i>	2152	59	2009 (93.4)	2	0.00355	97.6	14 (2.4)	0/4/10
95	<i>Usnea intermedia</i>	1536	70	1287 (83.8)	5	0.00466	97.8	13 (2.2)	0/0/13
96	<i>Usnea lapponica</i>	1585	50	1463 (92.3)	14	0.00311	96.3	22 (3.7)	0/1/21
97	<i>Usnea substerilis</i>	868	38	700 (80.6)	7	0.00205	97.8	11 (1.9)	0/1/10
98	<i>Violella fucata</i>	830	93	134 (16.1)	4	0.02164	98.6	12 (1.4)	0/0/12
99	<i>Violella fucata</i>	1780	122	646 (36.3)	7	0.00704	99.7	2 (0.3)	0/0/2
100	<i>Vulpicida pinastri</i>	874	93	387 (44.3)	9	0.00159	98.9	9 (1.1)	1/0/8

Note: Asterisk (*) denotes chimeric representative centroid.

^aClustering conducted at 95% using centroids function in USEARCH.

^bQuantity of transitions (TS), transversions (TV), and indels (Ind) between the generated consensus sequence (barcode) and representative centroid.

Fig. 2. Comparison of morphological species determination and the results from sequence megaBLAST search in the NCBI nucleotide database for the studied 100 species. Only the best match accession identity was considered, and the sequence similarity results were ignored. Diagram proportions are indicated for concordant results at the species or the genus level (= correct species / = correct genus but different species or species not present), divergent (= different genus and species), or neutral results (= unidentified or uncultured fungus). For one species, no sequences were recovered within the quality-sorted reads; this is noted as “no target”.

list of the barcode sequences (including the alternative versions), alignment matrices, phylogenetic trees—can be accessed through the PlutoF cloud database under project “Barcoding Swiss lichens using 454 pyrosequencing” (<https://plutof.ut.ee/#/study/view/31890>).

The barcodes from the targeted lichenized fungi were, in most cases, morphologically and molecularly identified to the same species ($n = 69$) or at least to the same genus ($n = 18$) using the NCBI nucleotide database as reference (Fig. 2; for details see Table S2²). Nine of the species correctly identified using GenBank BLAST showed less than 97% similarity, which could indicate wider ITS genetic variation of the species or even the presence of cryptic species. ITS sequences of 14 of the studied species were previously not available in GenBank, this being one of the reasons for imprecise identifications. For eight species, the best match in GenBank was an “uncultured fungus”, originating from environmental metabarcoding studies where morphology-based identifications and taxonomic assignments are often not performed.

Nine samples were identified to the same genus but to a different species than that based on morphology, and one sample to a different genus based on a high identity score ($\geq 97\%$). These species were *Anaptychia crinalis* (identified as *Anaptychia ciliaris*, 98.0%), *Bryoria capillaris* (identified as *Bryoria fuscescens*, 98.8%), *Lecanora impudens* (identified as *Lecanora allophana*, 99.2%), *Loxospora elatina* (identified as *Loxospora ochrophaea*, 99.7%), *Parmelia sulcata* (identified as *Myelochroa auruleanta*, 99.7%),

Parmotrema perlatum (identified as *Parmotrema crinitum*, 98.7%), and *Usnea barbata* and *Usnea substerilis* (identified as *Usnea intermedia/rigida*, >98.0%). We found that these mis-identifications had three main causes—(i) labelling or identification mistakes in the NCBI nucleotide database, as in the case of *Parmelia sulcata*; (ii) the incomplete reference database with missing species or partial sequences, as in the case of *Anaptychia crinalis*; (iii) biological reasons, such as low genetic variation in the ITS region (e.g., *Bryoria capillaris*, *Parmotrema perlatum*, *Usnea barbata*, and *Usnea substerilis*).

Besides the expected mycobiont, many other fungi were recovered within our samples. For 22 samples, the most-sequenced fungus was not the expected mycobiont but another fungus instead. Many of these fungi were identified as lichen-associated (facultative parasites/lichenicolous) or plant-associated (epi- or endophytes). However, the lichen-associated fungal diversity within our sequenced lichens is not in the scope of the present study and will not be discussed further.

We studied the nucleotide variation of samples where (a) less frequent version(s) of ITS constituted more than 10% of the expected mycobiont reads. Multiple barcodes per species were generated for 22 samples (Table 3; for the full list of barcodes of such samples refer to Table S3²). The number of generated barcodes, and distances between them, varied considerably from species to species. Barcode versions with only indels as differences showed zero distance (*Buellia arborea*, *Cladonia digitata*, *Hypotrachyna laevigata*). However, since the indels in these species were present in the non-coding regions (group I intron, ITS1, or ITS2), they cannot be completely ruled out as genuine mutations. In *Lecanora subcarpineae*, *Lepraria lobificans*, and *Menegazzia terebrata*, a poorly alignable, up to 10-bp-long region was detected in ITS1. These regions were positioned within or at the end of sequence regions with high G + C content (GC%), where formations of strong knots in RNA folds are likely. In *Lecanora subcarpineae*, a region of six ambiguous bases occurred at the end of a 38-bp region of 79.5% GC content. In *Lepraria lobificans*, five ambiguous bases occurred at the end of a 44-bp region with a GC% of 82.6%. In *Menegazzia terebrata*, in a 60-bp region, with a GC% of 86.0%, an ambiguous region of nine bases occurred within the sequencing reads. RNA secondary structure analyses revealed that the sequences in these areas form a secondary structure knot with probabilities of >95% for *Lecanora*, >99% for *Lepraria*, and >99% for *Menegazzia*. Figure S1² shows, by way of example, the ITS1 RNA secondary structure of the primary barcode version and ambiguous bases of this region in *Menegazzia terebrata* barcodes.

The dominant ITS version of *Physcia adscendens* is highly similar to sequences of this species (99%; Table S2²), while the second, less-frequent version included a high number of mostly unidirectional transitional mutations and was with low similarity to *Physcia adscendens* sequences (87%).

Table 3. List of samples where minor barcode version(s) constituted more than 10% of the target reads, with characteristics of nucleotide variation between the barcodes and comparison with available Sanger sequences of the species (where possible, otherwise marked with “—”).

No.	Species	No. of Barcodes	Alignment (bp)	Variable sites	Nucleotide diversity π	Max distance	Quantity of base differences TS/TV/Ind ^b						Same mutations in Sanger
							18S	Intron	ITS1	5.8S	ITS2	28S	
1	<i>Alectoria sarmentosa</i>	8	579	8	0.00795	0.016	0/0/0	n.a.	0/4/1	0/0/0	1/4/2	0/0/0	0
6	<i>Bacidina arnoldiana aggr.</i>^a	5	799	66	0.05519	0.087	0/0/0	n.a.	7/1/1	0/0/0	7/0/1	0/0/0	—
8	<i>Bryoria capillaris</i>	4	803	6	0.00498	0.008	0/0/0	0/1/0	2/1/0	0/0/0	2/0/0	0/0/0	3
9	<i>Buellia arborea</i>	2	586	0	0	0	0/0/0	n.a.	0/0/1	0/0/0	0/0/0	0/0/0	—
12	<i>Chaenotheca</i> cf. <i>stemonea</i> ^a	10	647	121	0.11473	0.253	0/0/0	0/0/0 ^c	2/0/0	1/0/1	3/1/3	0/0/0	—
15	<i>Cladonia digitata</i>	4	650	0	0	0	0/0/0	n.a.	0/0/0	0/0/0	0/0/3	0/0/0	0
19	<i>Fellhanera bouteillei</i> ^a	3	610	57	0.06421	0.102	0/0/0	n.a.	3/1/0	0/0/0	1/1/0	0/0/0	—
29	<i>Hypotrachyna laevigata</i>	2	583	0	0	0	0/0/0	n.a.	0/0/0	0/0/0	0/0/1	0/0/0	—
30	<i>Lecania</i> cf. <i>cyrtella</i>	2	802	25	0.03145	0.032	0/0/0	6/5/1	7/3/0	1/0/1	3/0/2	0/0/3	—
43	<i>Lecanora subcarpineae</i>	18	597	7	0.00445	0.01	0/0/0	n.a.	1/3/7	0/0/0	3/0/0	0/0/0	0
50	<i>Lecidella scabra</i>	20	837	10	0.00987	0.024	0/0/0	0/0/0 ^c	7/3/1	0/0/0	3/1/1	0/0/0	—
51	<i>Lecidella</i> sp.	9	597	13	0.01063	0.017	0/0/0	n.a.	8/1/1	0/0/0	3/1/0	0/0/0	—
54	<i>Lepraria lobificans</i>	6	565	4	0.00239	0.011	0/0/0	n.a.	0/6/3	0/0/0	0/0/1	0/0/0	1
55	<i>Lepraria rigidula</i>	6	601	13	0.00836	0.022	0/0/0	n.a.	5/2/1	0/0/0	3/3/2	0/0/0	12
64	<i>Melanohalea exasperata</i>	2	806	6	0.00752	0.008	0/0/0	0/0/1	3/1/1	0/0/0	2/0/3	0/0/1	5
65	<i>Menegazzia terebrata</i>	16	592	16	0.00836	0.035	0/0/0	n.a.	1/18/0	3/6/6	0/0/3	0/0/0	0
69	<i>Parmelia saxatilis</i>	2	586	3	0.00515	0.005	0/0/0	n.a.	0/0/0	0/0/1	0/3/3	0/0/0	0
70	<i>Parmelia sulcata</i>	2	593	3	0.00506	0.005	0/0/0	n.a.	2/0/0	0/0/0	1/0/0	0/0/0	2
79	<i>Physcia adscendens</i>	2	790	92	0.11735	0.137	4/0/0	32/0/0	22/0/0	9/0/0	18/0/4	7/0/2	3
86	<i>Pycnora sorophora</i>	2	565	4	0.00708	0.007	0/0/0	n.a.	0/0/0	0/0/0	0/4/0	0/0/0	0
95	<i>Usnea intermedia</i>	9	578	7	0.00596	0.01	0/0/0	n.a.	1/2/0	0/0/0	2/2/0	0/0/0	4
96	<i>Usnea lapponica</i>	6	579	8	0.00783	0.014	0/0/0	n.a.	2/2/0	0/0/0	3/1/0	0/0/0	4

Note: Nucleotide variation of specimens in bold more likely represents genuine mutations, while in others is probably due to PCR/sequencing errors.

^aIn presence of intraspecific variation, nucleotide variation within one of the “species” was compared: *Bacidina arnoldiana* subversions A1 & A2, *Chaenotheca* cf. *stemonea* subversions A1, A2, B & C, *Fellhanera bouteillei* subversions A1 & A2 (for reference see Figs. 3–5).

^bQuantity of transitions (TS), transversions (TV), and indels (Ind), divided into exons and introns.

^cVariation in intron presence.

However, no better alignment with any other sequence in GenBank was found. Altogether, 92 transitional mutations were present among the two ITS versions (4 in 18S, 32 in the group I intron, 22 in ITS1, 9 in 5.8S, 18 in ITS2, and 7 in 28S). These mutations were almost exclusively unidirectional, being mainly $G \Rightarrow A$ and $C \Rightarrow T$.

Besides artificial nucleotide variation caused by PCR/sequencing errors, nucleotide variation can occur for biological reasons, such as (i) incomplete concerted evolution within the ITS and (ii) intraspecific variation. For 13 species, distinct nucleotide variation could not be explained by PCR or CAFIE errors only (Table 3). By comparing the variation within the reads of the mycobiont with available Sanger sequences from GenBank of the same species (where available), we also found the same variable bases in Sanger sequences in eight species. Therefore, it seems probable that intragenomic and (or) intra-mycelial (i.e., allelic heterozygosity) variation is present at least in the following samples: #8 *Bryoria capillaris*, #55 *Lepraria rigidula*, #64 *Melanohalea exasperata*, #70 *Parmelia sulcata*, #79 *Physcia adscendens*, #95 *Usnea intermedia*, and #96 *Usnea lapponica*. For eight species, such comparisons could not be made due to a lack of Sanger sequences. Sequence variation possibly resulting from intragenomic or intra-mycelial variation were additionally detected in #6 *Bacidina arnoldiana*, #12 *Chaenotheca cf. stemonea*, #19 *Fellhanera bouteillei*, #30 *Lecania cf. cyrtella*, #50 *Lecidella scabra*, and #51 *Lecidella sp.*, of which the first three are seemingly also accompanied with intraspecific variation (Figs. 3–5). The number of barcodes, however, might not necessarily represent the true intragenomic variation of a species. For example, seven variable nucleotide positions were found in *Usnea intermedia* with no clear segregation into distinct ITS types. In ITS1, three mutation positions ($G \Leftrightarrow C$, $T \Leftrightarrow C$, $T \Leftrightarrow C$) generated three types, while in ITS2, four mutation positions ($T \Leftrightarrow C$, $G \Leftrightarrow A$, $A \Leftrightarrow C$, $T \Leftrightarrow A$) generated four types, of which three were dominant. These types were combined with each, generating seven different barcode versions. Incomplete concerted evolution within ITS is therefore possible. However, inflation of ITS types should also be considered due to recombination or chimera formation between different ITS1 and ITS2 versions. The more similar the parents, the harder it is to detect and identify chimeras. Therefore, it could just as well be possible that fewer than seven different ITS versions are natural, while others are artificial formations. The number of barcodes can also be inflated by systematic CAFIE errors, which we have tried to account for when interpreting the reasons of base variation of some species (e.g., #69 *Parmelia saxatilis*). For the full list of the alternative barcodes and their hypothetical origin refer to Table S3².

The highest number of variable sites, as well as the greatest genetic distances between barcodes, occurred in samples where multiple distinct lineages, probably representing different species, were identified in the pool.

These samples were crustose species *Bacidina arnoldiana* aggr., *Chaenotheca cf. stemonea*, and *Fellhanera bouteillei*.

In *Bacidina arnoldiana* aggr. (sample #6), five different ITS versions from two *Bacidina arnoldiana* clades were identified (Fig. 4). The morphologically homogenous, yet variable species *B. arnoldiana* is therefore polyphyletic, comprising in itself multiple lineages and possibly other taxa with unresolved taxonomy. The five ITS versions from *Bacidina arnoldiana* belong in two major clusters, *Bacidina arnoldiana* 1 and *Bacidina arnoldiana* 3, and group together with other *Bacidina arnoldiana* sequences. *Bacidina arnoldiana* 2 includes only GenBank data and is more closely related to *Bacidina arnoldiana* 3. Four ITS versions cluster within *Bacidina arnoldiana* 1, of which two are dominant, A1 and B1. These are also almost equally present in the sequence pool (A1 in 65 reads, 42.6%; B1 in 45 reads, 41.7%). The subversions A1 and B1 have a pairwise identity of only 93.8%, and based on genetic distances, clade *Bacidina arnoldiana* 1 could therefore include cryptic species. However, no final conclusions can be made of our limited sample size and data based on ITS markers only.

In *Chaenotheca cf. stemonea* (sample #12), sequences from two *Chaenotheca* “species” were present (Fig. 5)—*Chaenotheca cf. stemonea* and *Chaenotheca trichialis-xyloxena* group. The first includes four highly similar (>98%) barcodes differing in multiple mutations and in intron presence/absence towards the end of the 18S rDNA. The *Chaenotheca trichialis-xyloxena* group includes six barcodes that cluster further into three groups: versions B together with a subgroup of *C. trichialis*, version A1 clusters within *C. xyloxena*, and version A2 with a second subgroup of *C. trichialis* at the base of the clade. The distances within subversions B are low (pairwise identity >98%), while between subversions B, A1, and A2, distances are larger (pairwise identities between 96% and 97%).

Fellhanera bouteillei (sample #19) included three ITS versions, of which two highly similar types (identity 99%), A1 and A2, were dominant, while the third, less-frequent version B was only present in three sequences. The less-dominant version is, however, significant as it represents the clade of *Fellhanera beouteillei* s.str. together with sequences available in GenBank (Fig. 5). The dominant types A1 and A2 clustered distantly outside of *Fellhanera bouteillei* and could represent a different species (pairwise identity between barcode versions A1 and B was 89%).

Discussion

Barcoding lichens

In the scope of this study, we “barcoded” 99 out of 100 lichen mycobiont species using 454 pyrosequencing. Good success in generating barcodes shows the high potential of this method, especially concerning crustose lichens, which can be difficult to sequence with the Sanger technique. To our knowledge, this is the first attempt to apply pyrosequencing to lichen barcoding to such an extent.

Fig. 3. Maximum likelihood ITS gene tree of available *Bacidina arnoldiana* aggr. sequences, with extended outgroup of closely related *Bacidia* species. Barcodes from *Bacidina arnoldiana* aggr. (sample #6) are indicated with a shaded background. The number of reads per barcode is given in brackets at the end of the name. Branches leading to strongly supported nodes ($\geq 70\%$) are marked in bold. The scale bar shows the number of substitutions per site. [Colour online.]

The success of DNA-based identification depends greatly on the reference database, both in the quality and quantity of the barcodes included. Purely DNA-based identification of lichens—without considering morphological and chemical characters—is far from reach, especially regarding crustose lichens and samples from poorly investigated regions due to missing references and unknown diversity (Orock et al. 2012). Within the present study, we generated ITS barcodes for 12 described species whose sequences were previously not available in GenBank and for two morphologically newly characterized specimens (#41 *Lecanora* sp., #51 *Lecidella* sp.). Multiple species showed low intraspecific ITS sequence similarity to other available sequences in GenBank

(e.g., #10 *Bunodophoron melanocarpum*, #12 *Chaenotheca* cf. *stemonea*, #19 *Fellhanera bouteillei*, #30 *Lecania* cf. *cyrtella*, #66 *Micarea cinerea*) or a high similarity to a different taxon (e.g., #8 *Bryoria capillaris*, #13–#16 *Cladonia* species, #36 *Lecanora impudens*, #47 *Lecidella* cf. *elaeochroma*, #47 *Lecidella* cf. *leprothalla*, #58–#59 *Loxospora elatina*, #74 *Parmotrema perlatum*, #91–#92 *Usnea barbata*; refer to Table S2² and to the project in PlutoF for further information). Before specimen identification via DNA barcoding can be confidently applied, taxonomic studies in these groups are needed to confirm the species monophyly or, in cases of non-monophyly, propose segregation of a taxon into multiple species. However, our results also suggest that the NCBI nucleotide da-

Fig. 4. Maximum likelihood ITS gene tree of available *Chaenotheca* sequences, including the barcodes from *Chaenotheca* cf. *stemonea* (sample #12, all with shaded background). The number of reads per barcode is given in brackets at the end of the name. Branches leading to strongly supported nodes ($\geq 70\%$) are marked in bold. The scale bar shows the number of substitutions per site. [Colour online.]

tabase, currently the most complete database for lichen-forming fungi, could be used as a reference database to identify common species, as the majority of the analyzed lichens were identified correctly to the species or, at least, to the genus level.

When generating barcodes, we applied a rather conservative approach by generating consensus sequences

from target taxon alignments, rather than using centroid sequences. In most cases, centroid reads were sufficiently accurate for correct specimen identification to a species level. However, when producing a barcode database, even a few errors can cause systemic mistakes in future identifications. Even though it is more time consuming, this approach helps to better identify and avoid

Fig. 5. Maximum likelihood ITS gene tree of available *Fellhanera* sequences with extended outgroup of closely related species, including the barcodes from *Fellhanera bouteillei* (sample #19; all with shaded background). The number of reads per barcode is given in brackets at the end of the name. Branches leading to strongly supported nodes ($\geq 70\%$) are marked in bold. The scale bar shows the number of substitutions per site. [Colour online.]

mistakes, such as those caused by sequencing, undetected chimeras, and multiple ITS copies. However, the propagation of errors cannot be avoided completely; they can originate directly from PCR amplification or be hindered by systematic sequencing errors, independent from data processing methods.

Reasons for nucleotide variation

The source of nucleotide variation within a species can be the PCR, the sequencing, or be of biological origin. While the first two sources generate artifactual variation, biological origins represent genuine mutations.

We identified three main sources of artifactual variation in our samples.

- (1) Chimeras are hybrid products of PCR formed by different template sequences. Undetected chimeras lead to overestimated richness, artificial entities, and incorrect taxonomic identifications. It has been shown that chimera formation is especially strong in regions with alternating patterns between conserved and less-conserved regions, such as 16S rRNA

genes (Shin et al. 2014). In the ITS barcodes—including conserved 18S, 5.8S, and 28S rRNA exons, alternating with variable ITS1 and ITS2 regions—frequent chimeras with switched ITS1 or ITS2 regions were observed (data not shown). Reads with group I intron towards the end of 18S rRNA gene often included chimeras where the intron had been excluded. Longer templates require longer extension times, which can contribute to chimera formation (Haas et al. 2011; Shin et al. 2014). Chimera detection is reasonably applicable in a closed system, where recombining gene variants are known. While the vast majority of fungal diversity is still unknown, chimeras should be considered as a real issue in diversity analyses where multiple templates are present in the pool (Sharifian 2010).

- (2) High content of the nucleotides guanine and cytosine can impede amplification and result in low-quality sequencing with ambiguous bases. This difficulty is associated with poor denaturation of regions with high GC concentration, while template

secondary structure hinders the access of PCR primers and enzyme work at elongation (Dutton et al. 1993). Even though high-temperature denaturation (98 °C) and high-fidelity heat-stable polymerase were used, the formation of strong knots in RNA structure that impede correct amplification cannot be ruled out and might have affected the ambiguous regions in the species *Lecanora subcarpineae*, *Lepraria lobificans*, and *Menegazzia terebrata*.

- (3) 454 pyrosequencing is notorious for its high indel error rate in homopolymeric regions and carry-forward-incomplete-extension (CAFIE) errors (Margulies et al. 2005, Huse et al. 2007, Gilles et al. 2011). Incomplete extension creates shorter homopolymers due to insufficient dNTPs. Carry-forward errors are the result of incomplete dNTP flushing, which then causes a nucleotide from the end of the homopolymer to be read some bases later (e.g., a true sequence AAAATCG, can be read as AAATCGA). A comparison between the consensus barcodes and a representative read (the centroid sequence of the biggest cluster) showed a mean difference of 1.46%. About 90% of the errors were indels. These were most prominent in homopolymeric regions and were especially frequent towards the end of the sequence, while the rest were nucleotide substitutions. Considering that in closely related species complexes, less than 2% variation could already result in a different identification, the necessity to reliably separate artificial from genuine variation becomes clear.

Nucleotide variation in the ITS can represent genuine mutations when a species is characterized by incomplete concerted evolution (Feliner and Rosselló 2007). We have highlighted 12 species in Table 3 where detected nucleotide variation could represent intragenomic variation within ITS, or alternatively, allelic heterozygosity caused by differing nuclei within a single mycelium (Huang et al. 2010; Hyde et al. 2013). The latter is more likely when only two primary allele variants in approximately equal ratios are observed (Lindner et al. 2013). Two more or less equally represented ITS paralogs were found in #1 *Alectoria sarmentosa*, #6 *Bacidina arnoldiana*, #8 *Bryoria capillaris*, #43 *Lecanora subcarpineae*, and #70 *Parmelia sulcata*, while one dominant ITS version together with relatively rare haplotypes was found in #5 *Bacidia vermifera*, #12 *Chaenotheca cf. stemonea*, #19 *Fellhanera bouteillei*, #30 *Lecania cf. cyrtella*, #50 *Lecidella scabra*, #51 *Lecidella sp.*, #55 *Lepraria rigidula*, #64 *Melanohalea exasperata*, #95 *Usnea intermedia*, and #96 *Usnea lapponica*. The latter list includes representatives from a closely related species group in the genus *Usnea* (sect. *Usnea*; Mark et al. 2016), where low genetic variation and possible rapid radiation of species have been characterized (Truong et al. 2013; Kraichak et al. 2015). However, four other *Usnea* specimens from this closely related group were also included in our study, where such nucleotide variation was not

detected (#91–#92 *U. barbata*, #94 *U. intermedia*, and #97 *U. substerilis*). Whether or not intragenomic variation can be detected via pyrosequencing likely also depends on the random effects of PCR and the proportions of different ITS copies in a genome.

Xu et al. (2009) demonstrated a high level of intra-individual polymorphism in rDNA, including multiple functional genes, putative pseudo genes, and recombinants. They also found a systematic bias in the efficiency with which different sequences were amplified. In *Physcia adscendens*, a second ITS version with unidirectional transitions from cytosine to thymine and adenine to guanine was detected. The second ITS version of this species could represent a rare divergent ITS allele or a pseudogene, propagated to a higher frequency due to a PCR bias. Ribosomal DNA pseudogenes are characterized by low stability in the predicted secondary structure, a higher substitution rate, and deamination-driven substitutions (Buckler et al. 1997). A large multigene family of 5S rRNA, including pseudogenes, has also been identified in other filamentous fungi (Margolin et al. 1998; Rooney and Ward 2005). A duplication event of the ribosomal DNA cistron could be a plausible explanation for the detected nucleotide variation in *Physcia adscendens* and is in concordance with the findings of Lücking et al. (2012).

Intraspecific variation, resulting from possible lichen chimeras or microcosms—where diverse communities live mixed and alongside—is illustrated by the phylogenies for three crustose species. For example, in a single sample of *Bacidina*, constituting a minute quantity of lichen soredia, four distinct lineages of *Bacidina* were identified in a visually inseparable community. In these species, the problem of distinguishing between intra- and interspecific variation arises. This variation within a specimen cannot be detected in Sanger sequencing environmental samples, and this could be one of the reasons for the taxonomic mess in the *Bacidina arnoldiana* aggregate, as also illustrated in Fig. 4. Further studies with more data are needed to investigate such phenomena.

Whatever the reason, nucleotide variation detected through 454 pyrosequencing must be interpreted with caution, both with respect to the acquired barcode database as well as answering biological questions. Using other, less error-prone sequencing platforms and (or) combining a large number of reads per taxon could help to avoid random sequencing errors. Sanger references can aid in distinguishing between CAFIE errors and biological sequence variation. However, Sanger sequencing may remain insufficient in some cases, as it is unable to detect intragenomic sequence variation (e.g., to investigate species and genome evolution) and fails to give useable results in a mixture of templates.

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