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Phylogenetic placement of *Lepraria cryptovouauxii* sp. nov. (Lecanorales, Lecanoromycetes, Ascomycota) with notes on other *Lepraria* species from South America

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Abstract

Lepraria cryptovouauxii is described as a new semicryptic species similar to *L. vouauxii*, from which it differs geographically (South America) and phylogenetically; both species differ in nucleotide position characters in nucITS barcoding marker. *Lepraria harrisiana* is reported as new to South America and *L. nothofagi* as new to Antarctica, Bolivia, and Peru. *Lepraria incana* (South American records are referred to *L.* aff. *hodkinsoniana*) and *L. vouauxii* (most South American records are referred to *L. cryptovouauxii*) should be excluded at least temporarily from the lichen list of South America. All records previously referred to as *L. alpina* from Bolivia and Peru belong to *L. nothofagi*. Most of Bolivian records of *L. pallida* belong to *L. harrisiana*. *Lepraria borealis* and *L. caesioalba* should be included in *L. neglecta*. *Lepraria achariana*, *L. impossibilis*, and *L. sipmaniana* are sequenced for the first time.

Keywords

lichenized fungi, morphology, Neotropics, nucITS rDNA, secondary metabolites, taxonomy

Introduction

Traditionally phenotypic characters have been used to separate lichen species; however, in numerous cases DNA based phylogenetic approaches suggested relationships that differ from traditional systematics. In some groups of lichens the absence of easily recognizable morphological or chemical characters in some lineages supported by phylogenetic signal lead to description of cryptic species (for discussion see Crespo and Perez-Ortega 2009; Crespo and Lumbsch 2010). Furthermore, Vondrák et al. (2009) introduced the semicryptic species concept for taxa that cannot be diagnosed based on their morphology but are determined based on their ecology and distribution. Moreover, for taxa with limited number of phenotypical characters due to the lack of sexual structures, the morphological species concept is especially challenging in discrimination of species (e.g., Lendemer 2011a, 2013a; Lendemer and Hodkinson 2013).

Lepraria Ach. (Lecanorales, Lecanoromycetes, Ascomycota) is a genus of crustose to fruticose lichen species, which during evolution apparently totally lost the ability of sexual reproduction and are always sterile (e.g., Ekman and Tønsberg 2002; Fehrer et al. 2008; Lendemer 2011a, 2013a; Lendemer and Hodkinson 2013). However, despite of that, they continued to speciate and 74 species are known worldwide (Ekman and Tønsberg 2002; Fehrer et al. 2008; Wijayawardene et al. 2017).

Thalli of *Lepraria* consist of soredia-like granules laying directly on a substrate or on a layer of hypothalline hyphae in case of crustose species or, in species with fruticose thalli, granules are produced also on short pseudopodetia (Lendemer 2011a; Lendemer and Hodkinson 2013). The edge of crustose thalli may be diffuse or obscurely to markedly lobate (Sipman 2004; Lendemer 2011a). Secondary chemistry is one of the most important characters in the determination and taxonomy of *Lepraria*, as morphological characters are scarce (e.g., Tønsberg 1992; Kukwa and Flakus 2009; Saag et al. 2009; Lendemer 2011a, 2013a). However, morphology and lichen substances must be taken under consideration together when identifying lichens of this genus as some species share the same (or very similar) morphologies or secondary chemistry (Tønsberg 1992; Leuckert et al. 1995; Elix and Tønsberg 2004; Sipman 2004; Flakus and Kukwa 2007, 2011; Fehrer et al. 2008; Kukwa and Flakus 2009; Saag et al. 2009; Lendemer 2013a, b; Lendemer and Hodkinson 2013).

Until recently the taxonomy of the genus has been based almost solely on morphological features and the content of secondary metabolites, and it included only those species having crustose thalli with elobate margins and lacking dibenzofurans (Laundon 1989, 1992; Tønsberg 1992). Subsequent studies demonstrated that *Leproloma* Nyl. ex Cromb. should also be included in *Lepraria* (Ekman and Tønsberg 2002; Kukwa 2002). On the other hand, *Lepraria lesdainii* (Hue) R.C.Harris was transferred to the newly established genus *Botryolepraria* Canals et al. (Canals et al. 1997), which appeared to be more closely related to Verrucariaceae in Eurotiomycetes (Kukwa and Pérez-Ortega 2010) and *Lepraria moroziana* Lendemer and *L. obtusatica* Tønsberg to *Andreiomyces* Hodkinson & Lendemer (Hodkinson and Lendemer 2013) within Arthoniomycetes. Also, few fruticose species previously belonging to *Leprocaulon* Nyl. have been transferred to *Lepraria* as well, but few *Lepraria* species to *Leprocaulon* and other genera (Grube et al.

2004; Bungartz et al. 2013; Lendemer and Hodkinson 2013). The status of several other species remains unsettled, especially of those containing usnic acid, as no molecular data are available for some taxa and their phylogenetic position has not yet been determined (Kukwa 2006a; Osyczka et al. 2010; Fryday and Øvstedal 2012; Orange et al. 2017).

In this paper we present new molecular data on *Lepraria* based on specimens collected in South America. Three species have been sequenced for the first time and sequences of other species made possible to clarify status of some taxa in Bolivia and other South American countries. *Lepraria cryptovouauxii* is described as new to science.

Material and methods

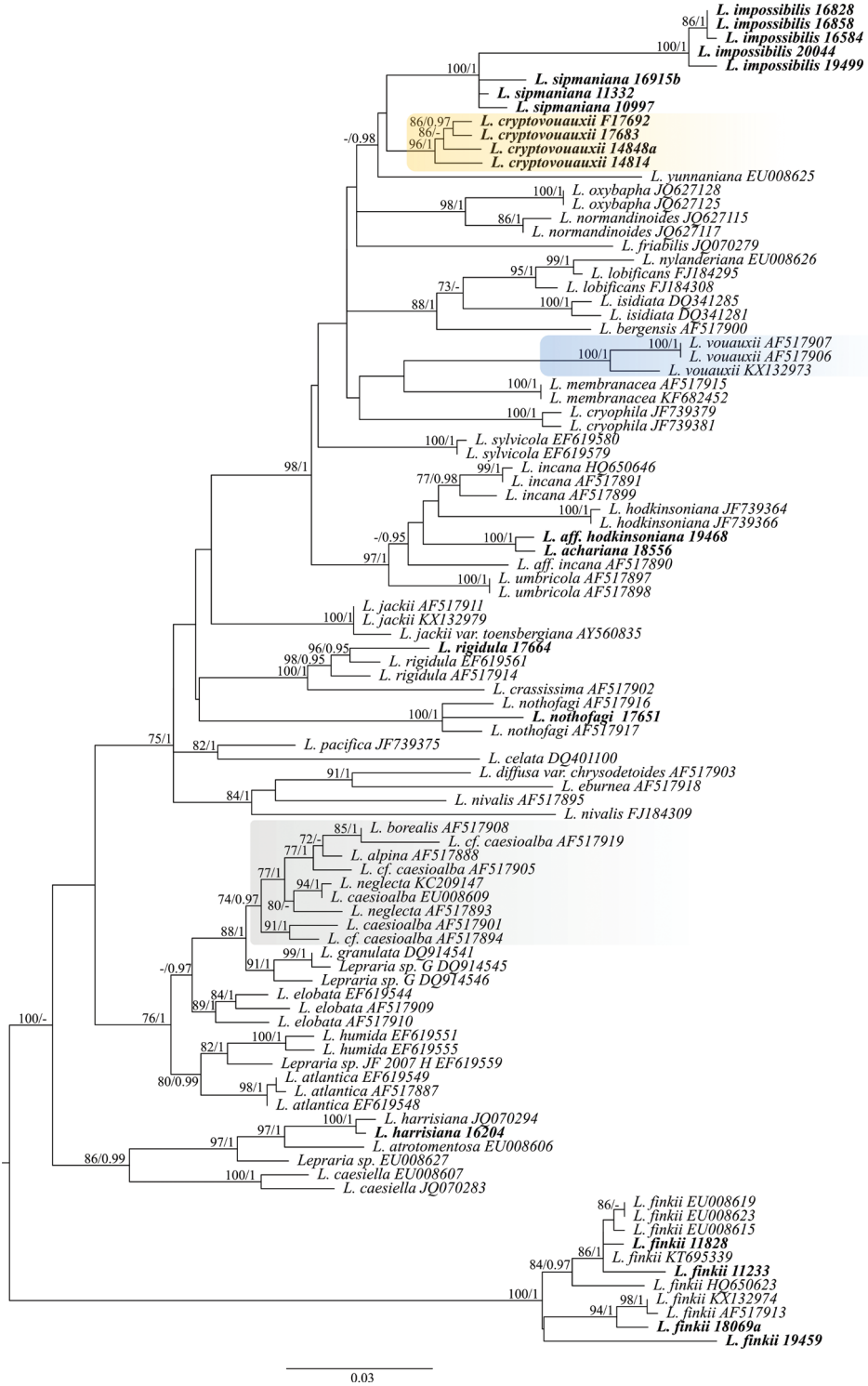
Taxon sampling

The studied specimens from South America and Antarctica are deposited in the following herbaria: C, KRAM, LPB, S, and UGDA. The measurements of thallus structures of the new species were taken in water, often with addition of ethanol. This procedure, used by Olszewska et al. (2014), reduced the hydrophobic properties of lichen substances present in the thallus and made all structures easier to observe. Ethanol was selected as it did not affect the size of the granules, which was empirically tested. The secondary chemistry of all samples was studied by thin layer chromatography (TLC) following methods by Orange et al. (2001). Confirmation of identified substances was achieved in some cases by running the extracts adjacent to an extract containing known substances.

In addition, specimens of *Lepraria nothofagi* Elix & Kukwa reported as *Lepraria* sp. 1 and sequenced by Ekman and Tønsberg (2002) were reinvestigated and their sequences, along with other sequences of *Lepraria* spp., were downloaded from GenBank. Their accession numbers are given in Figure 1. In the preliminary analysis we used all available sequences of *Lepraria* spp. for the alignment which was further reduced to representatives of each species. We excluded from the dataset very short sequences, and numerous identical or very similar sequences. Finally, each species or clade is represented by at least one or two representatives, except *L. neglecta* (Nyl.) Erichsen and *L. finkii* (de Lesd.) R.C.Harris that were better sampled due to their high variation in nucITS rDNA marker. Sequences of *L. lobificans* Nyl. non auct. were originally named as *L. santosii* Argüello & A.Crespo, but the latter name was synonymized with *L. lobificans* by Lendemer (2013a), and therefore the last name was used in Figure 1.

DNA extraction, PCR amplification, and DNA sequencing

Specimens were selected after detailed analyses of morphology and chemistry and only uncontaminated samples were used for molecular studies. Samples of all *Lepraria* species reported from South America by Flakus and Kukwa (2007), Flakus et al. (2011a),


L. impossibilis 16828
L. impossibilis 16858
L. impossibilis 16584
L. impossibilis 20044
L. impossibilis 19499
L. sipmaniana 16915b
L. sipmaniana 11332
L. sipmaniana 10997
L. cryptovouauxii F17692
L. cryptovouauxii 17683
L. cryptovouauxii 14848a
L. cryptovouauxii 14814
L. yunnaniana EU008625
L. oxybapha JQ627128
L. oxybapha JQ627125
L. normandinoides JQ627115
L. normandinoides JQ627117
L. friabilis JQ070279
L. nylanderiana EU008626
L. lobificans FJ184295
L. lobificans FJ184308
L. isidiata DQ341285
L. isidiata DQ341281
L. bergensis AF517900
L. vouauxii AF517907
L. vouauxii AF517906
L. vouauxii KX132973
L. membranacea AF517915
L. membranacea KF682452
L. cryophila JF739379
L. cryophila JF739381
L. sylvicola EF619580
L. sylvicola EF619579
L. incana HQ650646
L. incana AF517891
L. incana AF517899
L. hodkinsoniana JF739364
L. hodkinsoniana JF739366
L. aff. hodkinsoniana 19468
L. achariana 18556
L. aff. incana AF517890
L. umbricola AF517897
L. umbricola AF517898
L. jackii AF517911
L. jackii KX132979
L. jackii var. toensbergiana AY560835
L. rigidula 17664
L. rigidula EF619561
L. rigidula AF517914
L. crassissima AF517902
L. nothofagi AF517916
L. nothofagi 17651
L. nothofagi AF517917
L. pacifica JF739375
L. celata DQ401100
L. diffusa var. chrysodetoides AF517903
L. eburnea AF517918
L. nivalis AF517895
L. nivalis FJ184309
L. borealis AF517908
L. cf. caesioalba AF517919
L. alpina AF517888
L. cf. caesioalba AF517905
L. neglecta KC209147
L. caesioalba EU008609
L. neglecta AF517893
L. caesioalba AF517901
L. cf. caesioalba AF517894
L. granulata DQ914541
Lepraria sp. G DQ914545
Lepraria sp. G DQ914546
L. elobata EF619544
L. elobata AF517909
L. elobata AF517910
L. humida EF619551
L. humida EF619555
Lepraria sp. JF 2007 H EF619559
L. atlantica EF619549
L. atlantica AF517887
L. atlantica EF619548
L. harrisiana JQ070294
L. harrisiana 16204
L. atrotomentosa EU008606
Lepraria sp. EU008627
L. caesiella EU008607
L. caesiella JQ070283
L. finkii EU008619
L. finkii EU008623
L. finkii EU008615
L. finkii 11828
L. finkii KT695339
L. finkii 11233
L. finkii HQ650623
L. finkii KX132974
L. finkii AF517913
L. finkii 18069a
L. finkii 19459
0.03

and Kukwa and Flakus (2009) were subjected to DNA extraction and sequencing, but nucITS rDNA sequences were obtained for only nine species. We assumed that DNA degraded in samples collected more than three years prior to DNA extraction procedure.

DNA was extracted using a modified CTAB method (Guzow-Krzemińska and Węgrzyn 2000) or the E.Z.N.A.SP Fungal DNA Kit (Omega Bio-tek, Inc.) following manufacturer's protocol.

Fungal nucITS rDNA was amplified using the following primers ITS1F (Gardes and Bruns 1993) and ITS4A (Kroken and Taylor 2001) or ITS5 and ITS4 (White et al. 1990). The same primers were used for sequencing.

PCR was carried out in a volume of 25 µl using Color Perpetual *Taq* DNA Polymerase (Eurx) or StartWarm HS-PCR Mix (A&A Biotechnology) following the manufacturer's protocols. In each case 2 or 3 µl of genomic DNA was used for amplification. The following PCR cycling parameters were applied to amplify nuclear ITS region: an initial denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, 54 °C for 30 s (for ITS1F and ITS4 primers) or 52 °C for 30 s (for ITS5 and ITS4 primers), and 72 °C for 1 min, with a final extension at 72 °C for 10 min. In case of ITS1F and ITS4A primers the following parameters were used: an initial denaturation at 94 °C for 2 min, followed by 35 cycles at 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 7 min.

PCR products were visualized on 1% agarose gels stained with ethidium bromide or SimplySafe (Eurx) dyes in order to determine DNA fragment lengths. Subsequently, PCR products were purified using High Pure PCR Product Purification Kit (Roche Diagnostic GmbH) or Wizard SV Gel and PCR Clean-Up System (Promega).

The purified PCR products were sequenced using Big Dye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems) and primers as listed above: 1 µl of Big Dye Terminator, 2 µl of sequencing buffer, 0.5 µl DMSO with 1.5 µl (1 M) of primer, 1–4 µl of amplified products and dd H_2O in a total reaction volume of 10 µl. Cycle sequencing profile in Eppendorf thermal cycler consisted of: 20 s of initial denaturation, followed by 25 cycles, each with denaturation at 94 °C for 15 s, annealing at 52 °C for 20 s and elongation at 60 °C for 4 min. The sequences were read in Genomed (Warsaw). Alternatively, the sequencing was performed in Macrogen (the Netherlands/South Korea). The DNA sequences were assembled and manually adjusted in Auto Assembler v. 1.4.0 (Parker 1997) and SeaView v. 4.1 (Galtier et al. 1996; Gouy et al. 2010). The newly obtained sequences were deposited in GenBank database and their accession numbers are listed in Table 1.

Figure 1. ML tree based on nucITS rDNA dataset for *Lepraria* spp. with midpoint rooting. Newly sequenced specimens of *Lepraria* are in bold and their names are followed with collection number of specimens. In case of the sequences obtained from GenBank the taxa names are followed with accession numbers. Bootstrap supports from ML analysis ≥ 70 (first value) and posterior probabilities from BA ≥ 0.95 (second value) are indicated near the branches. The newly described *L. cryptovouauxii* is highlighted in orange, *L. vouauxii* is highlighted in blue, and *L. neglecta* is highlighted in grey.

Table 1. List of *Lepraria* specimens newly sequenced for this study with their nucITS rDNA GenBank Accession numbers. All samples were collected in Bolivia.

Species	Voucher	GenBank Accession No.
L. achariana	Kukwa 18556 (UGDA)	MK629283
L. cryptovouauxii	Flakus 17683, Rodriguez (KRAM)	MK629272
	Flakus 17692, Rodriguez (KRAM)	MK629270
	Kukwa 14848a, holotype (UGDA)	MK629273
	Flakus 14814, Rodriguez (KRAM)	MK629271
L. finkii	Kukwa 11233 (UGDA)	MK629288
	Flakus 11828, Kukwa (KRAM)	MK629285
	Kukwa 18069a (UGDA)	MK629287
	Kukwa 19459 (UGDA)	MK629286
L. harrisiana	Kukwa 16204 (UGDA)	MK629284
L. aff. *hodkinsoniana*	Kukwa 19468 (UGDA)	MK629282
L. impossibilis	Kukwa 16584 (UGDA)	MK629279
	Kukwa 16828 (UGDA)	MK629281
	Kukwa 16858 (UGDA)	MK629280
	Kukwa 19499 (UGDA)	MK629278
	Flakus 20044, Quisbert (KRAM)	MK629277
L. nothofagi	Flakus 17651, Rodriguez (KRAM)	MK629268
L. rigidula	Flakus 17664, Rodriguez (KRAM)	MK629269
L. sipmaniana	Kukwa 10997 (UGDA)	MK629274
	Kukwa 11332 (UGDA)	MK629275
	Kukwa 16915b (UGDA)	MK629276

Sequence alignment and phylogenetic analysis

The newly generated nucITS rDNA were compared to the sequences available in the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/) using BLASTn search (Altschul et al. 1990). The alignment was generated using Seaview software (Galtier et al. 1996; Gouy et al. 2010) employing muscle option followed with Gblocks selection of poorly aligned sites using less stringent parameters (Castresana 2000). In the final dataset, we analyzed 97 sequences of different *Lepraria* spp. The final alignment consisted of 546 unambiguous sites of which 282 were constant.

Moreover, all available sequences of *Lepraria cryptovouauxii* and *L. vouauxii* were aligned using Seaview software (Galtier et al. 1996; Gouy et al. 2010) employing muscle option and followed with trimming of terminal ends. The final alignment consisted of seven sequences and 515 sites. Then variable sites were selected and presented in Table 2. Finally, we chosen those nucleotide position characters from alignment that support the distinction of *L. cryptovouauxii* (Table 2).

We used Partition Finder 2 (Lanfear et al. 2016) implemented at CIPRES Science Gateway (Miller et al. 2010) to determine the best substitution model for each partition under Akaike Information Criterion (AIC) and greedy search algorithm (Lanfear et al. 2012). Two different models were found for two partitions, i.e. K80+I for 5.8S and GTR+G+X for ITS1 and ITS2 regions. The phylogenetic analyses were performed using Markov Chain Monte Carlo (MCMC) as implemented in MrBayes v. 3.2.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) at CIPRES Science Gateway (Miller et al. 2010). The dataset was analysed employing K80+I

Table 2. Variable positions in the alignment of *Lepraria cryptovouauxii* (marked in bold) and *L. vouauxii*. First sequence, i.e. KX132973 is treated as a reference sequence and dots represent nucleotides identical to reference sequence. Diagnostic nucleotide position characters in the fungal barcoding marker, nucITS, to distinguish *L. cryptovouauxii* from *L. vouauxii* are highlighted in yellow.

Position in the alignment/ Species	**1 0**	**1 4**	**2 7**	**2 8**	**3 8**	**3 9**	**4 0**	**6 3**	**6 4**	**8 1**	**8 7**	**8 8**	**9 2**	**9 4**	**1 0 0**	**1 0 6**	**1 1 8**	**1 1 9**	**1 2 3**	**1 2 4**	**1 3 9**	**1 4 0**	**1 5 7**	**1 5 5**	**1 6 5**
L. vouauxii KX132973	C	G	C	T	C	C	T	T	G	G	C	C	T	C	C	C	C	T	C	C	G	A	C	G	G
L. vouauxii AF517906	.	.	.	C	T	T	.	.	.	.	.	T	C	.	.	.	.	C	.	T	.	.	.	.	.
L. vouauxii AF517907	.	.	.	C	T	T	.	.	.	.	.	T	C	.	.	.	.	C	.	T	.	.	.	.	.
***L. cryptovouauxii* 17692**	**A**	**.**	**T**	**A**	**T**	**.**	**.**	**G**	**A**	**A**	**T**	**T**	**.**	**T**	**T**	**A**	**.**	**A**	**A**	**.**	**.**	**.**	**A**	**T**	**A**
***L. cryptovouauxii* 14814**	**A**	**.**	**T**	**C**	**T**	**.**	**.**	**G**	**A**	**A**	**T**	**T**	**.**	**T**	**T**	**A**	**A**	**A**	**A**	**.**	**A**	**.**	**A**	**T**	**A**
***L. cryptovouauxii* 17683**	**A**	**T**	**T**	**A**	**T**	**.**	**.**	**G**	**A**	**A**	**T**	**T**	**.**	**T**	**T**	**A**	**.**	**A**	**A**	**.**	**.**	**.**	**A**	**T**	**A**
***L. cryptovouauxii* 14848a**	**A**	**.**	**T**	**A**	**T**	**.**	**C**	**G**	**A**	**A**	**T**	**T**	**.**	**T**	**T**	**A**	**.**	**A**	**A**	**.**	**.**	**C**	**A**	**T**	**A**
Position in the alignment/ Species	**1 7 0**	**1 7 1**	**1 8 5**	**1 8 6**	**1 8 7**	**1 8 8**	**1 9 1**	**3 3 2**	**3 3 4**	**3 3 8**	**4 1 1**	**4 3 0**	**4 4 5**	**4 6 0**	**4 6 3**	**4 7 1**	**4 7 2**	**4 7 3**	**4 7 4**	**4 7 9**	**4 8 5**	**4 8 7**	**4 9 3**	**4 9 5**	**5 1 4**
L. vouauxii KX132973	G	T	G	T	C	A	G	C	T	A	G	G	G	A	G	A	C	A	C	C	T	G	C	T	C
L. vouauxii AF517906	.	.	.	.	.	.	.	.	C	.	A	.	.	.	C	.	.	.	.	.	.	.	T	.	.
L. vouauxii AF517907	.	.	.	.	.	.	.	.	C	.	A	.	.	.	C	.	.	.	.	.	.	.	T	.	.
***L. cryptovouauxii* 17692**	**A**	**G**	**.**	**.**	**.**	**.**	**A**	**A**	**.**	**G**	**.**	**T**	**T**	**T**	**T**	**.**	**A**	**C**	**A**	**G**	**A**	**.**	**T**	**A**	**A**
***L. cryptovouauxii* 14814**	**A**	**G**	**T**	**.**	**A**	**C**	**A**	**A**	**.**	**G**	**.**	**T**	**T**	**T**	**T**	**G**	**A**	**.**	**A**	**G**	**A**	**.**	**T**	**A**	**A**
***L. cryptovouauxii* 17683**	**A**	**G**	**.**	**.**	**A**	**.**	**A**	**A**	**.**	**G**	**.**	**T**	**T**	**T**	**T**	**.**	**A**	**C**	**A**	**G**	**A**	**.**	**T**	**A**	**.**
***L. cryptovouauxii* 14848a**	**A**	**G**	**.**	**G**	**A**	**.**	**A**	**A**	**.**	**G**	**.**	**T**	**T**	**T**	**T**	**.**	**A**	**.**	**A**	**G**	**A**	**A**	**T**	**A**	**A**

and GTR+G+X models for 5.8S and ITS partitions with 10 M generations, 2 independent runs, each with four chains. The output of MrBayes was analyzed with the program Tracer v. 1.5 (Rambaut and Drummond 2007) and the initial 25% of trees were discarded as burn-in and the majority-rule consensus tree was calculated to obtain posterior probabilities (PP).

Maximum likelihood (ML) analyses were performed using RaxML HPC v. 8 on XSEDE (Stamatakis 2014) under the GTRGAMMAI model at CIPRES Science Gateway (Miller et al. 2010). Rapid bootstrap analyses were performed with 1000 bootstrap replicates.

The phylogenetic trees were drawn using FigTree v. 1.4.2 (Rambaut 2009). RaxML bootstrap support (BS values ≥ 70) and PP values (values ≥ 0.95) are given near the branches on phylogenetic tree.

The alignments and trees are deposited at TreeBASE database under accession 24193.

Haplotype networks

Sequences of nucITS rDNA marker from specimens of the newly described *Lepraria cryptovouauxii* as well as *L. impossibilis* Sipman and *L. sipmaniana* (Kümmerl. & Leuckert) Kukwa were aligned using Seaview software (Galtier et al. 1996; Gouy et al. 2010) and the terminal ends were trimmed. The alignment consisted of 12 sequences and 530 sites.

The nucITS rDNA sequences of *Lepraria finkii* downloaded from GenBank were aligned together with newly generated sequences of this species using Seaview software

(Galtier et al. 1996; Gouy et al. 2010) and terminal ends were deleted. The final alignment consisted of 21 sequences and 464 sites.

For both datasets TCS networks (Clement et al. 2002) were created with 95% connection limit and gaps treated as missing as implemented in PopART software (http://popart.otago.ac.nz).

Results and discussion

Twenty-one new nucITS rDNA sequences were generated from nine *Lepraria* species for this study (Table 1). Among them *Lepraria achariana* Flakus & Kukwa, *L. impossibilis*, and *L. sipmaniana* as well as the newly described *L. cryptovouauxii* (see taxonomic part) were sequenced for the first time.

Based on nucITS rDNA dataset, topologically congruent trees were generated using maximum likelihood method (ML; best tree likelihood LnL = –5906.670489) and Bayesian approach (BA; harmonic mean was –5936.28). In Bayesian analysis, the average standard deviation of split frequencies was 0.002901 and the average PSRF for parameter values was 1.000. The ML tree was presented in Figure 1 with added bootstrap supports (BS) from ML analysis and posteriori probabilities (PP) from BA.

The newly sequenced specimens collected in Bolivia were resolved in different clades within the phylogenetic tree of *Lepraria* (Fig. 1). Five sequences of *L. impossibilis* form a highly supported clade (100 in ML and 1 in BA), which is closely related to *L. sipmaniana* represented by three newly sequenced specimens (which however do not form a well-supported group), *L. cryptovouauxii* represented by four sequenced specimens forming a well-supported clade (96 in ML and 1 in BA) and one sequence of *L. yunnaniana* (Hue) Zahlbr. All those species, except *L. yunnaniana* which contains divaricatic acid, produce pannaric acid 6-methylester.

To better understand phylogenetic position and genetic variation of nucITS rDNA marker within group of taxa containing pannaric acid 6-methylester, we generated haplotype network for specimens of all three species (Fig. 2). *Lepraria cryptovouauxii* differs in at least 19 positions from *L. sipmaniana* and 39 positions from *L. impossibilis*. This analysis showed that each of the species is well separated from others; however, we observed some infraspecific variation. In our dataset the haplotypes of newly described *L. cryptovouauxii* differ in at least seven to 10 mutational steps from each other and in case of *L. sipmaniana* in five to seven steps. The lowest variation was found in *L. impossibilis* for which two specimens share the same nucITS rDNA haplotype while other haplotypes differ in one to five positions from each other. Our study showed that nucITS rDNA marker is variable in this group of species at the infra and interspecific levels.

Lepraria cryptovouauxii was previously assigned to *L. vouauxii* (Hue) R.C.Harris as it shares secondary chemistry and very similar morphology with the latter (Flakus and Kukwa 2007; Flakus et al. 2011a). Molecular data, however, have shown that four South American specimens with obscurely lobate thalli containing pannaric acid 6-methylester and thus assignable to *L. vouauxii* represent a very different taxon form-

ing a separate clade unrelated to the three sequences of *L. vouauxii* obtained from European specimens (Ekman and Tønsberg 2002; Mark et al. 2016). *Lepraria vouauxii* is resolved in a highly supported clade (100 in ML and 1 in BA (Fig. 1). Moreover, their nucITS rDNA sequences differ in numerous positions of which some may be used as diagnostic characters to distinguish those taxa (Table 2). *Lepraria cryptovouauxii* and *L. vouauxii* can be treated as semicryptic species (Vondrák et al. 2009; Lendemer 2011a) as they differ in the distribution ranges (see the taxonomic part). Based on the new results, we assume that *L. vouauxii* should be at least temporarily excluded from the South American list of lichens (Table 3), but its occurrence there is not improbable (see the taxonomic part).

Lepraria impossibilis was described by Sipman (2004) as having lobate thallus, lobes with raised marginal rim, and producing pannaric acid 6-methylester and lecanoric acid. Flakus and Kukwa (2007) and Kukwa and Flakus (2009) assigned to this species also samples with thalli having diffuse margins. For our molecular analyses we used specimens with diffuse and lobate thalli and they all clustered together in a highly supported clade (100 in ML and 1 in BA) confirming that the morphology of *L. impossibilis* may vary and that the unique secondary chemistry is a diagnostic character.

Lepraria hodkinsoniana Lendemer was described to accommodate the material containing divaricatic acid and zeorin, which was previously referred to as *L. incana* (L.) Ach. in North America. Due to that, the latter species was excluded from the list of North American lichens (Lendemer 2011a). *Lepraria incana* was also reported from South America (Flakus and Kukwa 2007; Flakus et al. 2011a, b, 2015); however, after the description of *L. hodkinsoniana*, we doubted it can represent the former species. Therefore, we sequenced one specimen morphologically and chemically consistent with the description of this species, but the new sequence appeared to be more closely related to the sequences of *L. hodkinsoniana* than to those of *L. incana* obtained from European specimens (Ekman and Tønsberg 2002; Schmull et al. 2011). However, the inclusion of the sequence of *L. achariana* (this species contains lecanoric acid as the main secondary metabolite) to the data set revealed that the latter forms a highly supported clade (100 in ML and 1 in BA) with Bolivian specimen similar to *L. hodkinsoniana* (Fig. 1). Due to that, we decided to name the Bolivian material with divaricatic acid and zeorin as *L.* aff. *hodkinsoniana* and additionally we propose to exclude *L. incana* from the list of South American lichens (Table 3). Whether the specimen of *L.* aff. *hodkinsoniana* is only a chemotype of *L. achariana* or represents another semicryptic species with chemistry similar to *L. incana*, cannot be solved now and more specimens of both, *L. achariana* and *L.* aff. *hodkinsoniana*, need to be sequenced. Sequence named as *L.* aff. *incana* (GenBank Acc. no. AF517890; Fig. 1) was originally assigned to *L. incana* (Ekman and Tønsberg 2002), but after the inclusion of *L. achariana* and *L. hodkinsoniana* to the data set, it is clear that this specimen may represent yet undescribed taxon and the whole group requires further studies.

Sample of *L. rigidula* (B. de Lesd.) Tønsberg collected in Bolivia clustered together with other samples of this species from Norway and Ukraine obtained from GenBank (Ekman and Tønsberg 2002; Fehrer et al. 2008) and was found to be closely related to

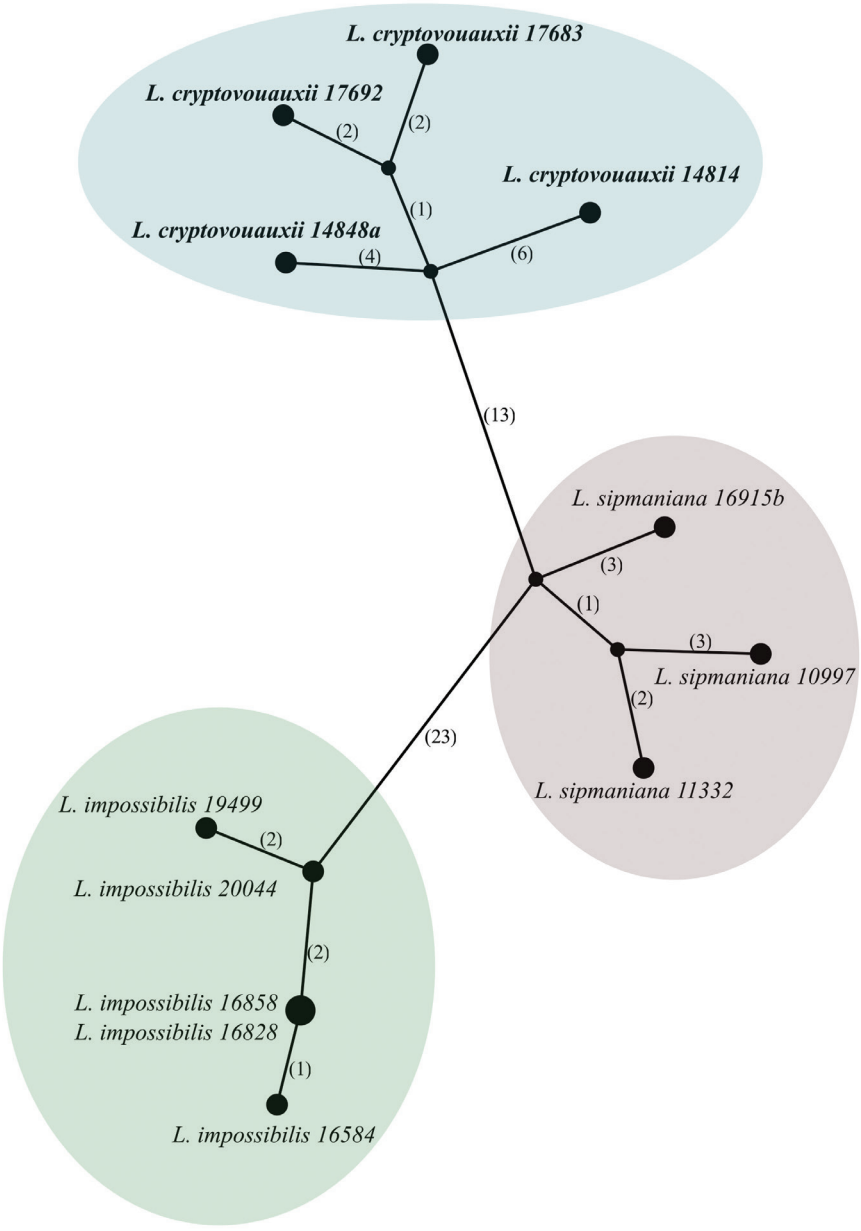


Figure 2. Haplotype network showing relationships between nucITS rDNA sequences from selected *Lepraria* spp. Newly generated nucITS rDNA sequences from *L. cryptovouauxii*, *L. impossibilis* and *L. sipmaniana* were analyzed. The names of species are followed with herbarium numbers of specimens. Mutational changes are presented as numbers in brackets near lines between haplotypes. Haplotypes corresponding to each of species are highlighted with separate elipses. The newly described *L. cryptovouauxii* is given in bold.

Table 3. Records of *Lepraria* from South America revised in this paper. Some samples of taxa marked with asterisk still need to be revised to clarify their identity (for more data see under each species).

Previously	In this paper
*L. alpina**	*L. nothofagi*
L. borealis	*L. neglecta*
L. caesioalba	*L. neglecta*
L. incana	*L.* aff. *hodkinsoniana*
*L. pallida**	*L. harrisiana* and *L. pallida*
*L. vouauxii**	*L. cryptovouauxii*

L. crassissima (Hue) Lettau with high support (100 in ML and 1 in BA) (Fig. 1). This finding confirms the occurrence of *L. rigidula* in South America (Flakus and Kukwa 2007; Flakus et al. 2015).

Lepraria nothofagi has been described from *Nothofagus* bark in Argentina (Flakus et al. 2011a). Here it is reported as new to Antarctica, Bolivia, and Peru, and for the first time, it is reported from rocks and terricolous bryophytes. The samples from Antarctica were previously included in the phylogeny of *Lepraria* by Ekman and Tønsberg (2002) as *Lepraria* sp. 1. The re-examination of those specimens revealed the chemistry characteristic for *L. nothofagi* (atranorin, strepsilin, and porphyrilic acid). The two sequences from those specimens form a highly supported clade (100 in ML and 1 in BA) with newly obtained sequence of *L. nothofagi* from Bolivia. That latter sample (Fig. 4D) was initially determined as *L. alpina* (B. de Lesd.) Tretiach & Baruffo, but the re-examination of the chemistry and morphology of this specimen as well as all other samples reported from South America by Flakus and Kukwa (2007) and Flakus et al. (2011a) revealed that they represent *L. nothofagi* (see the taxonomic part). Flakus and Kukwa (2007) have already pointed that the South American specimens of *L. alpina* studied by them had more powdery appearance than those examined from Europe. Moreover, according to Lendemer (2013b), sequences of samples with aggregate thalli containing porphyrillic acid, and thus referable to *L. alpina*, are nested within the *L. neglecta* group (Fig. 1), and the name is treated as a synonym of *L. neglecta* (Lendemer 2013a, b). Sequences representing members of the *L. neglecta* group are named in their original version in Figure 1; however, all those names are synonymous with *L. neglecta* (Lendemer 2013a, b). Because of that, *L. borealis* Lohtander & Tønsberg reported from Chile by Flakus et al. (2011a) and *L. caesioalba* (B. de Lesd.) J.R.Laundon reported from South America by Flakus and Kukwa (2007) and Flakus et al. (2011a) should be excluded from the lists of lichens occurring in South America and placed as synonyms of *L. neglecta* (Table 3).

Lepraria harrisiana Lendemer is reported in this paper as new to South America. The specimen of *L. harrisiana* used in phylogenetic analyses was at first assigned to *L. pallida* Sipman to which it is chemically similar in producing atranorin, zeorin, and fatty acids (Sipman 2004; Lendemer 2012). However, molecular data placed this specimen in the same highly supported clade (100 in ML and 1 in BA) with *L. harrisiana* from North America. Revision of Bolivian material previously assigned to *L. pallida* by Flakus and Kukwa (2007) and Flakus et al. (2011a, 2015) revealed that most Bolivian

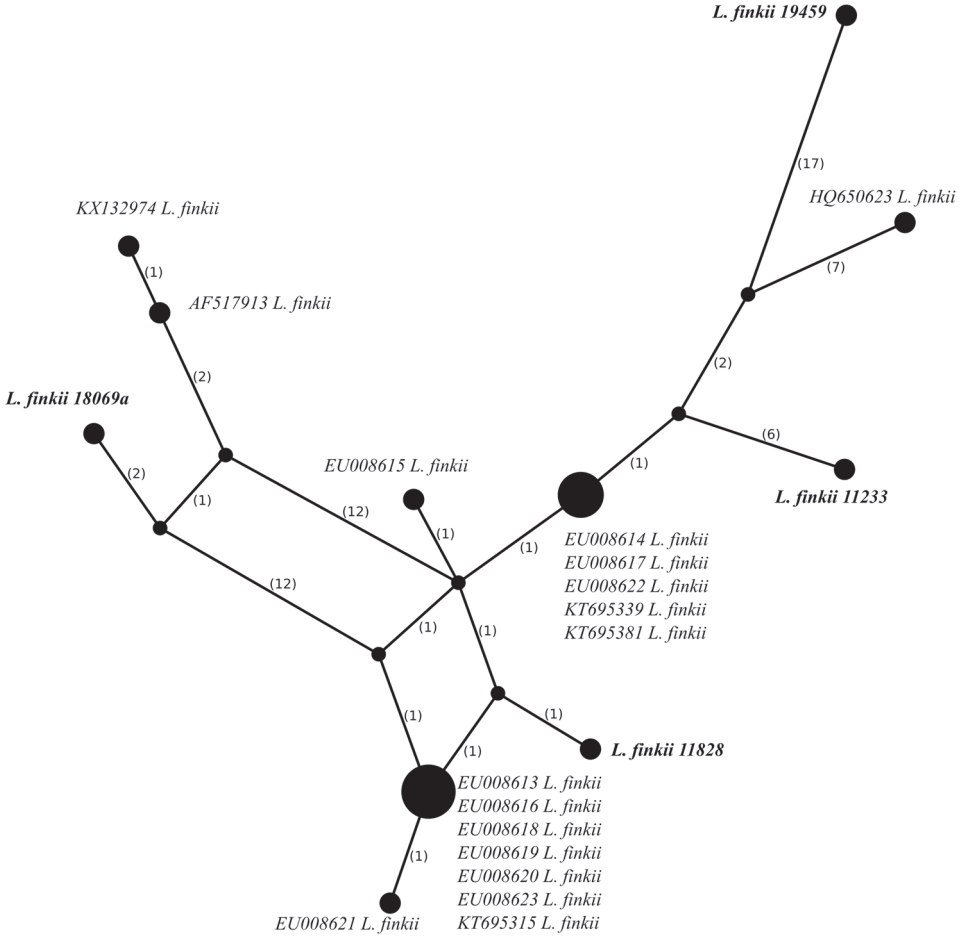


Figure 3. Haplotype network showing relationships between nucITS rDNA sequences from *Lepraria finkii.* Newly generated nucITS rDNA sequences are given in bold. The names of species are followed with herbarium numbers of specimens or accession numbers precede species names in case of sequences obtained from Genbank. Mutational changes are presented as numbers in brackets near lines between haplotypes.

specimens of this species belong to *L. harrisiana* (Table 3), but one sample represents *L. pallida* s. str. (see taxonomic part).

Additionally, four specimens of *L. finkii* from Bolivia were sequenced and were found to belong to a highly supported clade (100 in ML and 1 in BA) together with *L. finkii* specimens from GenBank (Fig. 1). The specimens resolved in this clade were collected in different geographical areas, i.e. South America (Bolivia), North America (Canada and USA), and Europe (Norway and Switzerland). This clade clusters together genetically highly variable specimens. Haplotypes of *L. finkii* from Bolivia are unique and significantly differ from each other and other haplotypes (Fig. 3). The haplotype identified in specimen Kukwa 19459 differs from other known haplotypes in at least 24

mutational steps. The haplotype identified in specimen Kukwa 18069a is most similar to European records (Norway and Switzerland), from which it differs in five or six sites, respectively. Sequences from specimens Kukwa 11233 and Flakus 11828 are most similar to North American haplotypes (Canada and USA), from which they differ in at least seven or two positions, respectively. Those Bolivian specimens may represent cryptic taxa; however, this requires further study, which is beyond the scope of this paper.

Taxonomy

***Lepraria cryptovouauxii* Kukwa, Flakus & Guzow-Krzemińska, sp. nov.**
MycoBank MB830289
Fig. 4A–C

Diagnosis. Species very similar to *Lepraria vouauxii*, but differing in the distinct phylogenetic position within the genus (Fig. 1), in substitution of several nucleotide positions in nucITS (Table 2) and the occurrence in high altitudes of the Andes in South America.

Type. Bolivia. Dept. La Paz; Prov. Franz Tamayo, Área Natural de Manejo Integrado Nacional APOLOBAMBA, road Pelechuco-Keara, 14°41'23"S, 69°08'02"W, elev. 4370 m, open high Andean vegetation, terricolous, 17 Nov. 2014, M. Kukwa 14848a (holotype UGDA, isotype LPB).

Description. Thallus crustose, continuous, leprose, placodioid, up to 0.4 mm thick, distinctly grey-yellow, orange-yellow to brownish orange in colour; crisped margins absent, but some parts obscurely lobate; prothallus disappearing with age; hypothallus as layer of densely intertwined hyphae, hyphae hyaline, c. 3 µm wide; rhizohyphae present, brown pigmented, 3–3.5 µm wide; granules globose or subglobose, 20–70 µm in diameter, discrete, ecorticate, with outer part consisting of incomplete layer of hyphae (c. 3 µm wide) and incrusted with irregular groups of crystals insoluble in K, granules often forming compound units up to 100 µm in diameter (in one sample, Flakus 17682, up to c. 300 µm, Fig. 4C).

Photobiont green, coccoid, cells globose to subglobose, 5–11 µm.

Chemistry. Pannaric acid-6-methylester (+, major), 4-oxypannaric acid-6-methyl ester (+, minor), vouauxii unknown 1 sensu Tønsberg (1992) (±, trace) and rarely traces of anthraquinones and atranorin (only in Flakus 8673).

Habitat and distribution. *Lepraria cryptovouauxii* grows on soil, rocks, or terricolous and saxicolous bryophytes in open and dry to moderately humid habitats at elevations between c. 3350 and 4790 m a.s.l.

Molecular data are available only for four Bolivian samples, but, judging on the basis of the ecological characteristic of other specimens and the altitudes they were collected in South America, we assume that *L. cryptovouauxii* occurs also in Chile, Ecuador, and Peru; the previous terricolous, muscicolous, or saxicolous records from the high Andes in South America belong here (Laundon 1989; Leuckert and Kümmerling 1991; Flakus and Kukwa 2007; Flakus et al. 2011a, 2015).

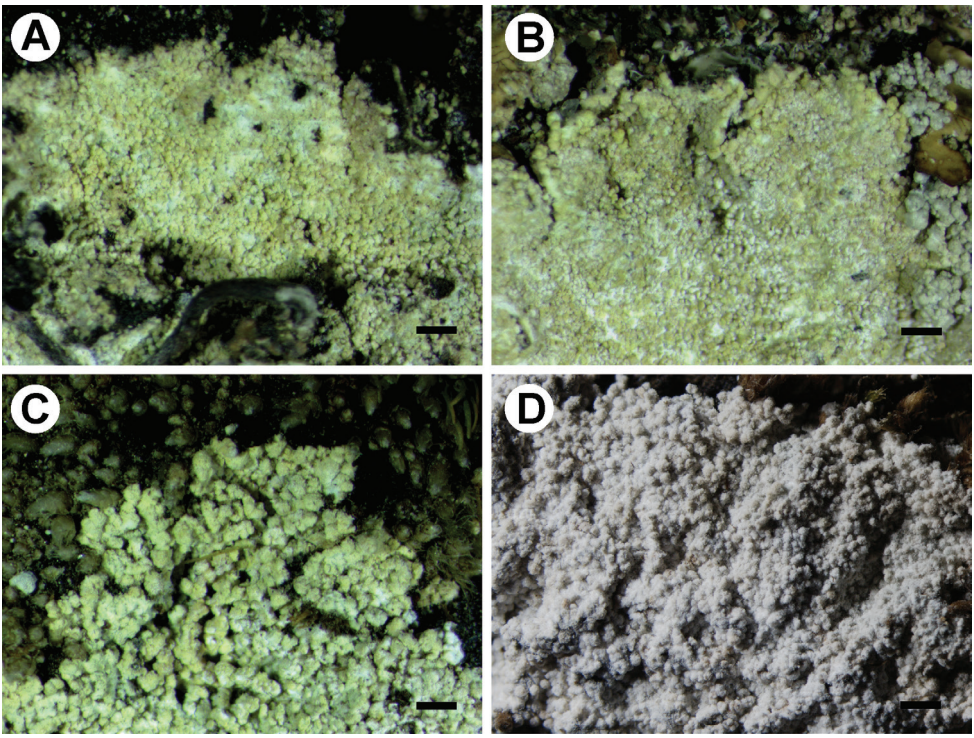


Figure 4. Morphology of *Lepraria cryptovouauxii* (**A–C**) and *L. nothofagi* (**D**). **A** Holotype (M. Kukwa 14848a) **B** Thallus with obscurely lobate margins (Flakus 14814) **C** Thallus with large and compacted aggregations of granules (Flakus 17682) **D** Details of thallus (Flakus 17651 & Rodriguez). Scale bars: 500 μm (**A–C**), 300 μm (**D**).

Few specimens with the same chemistry and similar morphology were collected on wood and tree bark (Flakus 7872, 8381; see Flakus and Kukwa 2007, Flakus 18440 and Kukwa 16829 collected in Dept. Tarija) are excluded from specimen list of *L. cryptovouauxii* due to the different habitat (cloud forests) and lower altitudes (up to c. 2300 m a.s.l.) on which they were collected. They may represent *L. vouauxii* or another undescribed taxon, but their nucITS sequences have not been obtained yet.

Etymology. The name refers to the similarity in morphology and secondary chemistry to *Lepraria vouauxii*.

Additional specimens examined. BOLIVIA. Dept. La Paz: Prov. Bautista Saavedra, Área Natural de Manejo Integrado Nacional Apolobamba, between la Curva and Charazani, 15°08'09"S, 69°02'03"W, 3780 m alt., open area with shrubs, terricolous, 15 Nov. 2014, M. Kukwa 14675 (LPB, UGDA); Prov. Franz Tamayo, Área Natural de Manejo Integrado Nacional Apolobamba, near Puyo Puyo village, 14°56'55"S, 69°07'58"W, 4795 m alt., high Andean open vegetation, terricolous, 5 July 2010, A. Flakus 17683, 17692, P. Rodriguez (KRAM, LPB); Prov. Manco Kapac, Horca del Inca Mt. near Copacabana village, 16°10'15"S, 69°05'05"W, 3974 m alt., 18 June

2006, A. Flakus 8671.1, 8673 (KRAM, LPB); Prov. Murillo, near Cumbre pass, Puna, 16°19'18"S, 68°04'42"W, 4450 m alt., 17 June 2006, A. Flakus 8593.1 (KRAM, LPB, UGDA); Prov. Murillo, near Cumbre pass, Puna, 4672 m alt., 16°20'14"S, 68°02'20"W, 13 May 2006, A. Flakus 5729, 5730, 5731, 5733, 5738, 5740 (KRAM, LPB); ibidem, alt. 4604 m, 16°21'59"S, 68°02'37"W, 13 May 2006, A. Flakus 5791, 5798 (KRAM, LPB); near Cumbre pass, 4550 m alt., 16°19'18"S, 68°04'42"W, high Andean Puna vegetation, on mosses, June 2006, A. Flakus 8584.1, 8586, 8597.1 8600, 8603, 8605, 8606 (KRAM, LPB, UGDA); Prov. Omasuyos, El Dragon hill near Chahualla, 15°51'17"S, 69°00'40"W, 3850 m alt., Puna Húmeda vegetation, saxicolous, 6 July 2010, A. Flakus 17812, P. Rodriguez (KRAM, LPB); Dept. Potosí: Prov. Nor Lípez, Pinturas Rupestres near Villamar Mallcu village, 21°46'20"S, 67°29'05"W, 4038 m alt., open semi-desert high Andean area, terricolous, 6 Dec 2009, A. Flakus 14814, P. Rodriguez (KRAM, LPB). Chile. Terr. Magallanes, Lago del Toro (L. Maravilla), Estancia Río Payne, above the river, on soil, 15 March 1941, R. Santesson 6594 (S). Ecuador. Prov. León: Railway station Cotopaxi, alt. 3550 m, on bare soil in Páramo, 26 Apr 1939, E. Asplund L 63 (S). PERU. Dept. Ancash: Prov. Huaraz, Huaraz, 3500 m alt., on soil, 22 Nov 1972, C. de Graaf (UGDA); Dept. Arequipa: Prov. Caylloma, near Cabanaconde village, semi-desert open mountain area, 3462 m alt., 15°37'56"S, 71°57'49"W, terricolous, 2006, A. Flakus 9531, 9532, 9533, 9644 (KRAM); Valle del Colca, above Tapay village, open mountain area, alt. 3705 m, 15°33'56"S, 71°55'32"W, terricolous, 2006, A. Flakus 9692, 9693, 9766 (KRAM); near Socorro village, 3349 m alt., 15°38'32"S, 71°43'22"W, terricolous, 2006, A. Flakus 9416, 9419 (KRAM); between Soro and Llahuar villages, 15°34'41"S, 72°01'01"W, 2100 m alt., open semi-desert montane area, on soil and bryophytes over rocks, 6 July 2008, A. Flakus 10135, 10139, M. Kukwa 6107, 6108 (KRAM, UGDA); Dept. Cuzco: prov. Urubamba, valley of Rio Piri, NW of Ollantaytambo, 13°06'S, 72°22'W, 3400 m alt., on soil, 23 March 1981 R. Santesson P86: 17 (S); Dept. Lima: Prov. Huarochiri, valley of Rio Santa Eulalia, NE of Carampoma, 11°38'S, 76°27'W. c. 3700 m alt., on bryophytes, 15 Feb 1981, R. Santesson P24: 5, R. Moberg (S); Dept. Junin: Prov. Tarma, c. 10 km (road distance) NNE of Palca, 11°18'S, 75°32'W, c. 2600 m alt., on soil, 7 Feb 1981, R. Santesson P12: 60, R. Moberg (S – specimen of *Lepraria diffusa*).

Selected specimens of *Lepraria vouauxii* examined for comparison. Canada. Canadian Arctic Archipelago: Ellesemere I., Eureka, East Wind Lake, 80°05'N, 85°37'W, on terricolous mosses, 31 July 1999, F. Daniels s.n. (UGDA L-15825). Italy. Umbria: Monte Corona, vicinity of Eremo dell'Assunta Incoronata, 700 m alt., on rock, Jan 2001, A. Zwolicki s.n. (UGDA L-10052); ibidem, on *Quercus* sp., Jan 2001, A. Zwolicki s.n. (UGDA L-10148). Poland. Pojezierze Iławskie: Szymbark, Teutonic castle, 53°38'38"N, 19°28'57"E, on brick, 4 July 2003, J. Boczkaj, M. Kukwa s.n. (UGDA L-10020); Bory Dolnośląskie: Przewóz, on brick, 14 Sept 2000, Š. Bayerová et al. (UGDA L-10720). Ukraine. Opilya: Ivano-Frankivsk region, Halych district, Kosova Hora near Burshtyn, 49°13'25,7"N, 24°42'07,6"E, 300 m alt., steppe vegetation, on gypsum, 27 June 2003, L. Śliwa 1991 (UGDA L-11320).

Notes. *Lepraria cryptovouauxii* and *L. vouauxii* are practically indistinguishable in morphology and secondary chemistry. The only difference we could observe is the colour of thallus, which is more intensively orange-yellow in *L. cryptovouauxii*, while *L. vouauxii* tends to be more greyish green. However, Lendemer (2013a) mentioned similar more distinctly coloured specimens for *L. vouauxii* in material from North America. Whether those samples represent another yet undescribed species has not been resolved. The brighter colour observed in *L. cryptovouauxii* can be caused by a higher concentration of dibenzofurans which may act as a sunscreen in the very sunny habitats of the Andes. Similar tendency was observed also for *L. diffusa* (J.R.Laundon) Kukwa, in which thalli were more intensively coloured in sunny places in comparison to samples from shaded situations (Kukwa 2006b). Dibenzofurans, when in high concentration, can have a colour visible on TLC plates (before spraying with sulphuric acid), which suggest that they may play a sunscreen role as other pigmented substances and determine the colour of thallus (Kukwa 2006b).

According to Lendemer (2013a), *L. vouauxii* lacks brown rhizohyphae, which are present in the new species. To confirm this character as a possible discriminating feature, several specimens of *L. vouauxii* were studied to check the colour of rhizohyphae. We found that, similar to *L. cryptovouauxii*, rhizohyphae can be brown, but in some specimens they were very sparse, in some formed well-visible layer between the thallus and substrate, but some thalli lacked those hyphae. Tønsberg (1992) also mentioned the presence of brown rhizohyphae (as hypothallus). Apparently, *L. vouauxii* shows variation in the development and colour of this structure.

Despite the lack of morphological and chemical differences, *L. cryptovouauxii* can be distinguished on the basis of its distribution as it occurs in the high Andes in South America, whereas *L. vouauxii* remains unconfirmed from South America and genetically known only from Europe (Fig. 1; Ekman and Tønsberg 2002; Mark et al. 2016). The habitat preferences also differ to some extent as *L. cryptovouauxii* grows only on soil, rocks, or saxicolous and terricolous bryophytes, whereas *L. vouauxii* occurs on various substrates, including tree bark, rocks, and soil (Tønsberg 1992; Kukwa 2006b; Flakus and Kukwa 2007; Lendemer 2013a).

Lepraria diffusa is morphologically somewhat similar and also produces dibenzofurans; however, it has aggregate thallus (sensu Lendemer 2011b), and it produces oxypannaric acid-2-methylester (Laundon 1989; Leuckert and Kümmerling 1991; Leuckert et al. 1995; Elix and Tønsberg 2004; Lendemer 2013a).

Lepraria xerophila Tønsberg is the species which also contains pannaric acid-6-methylester as the major secondary metabolite, but it differs in placodioid thalli with crisped margins (Tønsberg 2004; Lendemer 2011b, 2013a). This metabolite is present also in some chemotypes of *L. tenella* (Tuck.) Lendemer & B.P. Hodk. (syn. *Leprocaulon tenellum* (Tuck.) Nyl.), but this species differs in the almost constant production of lecanoric acid (at least always present together with pannaric acid-6-methylester) and atranorin and the development of pseudopodetia (Lamb and Ward 1974; Bungartz et al. 2013; Lendemer and Hodkinson 2013).

Lepraria harrisiana Lendemer

Remarks. Most Bolivian records (except one cited below) of *L. pallida* presented in Flakus and Kukwa (2007) and Flakus et al. (2011a, 2015) were revised and belong to *L. harrisiana*. Here only the new record is presented.

Specimens of examined. Bolivia. Dept. Chuquisaca: Prov. Zudañez, Área Natural de Manejo Integrado El Palmar, La Cascada bajo de El Palmar, 18°41'23"S, 64°54'26"W, 2740 m atl., Boliviano-Tucumano forest with *Podocarpus*, Lauraceae and palms, corticolous, 15 July 2015, M. Kukwa 16204 (LPB, UGDA).

Lepraria aff. *hodkinsoniana* Lendemer

Remarks. All known Bolivian records of *L. incana* presented in Flakus and Kukwa (2007), and Flakus et al. (2011a, b, 2015) should be assigned to *L.* aff. *hodkinsoniana*. Here two new records are presented.

Specimens of examined. Bolivia. Dept. Cochabamba: Prov. Carrasco, Parque Nacional Carrasco, Meruvia, 17°34'59"S, 65°15'06"W, 3215 m alt., upper montane Yungas forest, corticolous, 4 Nov. 2016, M. Kukwa 18041 (LPB, UGDA); Dept. Santa Cruz: Prov. Comarapa, Parque Nacional y Área Natural de Manejo Integrado Amboró, Remate, 17°51'39"S, 64°21'15"W, 2270 m alt., natural Yungas forest, on dead tree fern, 15 May 2017, M. Kukwa 19468 (LPB, UGDA).

Lepraria nothofagi Elix & Kukwa

Remarks. Records of *L. alpina* from Bolivia and Peru (Flakus and Kukwa 2007; Flakus et al. 2011a) and *Lepraria* sp. 1 from Antarctica (Ekman and Tønsberg 2002) belong to *L. nothofagi*. Here only new record is presented.

Some other records of *L. alpina* (Flakus et al. 2011a) should be revised to assess if they represent *L. neglecta* or *L. nothofagi*.

Specimens examined. Bolivia. Dept. La Paz; Prov. Franz Tamayo, Área Natural de Manejo Integrado Nacional APOLOBAMBA, near Puyo Puyo village, 14°56'55"S, 69°07'58"W, 4795 m alt., high Andean open vegetation, on bryophytes, 5 July 2010, A. Flakus 17651 & P. Rodriguez (KRAM, LPB).

Lepraria pallida Sipman

Remarks. This is the only so far known Bolivian record of this species.

Some other records from Brazil and Peru (Flakus and Kukwa 2007; Flakus et al. 2011a) still need to be revised.

Specimens of examined. Bolivia. Dept. La Paz; Prov. Nor Yungas, near Pacallo village, 16°12'10"S, 67°50'39"W, 1360 m alt., Yungas montane forest, on rocks and saxicolous bryophytes, 3 Aug. 2008, M. Kukwa 7172 (LPB, UGDA).

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RESEARCH ARTICLE

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A regional study of the genus *Phyllopsora* (Ramalinaceae) in Asia and Melanesia

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Abstract

Phyllopsora is a crustose to squamulose lichen genus inhabiting the bark of trees in moist tropical forests and rainforests. Species identification is generally challenging and is mainly based on ascospore morphology, thallus morphology and anatomy, vegetative dispersal units, and on secondary chemistry. While regional treatments of the genus have been conducted for Africa, South America and Australia, there exists no study focusing on the Asian and Melanesian species. Previously, 24 species of *Phyllopsora* s. str. have been reported from major national studies and checklists representing 13 countries. We have studied herbarium material of 625 *Phyllopsora* specimens from 18 countries using morphology, anatomy, secondary chemistry, and molecular data to investigate the diversity of *Phyllopsora* species in Asia and Melanesia. We report the occurrence of 28 species of *Phyllopsora* including the following three species described as new to science: *P. sabahana* from Malaysia, *P. siamensis* from Thailand and *P. pseudocorallina* from Asia and Africa. Eight species are reported as new to Asia. A key to the Asian and Melanesian species of *Phyllopsora* is provided.

Keywords

Malaysia, Sri Lanka, Thailand, rainforest, TLC, phylogeny, identification key

Introduction

The genus *Phyllopsora* Müll. Arg. consists of 54 crustose or squamulose species (Kistenich et al. in press). They grow mostly on bark of trees in (sub-)tropical rainforests or moist woodlands. The genus was described in 1894 from New Zealand (Müller 1894), but the first modern revision of the pantropical genus was conducted 87 years later by Swinscow and Krog (1981) focusing on the East African species. Ten years later, Brako (1991) monographed the Neotropical species, while Elix (2009) summarized the Australian species and their occurrence. Additional reports and regional studies of the genus and its species, distribution can be found from Eastern Africa (Timdal and Krog 2001), Peru (Timdal 2008b) and the West Indies (Timdal 2011). From Asia, however, only a few reports exist for selected countries. Upreti et al. (2002) listed five *Phyllopsora* species from India. Later, Mishra et al. (2011) described two species and one variety from India as new to science. Recently, Kondratyuk et al. (2016) described a new species from South Korea. Phyllopsoroid specimens have been reported in additional checklists and geographical studies from, for example, Bangladesh (Aptroot and Iqbal 2011), Northeast India (Logesh et al. 2017), Sri Lanka (Weerakoon and Aptroot 2014), South Korea (Joshi et al. 2011) and Thailand (Aptroot et al. 2007). A general Asian, transnational study focusing on *Phyllopsora* has to date not been published. So far, 24 of the 54 accepted *Phyllopsora* species have been reported to occur in Asia and Melanesia (Table 1). An additional nine species reported from Asia represent either synonyms or have recently been excluded from the genus (Kistenich et al. in press; 2018b; Table 1).

Species of *Phyllopsora* are generally challenging to identify by morphology only. In a molecular phylogeny of the lichen family Ramalinaceae C. Agardh, Kistenich et al. (2018b) showed the genus *Phyllopsora* to be polyphyletic. Consequently, they excluded ten species from the genus. Three of the excluded species most likely belong in the family Malmideaceae Kalb, Rivas Plata & Lumbsch. An additional three of the excluded species were transferred to the new genus *Parallopsora* Kistenich, Timdal & Bendiksby. The species of *Parallopsora* grouped together in a poorly resolved clade with a number of tropical genera, such as *Eschatogonia* Trevis., *Krogia* Timdal and *Physcidia* Tuck. (Kistenich et al. 2018b). Little is known about these genera in Asia, which are generally very similar to *Phyllopsora* in their macromorphology. They often differ, however, from *Phyllopsora* in ascospore size and arrangement, presence of prothallus, thallus construction and chemistry (Kalb and Elix 1995; Kistenich et al. 2018b; Timdal 2008a; 2009). Recently, Kistenich et al. (2018a) described three new species of *Krogia* from Asia and Oceania, which were all tentatively identified as *Phyllopsora* sp., indicating the morphological similarity between these two genera.

The scope of the present study is to revise Asian and Melanesian *Phyllopsora* specimens mainly collected between 1990 and 2017 by the authors. Herein, we provide an overview of the species of *Phyllopsora* occurring in the Asian countries with an updated taxonomy based on multiple sources of evidence, including DNA sequence data. We describe three new species and provide a key to the Asian and Melanesian species of *Phyllopsora*.

Materials and methods

The specimens

We investigated material from 18 different countries in Asia and Melanesia (Table 1) based on herbarium collections made mainly between 1990 and 2017. Older material of *Phyllopsora* is generally not suitable for DNA sequencing (Kistenich et al. in press). In addition to material from our own herbaria directly available to us (BM, BORH, O, PDA), we received loans from the institutional herbaria B, E, H, TNS, and UPS, as well as from the private herbarium of P. Diederich. In total, we investigated 908 specimens of *Phyllopsora* and related genera. Author names for the studied species are provided in Tables 1 and 2.

The definition of Melanesia follows the United Nations geoscheme for Oceania as devised by the United Nations Statistics Division based on the M49 coding classification (https://unstats.un.org/unsd/methodology/m49/). Accordingly, it includes the five countries Fiji, New Caledonia, Papua New Guinea, Solomon Islands, and Vanuatu.

Morphology and secondary chemistry

All specimens were studied morphologically and when necessary, also anatomically. Microscope sections were prepared using a freezing microtome and mounted in water, 10% KOH (K), lactophenol cotton blue, and a modified Lugol's solution in which water was replaced by 50% lactic acid. The types of upper cortex referred to in this paper (types 1 and 2) are those described by Swinscow and Krog (1981). Amyloid reactions in the apothecium were observed in the modified Lugol's solution after pretreatment in K, and crystals of lichen substances were observed using polarized light. Ascospore measurements are given as $X \pm 1.5 \times SD$ rounded to 0.5 µm, where X is the arithmetic mean and SD the standard deviation.

We performed thin-layer chromatography (TLC) as routine investigation for identification of lichen substances in accordance with the methods of Culberson (1972), modified by Menlove (1974) and Culberson and Johnson (1982). Generally, we examined the acetone-extracts in solvent system B'; fatty acids were not examined. In difficult cases, we additionally used solvent systems A and C for lichen substance identification.

Molecular methods and phylogenetic analysis

For DNA extraction, PCR amplification and DNA sequencing of the mitochondrial ribosomal small subunit (mtSSU) and the nuclear ribosomal internal transcribed spacer region (ITS: ITS1, 5.8S, ITS2), we followed the protocols outlined in Kistenich et al. (2018a). For sequence assembly and preliminary alignment, we used Geneious R9 (Kearse et al. 2012).

Table 1. Species of *Phyllopsora* reported from Asia and Melanesia.

Species	Authorship	Cambodia	China	Fiji	India	Indonesia	Japan	Malaysia	Nepal	New Caledonia	Papua New Guinea	Philippines	Solomon	South Korea	Sri Lanka	Taiwan	Thailand	Vanuatu	Vietnam
Accepted species																			
P. africana	Timdal & Krog					**	**	**			*	**	**		**		**	*	
P. breviuscula	(Nyl.) Müll. Arg.				* [10]					**		**			** [17]				*
P. buettneri	(Müll. Arg.) Zahlbr.		*		[16]		**				* [1, 5, 15]	[5]			*	[3]	** [6]		[4]
P. castaneocincta	(Hue) Kistenich & Timdal	*			* [16]		* T	**	**	*	*	* T	*		** [17]	*	**		
P. chodatinica	Elix							**		**	* [5]							*	
P. cinchonarum	(Fée) Timdal						** [12]									* T	** [6]		
P. confusa	Swinscow & Krog				[10]	*	*	**			** [1, 15]				** [17]	*	**		
P. cuyabensis	(Malme) Zahlbr.																**		
P. dolichospora	Timdal & Krog						*				**				** [17]				
P. foliata	(Stirt.) Zahlbr.						**								** [17]				
P. furfuracea	(Pers.) Zahlbr.				[10]	[5]				[8]	* [2, 7]	T			* [17]	[3]	** [6, 19]		[4]
P. gossypina	(Sw.) Kistenich et al.						[12]	[13]		[8]	[14, 15]				** [17]		[6]		[4]
P. halei	(Tuck.) Zahlbr.										[1, 2]				**	[3]			
P. himalayensis	G.K. Mishra et al.				T														
P. isidiosa	Kistenich & Timdal					**		**	**			**					**		
P. kalbii	Brako				[10]												**		
P. loekoesii	S.Y. Kondr. et al.						**		**					* T					
P. longiuscula	(Nyl.) Zahlbr.														**		**		*
P. mediocris	Swinscow & Krog														*				
P. neofoliata	Elix														*				
P. parvifolia	(Pers.) Müll. Arg.		[18]		[16]					[7, 8]	[14]				*				
P. parvifoliella	(Nyl.) Müll. Arg.					**					*	*					**		
P. porphyromelaena	(Vain.) Zahlbr.			*	* [10]	*	**	**		*	*	* T		*	**	* T	**		
P. pseudocorallina	Kistenich & Timdal	**						**			**								
P. sabahana	Kistenich & Timdal							**											
P. santensis	(Tuck.) Swinscow & Krog						[12]				[2]	[5]					*		
P. siamensis	Kistenich & Timdal																**		
P. subhispidula	(Nyl.) Kalb & Elix														**				
Reported, not confirmed species																			
P. chlorophaea	(Müll. Arg.) Zahlbr.				[10]											[3]			
P. corallina	(Eschw.) Müll. Arg.				[9, 16]									[11]	[17]	[3]			[4]
P. isidiotyla	(Vain.) Riddle				[10]														

Species	Authorship	Cambodia	China	Fiji	India	Indonesia	Japan	Malaysia	Nepal	New Caledonia	Papua New Guinea	Philippines	Solomon	South Korea	Sri Lanka	Taiwan	Thailand	Vanuatu	Vietnam
P. mauritiana	(Taylor) Swinscow & Krog				[10]														
P. nemoralis	Timdal & Krog				[10]														
P. pyxinoides	(Nyl.) Kistenich et al.																[6, 19]		
P. swinscowii	Timdal & Krog				[10]														
Excluded species																			
P. catervisorediata	G.K. Mishra et al.				T														
P. densiflorae	(Vain.) Gotth. Schneid.						T												
P. griseocastanea	(Vain.) Gotth. Schneid.											T							
P. manipurensis	(Müll. Arg.) Müll. Arg.				T														
P. subcrustacea	(Malme) Brako				[10]														
P. viridis	Paulson																T		
P. borbonica	Timdal & Krog														[17]				
P. sorediata	(Aptroot & Sparrius) Timdal																[6]		
P. soralifera	Timdal				[9]														

T: Type material (of the accepted name or a synonym); *: Identified in this study, based on morphology/chemistry; **: Identified in this study, based on DNA; [1]: Aptroot 1997; [2]: Aptroot et al. 1997; [3]: Aptroot and Sparrius 2003; [4]: Aptroot and Sparrius 2006; [5]: Brako 1991; [6]: Buaruang et al. 2017; [7]: Elix 2009; [8]: Elix and McCarthy 1998; [9]: Logesh et al. 2017; [10]: Mishra et al. 2011; [11]: Moon 2013; [12]: Ohmura and Kasiwadani 2018; [13]: Sipman 1993; [14]: Streimann 1986; [15]: Streimann and Sipman 1994; [16]: Upreti et al. 2002; [17]: Weerakoon and Aptroot 2014; [18]: Wei 1991; [19]: Wolseley et al. 2002.

As many of the specimens, from which we generated sequences, had not been previously identified, we needed to find out, which specimens belonged in *Phyllopsora* s. str. and consequently, which sequences to use in the final phylogenetic analyses. Hence, we phylogenetically analysed a combined alignment of our Ramalinaceae dataset (Kistenich et al. 2018b) and the newly generated sequences using standard RAxML (i.e., applying the GTR substitution model for each pre-defined partition [mtSSU, ITS1, 5.8S and ITS2] with 100 rapid bootstrap inferences and the GAMMA model for evaluating and optimizing the likelihood of the final tree; Stamatakis 2014). Based on these RAxML trees, we selected those specimens falling into *Phyllopsora* s. str. and incorporated them into our *Phyllopsora* dataset (Kistenich et al. in press). This dataset was analysed phylogenetically in more detail (see below) to provide evidence for undescribed species.

Each marker was aligned separately using MAFFT v.7.408 (Katoh and Standley 2013) with the E-INS-i algorithm and the nucleotide scoring matrix set to 1PAM / κ=2. We trimmed the ends of the ITS alignment to comprise only the ITS-region and deleted the residual 18S and 28S sequence information. Each dataset was initially analysed by IQ-TREE v.1.6.7 (Nguyen et al. 2015) to infer a maximum likelihood tree using 1000 ultrafast bootstrap repetitions (Hoang et al. 2018). We checked for gene-tree incongruence using compat.py (Kauff and Lutzoni 2002) with a cut-off of 90.

As we did not find any strongly supported incongruences, which would affect the circumscription of the new species, we concatenated the mtSSU and ITS alignments. We ran a detailed IQ-TREE analysis to find the best-fitting nucleotide substitution models and partitioning schemes (Chernomor et al. 2016; Kalyaanamoorthy et al. 2017) among models implemented in MrBayes (i.e., 1-, 2-, and 6-rate models) and to infer a maximum likelihood tree using 1000 standard non-parametric bootstrap repetitions (BS). We defined four subsets, one for mtSSU and three for ITS corresponding to the ITS1, 5.8S and ITS2 regions, and analysed those with the TESTMERGE function resembling PartitionFinder2. In addition, we analysed the dataset with MrBayes v.3.2.6 (Altekar et al. 2004; Ronquist and Huelsenbeck 2003) as described in Kistenich et al. (2018b). The temperature increment parameter was set to 0.05. We projected the BS values from the IQ-TREE analysis onto the MrBayes consensus tree with posterior probabilities (PP) and collapsed branches with BS < 50 and PP < 0.7. The resulting trees were edited in TreeGraph2 (Stöver and Müller 2010) and FigTree v.1.4.4 (http://tree.bio.ed.ac.uk/software/figtree).

Results

Morphology and secondary chemistry

Morphological identification of many specimens was challenging, but with data obtained by TLC, many specimens could be identified to species level. Of the 908 studied specimens, we found 625 specimens to belong in *Phyllopsora*, while 283 specimens were found to belong in other genera of the Malmideaceae and Ramalinaceae (not treated in this study). Of the 625 *Phyllopsora* specimens, 480 were identified to species level in *Phyllopsora* (Table 2, Suppl. material 2: Table S1), while 141 specimens (23%) were left unidentified (not included in Suppl. material 2: Table S1), most of which were not sequenced and did not contain lichen substances. The morphology and anatomy of the *Phyllopsora* species have been described in detail by Swinscow and Krog (1981) and Brako (1991), and are not repeated here. We often found the distinction between cortex type 1 and type 2 useful for species identification; however, in many species the cortex type is intermediate (type 1–2). The chemistry of the 54 accepted *Phyllopsora* species is summarized in Kistenich et al. (in press).

Information about all *Phyllopsora* species may also be found on our *Phyllopsora* website: http://nhm2.uio.no/lichens/Phyllopsora.

Molecular data and phylogenetic analysis

We obtained sequences for 140 phyllopsoroid specimens with 132 mtSSU and 106 ITS sequences (Tables 2, 3). Based on the initial RAxML analyses (not shown), 93 specimens were found to belong in *Phyllopsora* s. str. (Table 2) and were used in the subse-

Table 2. Specimens used in this study with voucher information and GenBank accession numbers. New sequences are indicated by accession numbers in bold. – indicates missing data.

Species	Extract #	mtSSU	ITS	Country	Year	Voucher	Herbarium
Biatora beckhausii (Körb.) Tuck.	–	MG925858	AF282071	Norway	1995	Holien, H. 6744	TRH
B. vacciniicola (Tønsberg) Printzen	–	MG925861	MG925960	Norway	2013	Klepsland, J. JK13-L330	O
Crocynia molliuscula (Nyl.) Nyl.	7359	MK352275	–	La Réunion	1996	Krog, H. & Timdal, E. RE18/03	O
	7360	MK352276	–	Mauritius	1991	Krog, H. & Timdal, E. MAU58/02	O
Phyllopsora africana Timdal & Krog ch1	470	**MK412413**	**MK412480**	Thailand	1993	Aguirre, James & Wolseley 2475a	BM
	471	**MK412414**	**MK412481**	Thailand	1992	Aguirre–Hudson, B. & Wolseley, P.A. 1327	BM
	509	MK352138	MK352317	La Réunion	1996	Krog, H. & Timdal, E. RE08/13	O
	1436	MK352175	MK352348	La Réunion	1996	Krog, H. & Timdal, E. RE22/09	O
	4037	MK352199	MK352370	Thailand	2012	v.d. Boom, P. 46982	hb. v.d. Boom
	7224	**MK412469**	**MK412512**	Sri Lanka	2017	Kistenich S. & Weerakoon, G. SK1-517	PDA
P. africana ch1?	1012	**MK412425**	–	Indonesia	2000	Wolseley, P. T15	BM
P. africana ch2	477	MK352122	MK352301	Japan	1995	Thor, G. 13199	UPS
	6770	**MK412461**	**MK412504**	Sri Lanka	2017	Weerakoon, G. Ri056	PDA
P. africana ch3	472	**MK412415**	–	Solomon Islands	1965	Hill, D.J. 9242	BM
	1416	**MK412435**	–	Malaysia	2012	Wolseley, P., Thüs, H. & Vairappan, C. D.8.04.oQ	BORH
	1427	**MK412443**	–	Indonesia	2000	Wolseley, P. T22 OQ	BM
	6348	MK352231	MK352401	Philippines	1994	Diederich, P. 13345	hb. Diederich
	6351	**MK412447**	–	Philippines	1994	Diederich, P. 13213	hb. Diederich
	6352	**MK412448**	–	Philippines	1994	Diederich, P. 13119	hb. Diederich
	6772	**MK412462**	**MK412505**	Sri Lanka	2017	Weerakoon, G. Im015	PDA
	7205	**MK412463**	**MK412506**	Sri Lanka	2017	Kistenich S. & Weerakoon, G. SK1-543	PDA
P. amazonica Kistenich & Timdal	3619	MK352194	MK352365	Brazil	2014	Barbosa, R.S., Haugan, R. & Timdal, E. 90	O
	4155	MK352208	MK352379	Brazil	2015	Kistenich, S. & Timdal, E. SK1-85	MPEG
P. breviuscula (Nyl.) Müll. Arg.	528	MG925892	MG925990	La Réunion	1996	Krog, H. & Timdal, E. RE36/18	O
	1305	MG925893	MG925991	Brazil	1980	Kalb, K. & Marcelli, M. in: Kalb, Lich. Neotropici 515	GZU
	1432	**MK412445**	–	Sri Lanka	2007	Jayalal, U. A4-5-8-5	PDA
	2100	–	MK352355	Philippines	1992	Tan, B.C. 92-187	B
	6752	MK352245	MK352412	New Caledonia	2016	Rikkinen, J. 35509	H
	6754	**MK412456**	**MK412499**	New Caledonia	2016	Rikkinen, J. 35503	H
	6760	**MK412457**	**MK412500**	Sri Lanka	2017	Weerakoon, G. Im042	PDA

Species	Extract #	mtSSU	ITS	Country	Year	Voucher	Herbarium
P. breviuscula	6764	**MK412458**	**MK412501**	Sri Lanka	2017	Weerakoon, G. Mn093	PDA
	6765	**MK412459**	**MK412502**	Sri Lanka	2017	Weerakoon, G. Mo81	PDA
	7212	**MK352256**	**MK352422**	Sri Lanka	2017	Kistenich, S. & Weerakoon, G. SK1-642	PDA
	7213	**MK412465**	**MK412508**	Sri Lanka	2017	Kistenich S. & Weerakoon, G. SK1-601	PDA
	7217	**MK412466**	**MK412509**	Sri Lanka	2017	Weerakoon, G. 982	PDA
	7218	**MK412467**	**MK412510**	Sri Lanka	2017	Weerakoon, G. 1013	PDA
	7229	**MK412470**	**MK412513**	Sri Lanka	2017	Kistenich S. & Weerakoon, G. SK1-649	PDA
	7234	–	**MK412516**	Sri Lanka	2017	Kistenich S. & Weerakoon, G. SK1-648	PDA
	7235	**MK412472**	**MK412517**	Sri Lanka	2017	Kistenich S. & Weerakoon, G. SK1-640	PDA
P. buettneri (Müll. Arg.) Zahlbr. ch1	428	MK352103	MK352283	Thailand	1994	Wolseley, P. & Kanajriavanit, S. s.n.	BM:734816
	995	**MK352146**	**MK352322**	Thailand	1993	James, P.W. & Wolseley, P.A. 2466a	BM
	1041	MK352160	MK352335	Kenya	2007	Divakar, Lumbsch & Mangold 19553D	hb. Pérez-Ortega
P. buettneri ch2	6464	MK352239	MK352406	Brazil	2015	Dahl, M.S., Kistenich, S., Timdal, E. & Toreskaas, A.K. AM-37	O
	7177	MK352252	–	Venezuela	1984	Brako, L. 8110	GZU
P. buettneri ch3	429	MK352104	MK352284	Thailand	1993	Aguirre, B., James, P.W. & Wolseley, P. 2736	BM
	493	**MK352131**	**MK352311**	Thailand	1994	Wolseley, P. & Kanajriavanit, S. s.n.	BM:1104011
	6462	MK352238	–	Japan	1995	Thor, G. 13183	UPS
P. byssiseda (Nyl.) Zahlbr.	4737	MK352211	MK352382	Venezuela	2015	Dahl, M.S., Kistenich, S., Timdal, E. & Toreskaas, A.K. SK1-220	VEN
	4739	MK352212	MK352383	Venezuela	2015	Dahl, M.S., Kistenich, S., Timdal, E. & Toreskaas, A.K. SK1-229	VEN
P. canoumbrina (Vain.) Brako	3627	MK352195	MK352366	Brazil	2014	Barbosa, R.S., Haugan, R. & Timdal, E. 166	O
P. castaneocincta (Hue) Kistenich & Timdal	460	MK352116	MK352295	Tanzania	2008	Timdal, E. 10912	O
	461	**MK412412**	**MK412479**	Thailand	1993	Aguirre, James & Wolseley 2482B	BM
	998	**MK412420**	–	Thailand	1991	Wolseley, P.A. & Aguirre–Hudson, B. 5564	BM
	999	**MK412421**	**MK412486**	Thailand	1993	Wolseley, P.A. & David, F. 3314	BM
	1022	**MK412427**	**MK412490**	Thailand	1992	Wolseley, P.A. & Aguirre–Hudson, B. 5583	BM

Species	Extract #	mtSSU	ITS	Country	Year	Voucher	Herbarium
P. castaneocincta	1032	**MK412429**	–	Nepal	2007	Sharma, L.R., Olley, L., Cross L7.1	E
	1045	**MK412431**	–	Thailand	1993	James, P.W. & Wolseley, P.A. 2466b	BM
	1264	**MK412433**	**MK412492**	Malaysia	2012	Wolseley, P., Thüs, H. & Vairappan, C. M.3.10.1	BORH
	1420	**MK412439**	–	Malaysia	2012	Wolseley, P., Thüs, H. & Vairappan, C. M.3.10.2a	BORH
	1421	**MK412440**	**MK412493**	Malaysia	2012	Thüs, H., Wolseley, P. & Vairappan, C. M110	BORH
	3560	MK352186	MK352358	South Africa	2014	Burrows, J. & Timdal, E. 14280	O
	4032	MK352196	MK352367	Thailand	2012	v.d. Boom, P. 47239	hb. v.d. Boom
	6743	MK352243	MK352410	Kenya	2013	Kirika, P., Mugambi, G. & Lumbsch, H.T. 3011	O
	7232	–	**MK412515**	Sri Lanka	2017	Kistenich S. & Weerakoon, G. SK1-594	PDA
	7255	MK352270	MK352434	Australia	1992	Elix, J.A. 32834	CANB
P. chlorophaea (Müll. Arg.) Zahlbr.	529	MK352145	MK352321	La Réunion	1996	Krog, H. & Timdal, E. RE36/17	O
	1051	MK352165	MK352340	Kenya	2002	Killmann, D. & Fischer, E. s.n.	hb. Killmann
	1309	MK352172	–	Venezuela	1986	Brako, L. & Berry, P.E. 8685	GZU
	SE382	MG925894	MG925992	La Réunion	1996	Krog, H. & Timdal, E. RE08/10	O
P. chodatinica Elix	513	MK352139	–	Australia	1986	Elix, J.A. & Streimann, H. 21023	O
	1539	MK352177	MK352350	New Caledonia	2005	Elvebakk, A. 05:691	O
	6456	MK352237	MK352405	Malaysia	2014	Paukov, A. 2232	B
P. cinchonarum (Fée) Timdal	439	MK352105	–	Thailand	2002	Sipman, H. 48664	B
	440	MK352106	MK352285	Japan	2006	Thor, G. 21521	UPS
	4168	MK352210	MK352381	Venezuela	2015	Dahl, M.S., Kistenich, S., Timdal, E. & Toreskaas, A.K. SK1-201	VEN
	6063	MK352227	–	Guatemala	2004	v.d. Boom, P. 33395	hb. v.d. Boom
P. concinna Kistenich & Timdal	4041	MK352202	MK352373	Panama	2010	v.d. Boom, P. 43947	hb. v.d. Boom
	4776	MK352224	MK352395	Brazil	2015	Dahl, M.S., Kistenich, S., Timdal, E. & Toreskaas, A.K. SK1-445	O
	6455	MK352236	MK352404	Venezuela	2015	M.S. Dahl, J.E. Hernández M., S. Kistenich, E. Timdal & A.K. Toreskaas SK1-225	O
	7176	MK352251	MK352418	Guatemala	2002	Andersohn, C. s.n.	B
P. confusa Swinscow & Krog	514	MK352140	MK352318	Kenya	1972	Krog, H. & Swinscow, T.D.V. K48/177	O
	1018	**MK412426**	**MK412489**	Thailand	1991	Wolseley, P.A. 1049	BM

Species	Extract #	mtSSU	ITS	Country	Year	Voucher	Herbarium
P. confusa	1024	MK352150	MK352325	Cuba	2007	Tønsberg, T. 37813	BG
	1300	MK352169	MK352343	Venezuela	1969	Oberwinkler, B., Oberwinkler, F. & Poelt, J. s.n.	GZU
	1417	**MK412436**	–	Malaysia	2012	Wolseley, P., Thüs, H. & Vairappan, C. M.3.10.6	BORH
	3571	MK352190	MK352362	Ecuador	2014	Prieto, M. s.n.	HUTPL
	4741	MK352214	MK352385	Venezuela	2015	Dahl, M.S., Kistenich, S., Timdal, E. & Toreskaas, A.K. SK1-237	VEN
	6360	**MK412451**	–	Papua New Guinea	1992	Diederich, P. 11056	hb. Diederich
	6361	**MK412452**	–	Papua New Guinea	1992	Diederich, P. 10319	hb. Diederich
	6766	**MK412460**	**MK412503**	Sri Lanka	2017	Weerakoon, G. Ri030	PDA
	7185	MK352253	MK352419	Cameroon	1999	Frisch, A. & Tamnjong Idi 99/ Ka1213	hb. Frisch
	7220	**MK412468**	**MK412511**	Sri Lanka	2017	Weerakoon, G. 176	PDA
	7236	**MK352260**	**MK352426**	Sri Lanka	2017	Kistenich, S. & Weerakoon, G. SK1-609	PDA
	7239	**MK412473**	**MK412518**	Sri Lanka	2017	Kistenich S. & Weerakoon, G. SK1-567	PDA
	7240	**MK412474**	–	Sri Lanka	2017	Kistenich S. & Weerakoon, G. SK1-532	PDA
P. corallina (Eschw.) Müll. Arg.	1316	MK352173	MK352346	Venezuela	1986	Brako, L. & Berry, P.E. 8659	GZU
	4164	MK352209	MK352380	Venezuela	2015	Dahl, M.S., Kistenich, S., Timdal, E. & Toreskaas, A.K. SK1-185	VEN
	4762	MK352220	MK352391	Brazil	2015	Dahl, M.S., Kistenich, S., Timdal, E. & Toreskaas, A.K. SK1-377	O
	4775	MK352223	MK352394	Brazil	2015	Dahl, M.S., Kistenich, S., Timdal, E. & Toreskaas, A.K. SK1-430	O
P. cuyabensis (Malme) Zahlbr.	449	MK352107	MK352286	Peru	2006	Timdal, E. 10258	O
	450	**MK352108**	**MK352287**	Thailand	1993	Aguirre, B., James, P.W. & Wolseley, P. 2467a	BM
	1290	MK352166	MK352341	Venezuela	1996	Hafellner, J. 53910	GZU
	1291	MK352167	MK352342	Guatemala	1979	Kalb, K. & Plöbst, G. s.n.	GZU
	2048	MK352180	MK352352	Bolivia	2008	Flakus, A. & Rodriguez, P. 12792	O
P. dolichospora Timdal & Krog	515	MK352141	MK352319	Mauritius	1991	Krog, H. & Timdal, E. MAU65/22	O
	6357	MK352233	–	Papua New Guinea	1992	Diederich, P. 10847	hb. Diederich

Species	Extract #	mtSSU	ITS	Country	Year	Voucher	Herbarium
P. dolichospora	6359	**MK412450**	–	Papua New Guinea	1992	Diederich, P. 10846	hb. Diederich
	6763	**MK352247**	**MK352414**	Sri Lanka	2017	Weerakoon, G. Hg40	PDA
	6767	**MK352248**	**MK352415**	Sri Lanka	2017	Weerakoon, G. Si113B	PDA
	7258	**MK352271**	**MK352435**	Sri Lanka	2017	Kistenich, S. & Weerakoon, G. SK1-643	PDA
P. fendleri (Tuck. & Mont.) Müll. Arg.	2098	MK352183	MK352354	Costa Rica	1985	H. Sipman & A. Chaverri 20806	B
	7473	MK352277	MK352437	Venezuela	1979	Sipman, H. 10688	B
P. foliata (Stirt.) Zahlbr.	1035	MK352157	MK352332	Japan	2004	Kashawadani, H. 46389	TNS
	7238	**MK352261**	**MK352427**	Sri Lanka	2017	Kistenich, S. & Weerakoon, G. SK1-627	PDA
	7247	MK352265	MK352431	Australia	2006	Elix, J.A. 38235	CANB
P. foliatella Elix	7243	MK352262	MK352428	Australia	1986	Elix, J.A. & Streimann, H. 20241	CANB
	7246	MK352264	MK352430	Australia	1986	Elix, J.A. & Streimann, H. 20203	CANB
	7253	MK352268	–	Australia	2005	Elix, J.A. 37286	CANB
	7254	MK352269	–	Australia	1998	Streimann, H. 61609	CANB
P. furfuracea (Pers.) Zahlbr.	451	**MK412411**	**MK412478**	Thailand	1993	Aguirre, James & Wolseley 2918	BM
	452	MK352109	MK352288	La Réunion	1996	Krog, H. & Timdal, E. RE36/22	O
	453	MK352110	MK352289	Trinidad And Tobago	2008	Rui, S. & Timdal, E. 10799	O
	455	MK352111	MK352290	Peru	2006	Timdal, E. 10183	O
P. furfurella Kistenich & Timdal	3570	MK352189	MK352361	Ecuador	2014	Prieto, M. s.n.	HUTPL
	4036	MK352198	MK352369	Dominican Republic	2008	v.d. Boom, P. 39069	hb. v.d. Boom
P. glaucella (Vain.) Timdal	1000	MK352147	MK352323	Dominican Republic	1987	Harris, R.C. 20779	BM
	2125	MK352184	MK352356	Argentina	2013	Ferraro, L.I., Aptroot, A. & Cáceres, M.E.S. 10761	O
	4766	MK352221	MK352392	Brazil	2015	Dahl, M.S., Kistenich, S., Timdal, E. & Toreskaas, A.K. SK1-393	O
	4780	MK352225	MK352396	Brazil	2015	Dahl, M.S., Kistenich, S., Timdal, E. & Toreskaas, A.K. AM-44	O
P. gossypina (Sw.) Kistenich et al.	–	AY584615	–	Costa Rica	2002	Lücking, R. 16052	DUKE
P. gossypina ch1	3575	MK352192	MK352363	Brazil	2014	Barbosa, R.S., Haugan, R. & Timdal, E. 141	O
	3576	MK352193	MK352364	Brazil	2014	Barbosa, R.S., Haugan, R. & Timdal, E. 34	O
	4160	MG925867	MG925967	Brazil	2015	Kistenich, S. & Timdal, E. SK1-108	O

Species	Extract #	mtSSU	ITS	Country	Year	Voucher	Herbarium
P. gossypina ch1	4746	MG925868	MG925968	Brazil	2015	Dahl, M.S., Kistenich, S., Timdal, E. & Toreskaas, A.K. SK1-287	O
	7201	**MK352254**	**MK352420**	Sri Lanka	2017	Kistenich, S. & Weerakoon, G. SK1-584	PDA
P. gossypina ch2	4750	MK352219	MK352390	Brazil	2015	Dahl, M.S., Kistenich, S., Timdal, E. & Toreskaas, A.K. SK1-297	O
P. halei (Tuck.) Zahlbr. ch2	457	MK352113	MK352292	Tanzania	2008	Timdal, E. 10931	O
	1044	MK352161	MK352336	Kenya	2007	Divakar, Lumbsch & Mangold 19574K	hb. Pérez–Ortega
P. halei ch3	7221	**MK352257**	**MK352423**	Sri Lanka	2017	Weerakoon, G. 1008	PDA
P. hispaniolae Timdal	1545	MK352178	–	Ecuador	1999	Palice, Z. 3875	hb. Palice
	3569	MK352188	MK352360	Ecuador	2014	Prieto, M. s.n.	HUTPL
	4039	MK352201	MK352372	Panama	2010	v.d. Boom, P. 44158	hb. v.d. Boom
P. imshaugii Timdal	3558	MK352185	MK352357	Ecuador	2014	Prieto, M. s.n.	HUTPL
	4043	MK352204	MK352375	Guatemala	2004	v.d. Boom, P. 33433	hb. v.d. Boom
	4744	MK352217	MK352388	Venezuela	2015	Dahl, M.S., Kistenich, S., Timdal, E. & Toreskaas, A.K. SK1-253	VEN
P. isidiosa Kistenich & Timdal	430	**MK412409**	**MK412476**	Thailand	1991	Wolseley, P.A. & Aguirre–Hudson, B. 5552	BM
	1027	MK352153	MK352328	USA	2006	Lendemer, J.C. 7765 dupl.	BG
	1030	MK352155	MK352330	Nepal	2007	Sharma, L.R., Olley, L., Cross, A., Joshi, M. & Regmi, B. M16	E
	1031	**MK412428**	–	Nepal	2007	Sharma, L.R., Olley, L., Cross L25-2	E
	1259	**MK412432**	–	Malaysia	2012	Wolseley, P., Thüs, H. & Vairappan, C. S.P.5	BORH
	2099	**MK412446**	**MK412494**	Indonesia	2003	L. Sudirman & H. Sipman 51474	B
	4035	MK352197	MK352368	Dominican Republic	2008	v.d. Boom, P. 39012	hb. v.d. Boom
	4781	MG925907	MG926004	Brazil	2007	Lücking, R & Rivas Plata, E. 23302	SP
	6349	MK352232	–	Philippines	1994	Diederich, P. 13210	hb. Diederich
	7251	MK352267	MK352433	Australia	2006	Elix, J.A. 38478	CANB
P. isidiotyla (Vain.) Riddle	1315	MG925906	MG926003	Brazil	1979	Kalb, K. & Plöbst, G. in: Kalb, Lich. Neotrop. 343	GZU
P. kalbii Brako	456	**MK352112**	**MK352291**	Thailand	1993	Aguirre, B., James, P.W. & Wolseley, P. 2695	BM
	458	MK352114	MK352293	Tanzania	2008	Timdal, E. 10913	O
	459	MK352115	MK352294	Venezuela	1989	Kalb, K. & A. s.n.	O
	1028	MK352154	MK352329	USA	2010	Lendemer, J.C. 25770	BG
	2052	MK352182	–	Bolivia	2010	Flakus, A. & Quisbert, J. 19221	O

Species	Extract #	mtSSU	ITS	Country	Year	Voucher	Herbarium
P. loekoesii S.Y. Kondr. et al.	1033	MK352156	MK352331	Nepal	2007	Sharma, L.R., Olley, L., Cross A. C5	E
	7478	MK352279	MK352439	Japan	1994	Thor, G. 12574	TNS
P. longiuscula (Nyl.) Zahlbr.	454	MG925899	MG925996	Peru	2006	Timdal, E. 10433	O
	467	MK352117	MK352296	Trinidad And Tobago	2008	Rui, S. & Timdal, E. 10730	O
	1011	**MK412424**	**MK412488**	Thailand	1992	Wolseley, P.A. & Aguirre–Hudson, B. 5580 p.p.	BM
	1039	MK352159	MK352334	Cuba	2006	Pérez–Ortega, S. s.n.	hb. Pérez–Ortega
	6761	**MK352159**	**MK352413**	Sri Lanka	2017	Weerakoon, G. Kn136	PDA
P. malcolmii Vezda & Kalb	1303	MK352170	MK352344	New Zealand	1994	Malcolm, W. in: Vezda, Lich. Rar. Exs. 200	GZU
P. martinii Swinscow & Krog	489	MK352129	MK352309	Tanzania	1989	Krog, H. 3T13/007	O
	6740	MK352242	MK352409	Kenya	2014	Kirika, P. & Lumbsch, H.T. 4087	O
P. mauritiana (Taylor) Swinscow & Krog	487	MK352128	MK352307	Tanzania	1988	Krog, H. 2T12/037	O
	488	–	MK352308	Mauritius	1991	Krog, H. & Timdal, E. MAU09/43	O
	SE386	MG925900	MG925997	Mauritius	1991	Krog, H. & Timdal, E. MAU09/44	O
P. mediocris Swinscow & Krog	527	MK352144	MK352320	Tanzania	1988	Krog, H. 2T06/023	O
	6346	MK352229	MK352399	Mauritius	2016	Diederich, P. 18571	hb. Diederich
	6347	MK352230	MK352400	Mauritius	2016	Diederich, P. 18573	hb. Diederich
P. melanoglauca Zahlbr.	1038	MK352158	MK352333	Cuba	2006	Pérez–Ortega, S. s.n.	hb. Pérez–Ortega
	4042	MK352203	MK352374	Guatemala	2004	v.d. Boom, P. 33408	hb. v.d. Boom
	4740	MK352213	MK352384	Venezuela	2015	Dahl, M.S., Kistenich, S., Timdal, E. & Toreskaas, A.K. SK1-232	VEN
	4743	MK352216	MK352387	Venezuela	2015	Dahl, M.S., Kistenich, S., Timdal, E. & Toreskaas, A.K. SK1-247	VEN
	6450	MK352235	MK352403	Brazil	2015	Dahl, M.S., Kistenich, S., Timdal, E. & Toreskaas, A.K. SK1-408	O
P. nemoralis Timdal & Krog	522	MK352142	–	La Réunion	1996	Krog, H. & Timdal, E. RE25/32	O
	1434	MK352174	MK352347	South Africa	1996	Nordin, A. 4622	UPS:L:92604
P. neofoliata Elix	6745	MK352244	MK352411	Kenya	2015	Kirika, P. & Lumbsch, H.T. 4728	O
	7245	MK352263	MK352429	Australia	1992	Elix, J.A. 32714	O
	7249	MK352266	MK352432	Australia	1989	Elix, J.A.	CANB
P. neotinica Kistenich & Timdal	505	MK352137	MK352316	Trinidad And Tobago	2008	Rui, S. & Timdal, E. 10774	O
	1023	MK352149	MK352324	Cuba	2007	Tønsberg, T. 37923	BG
	1438	MK352176	MK352349	Trinidad And Tobago	2008	Rui, S. & Timdal, E. 10763	O

Species	Extract #	mtSSU	ITS	Country	Year	Voucher	Herbarium
P. neotinica	4742	MK352215	MK352386	Venezuela	2015	M.S. Dahl, J.E. Hernández M., S. Kistenich, E. Timdal & A.K. Toreskaas SK1-246	O
	4769	MK352222	MK352393	Brazil	2015	Dahl, M.S., Kistenich, S., Timdal, E. & Toreskaas, A.K. SK1-402	O
P. ochroxantha (Nyl.) Zahlbr.	473	MK352118	MK352297	Peru	2006	Timdal, E. 10338	O
	474	MK352119	MK352298	Peru	2006	Timdal, E. 10389	O
	475	MK352120	MK352299	Trinidad And Tobago	2008	Rui, S. & Timdal, E. 10849	O
	4049	MK352206	MK352377	Brazil	2015	Kistenich, S. & Timdal, E. SK1-47	O
	4747	MK352218	MK352389	Brazil	2015	Dahl, M.S., Kistenich, S., Timdal, E. & Toreskaas, A.K. SK1-289	O
P. parvifolia (Pers.) Müll. Arg.	479	MK352124	MK352303	Tanzania	2008	Timdal, E. 10935	O
	480	MK352125	MK352304	Trinidad And Tobago	2008	Rui, S. & Timdal, E. 10867	O
	2049	MK352181	MK352353	Bolivia	2010	Flakus, A. & Quisbert, J. 20016	O
	3561	MK352187	MK352359	South Africa	2014	Burrows, J. & Timdal, E. 14244	O
	6365	MK352234	MK352402	Portugal	2015	v.d. Boom, P. 53877	hb. v.d. Boom
P. parvifoliella (Nyl.) Müll. Arg.	481	MK352126	MK352305	Peru	2006	Timdal, E. 10302	O
	482	**MG925902**	**MG925999**	Indonesia	2000	Wolseley, P.A. s.n.	BM:1104069
	483	**MK352127**	**MK352306**	Thailand	1993	James, P.W. & Wolseley, P.A. 2491	BM
	1004	**MK412422**	–	Thailand	1993	James, P.W. & Wolseley, P.A. 1847	BM
P. phaeobyssina (Vain.) Timdal	478	MK352123	MK352302	Trinidad And Tobago	2008	Rui, S. & Timdal, E. 10872	O
P. porphyromelaena (Vain.) Zahlbr. ch1	490	**MK412416**	**MK412482**	Thailand	1994	Wolseley, P. & Kanajriavanit, S. s.n.	BM:1104012
	498	MG925904	MG926001	La Réunion	1996	Krog, H. & Timdal, E. RE07/17	O
	502	MK352135	MK352314	Japan	1995	Thor, G. 12941	UPS
	1050	MK352164	MK352339	Kenya	2002	Killmann, D. & Fischer, E. s.n.	hb. Killmann
	1429	**MK412444**	–	Sri Lanka	2007	Jayalal, U. B9-4-3-3	PDA
	7207	**MK412464**	**MK412507**	Sri Lanka	2017	Kistenich S. & Weerakoon, G. SK1-634	PDA
P. porphyromelaena ch2	491	**MK412417**	**MK412483**	Thailand	1993	Aguirre–Hudson, B. & Wolseley, P.A. 1663	BM
	496	MK352133	–	Tanzania	1989	Krog, H. 4T16/019	O
	503	MK352136	MK352315	Japan	2006	Thor, G. 21238	UPS
	6436	**MK412454**	**MK412497**	Malaysia	2014	Paukov, A. 2233	B
	7208	**MK352255**	**MK352421**	Sri Lanka	2017	Kistenich, S. & Weerakoon, G. SK1-631	PDA
P. porphyromelaena ch2	7479	**MK412475**	**MK412519**	Japan	2017	Haugan, R. & Timdal, E. 16753	O:L:209897

Species	Extract #	mtSSU	ITS	Country	Year	Voucher	Herbarium
P. porphyromelaena ch3	492	**MK352130**	**MK352310**	Thailand	1993	Aguirre, B., James, P.W. & Wolseley, P. 2857	BM
	494	**MK352132**	**MK352312**	Thailand	1993	Aguirre, B., James, P.W. & Wolseley, P. 2481	BM
P. pseudocorallina Kistenich & Timdal	1034	**MK412430**	**MK412491**	Cambodia	2005	Kashiwadani, H. 47806	TNS
	1418	**MK412437**	–	Malaysia	2012	Thüs, H., Wolseley, P. & Vairappan, C. M001a	BORH
	1419	**MK412438**	–	Malaysia	2012	Thüs, H., Wolseley, P. & Vairappan, C. M005	BORH
	6356	**MK412449**	**MK412495**	Papua New Guinea	1992	Diederich, P. 11386	hb. Diederich
P. pyxinoides (Nyl.) Kistenich et al.	3574	MK352191	–	Brazil	2014	Cáceres, M., Haugan, R. & Timdal, E. 21024	O
	7358	MK352274	–	USA	1991	Ryan, B. 27530	O
P. rappiana (Brako) Elix	6737	MK352240	MK352407	Australia	2005	Elix, J. 36867	O
	7175	MK352250	MK352417	Panama	2010	v.d. Boom, P. 43820	hb. v.d. Boom
P. rosei Coppins & P. James	1299	MK352168	–	UK	1992	Coppins, B., James, P.W. & Poelt, J. Sc92/446	GZU
	6339	MK352228	MK352398	France	2000	Diederich, P. 14602	hb. Diederich
	7356	MK352272	MK352436	France	1990	Diederich, P. 9247	hb. Diederich
	7357	MK352273	–	UK	1992	Coppins, B., James, P.W. & Poelt, J. Sc92/193	GZU
P. sabahana Kistenich & Timdal	1265	**MK412434**	–	Malaysia	2012	Wolseley, P., Thüs, H. & Vairappan, C. S.B.oQ.3	BORH
	1423	**MK412441**	–	Malaysia	2012	Thüs, H., Wolseley, P. & Vairappan, C. M089	BORH
	1425	**MK412442**	–	Malaysia	2012	Wolseley, P., Thüs, H. & Vairappan, C. D.8.02.4	BORH
	6435	**MK412453**	**MK412496**	Malaysia	2014	Paukov, A. 2230	B
	6457	**MK412455**	**MK412498**	Malaysia	2014	Paukov, A. 2229	B
P. santensis (Tuck.) Swinscow & Krog	2043	MK352179	MK352351	Bolivia	2009	Flakus, A. & Rodriguez, P. 15581	O
	4038	MK352200	MK352371	Panama	2010	v.d. Boom, P. 44704	hb. v.d. Boom
	4051	MK352207	MK352378	Brazil	2015	Kistenich, S. & Timdal, E. SK1-79	O
P. siamensis Kistenich & Timdal	448	**MK412410**	**MK412477**	Thailand	1993	Wolseley, P.A. & Boonpragob, K. 3245	BM
	996	**MK412418**	**MK412484**	Thailand	1992	Wolseley, P.A. & Onsar 5590	BM
	997	**MK412419**	**MK412485**	Thailand	1993	Aguirre–Hudson, B. & Wolseley, P.A. 1643	BM
	1010	**MK412423**	**MK412487**	Thailand	1992	Wolseley, P.A. & Aguirre–Hudson, B. 5580	BM

Species	Extract #	mtSSU	ITS	Country	Year	Voucher	Herbarium
P. sp. 1	7230	**MK352259**	**MK352425**	Sri Lanka	2017	Kistenich, S. & Weerakoon, G. SK1-545	PDA
P. sp. 2	1017	**MK352148**	–	Malaysia	1997	Wolseley, P. s.n.	BM:1104019
P. sp. 3	7227	**MK352258**	**MK352424**	Sri Lanka	2017	Kistenich, S. & Weerakoon, G. SK1-555	PDA
P. sp. 4	7231	**MK412471**	**MK412514**	Sri Lanka	2017	Kistenich S. & Weerakoon, G. SK1-570	PDA
P. subhispidula (Nyl.) Kalb & Elix	501	MK352134	MK352313	Tanzania	1989	Krog, H. 4T15/007	O
	6738	MK352241	MK352408	La Réunion	1996	Krog, H. & Timdal, E. RE36/15	O
	6771	**MK352249**	**MK352416**	Sri Lanka	2017	Weerakoon, G. Hg29A	PDA
P. swinscowii Timdal & Krog	476	MK352121	MK352300	Peru	2006	Timdal, E. 10190	O
	525	MK352143	–	Mauritius	1991	Krog, H. & Timdal, E. MAU09/50	O
	1025	MK352151	MK352326	Cuba	2007	Tønsberg, T. 37817	BG
	1049	MK352163	MK352338	Kenya	2002	Killmann, D. & Fischer, E. s.n.	hb. Killmann
	4048	MK352205	MK352376	Brazil	2015	Kistenich, S. & Timdal, E. SK1-115	O
P. teretiuscula Timdal	1026	MK352152	MK352327	Cuba	2007	Tønsberg, T. 37814	BG
	1306	MK352171	MK352345	Costa Rica	2003	Hafellner & Emmerer 1490	GZU
	7474	MK352278	MK352438	Puerto Rico	1992	Harris, R.C. 27320	O
P. thaleriza (Stirt.) Brako	1048	MK352162	MK352337	Kenya	2003	Killmann, D. & Fischer, E. s.n.	hb. Killmann
	5465	MG925880	MG925982	South Africa	2014	Burrows, J. & Timdal, E. 14191	O
	5466	MG925881	MG925983	South Africa	2015	Rui, S. & Timdal, E. 13877	O
	5467	MK352226	MK352397	South Africa	2015	Rui, S. & Timdal, E. 13873	O

quent analyses. The remaining 47 specimens did not belong in *Phyllopsora* s. str. (Table 3) and are referred to at the family level only due to many problems of generic affiliation.

The concatenated alignment had a length of 1,825 bp with 264 accessions including one specimen of *Biatora beckhausii* (Körb.) Tuck. and one of *B. vacciniicola* (Tønsberg) Printzen for rooting of the phylogenetic trees. The alignment contained ca. 20% missing data and is available from TreeBase (study no. 23881).

The software IQ-TREE suggested the following substitution models for four subsets: GTR+I+Γ for mtSSU and SYM+I+Γ for ITS1, 5.8S and ITS2. Bayesian phylogenetic analysis halted automatically after 40×10^6 generations, when the ASDSF in the last 50% of each run had fallen below 0.01. Following a burnin of 50%, we used 80,004 trees for the final Bayesian majority-rule consensus tree. The phylogenetic results generated by IQ-TREE vs. MrBayes showed no incongruences. The extended majority-rule consensus tree (Fig. 1; see Suppl. material 1: Fig. S1 for the uncollapsed version of the tree), based on the Bayesian topology with all compatible groups (BS ≥ 50 and/or

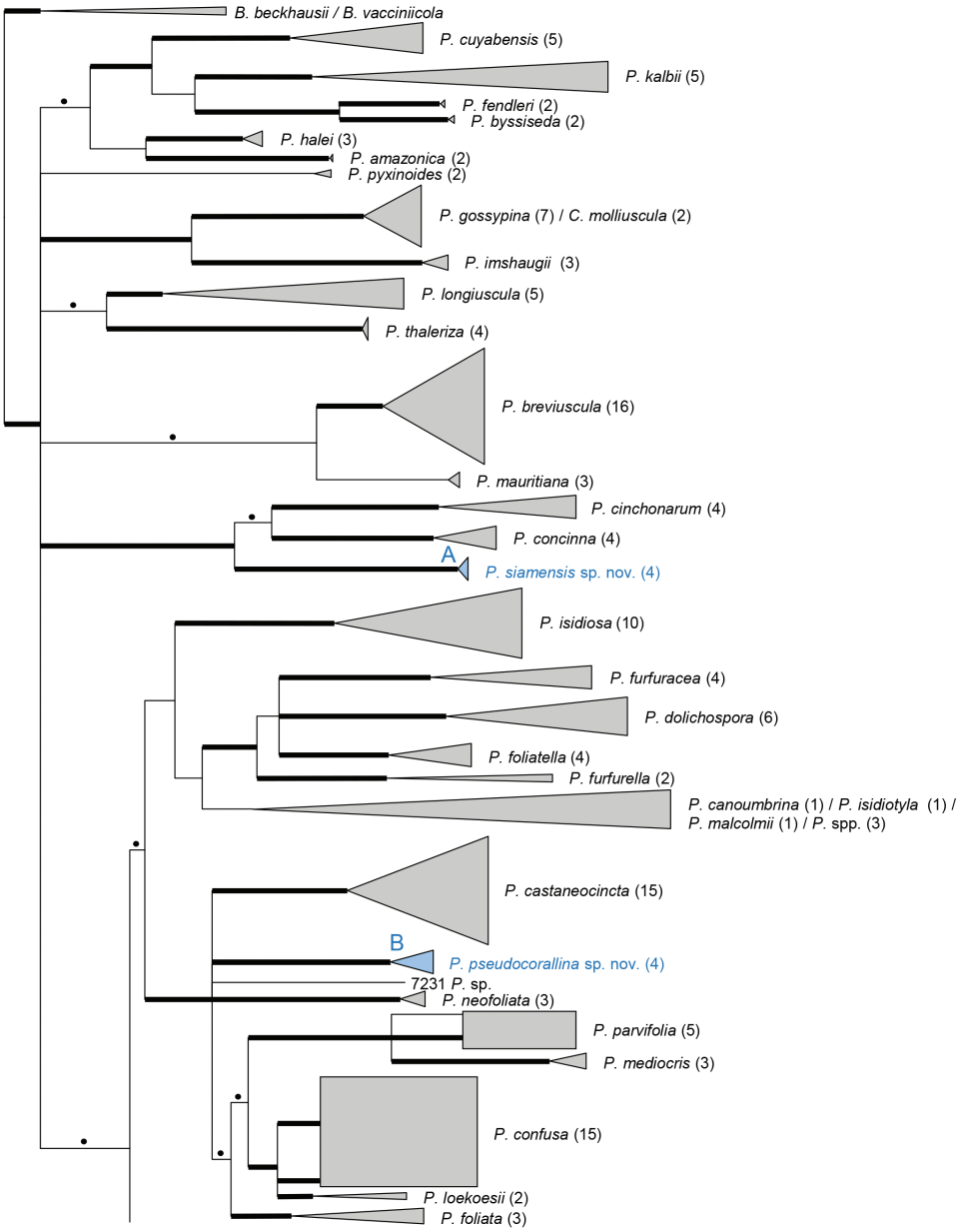

Figure 1. Extended majority-rule consensus tree resulting from the MrBayes analysis of the mtSSU and ITS alignment with Bayesian PP ≥ 0.7 and/or IQ-TREE maximum likelihood BS ≥ 50 and branch lengths. Strongly supported branches (PP ≥ 0.95 and BS ≥ 75) are marked in bold; branches only supported with PP ≥ 0.7 or BS ≥ 50 are marked with a dot above the branch. Two species of *Biatora* were used for rooting. Accessions belonging to the same species are collapsed for convenience. Three clades are distinguished to facilitate the discussion of new species (**A, B, C**). ch = chemotype.

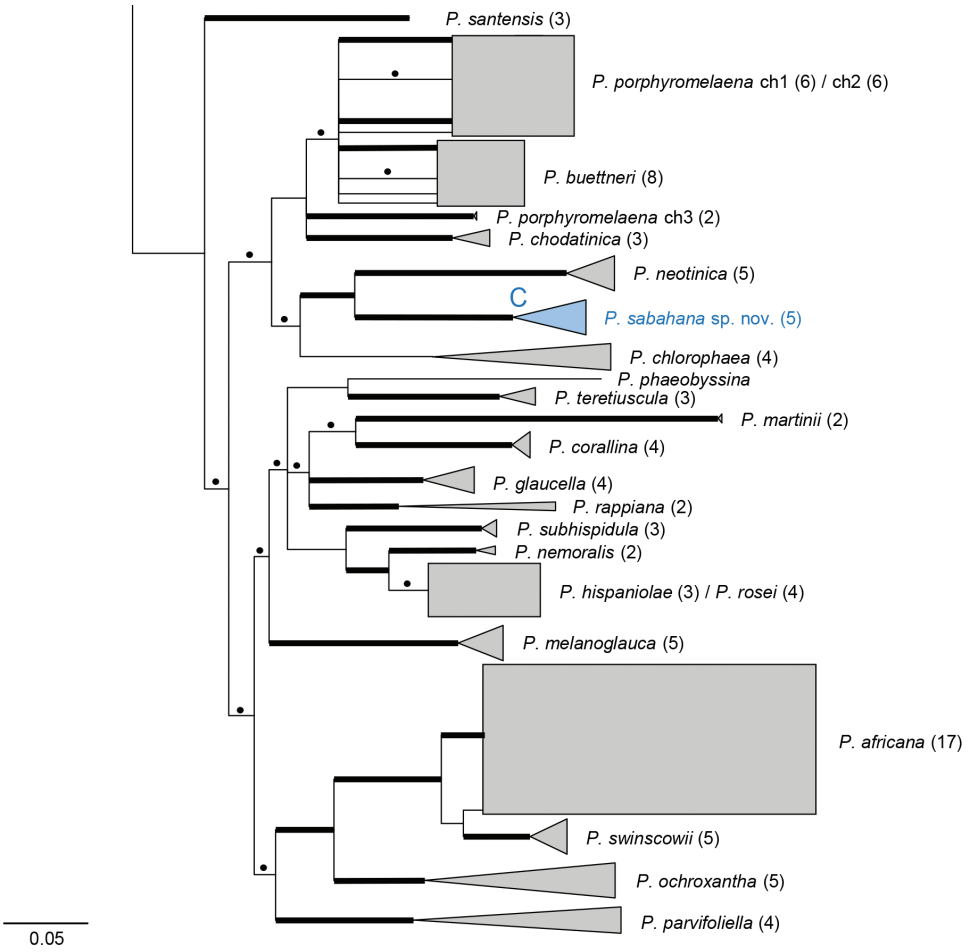

Figure 1. Continued.

PP ≥ 0.7), shows an overall good resolution of *Phyllopsora* species. Seventeen unidentified specimens did not associate with any known species in the phylogenetic tree (Fig. 1). Four unidentified specimens (1017, 7227, 7230, and 7231) were resolved on long branches, while the remaining 13 specimens grouped into three distinct, strongly supported clades (Fig. 1A–C). Clade A is resolved as sister to a clade consisting of *P. cinchonarum* and *P. concinna*, clade B is found in a clade with *P. castaneocincta*, *P. foliata* and *P. neofoliata* among others, and clade C is resolved as sister to *P. neotinica*.

Discussion

In this study, we present the first revision of the genus *Phyllopsora* for Asia and Melanesia based on the integrative study of morphology, chemistry and DNA sequence data.

We investigated 625 specimens of *Phyllopsora* collected from 18 countries and found the material to comprise at least 28 species of *Phyllopsora* s. str. (Figs 2–10) including three supported clades that we describe as species new to science. With this study, the genus *Phyllopsora* comprises 57 species.

Several species seem to be rather widespread throughout Asia and Melanesia, for instance, *P. castaneocincta*, *P. confusa*, *P. isidiosa*, and *P. porphyromelaena* (Table 1, Suppl. material 2: Table S1). In contrast, specimens of, for example, *P. cuyabensis*, *P. mediocris* and *P. neofoliata*, are rarely collected and reported from few countries (Table 1, Suppl. material 2: Table S1). Thus, their distribution range requires further studies.

Among the 28 species of *Phyllopsora*, eight are reported as new for Asia and Melanesia (Table 1). One of these new species is *P. africana* (Fig. 2A). This species has recently been found to be morphologically and chemically heterogeneous, comprising three chemotypes (Kistenich et al. in press). In addition to the known isidiate morph, a lacinulate morph was detected among *P. africana* material by Kistenich et al. (in press). Moreover, they described two new chemotypes. The lacinulate morph occurred in specimens of chemotype 1 and 3, but has so far never been found in those of chemotype 2. Specimens of chemotype 2, however, were shown to be morphologically cryptic to the sister species *P. swinscowii* (Kistenich et al. in press). In this study, we added twelve specimens of *P. africana* to our phylogeny (mainly lacinulate specimens of chemotype 3), but are not able to disentangle the difficult nature of this species complex. While we found most specimens of *P. africana* to roughly group according to chemotype in the phylogenetic tree (Suppl. material 1: Fig. S1), one specimen of *P. africana* chemotype 1 (7224) was more closely related to *P. swinscowii* (Suppl. material 1: Fig. S1). This raises the question of whether the two species should be synonymized based on their morphological and chemical similarity in combination with the short branches in the phylogenetic tree (Suppl. material 1: Fig. S1). We refrain from synonymizing them here, awaiting more data.

The two species *P. cuyabensis* (Fig. 4B) and *P. longiuscula* (Fig. 7B) are reported as new for the Asian continent. Specimens of both species are morphologically congruent with their Neotropical representatives. In the phylogenetic tree (Suppl. material 1: Fig. S1), however, the respective Asian accessions sit on rather long branches, clearly distinct from the Neotropical specimens. In these cases, there seem to exist genetically different populations for Neotropical and Asian specimens and more specimens should be collected to investigate the extent of genetic variation.

The genus *Phyllopsora* was recently shown to be polyphyletic by Kistenich et al. (2018b). The typical growth form, which characterizes this genus, has evolved multiple times independently in the family Ramalinaceae. These findings corroborate the morphological co-evolution in tropical lichens already indicated by Lakatos et al. (2006). Hence, molecular methods are often the only means of reliably assigning specimens to *Phyllopsora* or rather to its morphologically similar relatives (e.g., *Bacidia* De Not., *Bacidina* Vězda, *Eschatogonia*, *Parallopsora*). It is thus not surprising that several of our sequenced specimens (Table 3) were extraneous to *Phyllopsora* s. str. We did not assign those specimens to genus level, but all but one belong in the Ramalinaceae. The non-Ramalinaceae specimen appears to belong in the Malmideaceae. This indicates that correct taxonomic assignment even at family level using morphology may prove chal-

Table 3. Newly generated sequences for specimens not belonging to *Phyllopsora* with voucher information and GenBank accession numbers. – indicates missing data.

Family	Extract #	mtSSU	ITS	Country	Year	Voucher	Herb.
Malmideaceae	1268	MK400188	MK400239	Malaysia	2012	Wolseley, P., Thüs, H. & Vairappan, C. D.1.10.3	BORH
Ramalinaceae	417	MK400189	MK400240	Thailand	1993	Wolseley, P.A. & David, F. 3347	BM:749829
	423	MK400190	MK400241	Indonesia	2000	Wolseley, P. T9 LQ	BM:1104053
	427	MK400191	MK400242	Indonesia	2000	Wolseley, P. T13 LQ	BM:1104062
	432	MK400192	MK400243	Malaysia	1997	Wolseley, P. pkt. 8	BM:1104016
	433	MK400193	MK400244	Thailand	1991	Wolseley, P.A. & Aguirre–Hudson, B. 5548	BM:749824
	435	MK400194	MK400245	Indonesia	2000	Wolseley, P. T20 LMQ	BM:1104013
	1008	MK400195	–	Thailand	1993	Aguirre, James & Wolseley 2854	BM
	1013	MK400196	–	Thailand	1993	James, P.W. & Wolseley, P.A. 1700b	BM
	1014	MK400197	MK400246	Thailand	1993	Aguirre, James & Wolseley 2478a	BM:749861
	1015	MK400198	MK400247	Thailand	1993	Aguirre, James & Wolseley 2715	BM:749853
	1020	MK400199	–	Indonesia	2000	Wolseley, P. T6 LQ	BM:1104066
	1021	MK400200	–	Indonesia	2000	Wolseley, P. T1	BM:1104063
	1266	–	MK400248	Malaysia	2012	Wolseley, P., Thüs, H. & Vairappan, C. D.4.04.2	BORH
	1270	MK400201	MK400249	Malaysia	2012	Wolseley, P., Thüs, H. & Vairappan, C. M.1.12.oQ	BORH
	1275	MK400202	MK400250	Malaysia	2012	Wolseley, P., Thüs, H. & Vairappan, C. D+40	BORH
	1282	MK400203	MK400251	Malaysia	2012	Wolseley, P., Thüs, H. & Vairappan, C. S.B.10.2	BORH
	1284	MK400204	MK400252	Malaysia	2012	Wolseley, P., Thüs, H. & Vairappan, C. D.7.09.1	BORH
	1285	MK400205	MK400253	Malaysia	2012	Wolseley, P., Thüs, H. & Vairappan, C. M.3.08.oQ.2	BORH
	1287	MK400206	MK400254	Malaysia	2012	Wolseley, P., Thüs, H. & Vairappan, C. M.3.03.1	BORH
	1426	–	MK400255	Malaysia	2013	Vairappan, C. L261	BM
	1428	MK400207	MK400256	Thailand	1993	Aguirre, James & Wolseley 2477e	BM:1031544
	6056	MK400208	–	Malaysia	2014	Paukov, A. 2236	B
	6057	MK400209	–	Malaysia	2014	Paukov, A. 2235	B
	6762	MK400210	MK400257	Sri Lanka	2017	Weerakoon, G. Ne141	PDA
	6768	MK400211	MK400258	Sri Lanka	2017	Weerakoon, G. WL60	PDA
	6769	MK400212	MK400259	Sri Lanka	2017	Weerakoon, G. WL15/2	PDA
	7186	MK400213	MK400260	Sri Lanka	2017	Kistenich, S. & Weerakoon, G. SK1-651	PDA
	7187	MK400214	MK400261	Sri Lanka	2017	Kistenich, S. & Weerakoon, G. SK1-650	PDA
	7188	MK400215	MK400262	Sri Lanka	2017	Kistenich, S. & Weerakoon, G. SK1-564	PDA
	7189	MK400216	–	Sri Lanka	2017	Kistenich, S. & Weerakoon, G. SK1-566	PDA
	7190	MK400217	MK400263	Sri Lanka	2017	Kistenich, S. & Weerakoon, G. SK1-604	PDA
	7191	MK400218	MK400264	Sri Lanka	2017	Kistenich, S. & Weerakoon, G. SK1-602	PDA
	7192	MK400219	MK400265	Sri Lanka	2017	Kistenich, S. & Weerakoon, G. SK1-611	PDA
	7193	MK400220	MK400266	Sri Lanka	2017	Kistenich, S. & Weerakoon, G. SK1-558	PDA
	7195	MK400221	MK400267	Sri Lanka	2017	Kistenich, S. & Weerakoon, G. SK1-560	PDA
	7196	MK400222	MK400268	Sri Lanka	2017	Kistenich, S. & Weerakoon, G. SK1-673	PDA

Family	Extract #	mtSSU	ITS	Country	Year	Voucher	Herb.
Ramalinaceae	7198	MK400223	MK400269	Sri Lanka	2017	Kistenich, S. & Weerakoon, G. SK1-659	PDA
	7199	MK400224	MK400270	Sri Lanka	2017	Kistenich, S. & Weerakoon, G. SK1-587	PDA
	7202	–	MK400271	Sri Lanka	2017	Kistenich, S. & Weerakoon, G. SK1-573	PDA
	7204	–	MK400272	Sri Lanka	2017	Kistenich, S. & Weerakoon, G. SK1-524	PDA
	7206	MK400225	MK400273	Sri Lanka	2017	Kistenich, S. & Weerakoon, G. SK1-666	PDA
	7211	MK400226	MK400274	Sri Lanka	2017	Kistenich, S. & Weerakoon, G. SK1-561	PDA
	7215	–	MK400275	Sri Lanka	2017	Kistenich, S. & Weerakoon, G. SK1-592	PDA
	7222	MK400227	MK400276	Sri Lanka	2017	Weerakoon, G. 641 loc.31	PDA
	7226	–	MK400277	Sri Lanka	2017	Kistenich, S. & Weerakoon, G. SK1-628	PDA
	7228	MK400228	–	Sri Lanka	2017	Kistenich, S. & Weerakoon, G. SK1-667	PDA

lenging in certain cases. Furthermore, about a quarter of the total material investigated could not be identified, partly because many of those unidentified specimens were sterile and deficient in lichen substances. Unfortunately, we were not able to generate sequences of all unidentified specimens in the course of this study.

New species

The three new species, *P. pseudocorallina*, *P. sabahana* and *P. siamensis*, fall into distinct and well-supported clades in the phylogeny (Fig. 1A–C). They were originally assumed to comprise Asian populations of the species *P. porphyromelaena*, *P. corallina* and *P. imshaugii*, respectively, based on morphology and/or chemistry. Their sequence data, however, revealed them as separate species clearly distinct from their look-alikes (Fig. 1). *Phyllopsora pseudocorallina* (Fig. 9B) is distinguished from its namesake, i.e. *P. corallina*, by forming a partly more rosulate thallus. Poorly developed specimens, however, might be difficult to assign to the correct species. Specimens of *P. sabahana* (Fig. 9C) are challenging to identify based on morphology only. The species is morphologically and chemically almost identical to *P. porphyromelaena* chemotype 1. It differs only in forming slightly smaller ascospores. Thus, sterile specimens cannot be identified without DNA sequence data. *Phyllopsora siamensis* (Fig. 10B) is described from material collected in Thailand and we have not been able to detect this species in collections from other countries. The specimens resemble *P. imshaugii* in morphology and chemistry, but may be readily distinguished by forming larger ascospores. See also the remarks in the Taxonomy section.

In addition, we found sequences of the four unidentified specimens with extraction numbers 1017, 7227, 7230, and 7231 to be resolved on rather long branches (Fig. 1, Suppl. material 1: Fig. S1). Hence, we could not assign them to any other *Phyllopsora*

species, for which DNA sequences of the mtSSU or ITS region were available, based on molecular data, either. It is possible that these specimens represent several new species. In this study, however, we refrain from describing them as new species pending the collection of more material. Even though specimens 1017 and 7230 are clustered together in a clade with short branches (Suppl. material 1: Fig. S1), they are morphologically quite distinct and more specimens are needed to support the hypothesis that they belong to the same species.

Unconfirmed species records

Despite investigating about 600 phyllopsoroid specimens, we were not able to find in our material any specimens belonging to seven species (i.e., *P. chlorophaea*, *P. corallina*, *P. isidiotyla*, *P. mauritiana*, *P. nemoralis*, *P. pyxinoides*, and *P. swinscowii*) previously reported from India, South Korea, Sri Lanka, Taiwan, Thailand, and Vietnam (Table 1), respectively. We have only investigated a few collections from especially India, South Korea, Taiwan, and Vietnam (Suppl. material 2: Table S1), though. Also for the other countries, collections are limited to certain areas and we cannot exclude the species' occurrence in other parts of the respective countries. About 23% of the investigated material could not be identified to species level and it is possible that some of these unidentified specimens represent a poorly developed individual of any of these seven species. Regarding *P. corallina*, for instance, we found two candidate specimens from Papua New Guinea, but DNA sequence data is necessary to resolve their species status unambiguously. Alternatively, some of these species records might be based on misidentifications. In the case of *P. swinscowii*, we have shown the species to be morphologically identical to the isidiate morph of *P. africana*, a very widespread species. It is therefore possible that the records of *P. swinscowii* indeed represent *P. africana*. In general, we have repeatedly experienced difficulties in correctly identifying species of *Phyllopsora* based on morphology only. For many of the species records, it remains unclear whether anatomical studies and/or chemical investigations were performed as part of the identification process or not. Especially *P. chlorophaea*, *P. corallina* and *P. isidiotyla* may be difficult to identify without TLC or even sequence data.

Taxonomy

This taxonomy section is a result of the integrative species delimitation process primarily based on the conclusions from the statistically inferred species delimitation analyses combined with morphological and chemical evaluations as performed in the global *Phyllopsora* study by Kistenich et al. (in press). The additional material of the present study complements the global dataset for the phylogenetic analysis (Fig. 1, Suppl. material 1: Fig. S1) and revealed three new species, which were mainly delimited by forming separate clades on long branches compared to their neighboring clades.

Distribution references for Asia and Melanesia are cited in Table 1; for all other distributions, references are cited below.

Phyllopsora africana Timdal & Krog

Description. Timdal and Krog (2001), Elix (2009).

Distribution. Africa (Timdal and Krog 2001), Asia, Australia (Elix 2009).

Remarks. See discussion above and Kistenich et al. (in press) for taxonomic discussion. The species (Fig. 2A) is one of the most common in our material, represented by 59 collections (Suppl. material 2: Table S1). We found both isidate and lacinulate morphs as well as representatives of all three chemotypes (i.e., chemotype 1 contains chlorophyllopsorin and argopsin; chemotype 2 contains methyl 2,7-dichloropsoromate and methyl 2,7-dichloronorpsoromate; chemotype 3 contains chlorophyllopsorin, methyl 2,7-dichloropsoromate, methyl 2,7-dichloronorpsoromate, and argopsin) among the material. It is the phylogenetic sister to *P. swinscowii* (Fig. 1). The species is new to Asia and Melanesia, i.e. to Indonesia, Japan, Malaysia, Papua New Guinea, The Philippines, The Solomon Islands, Sri Lanka, Thailand, and Vanuatu.

Phyllopsora breviuscula (Nyl.) Müll. Arg.

Description. Timdal and Krog (2001), Elix (2009).

Distribution. Pantropical (Brako 1991, as *P. parvifolia* var. *breviuscula*; Timdal and Krog 2001; Elix 2009).

Remarks. Paleotropical material of this species (Fig. 2B) tends to be more narrow-lobed and ascending than Neotropical material. Species delimitation analyses by Kistenich et al. (in press) show that the taxon could be split into three or four entities, but as all sequenced specimens fell into one well-supported clade, and morphologically intermediate specimens exist, we still treat the taxon as one variable species. It is the phylogenetic sister to *P. mauritiana* (Fig. 1). The species is new to New Caledonia, The Philippines, and Vietnam.

Phyllopsora buettneri (Müll. Arg.) Zahlbr.

Description. Swinscow and Krog (1981), Timdal and Krog (2001), Timdal (2008b, as *P. buettneri* chemotypes 1 and 2), Elix (2009).

Distribution. Pantropical (Brako 1991, as *P. buettneri* var. *buettneri*; Timdal and Krog 2001; Elix 2009).

Remarks. We recognize five chemotypes of this species, three occurring in Asia and Melanesia (Fig. 2C). Chemotype 1 (pannarin and zeorin) was found in material from Japan, Sri Lanka, and Thailand; chemotype 3 (dechloropannarin, zeorin) in material from China, Japan, Sri Lanka, and Thailand; and chemotype 5 (pannarin and an unknown compound, no zeorin) in material from Papua New Guinea and Sri Lanka (Suppl. material 2: Table S1). The specimen of chemotype 1 from Japan, and one of the two from Sri Lanka, differed from typical specimens of the chemotype in lacking

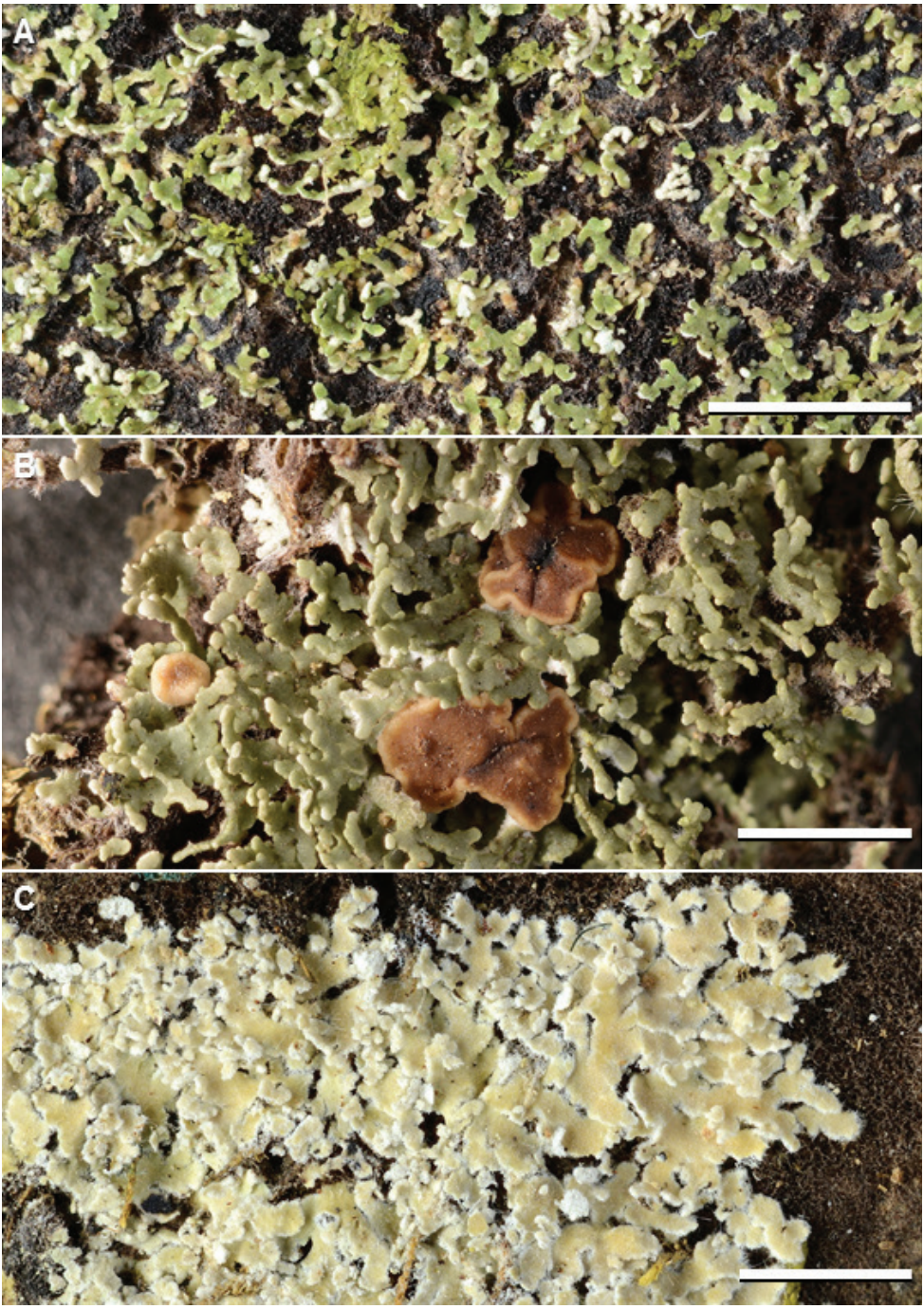

Figure 2. Species of *Phyllopsora* occurring in Asia and Melanesia. **A** *Phyllopsora africana* (Kistenich & Weerakoon SK1-517) **B** *P. breviuscula* (Kistenich & Weerakoon SK1-601) **C** *P. buettneri* (Thor 13183). Scale bars: 2 mm.

zeorin. Chemotype 5 is described here for the first time, and contains an unknown compound with R_f values similar to argopsin in solvent system B' but with a distinct blue UV_{366} fluorescence (not quenching) on the chromatograms after development. Chemotype 2 (pannarin, phyllopsorin, zeorin) is Neotropical and chemotype 4 (argopsin, norargopsin, zeorin) is known from the Norfolk Islands. We were unable to sequence specimens of chemotype 4 and 5, but specimens of chemotype 1–3 are resolved in a clade with chemotype 1 and 2 of *P. porphyromelaena* (Fig. 1, Suppl. material 1: Fig. S1). The species is new to China, Japan, and Sri Lanka.

Phyllopsora castaneocincta (Hue) Kistenich & Timdal

Description. Timdal and Krog (2001), Elix (2009), both as *P. kiiensis*.

Distribution. Africa (Timdal and Krog 2001), Asia, Australia (Elix 2009).

Remarks. This is one of the most common species in our material (Suppl. material 2: Table S1). It is usually easily recognized by the well-developed squamulose thallus on a reddish brown prothallus and by containing furfuraceic acid (Fig. 3A), but care is needed as about 10% of the examined specimens were actually deficient in lichen substances. It is new to Cambodia, Malaysia, Nepal, New Caledonia, Papua New Guinea, The Solomon Islands, Taiwan, and Thailand.

Phyllopsora chodatinica Elix

Description. Elix (2006, 2009).

Distribution. Australasia (Elix 2009) and Oceania.

Remarks. This species resembles *P. porphyromelaena* and *P. sabahana*, to which it is closely related in the phylogenetic tree (Fig. 1), but differs in the presence of xanthones and the absence of argopsin and norargopsin. Kistenich et al. (in press) showed that probably all Neotropical records of this species (e.g., by Timdal 2008b, 2011) belong in another species, *P. neotinica*. *Phyllopsora chodatinica* (Fig. 3B) is new to Malaysia, New Caledonia, and Vanuatu.

Phyllopsora cinchonarum (Fée) Timdal

Description. Brako (1989, as *Squamacidia janeirensis*), Timdal (2008b), Elix (2009, as *Triclinum cinchonarum*).

Distribution. Central and South America (Brako 1989; Timdal 2008b), Asia, Australia (Elix 2009).

Remarks. The species is recognized by the squamulose thallus on a white prothallus, long isidia, and the presence of lobaric acid (Fig. 3C). Several additional com-

Figure 3. Species of *Phyllopsora* occurring in Asia and Melanesia. A *Phyllopsora castaneocincta* (Kirika, Mugambi, & Lumbsch 3011) B *P. chodatinica* (Paukov 2232) C *P. cinchonarum* (Thor 21521). Scale bars: 2 mm.

pounds are reported, for example atranorin, fumarprotocetraric acid, and a scarlet pigment. In our Asian material, we have encountered only lobaric acid (always major), atranorin (minor to absent), and some unknown compounds (minor to absent). It is the phylogenetic sister to the Neotropical *P. concinna* (Fig. 1).

Phyllopsora confusa Swinscow & Krog

Description. Swinscow and Krog (1981), Timdal and Krog (2001), Elix (2009).

Distribution. Pantropical (Brako 1991; Timdal and Krog 2001; Elix 2009).

Remarks. This species is characterized by the small, lacinulate squamules lacking lichen substances (Fig. 4A), but may be difficult to separate from, for example, *P. foliata* and *P. mediocris*. It is also possible that some of the specimens we have left undetermined belong in this species. We have sequenced nine specimens from Asia and Melanesia (Table 2), in addition to the holotype from Kenya, and those specimens make up the core in our concept of this species. The accessions of *P. confusa* form a strongly supported clade with *P. loekoesii* in the phylogenetic tree (Fig. 1). The two specimens from Papua New Guinea (6360 and 6361) fall in between the *P. confusa* and *P. loekoesii* clade (Suppl. material 1: Fig. S1) and show an intermediate morphology. Further specimens are needed to investigate the possible synonymy of these two species. *Phyllopsora confusa* is new to Indonesia, Japan, Malaysia, Taiwan, and Thailand.

Phyllopsora cuyabensis (Malme) Zahlbr.

Description. Timdal (2008b).

Distribution. Central and South America (Brako 1991; Timdal 2008b), Asia.

Remarks. The species is represented by a single specimen from Thailand in our material (Fig. 4B). The Asian accession (450) falls into a strongly supported clade with sequences from four Neotropical specimens, although resolved on a long branch as sister to all Neotropical accessions (Suppl. material 1: Fig. S1). Being morphologically identical to the Neotropical specimens, it is unclear whether this specimen (450) represents a new species or merely genetic variation within *P. cuyabensis*. Additional sequences of Asian specimens are necessary to evaluate this possibility further. The species is sister to a clade comprising *P. byssiseda*, *P. fendleri* and *P. kalbii* (Fig. 1). The species is new to Asia.

Phyllopsora dolichospora Timdal & Krog

Description. Timdal and Krog (2001).

Distribution. Africa (Timdal and Krog 2001), Asia.

Remarks. This species (Fig. 4C) is morphologically and chemically (furfuraceic acid) similar to *P. furfuracea*, to which it is closely related (Fig. 1), but differs in forming

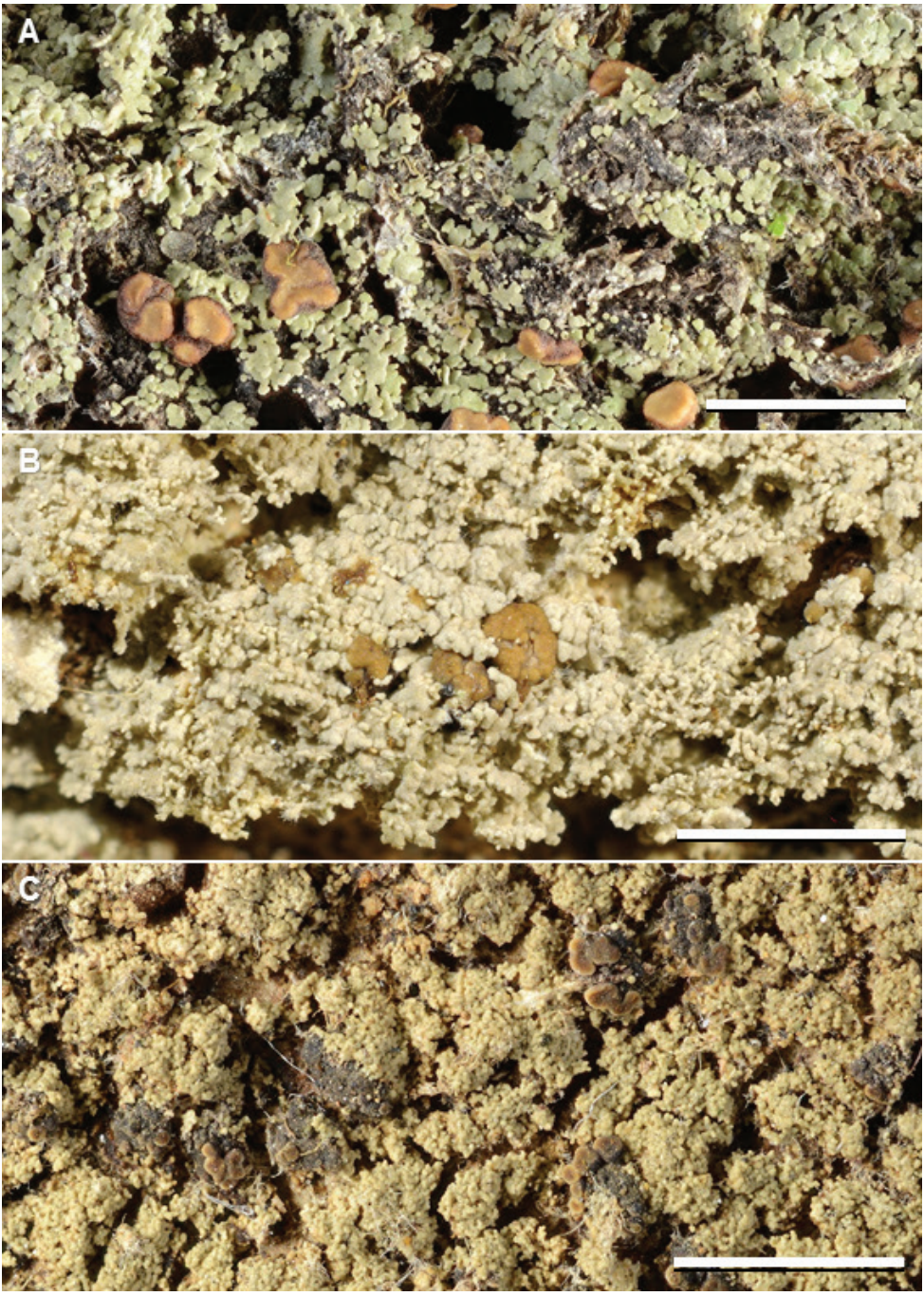

Figure 4. Species of *Phyllopsora* occurring in Asia and Melanesia. **A** *Phyllopsora confusa* (Kistenich & Weerakoon SK1-532) **B** *P. cuyabensis* (Aguirre, James & Wolseley 2467a) **C** *P. dolichospora* (Weerakoon Si113B). Scale bars: 2 mm.

longer ascospores and in containing additional substances (methyl furfuraceiate and methyl homofurfuraceiate). Judging from the number of examined specimens (Suppl. material 2: Table S1), *P. dolichospora* seems to be more common than *P. furfuracea* in Asia, although the number of reports (Table 1) suggests the opposite. This, however, might be a result of morphological misidentifications when TLC has not been run. The species is new to Japan and Papua New Guinea.

Phyllopsora foliata (Stirt.) Zahlbr.

Description. Elix (2009).

Distribution. Asia, Australia (Elix 2009).

Remarks. This rarely reported Australian species (Fig. 5A) is here confirmed from Japan and Sri Lanka mainly based on our DNA sequences (both mtSSU and ITS), which were compared with sequences obtained from Australian material (Table 2). It is new to Japan.

Phyllopsora furfuracea (Pers.) Zahlbr.

Description. Timdal and Krog (2001), Timdal (2008b), Elix (2009).

Distribution. Pantropical (Brako 1991; Timdal and Krog 2001; Elix 2009).

Remarks. Despite widespread reports in the literature, we were able to confirm the presence of this species (Fig. 5B) in Papua New Guinea, Sri Lanka, and Thailand, only. In the phylogenetic tree, *P. furfuracea* forms a clade with *P. dolichospora* and *P. foliatella* (Fig. 1).

Phyllopsora gossypina (Sw.) Kistenich, Timdal, Bendiksby & S. Ekman

Description. Hue (1909).

Distribution. Apparently pantropical.

Remarks. The species (Fig. 5C) was included in the genus *Crocynia* until recently (Kistenich et al. 2018b), and not originally a part of our taxon sampling; hence the few specimens examined. The accession from Sri Lanka (7201) clusters together with specimens of *C. molliuscula* (Suppl. material 1: Fig. S1), from which it is morphologically and chemically different. Further specimens need to be investigated to inform about its relationship to *C. molliuscula*. The species is the phylogenetic sister to *P. imshaugii* (Fig. 1).

Phyllopsora halei (Tuck.) Zahlbr.

Description. Swinscow and Krog (1981, as *P. pannosa*), Timdal and Krog (2001).

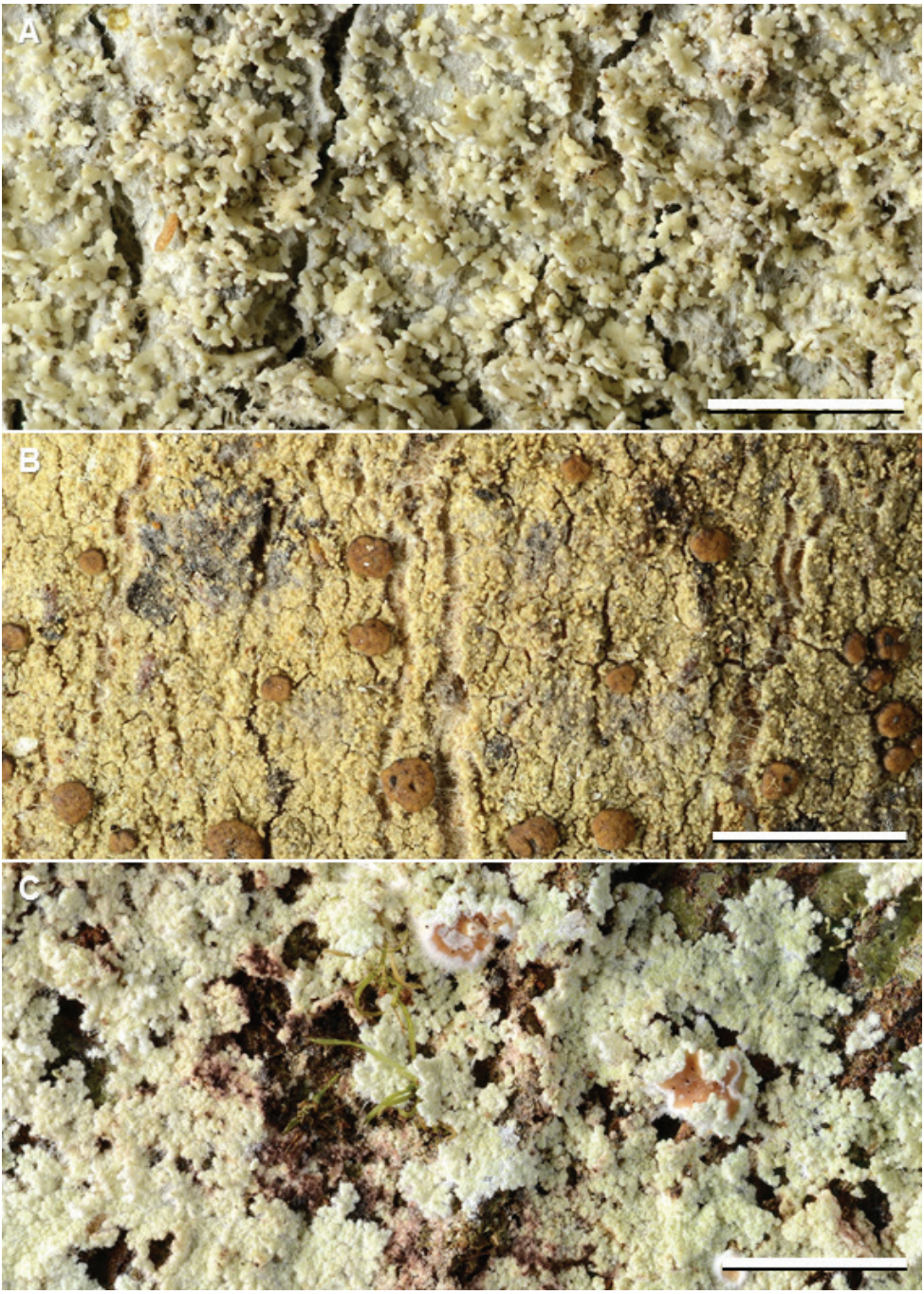

Figure 5. Species of *Phyllopsora* occurring in Asia and Melanesia. **A** *Phyllopsora foliata* (Kistenich & Weerakoon SK1-627) **B** *P. furfuracea* (Wolseley & Aguirre-Hudson 4025) **C** *P. gossypina* (Kistenich & Weerakoon SK1-524). Scale bars: 2 mm.

Distribution. North America (Brako 1991), Africa (Timdal and Krog 2001), Asia.

Remarks. This species (Fig. 6A) was previously known from the type collection from North America (Louisiana), East Africa (Ethiopia, Kenya, Tanzania), and a few reports from Asia (Table 1). We here confirm its presence in Asia, based on DNA sequences from material from Sri Lanka compared with sequences from Kenya and Tanzania (Suppl. material 1: Fig. S1). Three chemotypes of this species are known (Timdal and Krog 2001), differing in terpenoid patterns and presence of an unknown compound. Our two specimens from Sri Lanka belong in chemotype 3 of Timdal and Krog (2001). The species is the phylogenetic sister to *P. amazonica* (Fig. 1). It is new to Sri Lanka.

Phyllopsora himalayensis G.K. Mishra, Upreti & Nayaka

Description. Mishra et al. (2011).

Distribution. India (Mishra et al. 2011).

Remarks. The species was not studied by us due to lack of response from LWG to our repeated loan requests.

Phyllopsora isidiosa Kistenich & Timdal

Description. Kistenich et al. (in press).

Distribution. Pantropical, also occurring in temperate Asia and North America (Kistenich et al. in press).

Remarks. The species (Fig. 6B) is treated in detail by Kistenich et al. (in press). It is closely related to, for instance, *P. dolichospora*, *P. foliatella*, and *P. furfuracea* (Fig. 1).

Phyllopsora kalbii Brako

Description. Brako (1991), Timdal and Krog (2001).

Distribution. North, Central, and South America (Brako 1991), Africa (Timdal and Krog 2001), Asia.

Remarks. The species (Fig. 6C) was reported from India by Mishra et al (2011), and we confirm its presence in Asia by DNA sequences (mtSSU and ITS; 456) from material from Thailand. The species is sister to a clade comprising *P. byssiseda* and *P. fendleri* (Fig. 1). It is new to Thailand.

Phyllopsora loekoesii S.Y. Kondr., E. Farkas, S.–O. Oh & Hur

Description. Kondratyuk et al. (2016).

Distribution. Asia.

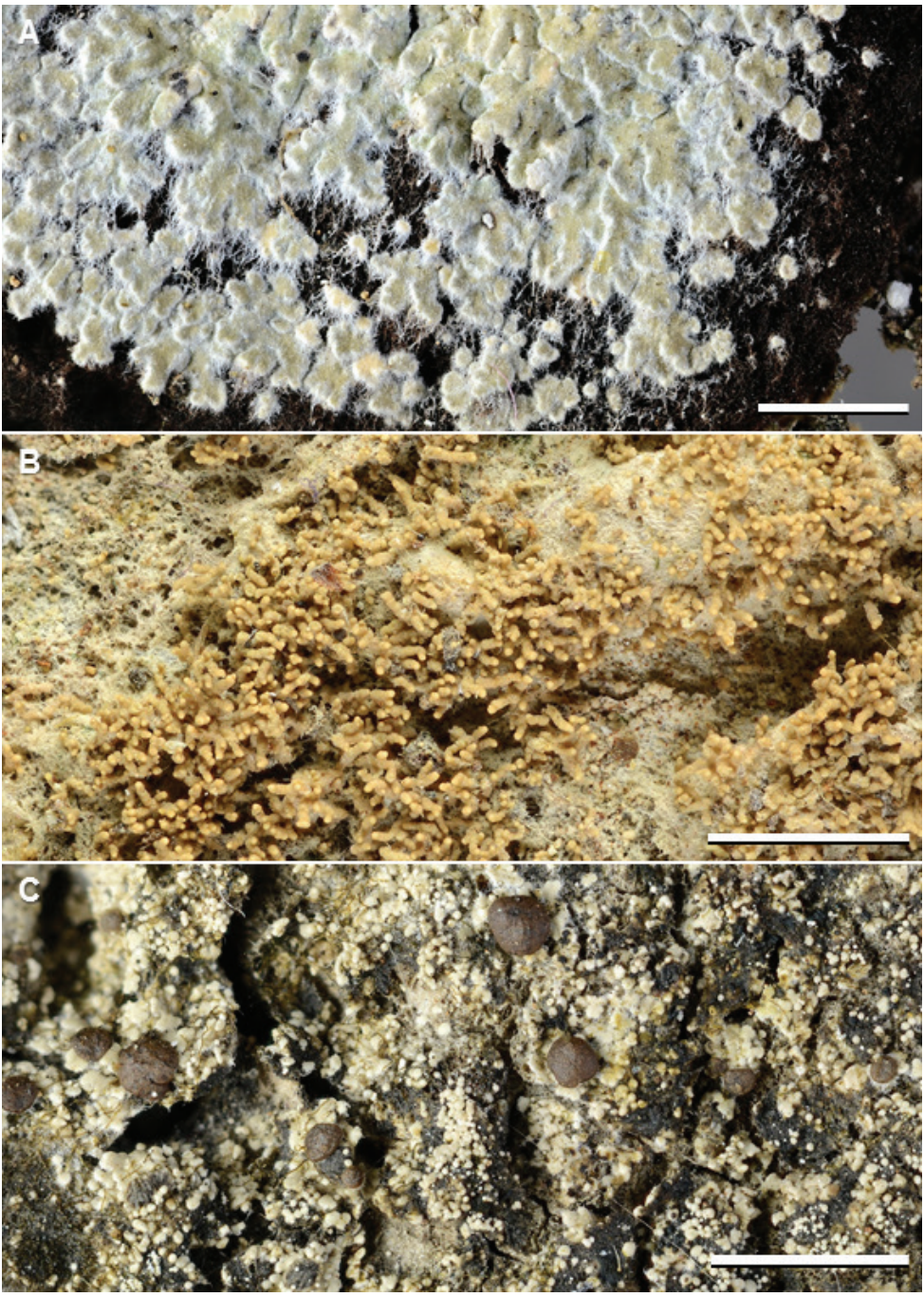

Figure 6. Species of *Phyllopsora* occurring in Asia and Melanesia. **A** *Phyllopsora halei* (Weerakoon 1008) **B** *P. isidiosa* (Diederich 13210) **C** *P. kalbii* (Aguirre, James & Wolseley 2695). Scale bars: 2 mm.

Remarks. The species (Fig. 7A) was recently described from South Korea by Kondratyuk et al. (2016), and we report it as new to Japan and Nepal. Our sequences were compared to unpublished sequences of the holo- and isotype kindly provided to us by Sergey Kondratyuk. Our accessions form a strongly supported clade together with accessions of *P. confusa* (Fig. 1), from which it is difficult to distinguish. See also remarks for *P. confusa*.

Phyllopsora longiuscula (Nyl.) Zahlbr.

Description. Brako (1991).

Distribution. Central and South America (Brako 1991), Asia, Australia (Kistenich et al. in press).

Remarks. In the concept of Brako (1991) and Timdal (2011), this species is lacinulate. Kistenich et al. (in press), however, extend the concept to include the isidiate species *P. intermediella*, which they synonymize. The Asian material we have examined is lacinulate. The species (Fig. 7B) is the phylogenetic sister to *P. thaleriza* (Fig. 1). It is new to Asia (Sri Lanka, Thailand, and Vietnam).

Phyllopsora mediocris Swinscow & Krog

Description. Swinscow and Krog (1981), Timdal and Krog (2001).

Distribution. Africa (Timdal and Krog 2001), Asia.

Remarks. The species (Fig. 7C) was previously known from East Africa and the Mascarenes (Timdal and Krog 2001). Although not sequenced, we here report it as new to Asia based on a specimen (Moberg 2750, UPS) from Sri Lanka (Suppl. material 2: Table S1). The species is the phylogenetic sister to *P. parvifolia* (Fig. 1).

Phyllopsora neofoliata Elix

Description. Elix (2006, 2009).

Distribution. Africa (Kistenich et al. in press), Asia, Australia (Elix 2009).

Remarks. This originally Australian species is reported as new to Africa (Kenya) by Kistenich et al. (in press) and here as new to Asia (Sri Lanka; Fig. 8A). Both the African and Sri Lankan specimens were sequenced (mtSSU and ITS) and found to conform with sequences of an isotype (O L-1319). The species is generally identified by the squamulose, lacinulate thallus containing furfuraceic acid.

Phyllopsora parvifolia (Pers.) Müll. Arg.

Description. Elix (2009).

Figure 7. Species of *Phyllopsora* occurring in Asia and Melanesia. **A** *Phyllopsora loekoesii* (Sharma, Olley & Cross AC5) **B** *P. longiuscula* (Weerakoon Kn136) **C** *P. mediocris* (holotype, Moberg 1481a-1, Tanzania). Scale bars: 2 mm.

Figure 8. Species of *Phyllopsora* occurring in Asia and Melanesia. **A** *Phyllopsora neofoliata* (Weerakoon WL21) **B** *P. parvifolia* (Kistenich & Weerakoon SK1-661) **C** *P. parvifoliella* (James & Wolseley 2491). Scale bars: 2 mm.

Distribution. Pantropical, but mainly Neotropical, extending into the temperate zones in North and South America and in Europe (Brako 1991, as *P. parvifolia* var. *parvifolia*; Kistenich et al. in press).

Remarks. Despite several reports from Asia and Melanesia (Table 1), we have seen only a single specimen of this species (Sri Lanka, Kistenich & Weerakoon SK1-661, PDA, not sequenced; Fig. 8B) from the area. The species is the phylogenetic sister to *P. mediocris* (Fig. 1). It is new to Sri Lanka.

Phyllopsora parvifoliella (Nyl.) Müll. Arg.

Description. Timdal (2008b).

Distribution. Central and South America (Brako 1991; Timdal 2008b), Asia.

Remarks. This squamulose, isidiate species contains atranorin and parvifoliellin (Fig. 8C); characters it shares with *P. concinna* and *P. rappiana*. The molecular phylogeny (Fig. 1) shows that the three species are not closely related, though; rather *P. parvifoliella* belongs in a clade together with *P. africana*, *P. ochroxantha*, and *P. swinscowii*. We have sequenced material from Indonesia and Thailand, and here report the species as new to Asia and Melanesia, i.e. from Indonesia, Papua New Guinea, The Philippines, and Thailand.

Phyllopsora porphyromelaena (Vain.) Zahlbr.

Description. Timdal and Krog (2001), Elix (2009), both as *P. albicans*.

Distribution. Pantropical (Brako 1991, as *P. buettneri* var. *glauca* chemical stains I and III; Timdal and Krog 2001; Elix 2009).

Remarks. This is the most common species in our material from Asia and Melanesia (Fig. 9A), despite previous records only from India, The Philippines, and Taiwan (mostly as *P. albicans*). Two chemotypes are previously recognized, and a third is recognized here. Chemotype 1 (argopsin and norargopsin) and chemotype 2 (argopsin and pannarin) are both widely distributed in Asia and Melanesia, but chemotype 3 (zeorin and three unknown compounds) is restricted to Thailand (Suppl. material 2: Table S1). The unknown compounds move in R_f-classes A:3–4, B':4–5, C:5 (major compound); A:6, B':6, C:5–6 (minor compound); and A:3, B':3, C:5 (minor compound).

In the phylogenetic tree (Fig. 1, Suppl. material 1: Fig. S1), accessions of chemotypes 1 and 2 group into a weakly supported clade with *P. buettneri*, while accessions of chemotype 3 form a clade with *P. chodatinica* and the *P. buettneri*/*P. porphyromelaena* clade. Additional specimens of chemotype 3 should be sequenced to find out whether it indeed represents a chemical strain of *P. porphyromelaena* or rather a distinct species.

The species is morphologically very similar to *P. sabahana*; see that species for discussion. It is possible that some specimens listed as *P. porphyromelaena* chemotype 1 in Suppl. material 2: Table S1, especially those from Malaysia, represent *P. sabahana*. It is new to Fiji, Indonesia, Japan, Malaysia, New Caledonia, Papua New Guinea, South Korea, Sri Lanka, and Thailand.

Phyllopsora pseudocorallina **Kistenich & Timdal, sp. nov**
MycoBank: MB829572
Fig. 9B

Diagnosis. Differs from *P. corallina* in having a more rosulate thallus and in substitutions in the mtSSU and ITS sequences.

Type. CAMBODIA, *Siem Reap*: around Ta Nei temple, Angkor Wats complex, 13°27'N, 103°53'E, ca. 30 m alt., on rock (sand stone), 2005-12-20, H. Kashiwadani 47806 (TNS!—holotype) [TLC: no lichen substances; DNA: MK412430 (mtSSU), MK412491 (ITS)].

Description. Thallus effuse or forming irregular rosettes up to 1 cm diam., squamulose; squamules medium sized, up to 1 mm wide, adnate to ascending, elongate, contiguous or partly imbricate, crenulate to incised, plane to weakly convex, medium green, glabrous on the upper side, faintly pubescent along the margin; isidia common, attached marginally to the squamules, cylindrical, simple or slightly branched, up to 0.1 mm wide and 0.6 mm long; upper cortex formed by thick-walled hyphae with rounded lumina (type 2), 20–30 µm thick; cortex and medulla not containing crystals (PD–, K–); prothallus indistinct to partly well developed, white.

Apothecia common, up to 1.5 mm diam., rounded when young, later often becoming irregular, simple or sometimes somewhat conglomerate, plane to moderately convex, yellowish to medium brown, with an indistinct, usually slightly paler, glabrous to finely pubescent margin; excipulum pale brown to colourless, K–; hypothecium pale brown to colourless, K–; epithecium colourless; no crystals in apothecium; ascospores narrowly ellipsoid to shortly bacilliform, simple, 6–10 × 2.5–3 µm (n=30). Conidiomata not seen.

Chemistry. No lichen substances.

Distribution. Cambodia, Malaysia, Papua New Guinea, The Seychelles.

Etymology. The specific epithet refers to its morphological and chemical similarity to *P. corallina*.

Remarks. The species is morphologically, anatomically, and chemically very similar to *P. corallina*. There is, however, a tendency of *P. pseudocorallina* being more rosulate, i.e., composed of more radiating and elongated marginal lobes. The phylogenetic tree (Fig. 1), however, shows the two species not to be closely related: *P. corallina* is resolved in a clade with *P. glaucella* and *P. rappiana* as sister to *P. martinii*, while *P. pseudocorallina* appears in a clade with *P. castaneocincta* and *P. neofoliata* among others (Fig. 1). The new species is widely distributed in Asia and also found on The Seychelles. It is unclear whether *P. corallina* occurs in Asia at all or if previously reported specimens of *P. corallina* rather represent specimens of *P. pseudocorallina*.

Additional specimens examined. MALAYSIA, *Sabah*: Malaysian Borneo, Maliau, "Knowledge Trail", pristine lowland dipterocarp forest; on stem (ca. 20 m high) of fallen tree on crushed bridge, 2012, H. Thüs, P. Wolseley & C. Vairappan M001a (BORH) [DNA: MK412437 (mtSSU)]; same locality data, H. Thüs, P. Wolseley & C. Vairappan M001b (BORH); same locality data, H. Thüs, P. Wolseley & C. Vairappan M005 (BORH) [DNA: MK412438 (mtSSU)]. PAPUA NEW GUINEA,

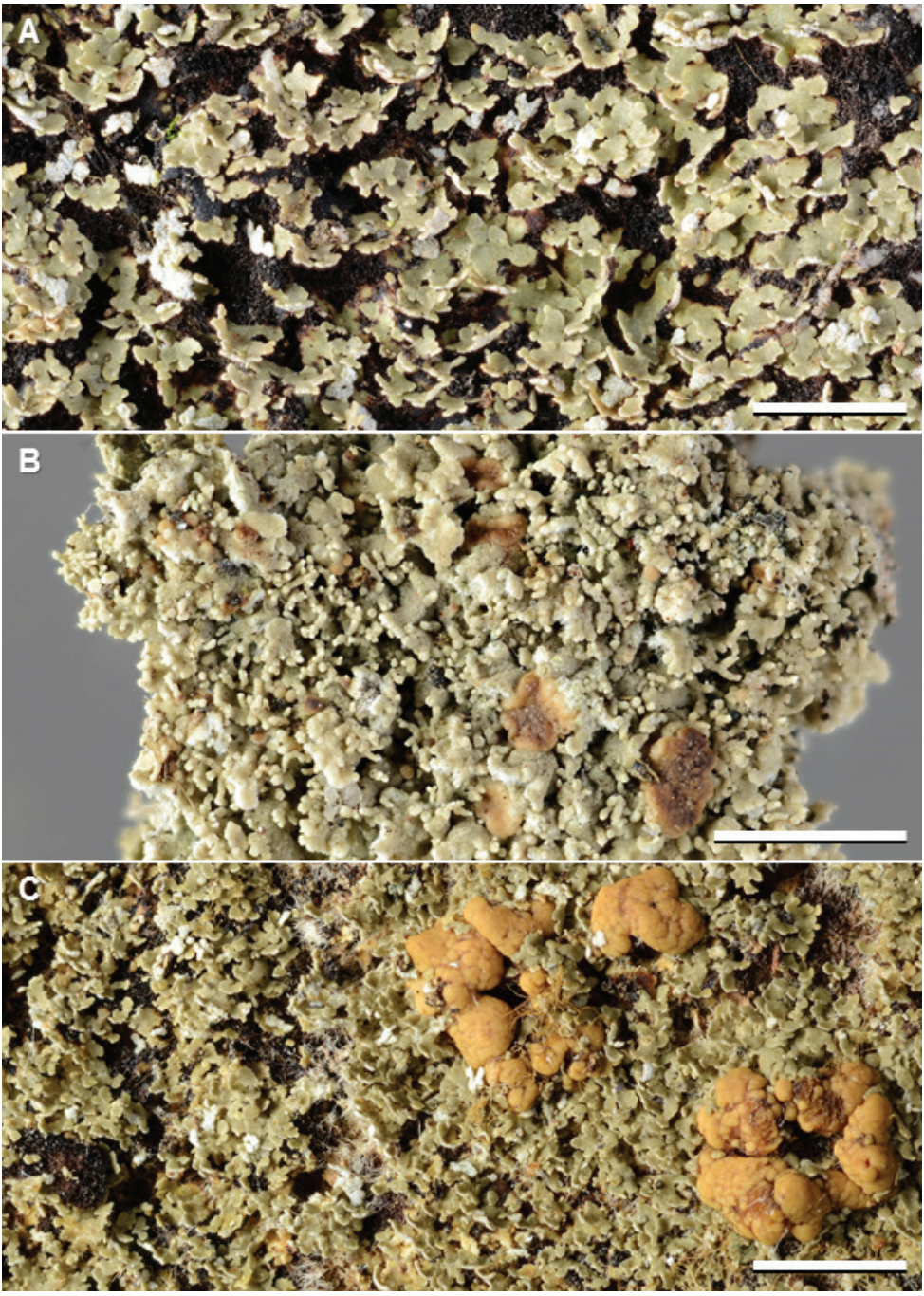

Figure 9. Species of *Phyllopsora* occurring in Asia and Melanesia. **A** *Phyllopsora porphyromelaena* (Kistenich & Weerakoon SK1-572) **B** *P. pseudocorallina* sp. nov. (holotype, Kashiwadani 47806) **C** *P. sabahana* sp. nov. (holotype, Wolseley, Thüs & Vairappan S.B.oQ.3). Scale bars: 2 mm.

Madang: Manam island, near Bogia, in gardens near Budua, 4°07'S, 145°00'E, 50 m alt., epiphytic, 1992-07-22, P. Diederich 11386 (hb. Diederich) [DNA: MK412449 (mtSSU), MK412495 (ITS)]. THE SEYCHELLES, *Mahé*: W of Anse Royale, Le Jardin du Roi, 4.74642S, 55.50297E, 150–200 m alt., parkland and neighbouring forest, on rock, 2015-07-26, P. Diederich 17809 (hb. Diederich); Port Glaud, near Sauzier Waterfall, 4.65847S, 55.41403E, 20–70 m alt., on tree, 2015-07-28, P. Diederich 17897 (hb. Diederich).

Phyllopsora sabahana **Kistenich & Timdal, sp. nov.**
MycoBank: MB829571
Fig. 9C

Diagnosis. Differs from *P. porphyromelaena* in having smaller ascospores and in substitutions in the mtSSU and ITS sequences.

Type. MALAYSIA, *Sabah*: Malaysian Borneo, SAFE-project Area, mostly Macaranga dominated secondary forest, 2012, P. Wolseley, H. Thüs & C. Vairappan S.B.oQ.3 (BORH!—holotype) [TLC: argopsin (major), norargopsin (minor); DNA: MK412434 (mtSSU)].

Description. Thallus effuse, squamulose; squamules medium sized, up to 0.8 mm wide, ascending, elongated, often imbricate, incised to deeply divided, plane to weakly convex; upper side pale green to medium green, glabrous, epruinose; margin concolorous with upper side, often finely pubescent; lacinules common, developing from lobe-tips; upper cortex formed by thick-walled hyphae with cylindrical lumina (type 1), 30–40 µm thick, containing crystals dissolving in K (PD+ orange, K–); medulla containing crystals partly dissolving in K (PD+ orange, K–); prothallus well developed, reddish brown.

Apothecia not common, up to 2 mm diam., rounded to irregular, simple or soon becoming conglomerate, weakly to moderately convex, yellowish brown, more or less immarginate even when young; excipulum pale brown to colourless, K–; hypothecium medium brown, K–; epithecium pale brown to colorless; no crystals in apothecia; ascospores narrowly ellipsoid, simple, 6–8 × 2–2.5 µm (n=20). Conidiomata not seen.

Chemistry. Argopsin (major), norargopsin (minor). Medulla and upper cortex PD+ orange, K–, C–, KC–.

Distribution. Malaysia (Borneo).

Etymology. The specific epithet refers to its occurrence in Sabah, Malaysia.

Remarks. The species is morphologically and chemically very similar to *P. porphyromelaena* chemotype 1, and is close to be regarded as a morphologically cryptic species. It may, however, be distinguished in forming smaller ascospores (6–8 × 2–2.5 vs. 8–13 × 2–4 µm). Apothecia are not common in neither species, however, and the measurements are based on only 20 spores from each species (the holotype of *P. sabahana* and two specimens of *P. porphyromelaena* from La Réunion). In the phylogenetic

tree (Fig. 1), the five accessions of *P. sabahana* form a strongly supported clade as sister to the Neotropical species *P. neotinica*, from which it may readily be distinguished in its composition of lichen substances (*P. neotinica* contains xanthones). So far, *P. sabahana* is only known from Borneo.

Additional specimens examined. MALAYSIA, *Sabah*: Malaysian Borneo: Maliau Basin, surroundings of Agathis Camp, pristine lowland Dipterocarp forest, 2012, P. Wolseley, H. Thüs & C. Vairappan, C. M089 (BORH) [DNA: MK412441 (mtSSU)]; Danum valley, pristine lowland Dipterocarp forest, 2012, P. Wolseley, H. Thüs & C Vairappan D.8.02.4 (BORH) [DNA: MK412442 (mtSSU)]; Ranau district, Kinabalu park, Tambuyukon trail, Kera camp (loc. T089), 6°12.742'N, 116°43.609'E, 728 m alt., epiphytic, 2014-12-08, A. Paukov 2229 (B) [DNA: MK412455 (mtSSU), MK412498 (ITS)] & 2230 (B) [DNA: MK412453 (mtSSU), MK412496 (ITS)].

Phyllopsora santensis (Tuck.) Swinscow & Krog

Description. Timdal (2008b), Elix (2009).

Distribution. North, Central, and South America (Brako 1991, as *P. corallina* var. *santensis*; Timdal 2008b), Asia, Australia (Elix 2009).

Remarks. The species was previously reported from Japan, Papua New Guinea, and The Philippines (Table 1), and is here reported from four localities in Thailand (Fig. 10A). We were unable to produce DNA sequences from our material, and the identification is based on typical morphology and presence of argopsin (major) and noragopsin (minor). New to Thailand.

Phyllopsora siamensis Kistenich & Timdal, sp. nov.

MycoBank: MB829573
Fig. 10B

Diagnosis. Differs from *P. imshaugii* in having more well developed squamules, larger ascospores, and in substitutions in the mtSSU and ITS sequences.

Type. THAILAND, *Lampang*: Doi Khun Tan National Park, loc. T118, 18°25'N, 99°14'E, 1000 m alt., hill evergreen forest, 1993-01-11, P.A. Wolseley & K. Boonpragob 3245 (BM 749856!—holotype) [TLC: norstictic acid; DNA MK412410 (mtSSU), MK412477 (ITS)].

Description. Thallus effuse, crustose to squamulose; squamules small, up to 0.4 mm wide, adnate, isodiametrical, more or less scattered when young, later contiguous or fusing, more or less crenulate, plane to weakly convex; upper side medium green, somewhat shiny, epruinose, glabrous; margin concolorous with upper side, often pubescent; isidia common, attached marginally to the squamules, cylindrical, simple or slightly branched, up to 0.15 mm wide and 1.5 mm long; upper cortex formed by

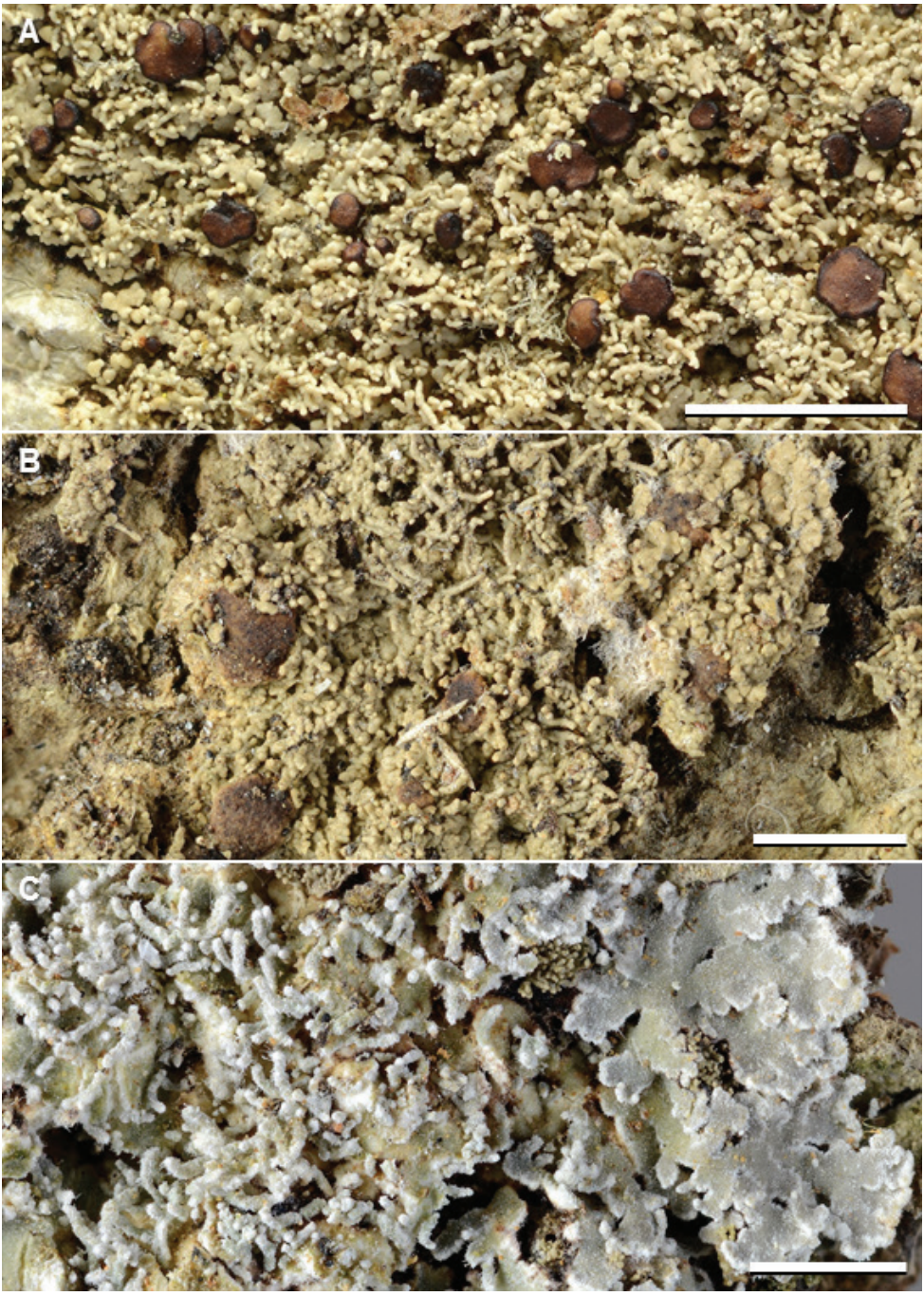

Figure 10. Species of *Phyllopsora* occurring in Asia and Melanesia. **A** *Phyllopsora santensis* (Aguirre, James & Wolseley 2485) **B** *P. siamenses* sp. nov. (holotype, Wolseley & Boonpragob 3245) **C** *P. subhispidula* (Weerakoon 1248). Scale bars: 2 mm.

thick-walled hyphae with rounded lumina (type 2), 15–30 µm thick, containing a few scattered crystals dissolving in K; medulla containing crystals dissolving in K and recrystallizing by forming acicular, red crystals, PD+ yellow, K+ red; prothallus well developed, thick, white.

Apothecia seen in the holotype only, up to 1.5 mm diam., more or less plane when young, soon becoming weakly to moderately convex, medium brown, rounded to irregular, simple, when young with a rather thick, paler, weakly pubescent margin, later becoming more or less immarginate; excipulum pale brown in the rim, darker brown in inner part; hypothecium dark brown, K–; crystals present in inner part of exciple and in hypothecium, dissolving in K and recrystallizing by forming acicular, red crystals; epithecium pale brown to colourless, K–; ascospores narrowly ellipsoid or fusiform to bacilliform, simple, 15–22 × 3.5–4.5 µm (n=20). Conidiomata not seen.

Chemistry. Norstictic acid (major), atranorin (minor to trace or absent). Medulla PD+ yellow, K+ red, C–, KC–.

Distribution. Thailand.

Etymology. The specific epithet refers to its occurrence in Thailand.

Remarks. The species is morphologically and chemically very similar to *P. imshaugii*. *Phyllopsora siamensis*, however, may be distinguished by forming slightly larger squamules and longer ascospores (15–22 × 3.5–4.5 vs 10.5–14.5 × 3–4 µm; the latter measurements are based on 40 spores in the type material from Jamaica) than *P. imshaugii*. So far, *P. imshaugii* is only known to occur in the Neotropics, while *P. siamensis* is solely known from Thailand. In the phylogenetic tree (Fig. 1), the four accessions of *P. siamensis* cluster in a strongly supported clade as sister to a clade comprising *P. cinchonarum* and *P. concinna*, from which the new species is readily distinguished by its chemistry. *Phyllopsora imshaugii* and *P. siamensis* are the only *Phyllopsora* species known to contain norstictic acid; the major compound of the two other species are lobaric acid and parvifoliellin, respectively.

Additional specimens examined. THAILAND, *Chiang Mai*: Doi Suthep National Park headquarters walk, loc. 62.4, 18°48'N, 98°54'E, 1050 m alt., tropical mixed deciduous forest, on Lauraceae, 1993-03-27, B. Aguirre–Hudson & P.A. Wolseley 1643 (BM 749866) [DNA: MK412419 (mtSSU), MK412485 (ITS)]; *Uthai Thani*: Khao Nang Rum, Cathouse site, 15°29'N, 99°18'E, 650 m alt., tropical mixed deciduous forest, 1992-01-07, P.A. Wolseley & B. Aguirre–Hudson 5580 p.p. (BM 1031552 p.p.) [DNA: MK412423 (mtSSU), MK412487 (ITS)]; Khao Nang Rum, Khao Kiew, 15°27'N, 99°20'E, 1250 m alt., oak/chestnut forest, 1992-01-23, P.A. Wolseley and Onsar 5590 (BM 749833) [DNA: MK412418 (mtSSU), MK412484 (ITS)].

Phyllopsora subhispidula (Nyl.) Kalb & Elix

Description. Timdal and Krog (2001).

Distribution. Africa (Timdal and Krog 2001), Asia.

Remarks. This species resembles closely the more common *P. buettneri*, but differs in forming isidia, not lacinules (Fig. 10C). It contains argopsin (major), norargopsin (minor), zeorin (major), and sometimes atranorin (trace), similar to chemotype 4 of *P. buettneri*. Phylogenetically (Fig. 1), the two species are not closely related, though. *Phyllopsora subhispidula* is sister to the clade comprising *P. nemoralis* and *P. hispaniolae*/*P. rosei* (Fig. 1). It is new to Asia (Sri Lanka).

Key to the phyllopsoroid genera in Asia and Melanesia

1	Apothecia zeorine, surrounded by a thalline sheath ..... ***Physcidia* p.p.**
–	Apothecia biatorin **2**
2(1)	Tholus non-amyloid or with an indistinct conical amyloid structure; ascospores filiform, spirally arranged in ascus; thallus and apothecia with red or purple patches caused by non-crystalline, acetone-insoluble pigment ..... ***Krogia***
–	Tholus with a distinct amyloid conical structure (*Bacidia* type); ascospores ellipsoid to filiform, not spirally arranged in ascus; thallus and apothecia without red patches **3**
3(2)	Upper and lower cortices formed by a single layer of isodiametric cells, continuous over the edge of the areolae/squamule ..... ***Eschatogonia***
–	Upper cortex multicellular or poorly differentiated; lower cortex absent..... **4**
4(2)	Ascospores ellipsoid to fusiform, simple or rarely pseudoseptate..... **5**
–	Ascospores bacilliform to filiform, septate or pseudoseptate **6**
5(4)	Apothecia and prothallus blackish brown to black; isidia lacking; thallus containing fumarprotocetraric acid..... ..... **'*Phyllopsora*' cfr. *nigrocincta* (Malmideaceae)**
–	Apothecia brown; prothallus white to dark reddish brown; isidia present or absent; if fumarprotocetraric acid present, then isidia present .... ***Phyllopsora***
6(4)	Thallus sorediat..... **7**
–	Thallus not sorediate..... **8**
7(6)	Squamules mostly adnate, bursting into convex soralia, containing atranorin and divaricatic acid ..... ***'Phyllopsora' sorediata***
–	Squamules ascending, with labriform soralia, containing methyl barbatate and often terpenoids ..... ***'Phyllopsora' glaucescens***
8(6)	Thallus large, subfoliose, isidiate ..... ***'Physcidia' cylindrophora***
–	Thallus crustose to squamulose, not isidiate **9**
9(8)	Thallus formed by ascending squamules, lacinulate, containing stictic acid... ..... ***Parallopsora* sp.**[*]
–	Thallus crustose or formed by adnate squamules, not lacinulate, not containing lichen substances..... **10**
10(9)	Thallus crustose ..... ***Sporacestra***
–	Thallus squamulose..... ***Aciculopsora***[*]

* unpublished data

Key to the species of *Phyllopsora* in Asia and Melanesia

1	Thallus pruinose, rosulate, broad-lobed **2**
–	Thallus not pruinose, effuse to rosulate, narrow to broad-lobed **3**
2(1)	Thallus lacinulate, containing pannarin, dechloropannarin or rarely argopsin ..... ***P. buettneri***
–	Thallus isidiate, containing argopsin ..... ***P. subhispidula***
3(1)	Upper cortex absent or poorly developed **4**
–	Upper cortex well developed **5**
4(3)	Species always apotheciate; isidia lacking; apothecia plain to concave, with a pale margin; lichen substances present ..... ***P. gossypina***
–	Species apotheciate or not; isidia often present; apothecia convex, more or less immarginate; lichen substances absent ..... ***P. cuyabensis***
5(3)	Medulla K+ red (norstictic acid) ..... ***P. siamensis***
–	Medulla K– **6**
6(5)	Medulla PD+ orange to red **7**
–	Medulla PD– **10**
7(6)	Prothallus white or absent; lacinules absent ..... ***P. santensis***
–	Prothallus brown; lacinules present or absent **8**
8(7)	Squamules isodiametrical or shortly elongate, more or less adnate, containing chlorophyllopsorin or methyl 2,7-dichloronorpsoromate; isidia or lacinules present ..... ***P. africana***
–	Squamules elongate, ascending, lacking chlorophyllopsorin and methyl 2,7-dichloronorpsoromate; lacinules present **9**
9(8)	Ascospores narrowly ellipsoid, 6–8 × 2–2.5 µm ..... ***P. sabahana***
–	Ascospores narrowly ellipsoid to fusiform or bacilliform, 8.0–12.5 × 2.5–3.5 µm ..... ***P. porphyromelaena***
10(6)	Thallus isidiate **11**
–	Thallus phyllidiate, lacinulate or apotheciate **20**
11(10)	Prothallus white or absent **12**
–	Prothallus brown **15**
12(11)	Isidia often more than 1 mm long, mainly simple; ascospores bacilliform to acicular, 26–41 × 2–3 µm; containing lobaric acid ..... ***P. cinchonarum***
–	Isidia shorter than 1 mm, globular to coralloid; ascospores ellipsoid to shortly bacilliform, less than 12 µm long; containing only atranorin or no lichen substance **13**
13(12)	Isidia globular; thallus containing atranorin; crystals present in medulla and hypothecium ..... ***P. himalayensis***
–	Isidia cylindrical to coralloid; thallus and apothecia lacking lichen substances and crystals **14**
14(13)	Isidia becoming coralloid; species crustose, effuse; areoles up to 0.1 mm diameter ..... ***P. isidiosa***
–	Isidia cylindrical or weakly branched; species squamulose, effuse or rosulate; squamules up to 1 mm diameter ..... ***P. pseudocorallina***

15(11) Thallus crustose, consisting of more or less scattered areoles or sometimes isidia only **16**
– Thallus squamulose **17**
16(15) Ascospores narrowly ellipsoid, 7–13 × 2–3 µm; containing furfuraceic acid only ..... ***P. furfuracea***
– Ascospores bacilliform, 16–25 × 2–3 µm; containing furfuraceic acid and 2–3 related compounds ..... ***P. dolichospora***
17(15) Prothallus thick, forming a cushion with colonizing areoles along the periphery **18**
– Prothallus thin, not forming a cushion **19**
18(17) Thallus containing atranorin and terpenoids ..... ***P. halei***
– Thallus containing furfuraceic acid or rarely no compounds ..... ***P. castaneocincta***
19(17) Isidia globular or shortly cylindrical; thallus pale green, containing atranorin or no lichen substances ..... ***P. kalbii***
– Isidia cylindrical; thallus dark green to brown, containing atranorin and parvifoliellin ..... ***P. parvifoliella***
20(10) Thallus containing xanthones ..... ***P. chodatinica***
– Thallus not containing xanthones **21**
21(20) Thallus containing furfuraceic acid ..... ***P. neofoliata***
– Thallus not containing lichen substances **22**
22(21) Prothallus brown, well developed **23**
– Prothallus white or absent **27**
23(22) Thallus rosulate or composed of elongated squamules **24**
– Thallus effuse, composed of more or less isodiametrical squamules **26**
24(23) Thallus phyllidiate; phyllidia mainly occurring in central part of thallus ..... ***P. parvifolia***
– Thallus lacinulate **25**
25(24) Squamules long, linear, deeply incised to branched ..... ***P. breviuscula***
– Squamules short, crenulate to narrowly incised ..... ***P. mediocris***
26(23) Thallus crustose, consisting of closely adnate areoles and ascending lacinules ..... ***P. longiuscula***
– Thallus squamulose, consisting of ascending squamules, breaking into lacinules ..... ***P. confusa***
27(22) Thallus phyllidiate; phyllidia mainly occurring in central part of thallus ..... ***P. parvifolia***
– Thallus lacinulate **28**
28(27) Squamules closely adnate, elongated, linear, somewhat branched ..... ***P. loekoesii***
– Squamules ascending, short, not branched **29**
29(28) Ascospores narrowly ellipsoid to fusiform, 11–20 × 2–3 µm ..... ***P. foliata***
– Ascospores narrowly ellipsoid to shortly bacilliform, 9–11 × 2–2.5 µm ..... ***P. confusa***

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Supplementary material 1

Uncollapsed version of the tree presented in Fig. 1

Authors: Sonja Kistenich, Mika Bendiksby, Charles S. Vairappan, Gothamie Weerakoon, Siril Wijesundara, Patricia A. Wolseley, Einar Timdal

Data type: phylogenetic data

Explanation note: Extended majority-rule consensus tree resulting from the MrBayes analysis of the mtSSU and ITS alignment with Bayesian PP ≥ 0.7 and/or IQ-TREE maximum likelihood BS ≥ 50 and branch lengths. Strongly supported branches (PP ≥ 0.95 and BS ≥ 75) are marked in bold; branches with PP ≥ 0.95 and BS < 75 or PP < 0.95 and BS ≥ 75 are marked in bold grey; branches only supported with PP ≥ 0.7 or BS ≥ 50 are marked with a dot above the branch. Two species of *Biatora* were used for rooting. Terminal names include DNA extraction number, species name and when relevant, chemotype (ch). Three clades are distinguished to facilitate the discussion of new species (A, B, C).

Link: https://doi.org/10.3897/mycokeys.53.33425.suppl1

Supplementary material 2

Specimens of *Phyllopsora* examined in this study

Authors: Sonja Kistenich, Mika Bendiksby, Charles S. Vairappan, Gothamie Weerakoon, Siril Wijesundara, Patricia A. Wolseley, Einar Timdal

Data type: specimens data

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PacBio amplicon sequencing for metabarcoding of mixed DNA samples from lichen herbarium specimens

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Abstract

The detection and identification of species of fungi in the environment using molecular methods heavily depends on reliable reference sequence databases. However, these databases are largely incomplete in terms of taxon coverage, and a significant effort is required from herbaria and living fungal collections for the mass-barcoding of well-identified and well-curated fungal specimens or strains. Here, a PacBio amplicon sequencing approach is applied to recent lichen herbarium specimens for the sequencing of the fungal ITS barcode, allowing a higher throughput sample processing than Sanger sequencing, which often required the use of cloning. Out of 96 multiplexed samples, a full-length ITS sequence of the target lichenised fungal species was recovered for 85 specimens. In addition, sequences obtained for co-amplified fungi gave an interesting insight into the diversity of endolichenic fungi. Challenges encountered at both the laboratory and bioinformatic stages are discussed, and cost and quality are compared with Sanger sequencing. With increasing data output and reducing sequencing cost, PacBio amplicon sequencing is seen as a promising approach for the generation of reference sequences for lichenised fungi as well as the characterisation of lichen-associated fungal communities.

Keywords

SMRT sequencing, high-throughput sequencing, long amplicon analysis (LAA), lichenised fungi

Introduction

Across the world, herbaria and fungal culture collections host large numbers of specimens and strains with the main goal being to document, preserve and classify the many species within the fungal kingdom. Among characters generally used to identify fungi, DNA sequences have been particularly useful for the numerous fungal species with plastic or convergent morphologies. Of the DNA markers traditionally used for fungal identification, the internal transcribed spacer region (ITS) was chosen as the primary barcode because it allowed species-level identification for the broadest range of fungi (Schoch et al. 2012). Following the formal acceptance of this fungal barcode, substantial effort was put into improving the curation of ITS sequences in several databases, including RefSeq (Schoch et al. 2014; O'Leary et al. 2015), UNITE (Kõljalg et al. 2013; Nilsson et al. 2018) and ISHAM-ITS (Irinyi et al. 2015), by selecting high-quality ITS sequences generated from reliably identified and preserved specimens. These reference databases play a critical role in the sequence-based species identification of herbarium specimens, cultured strains and fungal communities from environmental samples (Tedersoo and Nilsson 2016). However, linking sequences to names remains a challenge, partly because of the incompleteness of these reference datasets (Orock et al. 2012; Kõljalg et al. 2013; Crous et al. 2014; Nilsson et al. 2018). The molecular barcoding of herbarium specimens, including generic and species types therefore remains a priority (Crous et al. 2014; Yahr et al. 2016).

Although the characterisation of fungal diversity in environmental samples has benefited immensely from the development of next generation sequencing (NGS) technologies, the high-throughput generation of reference ITS sequences from herbarium specimens has been hindered by the short length of Illumina reads, the most commonly used NGS platform. With a size ranging from 500 up to 1200 bp, the full length of the ITS region cannot be sequenced as a single DNA fragment. For DNA extractions of lichen specimens, which harbor on their surface or within their thalli a plethora of other fungi, as well as for any other DNA samples of mixed fungal communities, assembling DNA markers from a pool of short reads belonging to several species carries a high risk of obtaining chimeric sequences (Hebert et al. 2018). As a consequence, fungal metabarcoding studies mostly use only half of the ITS region, either ITS1 or ITS2 (Nilsson et al. 2010; Mello et al. 2011; Blaalid et al. 2013). The generation of full length and high-quality barcodes from lichen specimens for reference databases or identification purposes generally involved Sanger sequencing (e.g., Kelly et al. 2011; Orock et al. 2012; Leavitt et al. 2014; Divakar et al. 2016; Xu et al. 2017). However, this method faces a similar challenge: that co-amplified non-lichenised fungal sequences often prevent the generation of readable target sequences (Kelly et al. 2011; Orock et al. 2012). Of the few available methods to separate sequences from target and non-target species (e.g., gel separation, group-specific primers), cloning has proven to be the most broadly applicable method for obtaining high quality sequences of the target lichenised fungus in mixed samples (Hofstetter et al. 2007). It does, however, significantly increase the cost and time required for generating high-quality sequences.

Long-read sequencing allows us to circumvent these challenges. For lichenised fungi, early long amplicon sequencing studies made use of Roche 454 pyrosequencing technology, either for the full ITS region (Hodkinson and Lendemer 2013; Mark et al. 2016) or for ITS1 only (Lücking et al. 2014). In Mark et al. (2016), with a target sequence recovered for 99 of the 100 samples studied, the application of this technology for lichen specimen metabarcoding seemed promising. However, the development of this sequencing technology was abandoned by Roche and is now obsolete. Long read sequencing using single molecule real-time (SMRT) sequencing technology (Pacific Biosciences, USA) is becoming more affordable, and has recently been applied to fungi for metabarcoding purposes (Chen et al. 2015; Cline and Zak 2015; James et al. 2016; Schlaeppi et al. 2016; Walder et al. 2017; Heeger et al. 2018). Some of the challenges usually associated with SMRT sequencing (high error rate, high rate of chimeric formation and high cost per sample) have been investigated and partly addressed. The high error rate of SMRT raw reads (about 15%; Goodwin et al. 2016) is significantly decreased due to the multiple passes obtained from a single polymerase read using circularised amplifications (Travers et al. 2010), as well as the correcting nature of high sequence coverage for randomly distributed errors (Koren et al. 2012). The error rate of circular consensus sequences (CCS) is now usually under 1% (Goodwin et al. 2016), and compares well with other sequencing platforms, including Illumina (Schlaeppi et al. 2016; Schloss et al. 2016) and Sanger (Goodwin et al. 2016). Using mock communities, the rates of formation of chimeric sequences (up to 16.3% in Heeger et al. 2018) were shown to be in the same range as the one obtained with short read sequencing (D'Amore et al. 2016). As for the cost per sample, it can be reduced thanks to multiplexing (Heeger et al. 2018) to a level where the cost of DNA extraction or PCR amplification becomes higher than the sequencing cost (Hebert et al. 2018).

The scarcity of automated pipelines to analyse SMRT amplicon data for different applications, including community analysis, remains an issue (Heeger et al. 2018; Tedersoo et al. 2018). Most software developed for sequencing-error correction and sequence assembly have been optimised for short reads and have not been evaluated for SMRT raw data (Heeger et al. 2018). During the SMRT sequencing primary analysis, image processing, base-calling and quality assessment are done in real-time on the instrument. Polymerase reads are then used to generate CCSs, which, in addition to the raw polymerase read files, are provided by the sequencing facility as fasta or fastq files. For community analyses, CCSs can then be analysed by various software or software packages, such as Mothur (Schloss et al. 2009) or PipeCraft (Anslan et al. 2017), in order to demultiplex, filter, cluster and assign consensus sequences to a taxonomic unit (see Anslan et al. 2018). Although PacBio (Pacific Biosciences) provides secondary analysis modules as part of the SMRT portal, none of these is specifically designed for community analysis. The Long Amplicon Analysis (LAA) pipeline available on SMRT Link and SMRT Portal attracted our interest, as it identifies differing clusters of sequencing reads within a single library and is capable of differentiating between underlying sequences that are 99.9% similar, such as haplotypes and pseudogenes (Bowman et al. 2014). The main application of LAA is allele phasing and detection

in diploid organisms (Bowman et al. 2014; Lleras et al. 2014) and it works as follows. Error-corrected subreads (CCS) obtained from polymerases reads are first demultiplexed (samples are separated using unique molecular indexes or combinations of indexes), then filtered depending on read quality and length. They are then aligned (overlap step) and clustered based on the alignment. Each cluster is iteratively phased in order to separate high-scoring mutations (alleles). Resulting sub-clusters are polished using a Quiver-based method to produce high-quality consensus sequences, which then go through a last filtering step to detect and remove PCR artefacts (e.g., chimeric sequences). This pipeline was designed to differentiate sequencing errors from true sequence variations, allowing the preservation of all sequence variants within a sample. For each index/sample barcode, the output includes all unique consensus sequences obtained, together with their coverage value and predicted accuracies. LAA can be performed on the instrument's SMRT portal, allowing the sequencing provider to send these demultiplexed consensus sequences directly to the customer, saving time and effort in software installation and raw data analyses.

In this study, the use of SMRT sequencing and the LAA pipeline for the production of ITS barcode sequences from lichen herbarium specimens was explored. The goals were: 1) to establish a high-throughput protocol to obtain indexed PCR products from lichen DNA extracts for SMRT sequencing; and 2) to investigate the ability of LAA to recover high-quality sequences for the target lichenised fungal species as well as for non-target fungal species.

Material and methods

DNA extractions

A total of 96 specimens were selected for their frequent need for cloning as well as their relevance to several ongoing taxonomic studies on Australian lichens at the Australian National Herbarium (see Suppl. material 1: Table S1). The genera represented were *Catillaria* (41 specimens from Australia and 16 from France), *Buellia* (39 specimens from Australia), *Endocarpon* (8 specimens from Australia) and *Verrucaria* (2 specimens from France). The specimens were 1–34 years old and are kept at CANB, MARSSJ and in the private herbarium of M. Bertrand. For crustose specimens (*Catillaria*, *Buellia* and *Verrucaria*), material (thallus and fruiting bodies) was detached from the substrate with a clean single-edge razor blade and a folded sheet of weigh paper was used to collect and transfer the material to a tube (8-strip cluster tubes, Corning Incorporated, Salt Lake City, USA). For *Endocarpon*, squamules were detached from the soil substrate using clean tweezers and transferred directly to a tube.

Each of the 96 cluster tubes contained a washed chrome steel bearing ball 3 mm in diameter (3MMCH/S/B grade 40, BSC Bearing & Power Transmission Solutions, Chullora, Australia). The samples were ground with a TissueLyser II (Qiagen, Hilden, Germany) in two cycles of 1 min and a frequency of 25/s. The cluster tubes were

centrifuged for 1 min at 6,000 rpm and the caps were removed with care to avoid cross-contaminations. A lysis buffer was added to each tube. Genomic DNA was extracted using the Invisorb® DNA Plant HTS 96 kit (STRATEC Molecular, Berlin, Germany) following the manufacturer's instructions, except for the last centrifugation step which was changed to 10 min at 2,000 rpm (instead of 5 min at 4,000 rpm) to avoid breaking the elution plates. A 1/10 dilution of the DNA extraction plate was prepared and subsequently used for amplification.

Amplification, normalisation and pooling

Indexed PCR products were generated using the PacBio Barcoded Universal Primers protocol (https://www.pacb.com/wp-content/uploads/2015/09/Procedure-and-Checklist-Preparing-SMRTbell-Libraries-PacB-Barcoded-Universal-Primers.pdf). The ITS barcode (internal transcribed spacer 1, 5.8S ribosomal RNA subunit and internal transcribed spacer 2) was the region targeted. With a first PCR, our target region was amplified using the primers ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990), both modified by adding a 5' block and a tail representing the PacBio universal sequences. In a 25 µl reaction, 5 µl of buffer, 1 µl of MyFi (Bioline, London, UK), 1 µl of 5 µM of each primer, 16 µl of water and 1 µl of DNA template (1/10 dilution) were added. The 96 PCR reactions were performed in strip tubes with individual caps to avoid cross-contaminations. The PCR programme was 5 min at 95 °C, then 20 cycles of 30 sec at 95 °C, 30 sec at 53 °C and 1:30 min at 72 °C, followed by a final elongation step for 7 min at 72 °C. Four strips were randomly selected and their PCR products run on to an agarose gel using the nucleic acid stain GelRed (Biotium, Fremont, CA, USA). Because the gel showed primer dimer bands in addition to the amplicon bands, the PCR products were cleaned using Sera-Mag magnetic beads (SpeedBead Magnetic Carboxylate Modified Particles, GE Healthcare) and a 96 well Alpaqua® magnetic plate. The cleaning was done by adding 0.8× volume of beads to each PCR product, followed by two washes with 200 µl of 70% ethanol. Dry beads were then resuspended in 25 µl of the elution buffer from the Invisorb® DNA Plant HTS 96 kit.

A second amplification was then performed using the Barcoded Universal F/R Primers Plate-96 available from PacBio (Millenium Science, Mulgrave, VIC, Australia). In a 25 µl reaction, 5 µl of buffer, 1 µl of MyFi, 2.5 µl of the barcoded primers, 15.5 µl of water and 1 µl of the product of the first round of PCR were added. The PCR programme was 3 min at 95 °C, then 20 cycles of 30 sec at 95 °C, 30 sec at 53 °C and 1:30 min at 72 °C, followed by a final elongation step for 7 min at 72 °C. All PCR products were checked on a gel as previously described. For samples showing no band, a new round of amplification (first PCR with ITS tailed primers and second PCR with barcoded universal primers) was performed using the non-diluted DNA extracts. Positive PCR products generated by this second round of amplification were used to substitute the negative samples on the original plate. The samples for which neither the dilution nor the original extract gave amplicons were left on the original plate. The 96 samples

were cleaned as described above and their concentration measured with a Nanodrop 8000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). All samples with a DNA concentration larger than 50 ng/µl were normalised manually to 50 ng/µl. One microliter of each of the 96 samples was pooled into a 1.5 ml Eppendorf tube.

Library preparation, sequencing and SMRT Portal primary analysis

The pooled sample (1.5 µg of DNA) was sent to the Ramaciotti Centre for Genomics (UNSW Sydney, Australia) for single molecular real-time (SMRT) sequencing. The sample met the quality control requirements and showed two clear peaks at 650 and 923 bp, which are within the expected size range for the ITS barcode. The library preparation was done using the PacBio Barcoded Universal Primers protocol (https://www.pacb.com/wp-content/uploads/2015/09/Procedure-and-Checklist-Preparing-SMRTbell-Libraries-PacB-Barcoded-Universal-Primers.pdf). The sample was sequenced in one SMRT cell on a PacBio RSII using a P6 chemistry with a four-hour movie. The raw data were analysed using Long Amplicon Analysis (LAA v. 1) with barcoding option on the SMRT Portal. This pipeline was run using the following settings: symmetric DNA barcodes with a minimum score of 22, a minimum subread length of 450 bp, a maximum number of subreads of 4,000, and default values for all other parameters. The phase alleles option was selected.

Molecular identification and validation

After demultiplexing, quality control and generation of consensus sequences using LAA, the amplicon data was provided in 96 files in fasta format. Sequences from the targeted lichenised fungal species were identified from fungal endophytes or contaminants based on sequence similarity using a BLASTN v 2.8.1 search on the nr database by excluding uncultured/environmental sample sequences (https://blast.ncbi.nlm.nih.gov/; search done on Dec 12–14 2018). The first BLASTN match (or highest bit score) was recorded unless it did not correspond to at least a fungal Class, in which case the next match was considered. For 12 samples, the target sequence obtained with PacBio was cross-validated by a sequence obtained with Sanger sequencing. The ITS barcode was amplified with the primers ITS1F and ITS4 using the DNA polymerase MyFi as previously described, and products were sent to Macrogen (Seoul, Korea) for purification and sequencing. PacBio and Sanger sequences were manually compared in Mesquite v 3.51 (Maddison and Maddison 2017). For samples for which multiple ITS barcodes were recovered for the target species, the sequence versions were compared using Mesquite and the BLASTN suite-2 sequences was used to estimate percentage identity between selected sequence pairs. Sequences with the highest coverage were selected as primary barcodes. Secondary barcodes were only taken into consideration when their coverage was above 30 and when they were less than 98% similar from

the primary barcode. Raw PacBio files and FASTQ files of primary and secondary barcodes obtained were deposited in the Sequence Read Archive on NCBI (BioProject ID PRJNA541190).

Results

Amplifications

After the two amplification steps, 19 of the 96 samples did not show any visible product. For these samples, a new round of amplifications was performed using the non-diluted DNA extracts instead of the 1/10 dilution. This second amplification round generated products for 13 samples that were previously negative. These additional products were then used to substitute the corresponding negative samples on the original plate. For the five samples for which none of the amplification rounds was positive (sample barcode numbers 41, 45, 73, 84, 92, 93), the initial sample were not substituted and were pooled together with the positive samples for final submission.

Sequencing

A first SMRT cell was run with a 0.02 nM sample, but resulting ZMW (Zero-Mode Waveguide) productivity was poor (P1=12%). After filtering, only 17,582 polymerase reads were recovered for this first run, which corresponded to 247,626 subreads (Table 1). The quality of the resulting subreads was, however, acceptable (mean of 14.68 passes). A second SMRT cell was then used with a 0.05 mM concentration of the same sample. The second run showed good ZMW productivity (P1=55%). After filtering, 82,501 polymerase reads were recovered (Table 1). The number of post-filter subreads obtained from this run was 1,095,489 and their quality was comparable to the first run (mean of 13.79 passes). Subreads from both SMRT cell runs were combined and analysed with the Long Amplicon Analysis protocol in the SMRT portal. After demultiplexing, the number of pre-filter subreads per barcode ranged from 91–115,311 (Suppl. material 1: Table S1). Barcodes with low subread numbers (<200) mostly corresponded to samples with negative (41, 45, 73, 93) or weak (53) amplification. The number of CCS obtained for each barcode ranged from

Table 1. Productivity and sequence output from two SMRT runs with different loading concentration.

Loading concentration	ZMW productivity			Post-filter polymerase reads			Subreads		
	P0	P1	P2	number	mean length	N50	number	mean length	number of passes
0.02 nM	86%	12%	2%	17,582	15,234 bp	27,799 bp	247,626	1038 bp	14.68
0.05 nM	29%	55%	16%	82,501	15,321 bp	21,997 bp	1,095,489	1111 bp	13.79

1–948. No chimeric sequences were detected in any of the barcoded samples, but a few low-quality sequences (noise) were found. After clustering and phasing, 0–32 final consensus sequences were obtained per barcode, with a length of 619–2,322 bp, a coverage of 8–500 (the default value of the maximum number of subreads to cluster is 500) and a predicted accuracy of 0.9525–0.9999. The predicted accuracy of the most represented sequence per barcode was above 0.9999 for the majority of the samples (93%).

Sequence identity

Sequences were recovered for 89 of the 96 samples (see Suppl. material 1: Table S1). For each positive sample, 1–32 sequences were obtained using LAA. A BLASTN search was used to identify the sequence of the target species (the lichenised fungi of interest) among the co-amplicons. Eighty-five samples had at least one blast hit that matched the target species. The search revealed that, despite no detection by the PacBio pipeline LAA, a few chimeric sequences (about 3% of the total number of obtained sequences) were obtained, seemingly consisting of two or three full and/or partial sequences, often of the same species, occurring in tandem. Samples for which either no sequence (e.g., samples 41 and 45) or no target species sequences (e.g., samples 92 and 95) principally derived from amplification reactions with no or weak PCR products. Some reactions with weak products did generate a sequence for the target species, although generally with a coverage lower than 100 (e.g., samples 29, 94 and 96).

For 46 samples, a single sequence was obtained for the target species among all the co-amplified fungal taxa. For the other samples, the clustering and phasing process resulted in 2–9 sequences that could be attributed to the target species using the BLASTN match. The similarity between these different sequence versions was investigated in an alignment. After reverse complement and barcode trimming, these sequence versions were in fact identical for an additional ten samples, bringing the total of successful target single-sequences to 56. A few sequence versions also differed with indels in long single nucleotide repeats (e.g., samples 27, 27, 28, 49, 64, 68, 69) and were the likely result of poor quality for low coverage consensus sequences. For the remaining samples, differences between sequence versions were more random (indels and single nucleotide mutations) and more likely due to genuine sequence heterogeneity within a sample, either due to biological reasons (concerted evolution or mixed individual) or carry-over contaminations. The percentage identity between the most divergent target sequence versions of each sample ranged between 94.78 and 99.98% (Suppl. material 1: Table S1).

In addition to the target species, sequences were also obtained for other fungi for 81 of the 96 samples. The great majority of these co-amplifying fungal sequences (479 out of 506 sequences) belonged to the ascomycetes, with Capnodiales and Chaetothyriales being the most commonly represented orders. Several sequences (23) belonged to the

basidiomycetes, two to the chytridiomycetes and one to the mucoromycetes. Finally, one sequence matched *Pirula salina*, a Xanthophyceae algae from the order Tribonematales.

Validation

Sanger sequences were obtained for 12 samples. For five of these (3, 6, 8, 61 and 76), the Sanger sequence was entirely identical to the PacBio sequence. For five additional samples (5, 7, 58, 60 and 74), the Sanger sequence was identical to the PacBio sequence, but contained ambiguous bases or was slightly shorter due to missing bases at the 5' or 3' ends. For two samples (4 and 57), the Sanger sequences comprised one nucleotide difference compared to the PacBio sequences in addition to several ambiguous bases. These differences were located at the start or the end of the sequence and were likely due to poor quality chromatograms in the 5' and 3' regions of the Sanger sequences.

Discussion

Collections of well-curated lichen specimens are important resources for species identification, whether done traditionally using morpho-anatomical together with chemical characters, or using DNA barcodes. For the latter, access to databases with high quality sequence data, detailed voucher information and reliable taxon names is critical. Although current reference sequence datasets exist, they are still incomplete. Thus, the molecular barcoding of lichen specimens for a broad range of species remains a priority (Yahr et al. 2016). In this study, a SMRT sequencing metabarcoding approach generated ITS sequences for 85 of the 96 lichen specimens investigated. This study shows that for lichen herbarium specimens up to 25 years old, full length and high-quality ITS sequences can be recovered for the target lichenised fungus using PacBio amplicon sequencing. Furthermore, sequences were also obtained for co-amplifying fungi, shedding light on the diversity of endolichenic fungi in the samples.

The protocol used in this study enabled the high-throughput processing of samples and the bioinformatic pipeline permitted the recovery of high-quality sequences with minimum time and effort, because both the primary and secondary analyses could be carried out by the sequencing provider. For mixed lichen DNA extracts, SMRT sequencing is a cheaper option than cloning and Sanger sequencing when high number of samples need to be processed. The cost of producing a barcode Sanger sequence is generally around AUD$15/sample (including DNA extraction, amplification and sequencing costs), but when cloning is required, this cost can increase to AUD$100/sample. With the PacBio amplicon protocol used in this study and the PacBio RSII platform, the cost per sample was estimated at AUD$37. This cost could be further reduced by co-amplifying more markers and/or multiplexing more samples. With the new PacBio platform Sequel, multiplexing 384 samples for one marker would bring

the cost down to AUD$12/sample. Moreover, with the recent increased output per SMRT cell (from 1 gigabase for RSII to 20 gigabases for the new PacBio platform Sequel), the cost per sample could fall even further with higher multiplexing levels.

The use of PacBio amplicon sequencing for high-throughput barcoding of lichen specimens is therefore very promising. Some challenges remain, both for the laboratory and the bioinformatic aspects of this method. Some considerations and suggestions for improvements based on this study are outlined below, especially with regard to the preparation of the amplicon library and the generation of ITS barcode sequences using the LAA pipeline.

Technical considerations for high-throughput DNA extraction and amplicon library preparation

A relatively high risk of carry-over contaminations is generally associated with two-step PCR amplicon protocols (Seitz et al. 2015), including the PacBio Barcoded Universal Primers protocol used in this study. In a preliminary test of this protocol (data not shown), output sequences suggested a significant number of potential carry-over contaminations. In light of these results, particular care was taken at various stages of the final run when handling the genomic DNA and PCR samples. More specifically, DNA extracts were stored in single tubes as opposed to a 96-well plate, and amplifications were carried out in strip tubes with individual caps instead of 96-well plates. In the final SMRT run, carry-over contaminations may have also occurred, but at a much lower level than the preliminary test run. Our current protocol could therefore be improved further. More specifically, the use of a PCR hood or post- and pre-PCR separation, as previously recommended (Urban et al. 2000; Jones and Kustka 2017), would be advisable for this two PCR step protocol. During DNA extraction, the use of 8-strip cluster tubes for the grinding of material should be replaced by single tubes, as opening the lids of these tubes causes a potential risk for sample cross-contamination. Minimising the number of pipetting steps and the opening of tubes after amplification is also important. In our protocol, a PCR clean-up step was required after the first PCR due to strong PCR dimer bands. Optimisation of primer concentrations in order to reduce primer dimer formation would eliminate this step. Finally, the inclusion of several replicates of the same sample within a SMRT run would facilitate the assessment of replicability between samples and potential carry-over contaminations.

The choice of polymerase has been shown to be important when using HT sequencing for rare allele detection (Hestand et al. 2016; Potatov and Ong 2017) as well as for community analysis (Oliver et al. 2015), because it impacts directly on the rate of PCR-induced errors. Polymerases with proofreading activities are usually recommended for library preparation protocols, including for SMRT amplicon sequencing. High-fidelity polymerases are, however, more expensive and, in our experience, are more difficult to optimise. A possible incompatibility between a high-fidelity polymerase and SMRT sequencing has even been suggested previously (Schlaeppi et al. 2016).

As argued by Hebert et al. (2018), for the molecular barcoding of reference specimens, the dominant sequence will match the source sequence even when a relatively high number of other sequences have PCR errors. In our preliminary test of this protocol, only a small number of PCR products could be recovered using the high-fidelity Phusion Hot Start II (Thermo Scientific). As the focus of our study was to obtain a barcode sequence from the target species, and not to characterise fungal communities using sequences, the polymerase MyFi was used to generate amplicons. It is marketed as being efficient with challenging templates and inhibitor-rich samples and has so far worked well for lichen specimens. Although not as accurate as a high-fidelity polymerase, MyFi comprises a proofreading component that allows a 3.5× higher fidelity than Taq DNA polymerase.

In addition to polymerase misincorporation, chimera formation is another common PCR-induced source of error. Low starting template concentration and low amplification cycle numbers tend to reduce the rate of chimera formation (Lahr and Katz 2009). Here, 25 cycles were used for both amplifications, and no chimeras were detected using the LLA pipeline. However, after manual inspection of the sequences obtained, several chimeric sequences (about 3%) were detected. Upon closer inspection, these chimeras were found to be formed of concatenated sequences of the full-length amplicon, together with primer sequences and barcodes. These concatemers have previously been reported in SMRT amplicon sequencing studies (e.g., Jones and Kustka 2017), and they are thought to be generated not during the PCR steps, but during the SMRTbell adaptor ligation step of the PacBio library preparation (Fichot and Norman 2013). They can readily be discarded by screening sequences of uncharacteristically large size (Jones and Kustka 2017).

Metagenomics and metabarcoding studies using two-step PCR protocols often include triplicate samples for amplification, which are then pooled and used as a template for the second PCR (e.g., Schlaeppi et al. 2016; Schloss et al. 2016). In community analysis, pooling triplicates is thought to be useful in decreasing PCR bias introduced by inherent differences or stochastic fluctuations in amplification efficiencies (Kennedy et al. 2014), although no study has confirmed that it influences the results of community analyses significantly (Kennedy et al. 2014; Smith and Peay 2014). Using replicates can also reduce the impact of PCR failure due to pipetting-related issues, but it is more expensive and time-consuming. Here, a 'cherry-picking' approach was used where negative samples were redone using a different genomic DNA concentration. Although additional positive samples were recovered with this approach, it may still be possible that PCR product could be recovered from the remaining negative samples using replicates and a broader range of genomic DNA concentrations. Due to large amounts and high diversity of secondary compounds in lichen specimens and their generally low genomic DNA yields, the window of concentration at which amplification is successful is often very narrow, and PCR results are sometimes difficult to reproduce. Using several dilutions of each genomic DNA extract and pooling their products after the first PCR could therefore help in maximising the number of positive samples.

Long Amplicon Analysis and the recovery of high-quality sequences for the target lichenised fungal species

The Long Amplicon Analysis pipeline has been developed by PacBio to phase and detect alleles in diploid organisms, and enables differentiation between underlying sequences that are 99.9% similar, such as haplotypes and pseudogenes (Bowman et al. 2014; Lleras et al. 2014). In this study, as an alternative to cloning, the LAA pipeline was used to recover high-quality barcode sequences from mixed DNA samples of target and non-target fungal species occurring in lichen specimens. The LAA pipeline is accessible on the SMRT portal and can be run by the sequencing service provider together with the primary analysis. The output data generated by LAA included fasta and fastq files with all sequence variants, as well as accuracy and coverage values for each of these sequences. Sequences with the highest coverage generally corresponded to the target species and for more than half of the samples, one single sequence was recovered for the target species. For the other samples, 2–8 sequence versions were recovered per target species. Most often the ITS variation within sample was caused by low quality consensus sequences, which could then be identified due to their low coverage. Occasionally, however, several versions of the ITS barcode with high coverage were recovered within a sample. The percentage identity between these versions mostly ranged between 95–99%. The presence of multiple versions is likely the result of sample heterogeneity (more than one target individual per sample), although carry-over contamination cannot be excluded. Additionally, intragenomic heterogeneity (several ITS copies per nucleus) and intramycelial heterogeneity (several nuclei per mycelium), as discussed in detail in Mark et al. (2016), are two other possible biological reasons for sequence variation. Other ITS sequences obtained corresponded to co-amplifying micro-organisms, predominantly fungi. The most common co-amplified fungi were from the orders Chaetothyriales (Eurotiomycetes) and Capnodiales (Dothideomycetes). The identity of these co-amplified fungi suggested two possible origins for these sequences: either the fungi occur naturally within the lichen thallus (endolichenic fungi), or lichenised or non-lichenised fungal species occur adjacent to the target species on the same substrate, and were accidentally co-sampled during the preparation of material for DNA extraction.

Most target sequences had high accuracy and coverage values, and a subset of these was validated with Sanger sequences obtained from the same DNA extracts. Initially, the size variation among amplicons recovered was more than the 10% recommended by PacBio and was of potential concern. An excess of sequences for the shorter amplicons relative to the longer ones could have been problematic, because lichenised fungi often have long ITS regions due to the presence of introns. However, at this low level of multiplexing (one marker for 96 samples), the difference in amplicon size did not prevent the generation of high-quality sequences for the target species. Moreover, at a higher multiplexing level (5 genes for 96 samples), the sequencing bias due to amplicon size variation did not seem to influence the results (Chen et al. 2015).

A recent study identified some problems with the LAA pipeline, including the formation of a few incorrect or truncated sequences even at relatively high read depths

(Francis et al. 2018). As a result, a new pipeline (C3S-LAA) was developed by these authors which differs from LAA by comparing similarity based on CCSs as opposed to uncorrected reads before the start of the clustering phase. Their new approach, which was used for the SMRT sequencing of long amplicons (4000–8000 bp), successfully eliminated these incorrect and truncated sequences (Francis et al. 2018). We have not observed these problems with our data. However, LAA did not detect chimeras that were formed by concatemers of amplicons with primers and barcodes, sometimes with the second sequence being the reverse complement of the first ("siamaeras", Hackl et al. 2014). However, these concatemers are easily detected with a BLAST comparison and filtered out because of their large size. In addition, some reverse complement sequences (or sequences with slightly truncated barcodes) were not recognised as being identical to other sequences by LAA and were therefore attributed to different clusters. This did not prevent LAA from recovering high-quality sequences for most target species, but it did add some time and effort in verifying whether or not they corresponded to true variants.

Conclusion

PacBio amplicon sequencing is a promising approach for the metabarcoding of lichen specimens, and can be applied to the generation of reference sequences and the characterisation of lichen-associated fungal communities. Although restricted to specimens for which the genomic DNA is not overly degraded, this approach succeeded in generating full-length and high-quality ITS barcodes for specimens up to 25 years old. By scaling up the multiplexing level, this approach could significantly reduce the cost of barcode/sample and compete with Sanger sequencing as well as other NGS approaches.

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Supplementary material 1

Table S1

Authors: Cécile Gueidan, John A. Elix, Patrick M. McCarthy, Claude Roux, Max Mallen-Cooper, Gintaras Kantvilas

Data type: measurement

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Two new species of *Amanita* sect. *Phalloideae* from Africa, one of which is devoid of amatoxins and phallotoxins

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Abstract

Two new species of *Amanita* sect. *Phalloideae* are described from tropical Africa (incl. Madagascar) based on both morphological and molecular (DNA sequence) data. *Amanita bweyeyensis* **sp. nov.** was collected, associated with *Eucalyptus*, in Rwanda, Burundi and Tanzania. It is consumed by local people and chemical analyses showed the absence of amatoxins and phallotoxins in the basidiomata. Surprisingly, molecular analysis performed on the same specimens nevertheless demonstrated the presence of the gene sequence encoding for the phallotoxin phallacidin (PHA gene, member of the MSDIN family). The second species, *Amanita harkoneniana* **sp. nov.** was collected in Tanzania and Madagascar. It is also characterised by a complete PHA gene sequence and is suspected to be deadly poisonous. Both species clustered together in a well-supported terminal clade in multilocus phylogenetic inferences (including nuclear ribosomal partial LSU and ITS-5.8S, partial *tef1*-α, *rpb2* and β-tubulin genes), considered either individually or concatenated. This, along with the occurrence of other species in sub-Saharan Africa and their phylogenetic relationships, are briefly discussed. Macro- and microscopic descriptions, as well as pictures and line drawings, are presented for both species. An identification key to the African and Madagascan species of *Amanita* sect. *Phalloideae* is provided. The differences between the two new species and the closest *Phalloideae* species are discussed.

Keywords

Ectomycorrhizal fungi, *Amanita*, phylogeny, taxonomy, mycotoxins, tropical Africa, 2 new species

Introduction

Most representatives of *Amanita* sect. *Phalloideae* (Fr.) Quél. are famous worldwide for their high, often deadly, toxicity. Currently, the section *Phalloideae* comprises nearly 60 described species, a number of which were described only recently, mainly from Asia (Li et al. 2015, Cai et al. 2016, Thongbai et al. 2017). Moreover, based on a multigene analysis and morphological data, Cai et al. (2014) identified 14 phylogenetic clades potentially representing new species. The phylogenetic analyses made by those authors also resulted in the transfer of several species from sect. *Phalloideae* to sect. *Lepidella* Corner & Bas and conversely.

Most of African mycodiversity remains under-explored with only ca. 1500 taxa described to date (Degreef 2018). Very few species belonging to sect. *Phalloideae* have been recorded from Africa and Madagascar (Walleyn and Verbeken 1998, Tulloss and Possiel 2017). The three poorly known *Amanita alliiodora* Pat., *A. murinacea* Pat. and *A. thejoleuca* Pat. were described from Madagascar, while *Amanita strophiolata* Beeli was described from DR Congo (we agree with Gilbert 1941:313 that the var. *bingensis* Beeli has no taxonomic value). The latter species is the only *Phalloideae* known from Central Africa, together with some doubtful mentions of the imported *A. phalloides* (Fr.: Fr.) Link. *Amanita phalloides* is only native to Europe, North Africa, Turkey (Kaya et al. 2013, 2015), a certain proportion of the Asian part of Russia and perhaps the West Coast of North America (Pringle and Vellinga 2006, Pringle et al. 2009, Wolfe et al. 2010). Mentions of the species in other regions of the world correspond to either introductions or misidentifications. The exact identity of *A. capensis* and its possible co-specificity with *A. phalloides* remain uncertain. The last species of *Phalloideae* known to Africa, *Amanita marmorata* Cleland & E.-J. Gilbert (syn.: *A. marmorata* subsp. *myrtacearum* O.K.Mill., Hemmes & G.Wong, *A. reidii* Eicker & Greuning, *A. phalloides* f. *umbrina* ss. African auct.), is also an introduced species. It was described from Australia, growing in association mainly with various species of *Eucalyptus* (e.g. *E. cephalocarpa* Blakely) and subsequently observed in South Africa under *Eucalyptus cloeziana* F.Muell. and *E.* sp. (Eicker et al. 1993, van der Westhuizen and Eicker 1994) and in Hawaii, under *Eucalyptus robusta* Sm., *E. saligna* Sm., *E.* sp., *Araucaria columnaris* Hook., *Melaleuca quinquenervia* (Cav.) S.T.Blake, as well as under pure *Casuarina equisetifolia* L. (Miller et al. 1996).

Amatoxins and phallotoxins are responsible for the high toxicity of *Amanita* sect. *Phalloideae*. Nevertheless, apart from *Amanita alliiodora*, considered toxic by the Madagascan people, and the deadly poisonous *A. phalloides* (incl. "*A. capensis*") and probably *A. marmorata*, no data are available attesting to the toxicity or the edibility of the Madagascan and African species.

In the framework of taxonomic and phylogenetic studies of *Amanita* sect. *Phalloideae*, specimens originating from tropical Africa were critically studied. Morphological and multigenic phylogenetic studies proved to be concordant and established the existence of two distinct species that could not be identified as any known taxa.

Amanita bweyeyensis from the western province of Rwanda and *A. harkoneniana* from the Tanzanian Miombo woodlands and Madagascar are described here as new. Their phylogenetic affinities with other *Amanita* species reported from Africa are discussed and a key to African species of *Amanita* sect. *Phalloideae* is provided.

Materials and methods

Specimens studied

African *Amanita phalloides*-related specimens held in BR were studied in depth (*Degreef* 653 from Burundi; *Degreef* 1257 and 1304, both from Rwanda). A picture appearing in Härkönen et al. (2003: 62, sub "*Amanita* species which looks very much like *Amanita phalloides*") convinced us to also check the specimen *Saarimäki* 591 (from Tanzania). We additionally obtained *Saarimäki* et al. 1061 (also from Tanzania) on loan from the University of Helsinki (H). Finally, P. Pirot sent us two unnumbered specimens he collected in Madagascar in 2014 and 2016.

We also examined for comparison the type specimen of *Amanita marmorata* subsp. *myrtacearum* (*O.K. Miller* 24545, VPI) collected in Hawaii and 3 specimens of *Amanita marmorata* collected in Australia: *H.D. Weatherhead* s.n. (= MEL 2028859A) and *J.B. Cleland* s.n. (= AD-C 3083 and 3085). We unsuccessfully tried to obtain the type specimen of *Amanita reidii* on loan. Braam Vanwyk informed us that the holotype preserved in PRU had unfortunately been destroyed and no longer exists. Although Miller et al. (1996: 144) mentioned having received a fragment of that type specimen on loan from PREM, it seems that no such material exists in the collections of that institution (Riana Jacob-Venter, in e-litt.), nor in K (Angela Bond, in e-litt.). We also received on loan the lectotypus of *Amanita murina* (Cooke & Massee) Sacc. (*Bailey* 651, K, correct name: *Amanita neomurina* Tulloss).

Macro- and microscopic studies

Macroscopic characters were deduced from herbarium specimens, as well as from specimen labels, field notes and pictures, when available. Microscopic examinations were carried out using an Olympus BX51 microscope, from herbarium material mounted in ammoniacal Congo Red or in Melzer's reagent. Measurements were made using a camera lucida and a calibrated scale. In the descriptions, figures between brackets are extreme values, underlined figures are averages, Q values are length/width ratios of spores, l/w values are the same ratios for other types of cells. Mentions like "[60/4/2]" after measurements of spores (or other microscopic structures) mean 60 spores measured, from 4 different basidiomata collected in 2 different places.

Molecular analyses

DNA extraction, amplification and sequencing

Genomic DNA was isolated from CTAB-preserved tissues or dry specimens using a CTAB isolation procedure adapted from Doyle and Doyle (1990). PCR amplification of the ITS region (nuclear ribosomal internal transcribed spacer) and LSU (large subunit ribosomal DNA) was performed using the primer pairs ITS4/ITS5 or ITS1-F/ITS4 and LR0R/LR5, respectively (http://biology.duke.edu/fungi/mycolab/primers.htm). Parts of the protein-coding genes β-tubulin, *rpb2* (second largest subunit of RNA polymerase II) and *tef-1* (translation elongation factor 1 alpha) were amplified using the primer pairs Am-β-tub-F/Am-β-tub-R, Am-6F/Am-7R and EF1-983F/EF1-1567R, respectively (Zhang et al. 2010). PCR products were purified by adding 1 U of Exonuclease I and 0.5 U FastAP Alkaline Phosphatase (Thermo Scientific, St. Leon-Rot, Germany) and incubating at 37 °C for 1 h, followed by inactivation at 80 °C for 15 min.

Sequencing was performed by Macrogen Inc. (Korea and The Netherlands) using the same primer combinations as for PCR, except for Am-β-tub-F, which was replaced by the shorter primer Am-β-tub-F-Seq (5'-CGGAGCRGGTAACAAYTG-3') following Thongbai et al. (2017). The sequences were assembled in Geneious Pro v. 6.0.6 (Biomatters).

Phylogenetic analysis

Thirty-five sequences of *Amanita* specimens were newly generated for this study and deposited in GenBank (http://www.ncbi.nlm.nih.gov/; Table 1). Initial BLAST searches (http://blast.ncbi.nlm.nih.gov) of both LSU and ITS-5.8S sequences were performed to estimate similarity with *Amanita* sequences already present in Genbank database (Table 1). Additional sequences were selected from previously published phylogenies and from GenBank (Table 1). The quality of the sequences was taken into account in selecting the sequences for the phylogenetic analyses. Materials and sequences used in this study are listed in Table 1.

A combined dataset (including nuclear ribosomal partial LSU and ITS-5.8S, partial *tef1*-α, *rpb2* and β-tubulin genes), comprising sequences from 94 collections including the outgroup and an ITS-5.8S / LSU dataset of 69 sequences, including several clones derived from the same collections and the outgroup, were constructed and used for further phylogenetic analyses.

Amanita cf. *spissacea* voucher OR1214 and *Amanita subjunquillea* voucher HKAS63418 were used as outgroups for the combined and ITS-LSU datasets, respectively (Thongbai et al. 2017, Cui et al. 2018).

Nucleotide sequences were automatically aligned using the MUSCLE algorithm (Edgar 2004) with default settings. The alignment was further optimised and manu-

Table 1. List of collections used for DNA analyses, with origin, GenBank accession numbers and references.

Species		GenBank accession no.				
Specimen voucher	**Country**	**LSU**	**ITS**	***rpb2***	***tef1*–α**	**βtubulin**
Sect. *Phalloideae*						
Amanita alliodora Pat. 1928						
DSN062	Madagascar	KX185612	KX185611	–	–	–
Amanita amerivirosa nom. prov.						
RET 397-8	USA	KJ466460	KJ466398	–	KJ481964	KJ466543
RET 480-1	USA	KJ466461	KJ466399	KJ466630	KJ481965	KJ466544
Amanita bisporigera G.F. Atk. 1906						
RET 377-9	USA	KJ466434	KJ466374	–	KJ481936	KJ466501
Amanita brunneitoxicaria Thongbai, Raspé& K.D. Hyde 2017						
BZ2015-01	Thailand	–	NR_151655	KY656879	–	KY656860
Amanita bweyeyensis Fraiture, Raspé & Degreef, sp. nov.						
clone Agar_8B_S114	Madagascar	–	KT200567	–	–	–
JD 1257	Rwanda	MK570926	MK570919	–	–	–
JD 1304	Rwanda	MK570927	MK570920	MK570931	MK570940	MK570916
TS 591	Tanzania	MK570928	MK570921	–	–	–
Amanita djarilmari E.M. Davison 2017						
EMD 008 cl_4	Australia	–	KU057382	–	–	–
EMD 008 cl_5	Australia	–	KU057383	–	–	–
EMD 008 cl_6	Australia	–	KU057384	–	–	–
EMD 50101_1	Australia	–	KU057393	–	–	–
EMD 50101_15	Australia	–	KU057392	–	–	–
EMD 50101_3	Australia	–	KU057391	–	–	–
EMD 50101_5	Australia	–	KU057390	–	–	–
EMD 50101_7	Australia	–	KU057389	–	–	–
EMD 80131_1	Australia	–	KU057399	–	–	–
EMD 80131_2	Australia	–	KU057400	–	–	–
EMD 80131_3	Australia	–	KU057401	–	–	–
EMD 80131_4	Australia	–	KU057402	–	–	–
EMD 80131_5	Australia	–	KU057403	–	–	–
PERTH08776040	Australia	KY977708	–	–	MF037234	MF000743
PERTH08776067 l_1	Australia	KY977704	KY977732	MF000755	MF000750	MF000742
PERTH08776067 l_2	Australia	KY977704	KY977733	MF000755	MF000750	MF000742
PERTH08776067 l_3	Australia	KY977704	KY977734	MF000755	MF000750	MF000742
PERTH08776067 l_4	Australia	KY977704	KY977735	MF000755	MF000750	MF000742
PERTH08776067 l_5	Australia	KY977704	KY977736	MF000755	MF000750	MF000742
PERTH08776075l_1	Australia	KY977706	KY977737	–	–	–
PERTH08776075l_2	Australia	KY977706	KY977738	–	–	–
PERTH08776075l_3	Australia	KY977706	KY977739	–	–	–
PERTH08776075l_4	Australia	KY977706	KY977740	–	–	–
PERTH08776075l_5	Australia	KY977706	KY977741	–	–	–
PERTH08776083 l_1	Australia	KY977710	KY977742	–	–	MF000744
PERTH08776083 l_2	Australia	KY977710	KY977743	–	–	MF000744
PERTH08776083 l_3	Australia	KY977710	KY977744	–	–	MF000744
PERTH08776083 l_4	Australia	KY977710	KY977745	–	–	MF000744
PERTH08776083 l_5	Australia	KY977710	KY977746	–	–	MF000744

Species		GenBank accession no.				
Specimen voucher	**Country**	**LSU**	**ITS**	***rpb2***	***tef1*–α**	**βtubulin**
Amanita eucalypti O.K. Mill. 1992						
PERTH8809828 cl_3	Australia	KY977707	KU057380	MF000758	MF000751	MF000746
PERTH8809828 cl_4	Australia	KY977707	KU057397	MF000758	MF000751	MF000746
PERTH8809828 cl_5	Australia	KY977707	KU057396	MF000758	MF000751	MF000746
PERTH8809828 cl_6	Australia	KY977707	KU057395	MF000758	MF000751	MF000746
PERTH8809828 cl_7	Australia	KY977707	KU057394	MF000758	MF000751	MF000746
PERTH8809828 l_2	Australia	KY977707	KU057398	MF000758	MF000751	MF000746
PERTH8809968 cl_3	Australia	KY977707	KU057380	MF000758	MF000751	MF000746
PERTH8809968 cl_4	Australia	KY977707	KU057381	MF000758	MF000751	MF000746
PERTH8809828 cl_1	Australia	KY977707	KU057398	MF000758	MF000751	MF000746
Amanita exitialis Zhu L. Yang & T.H. Li 2001						
HKAS74673	China	KJ466435	KJ466375	KJ466590	KJ481937	KJ466502
HKAS75774	China	JX998052	JX998027	KJ466591	JX998001	KJ466503
HKAS75775	China	JX998053	JX998026	KJ466592	JX998002	KJ466504
HKAS75776	China	JX998051	JX998025	KJ466593	JX998003	KJ466505
Amanita fuliginea Hongo 1953						
HKAS75780	China	JX998048	JX998023	KJ466595	JX997995	KJ466507
HKAS75781	China	JX998050	JX998021	KJ466596	JX997994	KJ466508
HKAS75782	China	JX998049	JX998022	KJ466597	JX997996	KJ466509
HKAS77132	China	KJ466436	KJ466375	KJ466598	KJ481939	KJ466510
HKAS79685	China	KJ466437	KJ466376	KJ466594	KJ481938	KJ466506
Amanita fuligineoides P. Zhang & Zhu L. Yang 2010						
HKAS52727	China	JX998047	JX998024	KJ466599	–	KJ466511
LHJ140722-13	China	KP691685	KP691696	KP691705	KP691674	KP691715
LHJ140722-18	China	KP691686	KP691697	KP691706	KP691675	KP691716
Amanita gardneri E.M. Davison 2017						
EMD 8-2010 cl_1	Australia	–	KU057387	–	–	–
EMD 8-2010 cl_3	Australia	–	KU057388	–	–	–
EMD 8-2010 cl_4	Australia	–	KU057386	–	–	–
EMD 8-2010 cl_6	Australia	–	KU057385	–	–	–
PERTH08776121	Australia	KY977712	–	MF000756	MF000752	MF000748
Amanita griseorosea Q. Cai, Zhu L. Yang & Y.Y. Cui 2016						
HKAS77334	China	KJ466476	KJ466413	KJ466661	KJ481994	KJ466580
HKAS77333	China	KJ466475	KJ466412	KJ466660	KJ481993	KJ466579
Amanita harkoneniana Fraiture & Saarimäi, sp. nov.						
P Pirot SN	Madagascar	MK570929	MK570922	MK570938	MK570941	MK570917
TS 1061	Tanzania	MK570930	MK570923	–	–	–
Amanita marmorata Cleland & E.-J. Gilbert 1941						
HW N	Australia	MK570931	MK570924	MK570939	MK570942	MK570918
PERTH 8690596 cl_1	Australia	KY977711	KU057408	–	–	MF000749
PERTH 8690596 cl_2	Australia	KY977711	KU057404	–	–	MF000749
PERTH 8690596 cl_3	Australia	KY977711	KU057405	–	–	MF000749
PERTH 8690596 cl_4	Australia	KY977711	KU057406	–	–	MF000749
PERTH 8690596 cl_5	Australia	KY977711	KU057407	–	–	MF000749
RET 623-7	Australia	KP757874	KP757875	–	–	–
RET 85-9	Australia	MG252697	MG252696	–	–	–
Amanita marmorata subsp. *myrtacearum* O.K. Mill., Hemmes & G. Wong 1996						
DED 5845	Hawai	AY325881	AY325826	–	–	–

Species		GenBank accession no.				
Specimen voucher	**Country**	**LSU**	**ITS**	***rpb2***	***tef1*–α**	**βtubulin**
Amanita millsii E.M. Davison & G.M. Gates 2017						
HKAS77322	Australia	KJ466457	KJ466395	KJ466643	KJ481978	KJ466557
HO5815331_2	Australia	KY977713	KY977715	MF000753	MF000759	MF000760
HO5815331_1	Australia	KY977713	KY977714	MF000753	MF000759	MF000760
HO5815331_3	Australia	KY977713	KY977716	MF000753	MF000759	MF000760
HO5815331_5	Australia	KY977713	KY977717	MF000753	MF000759	MF000760
Amanita molliuscula Q. Cai, Zhu L. Yang & Y.Y. Cui 2016						
HKAS75555	China	KJ466471	KJ466408	KJ466638	KJ481973	KJ466552
HMJAU20469	China	KJ466473	KJ466410	KJ466640	KJ481975	KJ466554
HKAS77324	China	NG_057038	NR_147633	KJ466639	KJ481974	KJ466553
Amanita ocreata Peck 1909						
HKAS79686	USA	KJ466442	KJ466381	KJ466607	KJ481947	KJ466518
Amanita pallidorosea P. Zhang & Zhu L. Yang 2010						
HKAS61937	China	KJ466443	KJ466382	KJ466609	KJ481949	KJ466520
HKAS71023	Japan	KJ466444	KJ466383	KJ466624	KJ481960	KJ466536
HKAS75483	China	KJ466445	KJ466384	KJ466623	KJ481959	KJ466535
HKAS75783	China	JX998055	JX998035	KJ466625	JX998010	KJ466537
HKAS75784	China	JX998056	JX998036	KJ466626	JX998009	KJ466538
HKAS75786	China	JX998054	JX998037	KJ466627	JX998011	KJ466539
HKAS77329	China	KJ466447	KJ466387	KJ466610	KJ481950	KJ466521
HKAS77348	China	KJ466448	KJ466387	KJ466611	KJ481951	KJ466522
HKAS77349	China	KJ466449	KJ466389	KJ466628	KJ481961	KJ466540
HKAS77327	China	KJ466446	KJ466386	KJ466608	KJ481948	KJ466519
Amanita parviexitialis Q. Cai, Zhu L. Yang & Y.Y. Cui 2016						
HKAS79049	China	NG_057092	–	KT971345	KT971343	KT971346
Amanita phalloides Secr. 1833						
HKAS75773	USA	JX998060	JX998031	KJ466612	JX998000	KJ466523
Amanita rimosa P. Zhang & Zhu L. Yang 2010						
HKAS75778	China	JX998045	JX998019	KJ466616	JX998006	KJ466527
HKAS75779	China	JX998046	JX998020	KJ466617	JX998004	KJ466528
HKAS77105	China	KJ466452	KJ466391	KJ466618	KJ481954	KJ466529
HKAS77120	China	KJ466453	KF479044	KJ466619	KJ481955	KJ466530
HKAS77279	China	KJ466454	KJ466392	KJ466620	KJ481956	KJ466531
HKAS77335	China	KJ466455	KJ466393	KJ466621	KJ481957	KJ466532
HKAS77336	China	KJ466456	KJ466394	KJ466622	KJ481958	KJ466533
HKAS75777	China	JX998044	JX998018	KJ466615	JX998005	KJ466526
Amanita sp. 10 ZLY2014						
HKAS77322	Australia	KJ466457	KJ466395	KJ466643	KJ481978	KJ466557
Amanita sp. 2 ZLY2014						
HKAS77350	China	KJ466462	KJ466400	KJ466631	KJ481966	KJ466545
Amanita sp. 3 ZLY2014						
HKAS77342	China	KJ466463	KF479045	KJ466632	KJ481967	KJ466546
HKAS77343	China	KJ466464	KJ466401	KJ466633	KJ481968	KJ466547
HKAS77344	China	KJ466465	KJ466402	KJ466634	KJ481969	KJ466548
HKAS77351	China	KJ466466	KJ466403	KJ466635	KJ481970	KJ466549
Amanita sp. 5 ZLY2014						
RET 422-8	USA	KJ466469	KJ466406	KJ466649	KJ481983	KJ466563
RET 493-6	USA	KJ466470	KJ466407	KJ466650	KJ481984	KJ466564

Species		GenBank accession no.				
Specimen voucher	**Country**	**LSU**	**ITS**	***rpb2***	***tef1*–α**	**βtubulin**
Amanita sp. 8 ZLY2014						
HKAS75150	Bangladesh	KJ466477	KJ466414	KJ466641	KJ481976	KJ466555
Amanita sp. 9 ZLY2014						
HKAS77323	China	KJ466478	KJ466415	KJ466642	KJ481977	KJ466556
Amanita suballiacea (Murrill) Murrill 1941						
RET 490-1	USA	KJ466485	KJ466420	KJ466601	KJ481941	KJ466513
RET 491-7	USA	KJ466486	KJ466421	KJ466602	KJ481942	KJ466514
RET 478-6	USA	KJ466484	KJ466419	KJ466600	KJ481940	KJ466512
Amanita subfuliginea Q. Cai, Zhu L. Yang & Y.Y. Cui 2016						
HKAS77347	China	KJ466468	KJ466405	KJ466637	KJ481972	KJ466551
HKAS77326	China	KJ466467	KJ466404	KJ466636	KJ481971	KJ466550
Amanita subjunquillea S. Imai 1933						
HKAS74993	China	KJ466489	KJ466424	KJ466652	KJ481987	KJ466570
HKAS75770	China	JX998062	JX998034	KJ466653	JX997999	KJ466571
HKAS75771	China	JX998063	JX998032	KJ466654	JX997997	KJ466572
HKAS75772	China	JX998061	JX998033	KJ466655	JX997998	KJ466573
HKAS77325	China	KJ466490	KJ466425	KJ466656	KJ481988	KJ466574
HKAS77345	China	KJ466491	KJ466426	KJ466657	KJ481989	KJ466575
HMJAU20412	China	KJ466492	KJ466427	KJ466658	KJ481990	KJ466576
HMJAU23276	China	KJ466493	KJ466428	KJ466659	KJ481991	KJ466577
HKAS63418	China	KJ466488	KJ466423	KJ466651	KJ481986	KJ466569
Amanita subpallidorosea Hai J. Li 2015						
LHJ140923-41	China	KP691692	KP691683	KP691701	KP691670	KP691711
LHJ140923-55	China	KP691693	KP691680	KP691702	KP691671	KP691712
LHJ140923-17	China	KP691691	KP691677	KP691700	KP691669	KP691713
Amanita virosa Secr. 1833						
HKAS71040	Japan	KJ466496	KJ466429	KJ466665	KJ481997	KJ466584
HMJAU20396	China	JX998059	JX998029	–	JX998008	KJ466585
HMJAU23303	China	KJ466497	KJ466430	KJ466666	KJ481998	KJ466586
HMJAU23304	China	KJ466498	KJ466431	KJ466667	KJ481999	KJ466587
HKAS56694	Finland	JX998058	JX998030	KJ466664	JX998007	KJ466583
Amanita halloides ar lba Costantin & L.M. Dufour 1895						
AF2322	Belgium	–	MK570925	–	–	–
Amanita halloides ar mbrina (Ferry) Maire 1937						
PREM 48618	South Africa	AY325882	AY325825	–	–	–
Amanita eidii Eicker & Greuning 1993						
PRU 4306	South Africa	AY325883	AY325824	–	–	–
Amanita p						
CM1309	New Caledonia	–	KY774002	–	–	–
Amanita p Kerala01						
RET 91-7	India	–	KC855219	–	–	–
Incertae sedis						
Amanita ballerina Raspé Thongbai & K.D. Hyde 2017						
OR1014	Thailand	–	KY747466	KY656883	–	KY656864.
OR1026	Thailand	MH157079	KY747467	KY656884	–	KY656865
Amanita franzii Zhu L. Yang, Y.Y. Cui & Q. Cai 201						
HKAS77321	China	KJ466481	MH508357	KJ466646	MH508798	KJ466560

Species		GenBank accession no.				
Specimen voucher	**Country**	**LSU**	**ITS**	***rpb2***	***tef1*–α**	**βtubulin**
HKAS91231	China	MH486525	MH508358	MH485994	MH508801	MH485516
Amanita pseudogemmata Hongo 1974						
HKAS85889	China	MH486768	–	MH486186	MH508995	MH485692
HKAS84744	China	MH486767	–	MH486185	MH508994	MH485691
Amanita zangii Zhu L. Yang, T.H. Li & X.L. Wu 2001						
GDGM29241	China	KJ466499	KJ466432	KJ466668	KJ482000	KJ466588
HKAS77331	China	KJ466500	KJ466433	KJ466669	KJ482001	KJ466589
Sect. *Validae*						
Amanita cf spissacea S. Imai 1933						
OR1214	Thailand	KY747478	KY747469	KY656886	–	KY656867

Note: cl_ stands for clone. References to sequences retrieved from GenBank: Cai et al. (2012), Cai et al. (2014), Cui et al. (2018), Davison et al. (2017), Henry et al. (2015), Houles et al. (2018), Li et al. (2015), Thongbai et al. 2017, Tulloss (continuously updated).

ally adjusted as necessary by direct examination with the software Se-Al v. 2.0a11 (University of Oxford).

The assignment of codon positions in the protein-coding sequences was confirmed by translating nucleotide sequences into predicted amino acid sequences using MacClade 4.0 (Maddison and Maddison 2000) and then compared with the annotated *Amanita brunnescens* sequences AFTOL-ID 673.

Potential ambiguously aligned segments, especially in the three introns present in *tef-1* and β-tubulin gene sequences and in the ITS-5.8S alignment, were detected by Gblocks v0.91b (Castresana 2000; http://molevol.cmima.csic.es/castresana/Gblocks.html) with the following parameter settings: minimum number of sequences for a conserved position = 24 (minimum possible); minimum number of sequences for a flank position = 24 (minimum possible); maximum number of contiguous non-conserved positions = 4 bp, minimum block size = 4 bp and gaps allowed within selected blocks in half of the sequences.

To detect the possible bias from substitution saturation and to evaluate the phylogenetic signal, we tested each partition of the combined dataset and the ITS-LSU dataset by using Xia's test (Xia et al. 2003, Xia and Lemey 2009), as implemented in DAMBE (Xia and Xie 2001). As the Iss.c is based on simulation results, there is a problem with more than 32 species. To circumvent this problem, DAMBE was used to randomly sample subsets of 4, 8, 16 and 32 OTUs multiple times and to perform the test for each subset to see if substitution saturation exists for these subsets of sequences. In order to confirm the results of the Xia's method, we also plotted the raw number of transversions and transitions against Tamura-Nei genetic distances with the aid of the DAMBE package, with an asymptotic relationship indicating the presence of saturation.

Models of evolution for BI were estimated using the Akaike Information Criterion (AIC) as implemented in Modeltest 3.7 (Posada and Crandall 1998).

The dataset was subdivided into 10 data partitions: *tef-1* 1st and -2nd codon positions, *tef-1* -3rd codon positions, *tef-1* introns and *rpb2* 1st and -2nd codon positions,

rpb2 -3rd codon positions, β-tubulin 1st and -2nd codon positions, β-tubulin -3rd codon positions, β-tubulin intron, ITS, LSU. Phylogenetic analyses were performed separately for each individual and concatenated loci using Bayesian Inference (BI) as implemented in MrBayes v3. 2 (Ronquist et al. 2012) and Maximum Likelihood (ML) as implemented in RAxML 7.2.7 (Stamatakis et al. 2008).

The best-fit models for each partition were implemented as partition specific models within partitioned mixed-model analyses of the combined dataset (Table 2). All parameters were unlinked across partitions. Bayesian analyses were implemented with two independent runs, each with four simultaneous independent chains for ten million generations, starting from random trees and keeping one tree every 1000th generation. All trees sampled after convergence (average standard deviation of split frequencies < 0.01 and confirmed using Tracer v1.4 [Rambaut and Drummond 2007]) were used to reconstruct a 50% majority-rule consensus tree (BC) and to calculate Bayesian Posterior Probabilities (BPP). BPP of each node was estimated based on the frequency at which the node was resolved amongst the sampled trees with the consensus option of 50% majority-rule (Simmons et al. 2004). A probability of 0.95 was considered significant. Maximum Likelihood (ML) searches conducted with RAxML involved 1000 replicates under the GTRGAMMAI model, with all model parameters estimated by the programme. In addition, 1000 bootstrap (ML BS) replicates were run with the same GTRGAMMAI model. We provided an additional alignment partition file to force RAxML software to search for a separate evolution model for each dataset. Clades with Maximum Likelihood bootstrap values of 75% or greater were considered supported by the data.

To detect topological conflicts amongst data partitions, the nodes between the majority-rule consensus trees obtained in the ML analysis from the individual datasets were compared with the software compat.py (available at www.lutzonilab.net/downloads). Paired trees were examined for conflicts only involving nodes with ML BS > 75% (Mason-Gamer and Kellogg 1996, Lutzoni et al. 2004, Reeb et al. 2004). A conflict was assumed to be significant if two different relationships for the same set of taxa (one being monophyletic and the other not) were observed in rival trees. Sequence data and statistical analysis for each individual dataset and combined analysis are provided in Table 2.

PCR amplification of *Amanita* toxins genes family members

Two major toxin-encoding genes, AMA1 and PHA1, directly encode for α-amanitin and the related bicyclic heptapeptide phallacidin, the lethal peptide toxins of poisonous mushrooms in the genus *Amanita*. α-Amanitin and phallacidin are synthesised as pro-proteins of 35 and 34 amino acids, respectively, in the ribosomes and are later cleaved by a prolyl oligopeptidase (Hallen et al. 2007, Luo et al. 2009, Li et al. 2014). In these pro-proteins, the amino acid sequences found in the mature toxins are flanked by conserved amino acid sequences, with an invariant Pro residue immediately upstream of the toxin regions and as the last amino acid in the toxin regions.

Table 2. Summary of data sets of ITS rDNA, nuc-LSU rDNA, *tef1*-α, *rpb2* and β-tubulin.

	Datasets									
Properties	*tef1* 1st & 2nd	*tef1* 3rd	*tef1* introns	*rpb2* 1st& 2nd	*rpb2* 3rd	β-tubulin 1st& 2nd	β-tubulin 3rd	β-tubulin introns	nucLSU	ITS
Alignment size	296	147	147	452	226	167	83	171	887	935
Excluded characters	–	–	–	–	–	–	–	–	–	557
Model selected	GTR+I+G	GTR+G	HKY+I	GTR+I	GTR+G	SYM+I+G	HKY+G	HKY+G	GTR+I+G	HKY+I+G
-Likelihood score	780.2892	1857.1256	1535.9010	1285.5159	3033.6099	1319.9380	1108.1555	1023.9042	3403.9714	4844.7563
Base frequencies										
Freq. A =	0.3179	0.1686	0.2479	0.2914	0.2478	Equal	0.1745	0.2254	0.2877	0.3023
Freq. C =	0.2276	0.3231	0.2175	0.2132	0.1956	Equal	0.3113	0.1690	0.1671	0.1846
Freq. G =	0.2536	0.2159	0.1807	0.2761	0.2541	Equal	0.2257	0.2228	0.2937	0.2068
Freq. T =	0.2010	0.2924	0.3540	0.2192	0.3025	Equal	0.2885	0.3827	0.2515	0.3062
Proportion of invariable sites	0.8042	–	0.0940	0.8283	–	0.4975	–	–	0.5726	0.2855
Gamma shape	0.7888	2.1595	-	-	2.7065	4.2837	3.7320	0.8697	0.5839	0.8470
Test of substitution saturation										
Iss	0.263	0.354	0.723	0.335	0.308	0.156	0.306	0.662	0.499	0.472
Iss.cSym	0.683	0.721	0.928	0.697	0.685	0.706	0.875	0.776	0.764	0.707
P (Sym)	< 0.0001	< 0.0001	0.2135	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.402	< 0.0001	< 0.0001
Iss.cAsym	0.354	0.668	0.802	0.502	0.458	0.407	0.711	0.535	0.675	0.645
P (Asym)	< 0.0001	< 0.0001	0.6284	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.354	< 0.0001	< 0.0001

Note: Iss: index of substitution saturation. Iss.cSym: critical value for symmetrical tree topology. Iss.cAsym: critical value for extremely assymetrical tree topology. P: probability that Iss is significantly different from the critical value (Iss.cSym or Iss.cAsym).

The toxins genes and MSDIN (cyclic peptide precursor) family members and related sequences were amplified from total genomic DNA with two consecutive PCR reactions, using the products of the first PCR as templates for the second one. For the first PCR, we used degenerated primers forward (5'ATGTCNGAYATYAAYGCNACNCG3') and the reverse primer (5'CCAAGCCTRAYAWRGTCMACAAC3'), following the cycling condition detailed in Li et al. (2014).

For the nested PCR amplification (using the PCR products above as the amplification template of AMA1 and PHA1 genes), primers targeting conserved regions of MSDINs family were obtained from previous studies (Hallen et al. 2007, Luo et al. 2009, Li et al. 2014, Wołoszyn and Kotłowski 2017) or designed *ad hoc* against the conserved upstream and downstream sequences of AMA1 and PHA1 available on Genbank and tested in different combinations. For α-amanitin, we used 5'CCATCTGGGGCATCGGTTGCAACC3' as forward primer (Li et al. 2014) in combination with the reverse primers 5'CTACGTYYGAGTCAGGACAACTGCC3' (Li et al. 2014) and the newly generated AMA-α-R2 (5'GTCAAAGTCAGTGCGACTGCCTTGT3') and AMA-α-R3 (5'CTGCATTTGAGTTAGGATAACGACA3'). We also tested primer pairs AMAF and AMAR 5 (Wołoszyn and Kotłowski 2017). For β-amanitin, we used forward primer AMA-β-F (5'CCATMTGGGGMATMGGTTGYRACC3') in combination with reverse primers AMA-β-R (5'GTCMACAACTYGTATYGKCCACTACT3'), AMA-β-R2 (5'GTCMACAACTYRTATYGKCCACMGCT3') and AMA-β-R3 (5'CCTRAYAWRGTCMACAACT3'). For PHA genes, we used forward primer 5'CCTGCYTGGCTYGTAGAYTGCCCA3' (Li et al. 2014) in combination with the reverse primers 5'CGTCCACTACTAYDTCMARGTCAGTAC3' (Li et al. 2014) and AMA-PHA-R2 (5'AGTCACGACTACATCGAGGTCAGTACA3'). Primer pairs FALF and FALR (Wołoszyn and Kotłowski 2017) were also tested for amplification of the phallotoxins genes.

Thermal cycling conditions were: initial denaturation at 94°C for 4 min, followed by 33 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s, extension at 72°C for 30 s and a final extension at 72°C for 7 min, for all reactions except for the ones involving primers from Wołoszyn and Kotłowski (2017), for which an annealing temperature of 68°C for 1 min was used.

Chemical analyses

Mushroom preparation

Two groups of dried mushrooms, i.e. with cuticle (n=3) and without cuticle (n=3), were analysed. For each specimen, 100 mg of dry tissues were ground and homogenised in 3 ml extraction medium (methanol:water:0.01 M HCl [5:4:1, v/v/v]) using a tissue homogeniser. After 1 hour of incubation, all extracts were centrifuged at 5000 rpm for 5 min, the supernatant was filtered using a 0.45 mm syringe filter and 20 µl of this supernatant was injected in the RP-HPLC device for toxin detection.

Standard solutions and chemicals

The α-amanitin and phalloidin standards were obtained from Sigma-Aldrich (USA). The β-amanitin, γ-amanitin and phallacidin standards were obtained from Enzo Life Sciences (Farmingdale, NY, USA). The solvents used in this study were all HPLC grade. Stock solutions of all toxins (100 μg/ml) were prepared in methanol. The calibration standards of all toxins were diluted in the extraction fluid in concentrations of 1, 5, 20, 100, 200, 500 ng/ml. Calibration curves were produced for each toxin; they were linear over the range of interest ($R^2 > 0.99$).

RP-HPLC analysis of toxins

Chromatography conditions for the procedure followed in this study were reported by Kaya et al. (2013, 2015). In short, the authors reported excellent separation of amatoxins and phallotoxins with the RP-HPLC and UV detection. In the laboratory, an RP-HPLC analysis of mushroom extracts was performed on a Shimadzu (Japan) HPLC system. The RP-HPLC analysis of standard solutions of α-amanitin, β-amanitin, γ-amanitin, phalloidin, phallacidin and subsequent quantification of mushroom extracts were performed on 150 × 4.6 mm, 5 mm particle, C18 column (Agilent Technologies, Palo Alto, CA) with 302 nm (for amatoxins) and 290 nm (for phallotoxins) at the UV detector. The mobile phase was used in isocratic profile with a flow rate of 1 ml/min. The content of the mobile phase was 0.05 M ammonium acetate (pH 5.5 with acetic acid)/acetonitrile (90:10 v/v). The detection limits were set at 0.6 ng/g for all toxins.

Results

Molecular analyses

Phylogenetic analysis

By comparing the tree topologies obtained for the individual datasets, no significant conflict, involving significantly supported nodes, was found using the 75% ML BP criterion; the datasets were therefore combined.

The test of substitution saturation (Table 2) showed that the observed index of substitution saturation (*Iss*) for the ITS-LSU dataset (ITS and LSU partition considered individually) the *tef-1*, *rpb2,* LSU and β-tubulin alignments of the combined dataset was significantly lower than the corresponding critical index substitution saturation (*Iss.c*), indicating that there was little saturation in our sequences ($P < 0.001$). On the other hand, the ITS partition of the combined dataset, the *tef-1* introns and the β-tubulin intron showed sign of substitution saturation, indicating the unsuit-

ability of these data for phylogenetic analysis. Nevertheless, re-analysing the ITS-LSU partition with DAMBE, after the exclusion of the 378 sites (40% of a total of 935 sites) retained by Gblocks, the substitution saturation test revealed an Iss value that was significantly ($P < 0.001$) lower than the Iss.c (Table 2), indicating the suitability of this data for further phylogenetic analysis. We therefore included an ITS partition, excluding the poorly aligned positions identified by Gblocks, in the combined dataset. Regarding the introns partitions, according to Thongbai et al. (2017), *A. zangii* and the *A. ballerina* clade (Fig. 1), should be considered to belong in a different section (*Amanita incertae sedis*), sister to the *Phalloideae* sensu Bas (1969). We therefore tested the combined dataset for substitution saturation by using *A. zangii* as the outgroup and excluding from the analysis the *A. ballerina* clade and the outgroup. In this case, no sign of saturation was evidenced, which supports the consistency of the phylogenetic signal in the main *Phalloideae* clade. We therefore decided to include the introns partitions in the phylogenetic analyses in order to increase the resolution at species level.

The ITS-LSU dataset and the final combined DNA sequence alignments of all loci (β-tubulin, *rpb2*, ITS, LSU, *tef-1*) alignments contained 15 and 35 OTUs and were 1575 and 3133 sites long including gaps, respectively. Sequence data and statistical analysis for each dataset are provided in Table 2.

The topologies obtained by analysing the combined dataset and the ITS-LSU dataset were highly congruent with published trees (Zhang et al. 2010, Cai et al. 2016, Thongbai et al. 2017), at least for what concerns significantly supported branches, and the Bayesian consensus trees (Figs 1 and 2) were almost identical to the optimal trees inferred under the Maximum Likelihood criterion. Several collections from tropical Africa clustered together in a well-supported clade. So far, this clade remains isolated but is notably distantly related to all other *Amanita* species, as yet reported from Africa (Zhang et al. 2010, Cai et al. 2016, Thongbai et al. 2017) or elsewhere and for which sequences are known (Figs 1 and 2), suggesting a common phylogenetic background. *Amanita alliiodora* clustered together with the two unnamed species from tropical Africa in all phylogenetic inferences considered individually or concatenated (i.e. phylogenetic species, Figs 1 and 2, shaded box).

Morphological examination showed combinations of morphological features unique to and characteristic of each, thereby defining two morphotypes. The critical morphological features that differentiate them are the following. The first species grows under Eucalyptus. Its bulb at stipe base is (sub-)globose, neither pointed nor rooting. The ring is striated and the smell sweetish and conspicuous. The second species is not bound with *Eucalyptus* and has been collected in Miombo woodland and in a garden. The bulb at the stipe base is turnip-shaped to rooting. The ring is smooth or vaguely plicate and the smell weak, resembling raw potato. We therefore concluded that these two morphotypes / clades represent two distinct new species, which we describe below resp. as *A. bweyeyensis* sp. nov. and *A. harkoneniana* sp. nov.

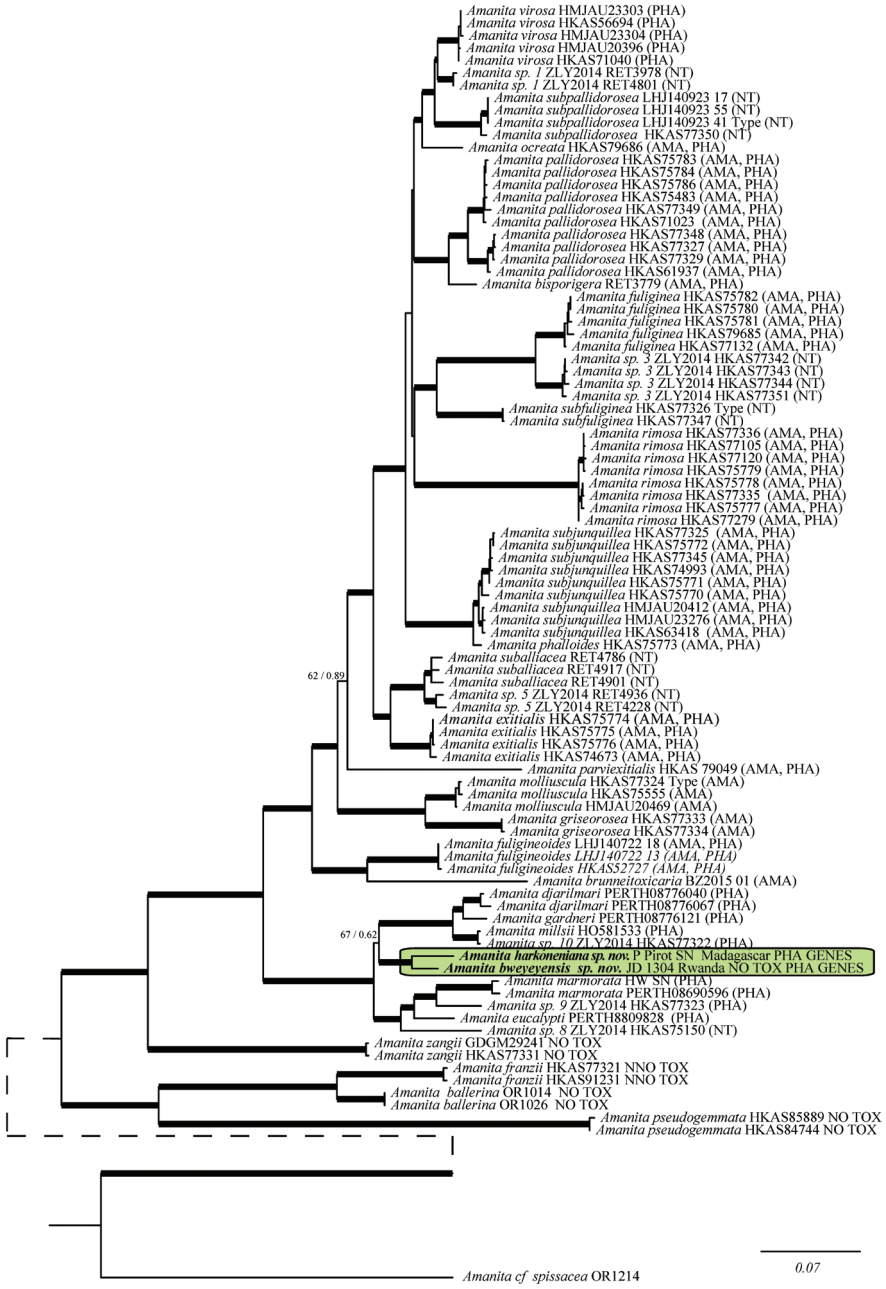

Figure 1. The 50% majority-rule consensus tree from Bayesian inference of the combined dataset. Thickened branches in bold represent ML BS support greater than 75% and BPP greater than 0.95; thickened branches in grey denote branches supported by either ML BS or BPP. For selected nodes ML BS support value and BPP are, respectively, indicated to the left and right of slashes. The new taxa are highlighted in the shaded box. AMA and PHA indicate the presence of amatoxins and phallotoxins, respectively, detected by HPLC. NT indicates not tested.

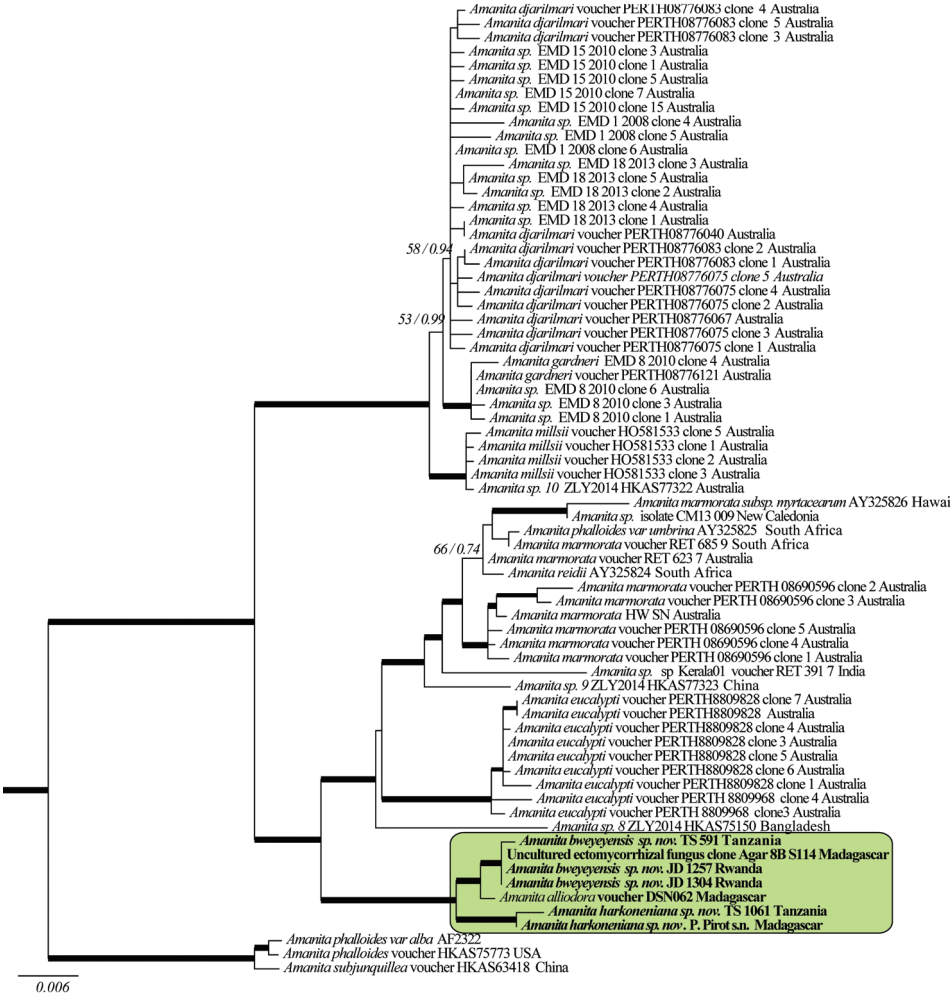

Figure 2. The 50% majority-rule consensus tree from Bayesian inference of the combined nuclear ITS-5.8S and LSU sequences. Thickened branches in bold indicate bootstrap support greater than 70% and Bayesian posterior probability greater than 0.95. For selected nodes, parsimony bootstrap support value and Bayesian posterior probabilities are, respectively, indicated to the left and right of slashes. The new taxa are highlighted in the shaded box.

PCR amplification of *Amanita* toxins genes family members

By using a combination of the degenerated primers cited above, we obtained a complete 17-mer sequence of phallacidin precursor for the three specimens of *A. bweyeyensis* and the two specimens of *A. harkoneniana* studied (Table 3), comprising the mature toxin region sequence of phallacidin (AWLVDCP) and both the invariant Pro residues immediately preceding the mature peptide sequence and the last amino acid of the toxin. Surprisingly, this is the first time that a complete PHA sequence has been found

Table 3. PCR products (phalloidin, PHA gene) amplified from *A. bweyeyensis* and *A. harkoneniana* with degenerate primers, compared to the PHA gene sequences available on GenBank.

						Phallacidin precursor (17-mer)											
											Phallacidin mature peptide						
	M	**S**	**D**	**I**	**N**	**A**	**T**	**R**	**L**	**P**	**A**	**W**	**L**	**V**	**D**	**C**	**P**
MK570933 *A. bweyeyensis* JD 1304	ATG	TCT	GAC	ATC	AAT	GCC	ACC	CGT	CTC	CCT	GCY	TGG	CTT	GTA	GAC	TGC	CCC
MK570932 *A. bweyeyensis* JD 1257	ATG	TCT	GAC	ATC	AAT	GCC	ACC	CGT	CTC	CCT	GCY	TGG	CTT	GTA	GAC	TGC	CCC
MK570934 *A. bweyeyensis* TS 591	ATG	TCT	GAC	ATC	AAT	GCC	ACC	CGT	CTT	CCT	GCT	TGG	CTT	GTA	GAC	TGC	CCC
MK570936 *A. harkoneniana* TS 1061	ATG	TCT	GAC	ATC	AAT	GCC	ACC	CGT	CTT	CCT	GCY	TGG	CTY	GTA	GAY	TGC	CCA
MK570935 *A. harkoneniana* P PIROT SN	ATG	TCT	GAC	ATC	AAT	GCC	ACC	CGT	CTT	CCT	GCY	TGG	CTY	GTA	GAY	TGC	CCC
KF387488 *A. exitialis*	ATG	TCT	GAC	ATC	AAT	GCC	ACC	CGT	CTT	CCT	GCC	TGG	CTC	GTA	GAC	TGC	CCA
EU196142 *A. bisporigera*	ATG	TCT	GAC	ATC	AAT	GCC	ACC	CGT	CTT	CCT	GCT	TGG	CTT	GTA	GAC	TGC	CCA
KF546298 *A. fuligineoides*	???	???	???	???	???	???	???	???	???	CCT	GCT	TGG	CTT	GTA	GAT	TGC	CCA
KF546296 *A. fuliginea*	???	???	???	???	???	???	???	???	???	CCT	GCT	TGG	CTT	GTA	GAC	TGC	CCA
KF552098 *A. pallidorosea*	ATG	TCT	GAT	ATT	AAT	GCT	ACG	CGT	CTT	CCC	GCC	TGG	CTT	GTA	GAC	TGC	CCA
KF546303 *A. phalloides*	???	???	???	???	???	???	???	???	???	CCT	GCT	TGG	CTT	GTA	GAT	TGC	CCA
KC778570 *A. oberwinklerana*	???	???	???	???	???	???	???	???	???	CCT	GCT	TGG	CTT	GTA	GAT	TGC	CCA
KC778568 *A. subjunquillea*	???	???	???	???	???	???	???	???	???	CCT	GCT	TGG	CTT	GTA	GAT	TGC	CCA
KF546306 *A. rimosa*	???	???	???	???	???	???	???	???	???	CCT	GCT	TGG	CTT	GTA	GAC	TGC	CCA

Table 3. (Continued) PCR products (phalloidin, PHA gene) amplified from *A. bweyeyensis* and *A. harkoneniana* with degenerate primers, compared to the PHA gene sequences available on GenBank.

	Phallacidin precursor (17-mer)														
	C	**V**	**G**	**D**	**D**	**V**	**N**	**P**	**V**	**L**	**T**	**R**	**G**	**Q**	**R**
MK570933 *A. bweyeyensis* JD 1304	TGC	GTC	GGT	GAC	GAC	TGC	AAC	CCC	GTA	CTC	ACT	CGT	GGG	CAG	AGG
MK570932 *A. bweyeyensis* JD 1257	TGC	GTC	GGT	GAC	GAC	TGC	AAC	CCC	GTA	CTC	ACT	CGT	GGG	CAG	AGG
MK570934 *A. bweyeyensis* TS 591	TGC	GTC	GGT	GAC	GAC	TGC	AAC	CCC	GTA	CTC	ACT	CGT	GGG	CAG	AGG
MK570936 *A. harkoneniana* TS 1061	TGC	GTC	GGT	GAC	GAC	TGC	AAC	CCC	GTT	CTC	ACT	CGT	GGG	CAG	AGG
MK570935 *A. harkoneniana* P PIROT SN	TGC	GTC	GGT	GAC	GAC	TGC	AAC	CCC	GTT	CTC	ACT	CGT	GGG	CAG	AGG
						V	N	R	L	L	T	R	G	E	S
KF387488 *A. exitialis*	TGC	GTC	GGT	GAC	GAC	GTC	AAC	CGC	CTC	CTC	ACT	CGT	GGC	GAG	AGC
						V	N	R	L	L	T	R	G	E	R
EU196142 *A. bisporigera*	TGC	GTC	GGT	GAC	GAC	GTC	AAC	CGT	CTC	CTC	ACT	CGT	GGT	GAG	AGG
						V	N	F	I	L	T	R	G	Q	K
KF546298 *A. fuligineoides*	TGC	GTT	GGT	GAC	GAT	GTC	AAC	TTC	ATC	CTC	ACT	CGT	GGC	CAG	AAG
						V	N	R	L	L	A	R	G	E	K
KF546296 *A. fuliginea*	TGC	GTC	GGT	GAC	GAC	GTT	AAC	CGC	CTC	CTC	GCT	CGT	GGC	GAG	AAG
						I	N	R	L	L	T	R	G	E	K
KF552098 *A. pallidorosea*	TGC	GTC	GGT	GAC	GAC	ATC	AAC	CGC	CTC	CTC	ACT	CGT	GGC	GAG	AAG
KF546303 *A. phalloides*	TGC	GTC	GGT	GAC	GAC	ATC	AAC	CGC	CTC	CTC	ACC	CGC	GGC	GAG	AAG
KC778570 *A. oberwinklerana*	TGC	GTC	GGT	GAC	GAC	ATC	AAC	CGC	CTC	CTC	ACT	CGT	GGC	GAG	AAG
						S	N	R	L	L	T	R	G	E	K
KC778568 *A. subjunquillea*	TGT	GTC	GGT	GAC	GAC	ATC	AGC	CGC	CTT	CTC	ACT	CGT	GGC	GAG	AAG
KF546306 *A. rimosa*	TGT	GTC	GGT	GAC	GAC	ATC	AGC	CGC	CTT	CTC	ACT	CGT	GGC	GAG	AAG

in a species of *Amanita* sect. *Phalloideae* that does not produce this toxin. This finding is in contrast with the study of Hallen et al. (2007), concluding that all of the species synthesising amatoxins and phallotoxins, but none of the other species, hybridised to AMA and PHA genes probes (based on the same primers used in this study). However, while successful PCR amplification proves the presence of a gene (PHA gene in this case), an unsuccessful PCR, possibly due to primer mismatches, cannot be used to prove the absence of the genes encoding α- and β-amanitin, whose exact DNA sequence for these specimens is not known.

Taxonomy

***Amanita bweyeyensis* Fraiture, Raspé & Degreef, sp. nov.**
Figs 3, 4
MycoBank no.: MB830175

Diagnosis. *Amanita bweyeyensis* differs from the closest *Amanita* species by: pileus first pale brownish-grey then entirely whitish or with a faintly yellowish or pale beige shade, basal bulb of the stipe globose, neither pointed nor rooting, basidiospores subglobose to widely ellipsoid (Q = 1.10–1.17–1.28), absence of α- and β-amanitin, phalloidin and phallacidin in its basidiomata, connection with the genus *Eucalyptus* and distribution in Burundi, Rwanda and Tanzania.

Holotypus. RWANDA. Western Prov.: buffer zone Nyungwe forest, Bweyeye (02°36.62'S; 29°14.04'E), ca. 2050 m alt., 16 Apr. 2015, J.Degreef 1304 (BR!).

Description. Primordium subglobose, smooth, whitish or with a weak olive tint. **Pileus** 40–73–120 mm diam., first hemispherical then expanding to regularly convex or applanate, without umbo; margin even, not striate nor appendiculate, in some mature specimens the pileipellis does not reach the edge of the pileus, leaving free the extreme tip of the lamellae; first pale brownish-grey (close to 6B2 or 6C2–3), then often entirely whitish or with a faintly yellowish or pale beige shade (between 4A2 and

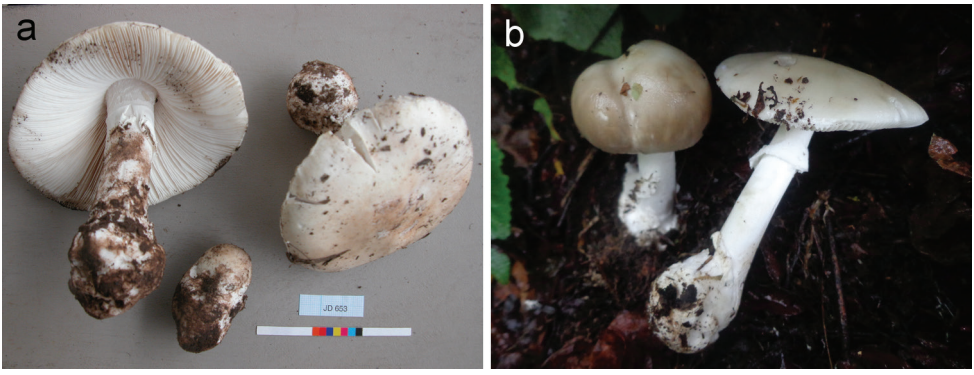

Figure 3. Basidiomata of *Amanita bweyeyensis*. **a** Degreef 653 **b** Degreef 1257.

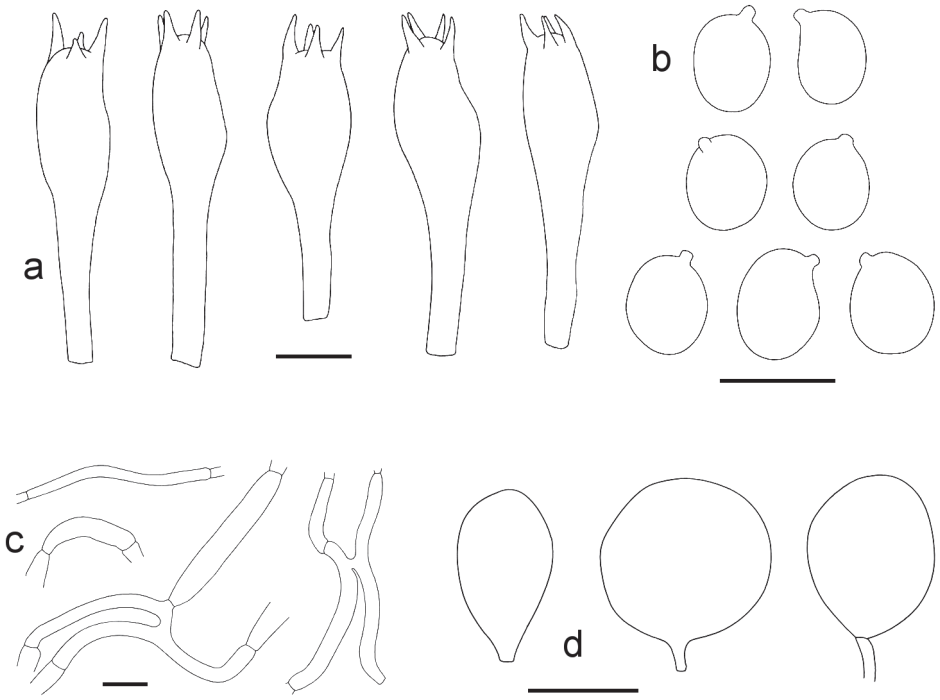

Figure 4. *Amanita bweyeyensis* **a** Basidia (from Degreef 1257, scale bar: 10 µm) **b** Spores (from Saarimäki et al. 591, scale bar: 10 µm) **c** Filamentous hyphae from the volva (from Degreef 1304, holotypus, scale bar: 20 µm) **d** Sphaerocysts from the volva (from Degreef 1304, holotypus, scale bar: 50 µm).

5B2); somewhat viscid, smooth, devoid of veil remnants. **Lamellae** free, white, becoming slightly yellowish when old and ochraceous, pinkish-beige to pale pinkish-brown on the exsiccates with a narrow white and fluffy edge; mixed with an equal number of lamellulae which are very variable in length and are usually truncated; sub-distant, 8–9 lamellae and lamellulae per cm at 1 cm from the edge of the pileus, about 120–160 lamellae and lamellulae in total (counts on 5 basidiomata), 3–14 mm broad, serrate when seen with a magnifying glass. **Stipe** 65–95–152 × 7–25 mm, ratio length of the stipe/diam. of pileus = 1.04–1.25–1.38; sub-cylindrical, slightly wider just under lamellae, gradually and slightly widened from top to bottom, white, with finely fibrillose surface, hollow (at least on exsiccates). Ring white, hanging, membranous but thin and fragile, finely fibrillose, smooth to somewhat plicate longitudinally, upper part adhering to the stipe and often more or less striate. Basal bulb of the stipe globose, sometimes a bit elongated but neither pointed nor rooting, up to 45 mm wide, surrounded by a white volva (also white inside), membranous, up to 30–35 mm high. **Context** white, soft; smell sweetish, conspicuous; taste not recorded.

Basidiospores hyaline, with thin, amyloid wall, (globose-) subglobose to widely ellipsoid (-ellipsoid), rather often with a mangiform or amygdaliform profile, (7.5-) 8.0–8.81–9.5 (-11.0) × (6.0-) 7.0–7.54–8.5 (-9.0) µm, Q = (1.00-) 1.10–1.17–1.28

(-1.58) [112/4/2]. **Basidia** 4-spored, without clamp, thin-walled, clavate, often rather abruptly swollen, 36–42.3–50 × (8.0-) 10.5–12.0–14 (-15) µm, l/w = 2.6–3.59–4.2 (-5.5) [66/4/2]. **Lamellar edge** sterile, composed of sphaeropedunculate marginal cells which are widely clavate to pyriform, hyaline, thin-walled, smooth, without clamp, 18–26.3–32 (-37) × 12–17.0–20 (-33) µm, l/w = (1.00-) 1.33–1.57–1.83 (-2.33) [40/4/2]. **General veil** (volva) mostly composed of cylindrical hyphae, with very different diameters, (15-) 35–80 (-110) × 2–8.5–15 (-26) µm, hyaline, with smooth and thin wall, septate, with rather frequent anastomoses between parallel hyphae, without clamps, branched, mixed with very few sphaerocysts, thin-walled, smooth, globose to ovoid, 33–76–125 × (25-) 32–56–95 µm, l/w = 1.00–1.52–2.25 [20/2/2].

Distribution. At present, the species is only known from Burundi, Rwanda and Tanzania but, according to its ecology, it could probably be observed in all *Eucalyptus* plantations in tropical Africa and possibly in South Africa as well. Consequently, if the species is collected for consumption, care should be taken to avoid confusion with *A. marmorata*, a species growing in the same biotopes and suspected to be highly toxic.

Ecology. On the ground, under *Eucalyptus*. The label of *Saarimäki* 591 indicates "in *Acacia* and *Eucalyptus* forest" whereas the legend of the associated picture (Härkönen et al. 2003: 62) indicates "growing in an *Acacia mearnsii* plantation". However, the litter visible on that picture does not correspond to the latter species but looks like *Eucalyptus* leaves.

Etymology. This species is named after the collection locality of the type specimen in Rwanda.

Specimens examined. BURUNDI. Muravya Prov.: Bugarama, 9 Jan. 2011, J.Degreef 653 (BR). – RWANDA. Western Prov.: buffer zone Nyungwe forest, Bweyeye (02°36.79'S; 29°14.01'E), ca. 2040 m alt., 20 Oct. 2014, J.Degreef 1257 (BR); Ibidem (02°36.62'S; 29°14.04'E), ca. 2050 m alt., 16 Apr. 2015, J.Degreef 1304 (holotype: BR!). – TANZANIA. Pare District: South Pare Mts., Mpepera, ca. 1600 m alt., 5 Dec. 1990, T.Saarimäki et al. 591 (H).

Notes. During collecting field trips in Rwanda, one of us (JD) was confused by observing local people (Abasangwabutaka) picking huge quantities of this mushroom in old *Eucalyptus* plantations and eating them (after removal of the cuticle) without experiencing any trouble. The species was not observed to be eaten in Burundi and is probably not used in Tanzania either.

It is quite likely that the specimen shown in a picture by van der Westhuizen and Eicker (1994: 38) under *Amanita phalloides* var. *alba* is *Amanita bweyeyensis*. This specimen was observed at Sabie (South Africa), growing in the leaf-litter under *Eucalyptus cloeziana* in early December and again in March. The pileus surface is described as "white and occasionally faintly yellowish over the central part" and the pileus margin as "very finely denticulate". Härkönen et al. (2003: 62) already drew attention to that picture.

A comparison with the closely related species is given in the chapter "discussion" below.

Amanita harkoneniana **Fraiture & Saarimäki, sp. nov.**
Figs 5, 6
MycoBank no.: MB830176

Diagnosis. *Amanita harkoneniana* differs from the closest *Amanita* species by: pileus first whitish to pale yellowish-beige then entirely whitish, devoid of veil remnants, basal bulb of the stipe turnip-shaped or irregularly elongated and more or less rooting, basidiospores subglobose to widely ellipsoid (Q = 1.04–1.13–1.25), basidia 34–37.5–41 µm long and growth without connection with the genus *Eucalyptus*, in Tanzania and Madagascar.

Holotypus. TANZANIA. Tabora District: ca. 10 km S of Tabora, Kipalapala, ca. 1200 m alt., 12 Dec. 1991, T.Saarimäki et al. 1061 (H!).

Description. Primordium smooth, subglobose but with a more or less conical or irregular rooting part; veil whitish; pileus with a weak brownish tint (around 4B2–3 and 5B2–3 but paler). **Pileus** 35–53–70 mm diam., first hemispherical, then largely conical or convex to nearly applanate, often with a deflexed margin, without umbo; margin even, neither striate (sometimes striate on exsiccates) nor appendiculate; first whitish to pale yellowish-beige (between 4A2 and 4B2) then entirely whitish; slightly viscid when young, smooth, devoid of veil remnants. **Lamellae** white, becoming slightly yellowish when old and pale to dark brownish in exsiccates with a narrow white and fluffy edge, free, mixed with an equal number of lamellulae which are very variable in length and are usually truncated, sub-distant, 8–10 lamellae and lamellulae per cm at 1 cm from the edge of the pileus, about 125–215 lamellae + lamellulae in total (counts on 2 basidiomata), ventricose, very finely serrate when seen with a magnifying glass. **Stipe** 65–130 × 8–14 mm, sub-cylindrical, slightly wider just under the lamellae, gradually and slightly widened from top to bottom, white, with finely fibrillose surface, hollow (at least in exsiccates) or stuffed. Ring white, hanging, membranous but thin and fragile, upper part adhering to the stipe. Basal bulb of the stipe turnip-shaped or irregularly elongated, more or less rooting, surrounded by a white volva (also white inside), membranous, up to 40–60 mm high. **Context** white, soft, very thin along the margin of the pileus, much thicker near the stipe; smell weak resembling raw potato

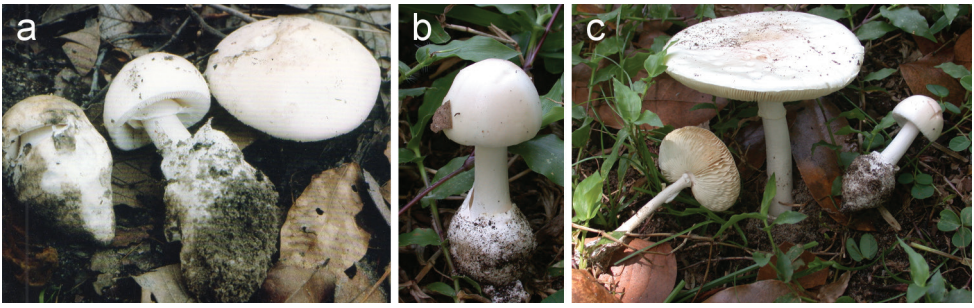

Figure 5. Basidiomata of *Amanita harkoneniana* **a** Saarimäki et al. 1061 (holotypus) **b** Pirot s.n. (coll. 2014) **c** Pirot s.n. (coll. 2014).

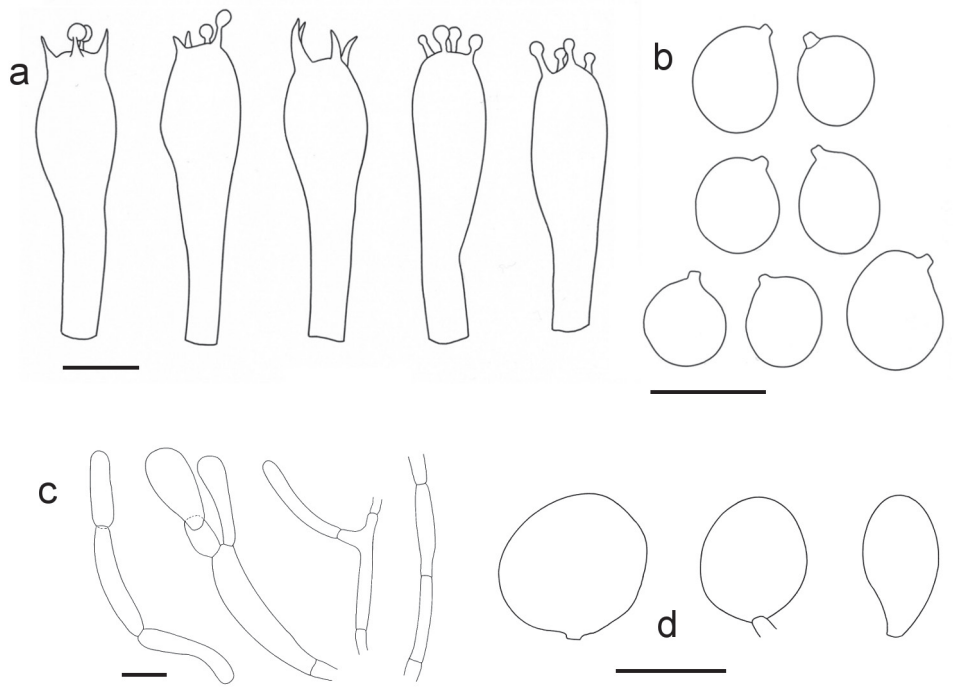

Figure 6. *Amanita harkoneniana* (all from Saarimäki et al. 1061, holotypus) **a** Basidia (scale bar: 10 µm) **b** Spores (scale bar: 10 µm) **c** Filamentous hyphae from the volva (scale bar: 20 µm) **d** Sphaerocysts from the volva (scale bar: 50 µm).

[Harkonen pers. comm.], very variable according to specimens but mostly of shellfish as in *Russula xerampelina*, especially for mature and old specimens [P. Pirot, pers. comm. about specimens from Madagascar], taste mild, then unpleasant [description of the Tanzanian specimen].

Basidiospores hyaline, with thin, rather weakly amyloid wall, (globose-) subglobose to widely ellipsoid (-ellipsoid), (6.5-) 7.0–8.07–8.6 (-10.0) × (6.0-) 6.5–7.15–8.0 (-8.5) µm, Q = (1.00-) 1.04–1.13–1.25 (-1.33) [53/3/2]. **Basidia** 4-spored, without clamp, clavate, often rather abruptly swollen, (30-) 34–37.5–41 (-46) × 9.0–10.4–11.0 (-13.0) µm, l/w = 3.00–3.60–4.40 (-4.90) [31/3/2]. **Lamellar edge** sterile, composed of marginal cells which are widely clavate to pyriform, hyaline, thin-walled, smooth, not clamped, 26–32.2–40 × 13–16.8–20 µm, l/w = 1.56–1.93–2.23 [10/1/1]. **General veil** (volva) mostly composed of cylindrical hyphae, with very different diameters, (20-) 33–50 (-110) × 4–11 (-15) µm, hyaline, with smooth and thin wall, septate but without clamps, with occasional anastomoses between parallel hyphae, branched, mixed with a few scattered hyaline sphaerocysts, globose to sphaeropedunculate or ellipsoid, 45–75–100 (-120) × (20-) 35–57–87 (-115) µm, l/w = 1.04–1.38–1.68 (-2.38), with a smooth and thin wall, rarely slightly thickened (< 1 µm) [18/1/1].

Distribution. Up to now, the species is only known from Tanzania and Madagascar. According to its ecology, it could potentially be observed in all regions occupied by the miombo woodland.

Ecology. In miombo woodland (Tanzania) and in a garden, next to *Cocos nucifera* L., *Citrus* sp. ("combava"), *Tambourissa* sp. and *Psidium guajava* L., along the Indian Ocean (Madagascar).

Etymology. This species is dedicated to Prof. Marja Härkönen in acknowledgment of her tremendous contribution to African mycology.

Specimens examined. MADAGASCAR. Prov. Toamasina: Mahambo, Dec. 2014, P.Pirot s.n. (BR); Ibidem, 2016, P.Pirot s.n. (BR). – TANZANIA. Tabora District: ca. 10 km S of Tabora, Kipalapala, ca. 1200 m alt., 12 Dec. 1991, T.Saarimäki et al. 1061 (holotype: H!).

Note. We believe that the picture of "*Amanita* cfr. *phalloides*" presented by Ryvarden et al. (1994: 76–77) could be *Amanita harkoneniana*. The macroscopic description and the picture given by the authors correspond to the characters of that species. From this description, the fruit-bodies have a nauseous odour, are soon decaying and grow in miombo woodlands or in association with pine trees in the middle of the rainy season; they are rarely seen. No precise locality is given but the book covers South Central Africa (mostly Malawi, Zambia and Zimbabwe).

A comparison with the closely related species is given in the chapter "discussion" below.

Chemical analyses

RP-HPLC analyses of the specimen Degreef *1304* (holotypus of *A. bweyeyensis*) was made by two of us (EK & IA). The analysis showed the complete absence of α-, β- and γ-amanitin as well as that of phallacidin and phalloidin. The results were below the limit of detection (0.6 ng/g) for all the toxins in all the analysed samples: 3 samples with cuticle and 3 samples without cuticle.

It is interesting to mention that another specimen of *A. bweyeyensis* (*Tiina Saarimäki et al.* 591), collected in Tanzania, had been analysed previously, in the Technical Research Centre of Finland in Espoo, and that neither amatoxins nor phallotoxins had been found in that specimen either (Harkonen pers. comm.).

Identification key to the African and Madagascan species of *Amanita* sect. *Phalloideae*

1 Spores elongated, Q > 1.45. Slender species, ratio stipe length / pileus diameter > 1.5. Ring funnel-shaped on young basidiomata, not striated. Pileus margin often striated because of the thinness of the flesh .....
.....***Amanita strophiolata*** [incl. var. *bingensis*]

Pileus 50–60 mm diam., dirty white, often with a yellowish or greenish centre. The original description of var. *bingensis* mentions a pungent taste. Spores (7-) 7.5–10.0 (-10.5) × (4.0-) 5.0–6.5 (-7.0) µm, Q = 1.40–1.75.

– Spores less elongated, Q < 1.45. Less slender species, ratio stipe length / pileus diameter < 1.5. Ring never ascending, striated or not. Pileus margin not striated **2**

2 Pileus greenish or olivaceous, sometimes yellowish-green or brownish-green, virgate (i.e. with fine darker radial stripes). Smell of old rose or rotten honey in age ..... ***Amanita phalloides***
Pileus 65–152 mm diam., ring striate. Spores 7.5–10.0 (-12.5) × (5.5-) 6.0–7.5 (-8.0) µm.

– Pileus whitish, greyish or pale brownish (sometimes olivaceous grey with a paler margin but then, strong smell of garlic), not virgate but sometimes radially marbled. Smell fungoid or different **3**

3 Strong garlic smell, persisting several months in herbarium specimens. Spores subglobose, mean Q < 1.5 ..... ***Amanita alliiodora***
Pileus viscid, olivaceous grey, with a pallid margin, about 50 mm diam., ring striated.

– Smell fungoid or different. Spores subglobose or more elongated **4**

4 Lamellae staining yellowish when bruised ..... ***Amanita thejoleuca***
Pileus 60–80 mm diam., pale yellowish-brown, darker in the centre. Ring rather fugacious, often missing on mature specimens. Spores 7–8 × 5–6 µm (original description), or 10–12 × 7.5–10 µm (after the spore drawings in Gilbert, 1941)

– Gills not yellowing when bruised **5**

5 Pileus white at first, soon radially marbled by pale brownish or greyish streaks. Species mostly associated with various species of *Eucalyptus*, also mentioned once under *Casuarina equisetifolia* ..... ***Amanita marmorata***
Pileus 25–95 mm diam., ring striated. Spores (6.5-) 7.5–9.5 (-11.5) × (5.5-) 6.0–8.0 (-10.0) µm, Q = 1.05–1.40

– Pileus not marbled, uniformly coloured or paler at margin, whitish to mouse grey or pale brownish. Species bound or not with *Eucalyptus* **6**

6 Pileus mouse grey, dry. Ring striated ..... ***Amanita murinacea***
Pileus 70–80 mm diam. Spores 7.5–8.5 × 7–8 µm, mean Q = 1.15

– Pileus whitish to pale brownish or greyish, often more or less viscid. Ring striated or not **7**

7 Species growing under *Eucalyptus*. Bulb at stipe base +/- globose, neither pointed nor rooting. Ring striated. Smell sweetish, conspicuous ..... ***Amanita bweyeyensis***

– Species not bound with *Eucalyptus*, found in Miombo woodland and in a garden. Bulb at stipe base turnip-shaped to rooting. Ring smooth or vaguely plicate. Smell weak resembling raw potato ..... ***Amanita harkoneniana***

Discussion

The fact that *A. bweyeyensis* seems to grow always in association with *Eucalyptus* species (Myrtaceae), which are not indigenous in Africa, suggests that the fungus has been introduced with the trees. Such introductions are well known (see for example Díez 2005). Vellinga et al. (2009) stress the fact that Pinaceae and Myrtaceae are the plant families which are the most frequently reported as hosts of introduced mycorrhizal fungi. They also mention that South Africa is the African country with the highest number of mycorrhizal introductions. We therefore compared *A. bweyeyensis* more specifically with the Australian species of *Amanita* sect. *Phalloideae* (Reid 1979, Miller 1991, Wood 1997, Davison et al. 2017, Tulloss 2018). We believe that conspecificity with any of these species can be excluded, because they present one or several of the following characters: spores too elongated (mean Q ≥ 1.4), pileus strongly coloured (brown or grey), pileus with patches of general veil, ring absent, stipe not bulbous, different host, toxin content etc.

Amanita marmorata Cleland & E.-J. Gilbert was described from New South Wales (Australia) (Gilbert 1941). It was subsequently re-described from South Africa, under the name *A. reidii* Eicker & Greuning (Eicker et al. 1993, Cai et al. 2014) and from Hawaii, sub *A. marmorata* subsp. *myrtacearum* O.K. Mill., Hemmes & G. Wong (Miller et al. 1996). Before the description of *A. reidii* in 1993, African collections of that taxon were often called *Amanita phalloides* var. or f. *umbrina* (see e.g. van der Westhuizen and Eicker 1994:41). The species is present in Africa and it grows in connection with the genus *Eucalyptus* but it can be separated from *A. bweyeyensis* by its whitish pileus marbled with grey brown radial streaks and by the presence of phalloidin and phallacidin in its basidiomata (Hallen et al. 2002, Davison et al. 2017). The presence of α- and β-amanitin in *A. marmorata* remains ambiguous. Hallen et al. (2002) stated that those toxins were present in the species (sub *A. reidii* and probably also sub *A. phalloides* f. *umbrina*), whilst Davison et al. (2017) could not detect them. The marbled colour, the globose bulb and the connection with *Eucalyptus* also exclude conspecificity with *A. harkoneniana*. *A. marmorata* is also well separated from our two new species in all the phylogenetic inferences (Figs 1 and 2).

Three new species of *Phalloideae* were recently found in Australia, namely *Amanita djarilmari* E.M.Davison and *A. gardneri* E.M.Davison from the south-west of Australia and *A. millsii* E.M.Davison & G.M.Gates from Tasmania (Davison et al. 2017). The three species have a white- or pale-coloured pileus and a white universal veil. They are quite similar to our two new species, but are however well separated from them in the phylogenetic trees (Figs 1 and 2). The following differences with *A. bweyeyensis* can also be cited: *A. djarilmari* has elongated spores (mean Q = 1.43) and contains phallacidin and phalloidin; *A. gardneri* has a fusiform bulb at stem base, becoming radicant, very elongated spores (mean Q = 1.81) and contains phallacidin and phalloidin; *A. millsii* is apparently not connected with *Eucalyptus* species and it contains phallacidin and phalloidin. The three species can be separated from *A. harkoneniana* by the following characters: *A. djarilmari* has a rounded bulb at stem base and elongated spores (mean

Q = 1.43); *A. gardneri* has very elongated spores (mean Q = 1.81); *A. millsii* shows persistent patches of universal veil on the pileus, its basidiomata have a more squat habit and its basidia are longer (43–61 µm).

Amanita capensis A. Pearson & Stephens is a nom. nud. which was published in Stephens and Kidd (1953) and quite largely used in South Africa ("Cape death cap"). It is usually considered as a stouter colour variant of *Amanita phalloides*, including specimens with a whitish pileus (Levin et al. 1985, Reid and Eicker 1991). Several cases of severe poisoning have been attributed to the "species", some of them fatal (Stephens and Kidd 1953, Steyn et al. 1956, Sapeika et al. 1960). It is therefore surprising that Hallen et al. (2002) did not find the toxins in a specimen identified as *A. capensis* but the exact identity of the fungus remains uncertain and confusion with another taxon cannot be excluded. Conspecificity with *A. bweyeyensis* can be rejected amongst others because of the toxicity and pileus colour of *A. capensis* as well as of its association with other trees than *Eucalyptus*. *Amanita capensis* differs from *A. harkoneniana* amongst others because it has a larger size than this latter species, a globose bulb and a striated ring.

Amanita alliiodora Pat. is a very poorly known species, described from Madagascar by Patouillard (1924, corrected version in 1928). The most important characteristics of the species are the following. Pileipellis pale olivaceous grey, whitish at the margin, viscid when moist. Ring striated. Spores subglobose, 7–8 µm diam. (Patouillard 1924, 1928) or 8–8.5 µm diam. (Dujarric de la Rivière and Heim 1938), or 8.5–9.6 × 7.8–8.7 µm (Tulloss 2017, after the spore drawings from the type specimen, published in Gilbert 1941). The species is also said to have a bitter taste and to produce a strong smell of garlic, still persisting on exsiccates. It is considered toxic and is not eaten by the local population, which however uses its odour to cure headaches. *A. alliiodora* is distinct from *A. bweyeyensis* because it has a grey pileus and a strong smell of garlic and also because it does not grow in association with *Eucalyptus* and is probably toxic. It is also distinct from *A. harkoneniana* because of the grey pileus, the striated ring and the smell of garlic. *A. alliodora* clustered in a sister position to *A. bweyeyensis* in the ITS-nucLSU based phylogenetic analysis, forming a two-species clade sister to *A. harkoneniana*, showing that these three species share a common phylogenetic background.

Within the genus *Amanita*, the genes encoding amatoxins (α- and β-amanitin) and phallotoxins (phallacidin and phalloidin) were found so far to be present only in species that produce these compounds (Hallen et al. 2007). The successful PCR amplification of the PHA gene for both *A. bweyeyensis*, a species which is regularly consumed by local people and *A. harkoneniana* was indeed surprising. Especially since the HPLC analysis did not show any sign of these compounds in the basidiomata. This is the first time that the presence of at least one of those genes (PHA gene) could be proven for species that seem to lack (or have lost) the ability to produce these toxins.

Very little is indeed known about the mechanisms behind the regulation of the fungal secondary metabolism. Many factors can play a key role in preventing the expression of phallacidin gene in these species. Several studies (Enjalbert et al. 1993, 1999; Brüggemann et al. 1996; Mcknight et al. 2010; Kaya et al. 2015) have shown

that phallotoxin amounts and distribution (localisation in the basidiome) in *A. phalloides* largely vary as a result of environmental and climatic conditions. Furthermore, several studies have shown that the toxin concentration in the pure cultured mycelium of deadly *Amanita* is about 10% of that in basidiomata and that it is indeed possible to increase the amatoxin production through optimisation of growth conditions, such as medium composition, pH and temperature etc. (Zhang et al. 2005, Hu et al. 2012). Furthermore, temporal and structural sequestration of secondary metabolites are common features in microorganisms. Amatoxins and phallotoxins are biologically active secondary metabolites and some mechanism of separation from primary metabolism seems to be essential to avoid their coming into contact with their sites of action (RNA polymerase II and F-actin, respectively). Having higher toxin concentrations only in the basidiome, or part of it, would invest resources for defence where it is especially needed, in the visible and vulnerable mushroom and not microscopic spores or mycelia.

Amatoxins and phallotoxins are encoded by members of the "MSDIN" gene family and are synthesised on ribosomes as short (34- to 35-mer) pro-proteins, with conserved upstream and downstream sequences flanking a hypervariable region of 7 to 10 amino acids (Hallen et al. 2007, Luo et al. 2012). The hypervariable region gives rise to the linear peptides corresponding to the mature toxins. The precursor peptides must undergo several post-translational modifications, including proteolytic cleavage, cyclisation, hydroxylation and formation of a unique tryptophan-cysteine cross bridge called tryptathionine. In particular, they are cleaved and macrocyclised into 7–10 amino acid cyclic peptides by a specialised prolyl-oligo-peptidase enzyme (POP), which is the key enzyme of the cyclic peptide pathway, catalysing both hydrolysis (Luo et al. 2009, 2014; Riley et al. 2014).

The genes of most secondary metabolite biosynthetic pathways tend to be clustered and co-regulated in fungi (e.g. fumonisin biosynthesis in *Fusarium*). Many, but not all, clusters contain cluster-specific transcription factors that regulate expression of the biosynthetic genes for their respective metabolites, thus allowing for multiple regulatory layers giving the producing fungus precise spatial and temporal control over metabolite expression. A mutation in each key protein involved in the biosynthetic/regulatory pathway of phallotoxins production could result in an altered expression of the toxin. The evolutionary persistence of toxins productions in *Amanita* sect *Phalloideae* suggests that it should confer some selective advantage to the producing fungi. Since the lack of toxins could be the result of an alteration of the expression of these genes due to environmental and climatic conditions, in our opinion *A. bweyeyensis* and *A. harkoneniana* should be considered to have the potential to be deadly poisonous.

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