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Phylogenetic studies uncover a predominantly African lineage in a widely distributed lichen-forming fungal species

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Abstract

A number of lichen-forming fungal species are widely distributed. Here, we investigate biogeographic patterns in a widely distributed isidiate taxon – *Parmelinella wallichiana* – using molecular sequence data. Our results revealed that *Parmelinella wallichina*, as currently circumscribed, is not monophyletic but falls into four clades, two of them represented by a sample only. A third clade, occurring in Africa and southern India is described as a new species, *Parmelinella schimperiana* Kirika & Divakar, **sp. nov.** Our study adds a further example of previously overlooked, geographically distinct, lineages that were discovered using molecular data.

Key words

Africa, genealogical criteria, molecular systematics, new species, Parmeliaceae, *Parmelinella*, parmelioid lichens, phylogeny, taxonomy

Introduction

The advent of DNA sequence technologies and advances in molecular phylogenetic methods have revolutionized our understanding on species delimitation and systematics in lichens and fungi in general (reviewed in Crespo and Lumbsch 2010; Lumbsch and Leavitt 2011; Divakar and Crespo 2015; Leavitt et al. 2015a). Molecular sequence data have led to the circumscription of previously overlooked species-level lineages and a number of taxonomic re-evaluations of widely distributed species in lichenized fungi. Diverse examples of widely distributed nominal taxa masking multiple, species-level diversity include: *Cladia aggregata* (Parnmen et al. 2012), *Melanelixia glabra* (Divakar et al. 2010a), *Melanelixia fuliginosa*/*M. glabratula* (Leavitt et al. 2012), *Melanohalea elegantula*, *M. exasperata* (Leavitt et al. 2013a), *Montanelia tominii* (Leavitt et al. 2015b), *Parmelia saxatilis* (Molina et al. 2011a), *P. sulcata* (Divakar et al. 2005, Molina et al. 2011b), *Parmelina quercina* (Argüello et al. 2007), *P. tiliacea* (Nuñez-Zapata et al. 2011), *Protoparmelia badia* (Singh et al. 2015), *Physconia distorta* (Divakar et al. 2007), *Rhizoplaca melanophthalma* (Leavitt et al. 2013b), and *Sphaerophorus globosus* (Högnabba and Wedin 2003). These studies demonstrate that some supposedly cosmopolitan taxa may include previously unrecognized diversity. Thus re-evaluation of widely distributed species applying molecular sequence data is prerequisite to understanding biogeographic patterns in broadly distributed taxa. Furthermore, inaccurate species assessment may have negative consequences for conservation purposes or understanding of diversification patterns.

Parmelinella is a small genus (ca. 10 species) and belongs to the parmelioid clade in the family Parmeliaceae (Divakar et al. 2015). The species included in this genus are characterized by a pored epicortex, isolichenan in the cell walls, subirregular lobes, cylindrical or bifusiform conidia, simple cilia and rhizines, and a yellow-grey upper cortex - containing secalonic acid derivatives and atranorin (Elix 1993; Crespo et al. 2010; Thell et al. 2012). Species in the genus are mainly distributed in subtropical to tropical regions of Africa, Asia, Australasia and South America. *Parmelinella chozoubae*, *P. manipurensis* and *P. nimandairana* are restricted to Asia; P. *salacinifera*, is reported from Southeast USA, central and south America, and Thailand; *P. simplicior* occurs in Asia and East Africa; and *P. cinerascens*, *P. lindmanii*, *P. mutata* and *P. versiformis* are endemic to South America (Elisaro et al. 2010; Benatti 2014). For a long time only four additional *Parmelinella* species were known from India (Divakar and Upreti 2005), but recent studies added six species to the genus, most of which had previously been known to occur only in South America (Elisaro et al. 2010; Benatti 2014). Of the ten species, only two, *P. simplicior* and *P. wallichiana*, have previously been reported from East Africa (Swinscow and Krog 1988; Alstrup et al. 2010).

Parmelinella wallichiana is the only widely distributed species in this genus and is known from Africa, Asia, Australia and South America. While it is widespread in East Africa and Asia, the species is known from a few localities in Australia and South America. *Parmelinella wallichiana* normally reproduces asexually by isidia and grows in wide range of ecological environments. The species is most frequently epiphytic but

also found rarely on rocks. Studies have demonstrated broad, intercontinental distributions of a number of lichen-forming fungi that reproduce via asexual propagules (see e.g. Divakar et al. 2005; Molina et al. 2011a and b; Leavitt et al. 2013a; Roca-Valiente et al. 2013; Divakar et al. 2016).

This study aims to assess biogeographic patterns in the widely distributed, isidiate, lichen-forming fungal species *Parmelinella wallichiana*. To this end, we generated DNA sequences of nuclear ribosomal internal transcribed spacer region (ITS1, 5.8S and ITS2), large subunit (nuLSU) and mitochondrial small subunit (mtSSU). Phenotypical features were re-evaluated and compared in light of the relationships inferred from the phylogenetic reconstructions.

Materials and methods

Taxon sampling

A DNA data matrix was assembled using sequences of nuclear ITS, nuLSU and mitochondrial SSU rDNA of 21 samples, representing 18 specimens of *P. wallichiana* s. lat. from Africa, Asia and S. America assembled together with DNA sequences of *P.* aff. *wallichiana* and *P. lindmanii* (Elisaro et al. 2010) downloaded from GenBank. GenBank accession numbers and information of studied materials are shown in Table 1. The data sets include 12 sequences from previous publications (Blanco et al. 2004; Divakar et al. 2004; Divakar et al. 2006; Divakar et al. 2010b: Eliasaro et al. 2010: Kirika et al. 2015), and 25 were newly generated for this study. Three specimens of *Bulbothrix isidiza* were used as an out-group since it has been shown to belong to a sister group in a previous study (Kirika et. al.2015).

DNA extraction and PCR amplification

Total genomic DNA was extracted from small pieces of thallus devoid of any visible damage or contamination using the USB PrepEase Genomic DNA Isolation Kit (USB, Cleveland, OH) in accordance with the manufacturer's instructions. We generated sequence data from nuclear ribosomal markers, the ITS region and a fragment of the nuLSU, in addition to a fragment of the mtSSU. Polymerase-chain-reaction (PCR) amplifications were performed using Ready-To-Go PCR Beads (GE Healthcare, Pittsburgh, PA, USA) using the dilutions of total DNA. Fungal ITS rDNA was amplified using ITS1F primers (Gardes and Bruns 1993), ITS4 and ITS4A (White et al. 1990; Larena et al. 1999); mtSSU rDNA was amplified using the primers mrSSU1, mrSSU3R and mrSSU2R (Zoller et al. 1999); nuLSU rDNA was amplified using LR0R and LR5 (Vilgalys and Hester 1990). PCR products were visualized on 1% agarose gel and cleaned using ExoSAP-IT (USB, Cleveland, OH, USA). Cycle sequencing of complementary strands was performed using BigDye v3.1 (Applied Biosystems,

Table 1. Specimens used in this study, with location, reference collection detail and GenBank accession numbers.

Species	Seq/DNA code	Locality	Collector(s)	Voucher specimen	GenBank accession numbers		
					ITS	mtSSU	nuLSU
Bulbothrix isidiza	15505	India: Sikkim	Divakar	MAF	KX341979	-	KX341998
Bulbothrix isidiza	BUIS318it	Congo	Mamush s/n	MAF-Lich 15511	GQ919262	GQ919210	GQ919237
Bulbothrix isidiza	BUIS1376I	Madagascar: Col de Tapia N Ambositra		Ertz 12878 (BR)	GQ919263	GQ919238	GQ919211
Parmelinella wallichiana	3122	Brazil: Curitiba	S. Eliasaro	UPCB	GQ267691	-	-
Parmelinella aff. *wallichiana*	BRYC56001	Cameroon: Ekona	Orock	BRYC56001	JQ673451	-	-
Parmelinella wallichiana	7653	India: Sikkim	Chatterjee & Divakar	MAF-7653	AY611106	AY611165	AY607819
Parmelinella wallichiana	322	India: Uttaranchal	Divakar	MAF	KX341980	KX341990	KX341999
Parmelinella wallichiana	3615	India: South India	Lumbsch et al.	F	KX341981	-	-
Parmelinella wallichiana	4678	Kenya: Eastern	Kirika & Lumbsch, 4678	EA, F, MAF	KX341982	KX341991	-
Parmelinella wallichiana	4715	Kenya: Eastern	Kirika & Lumbsch, 4715	EA, F, MAF	KX341983	KX341992	KX342000
Parmelinella wallichiana	9310	Kenya: Eastern	Kirika, 3432	EA, F	-	KX341993	KX342001
Parmelinella wallichiana	9311	Kenya: Eastern	Kirika, 3487	EA, F	KX341984	-	KX342002
Parmelinella wallichiana	9312	Kenya: Eastern	Kirika, Malombe & Matheka, 3703	EA, F	KX341985	-	KX342003
Parmelinella wallichiana	9397	Kenya: Rift Valley	Kirika, 3334	EA, F	KX341986	KX341994	KX342004
Parmelinella wallichiana	9398	Kenya: Rift Valley	Kirika, 3145	EA, F	-	-	KX342005
Parmelinella wallichiana	9552	Kenya: Rift Valley	Kirika, Mugambi & Lumbsch, 2815	EA, F	-	KX341995	KX342006
Parmelinella wallichiana	9592	Kenya: Rift Valley	Kirika, Mugambi & Lumbsch, 2974	EA, F	KX341987	-	-
Parmelinella aff. *wallichiana*	9647	Kenya: Coast	Kirika & Lumbsch, 4033	F,EA,MAF	KX341988	KX341996	KX342007
Parmelinella wallichiana	9693	Kenya: Eastern	Kirika, 4280	F,EA,MAF	KX341989	KX341997	KX342008
Parmelinella wallichiana	250204	China: Yunnan	Crespo, Blanco & Arguello	MAF-L-10411	DQ279532	DQ287842	-
Parmelinella lindmanii	3131	Brazil: Curitiba	S. Eliasaro	UPCB	GQ267190	-	-

Foster City, CA, USA) and the same primers used for PCR amplifications. Sequenced PCR products were run on an ABI 3730 automated sequencer (Applied Biosystems) at the Pritzker Laboratory for Molecular Systematics and Evolution at the Field Museum, Chicago, IL, USA.

Sequence editing and alignment

New sequences were assembled and edited using GENEIOUS v8.1.7 (Biomatters Ltd. 2005–2015). Multiple sequence alignments for each locus were performed using the program MAFFT v7 (Katoh et al. 2005; Katoh and Toh 2008). For the ITS and nuLSU sequences, we used the G-INS-i alignment algorithm and '20PAM / K=2' scoring matrix, with an offset value of 0.3, and the remaining parameters were set to default values. We used the E-INS-i alignment algorithm and '20PAM / K=2' scoring matrix, with the remaining parameters were set to default values for the mtSSU sequences. The program Gblocks v0.91b (Talavera and Castresana 2007) was used to delimit and remove ambiguous alignment nucleotide positions from the final alignments using the online web server (http://molevol.cmima.csic.es/castresana/Gblocks_server.html), implementing the options for a less stringent selection of ambiguous nucleotide positions, including the 'Allow smaller final blocks', 'Allow gap positions within the final blocks', and 'Allow less strict flanking positions' options.

Phylogenetic analyses

Phylogenetic relationships were inferred using maximum likelihood (ML), and Bayesian inference (BI). Exploratory phylogenetic analyses of individual gene topologies showed no evidence of well-supported (≥ 70% bootstrap values) topological conflict, thus relationships were estimated from a concatenated, three-locus (ITS, nuLSU, mtSSU) data matrix using a total-evidence approach (Wiens 1998). We used the program RAxML v8.1.11 (Stamatakis 2006; Stamatakis et al. 2008) to reconstruct the concatenated ML gene-tree using the CIPRES Science Gateway server (http://www.phylo.org/portal2/). We implemented the 'GTRGAMMA' model, with locus-specific model partitions treating all loci as separate partitions, and evaluated nodal support using 1000 bootstrap pseudoreplicates. Exploratory analyses using alternative partitioning schemes resulted in identical topologies and highly similar bootstrap support values. We also reconstructed phylogenetic relationships from the concatenated multilocus data matrix under BI using the program BEAST v1.8.2 (Drummond and Rambaut 2007). We ran two independent Markov Chain Monte Carlo (MCMC) chains for 20 million generations, implementing a relaxed lognormal clock, a birth-death speciation process prior. The most appropriate model of DNA sequence evolution was selected for each marker using the program PartitionFinder v1.1.1 (Lanfear et al. 2012), treating the ITS1, 5.8S, ITS2, nuLSU, and mtSSU as separate partitions. The

first 2 million generations were discarded as burn-in. Chain mixing and convergence were evaluated in Tracer v1.5 (Rambaut and Drummond 2009), considering ESS values >200 as a good indicator. Posterior trees from the two independent runs were combined using the program LogCombiner v1.8.0 (Drummond et al. 2012), and the final maximum clade credibility (MCC) tree was estimated from the combined posterior distribution of trees.

Morphological and chemical studies

Morphological characters, including lobe shape, size and width, cilia and rhizines were studied using a Leica Wild M 8 dissecting microscope. All the specimens of *P. wallichiana* included in the molecular analysis were evaluated (see Table 1). In the case of the new species, additional herbarium specimens were also studied.

Observations and measurements of ascospores were made in water, at 40× (objective) and 10× (eye piece) magnification with a Leica Leitz DM RB microscope. For each species at least 20 spores from different specimens were measured. Mean value (M) and standard deviation (SD) were calculated. In the description of the new species, the results of the measurements are given as (minimum value observed) M ± SD (maximum value observed). M, SD and n (number of spores measured) are expressed within parentheses. Chemical constituents were identified by thin layer chromatography using standard methods (Orange et al. 2010). Extraction of secondary metabolites for TLC analysis was done by pacing small pieces of the thallus in Eppendorf tubes and then adding a few drops of acetone in the tube. The resulting extract was then spotted on glass plates coated with Silica gel using capillary tubes. Plates were developed in Camag horizontal developing chamber (Oleico Lab Stockholm) using solvent system A (Toluene:Dioxane:acetic acid, 45:15:2), plates were then air dried, sprayed with 10% sulphuric acid and then heated in an oven at 110 degrees Celsius to visualize the spots. Substances were identified by comparing the spots with controls (Orange et al. 2010).

Results and discussion

A total of 28 new DNA sequences of *Parmelinella wallichiana* were generated for this study (Table 1). These were deposited in GenBank under accession numbers KX341978-KX342008. The dataset included samples from wide geographic regions as Asia, East Africa and South America. The final alignment of the combined data set was 2174 positions in length and was comprised of 458 unambiguously aligned nucleotide position characters in ITS, 844 in the nuLSU, and 872 in the mtSSU. As the topologies of the single locus phylogenies did not show any conflicts they were analyzed in a concatenated data matix (data not shown). The ML and BI analyses were identical in their topology and hence only the ML tree with support values of both analyses is depicted in Figure 1.

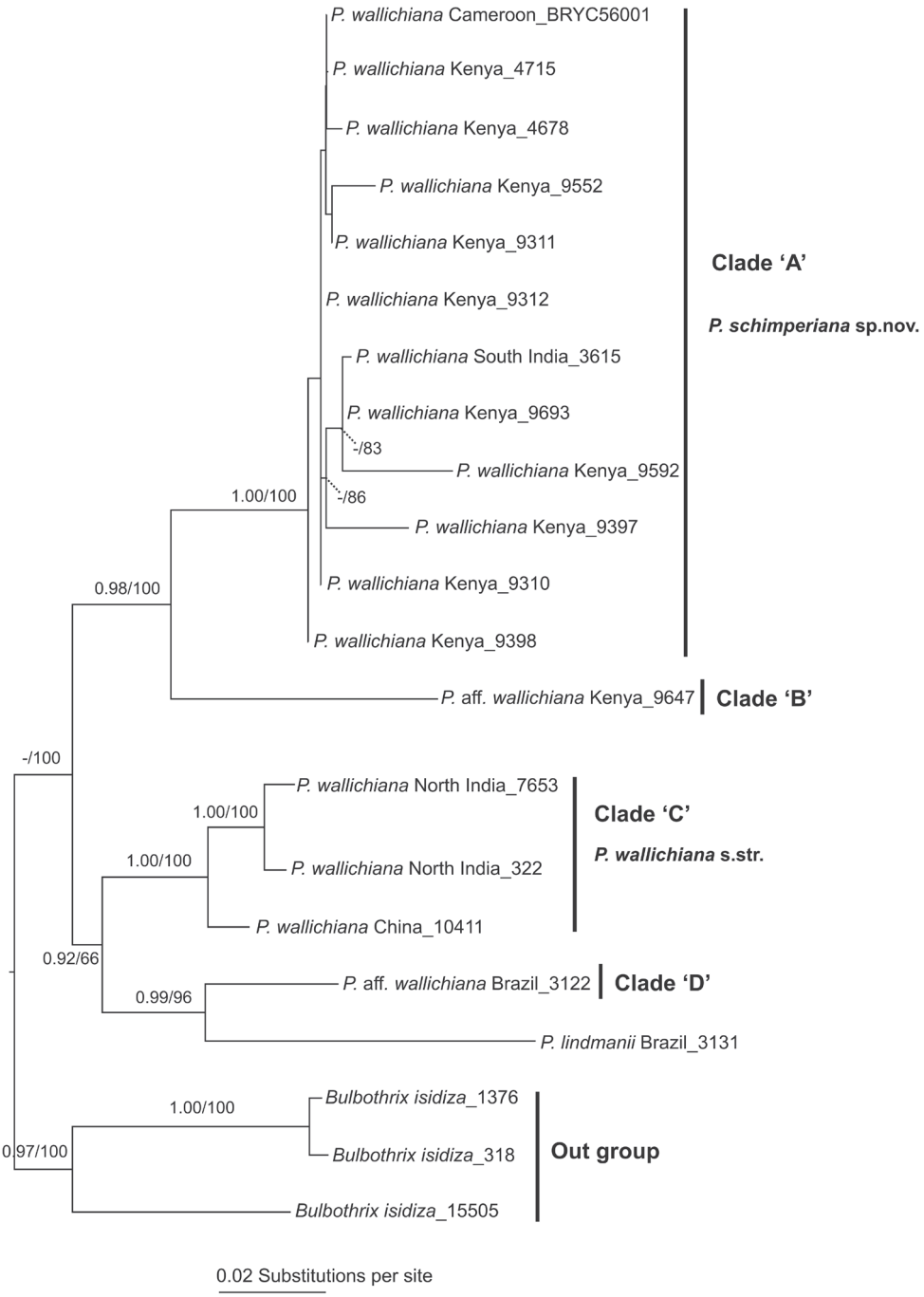

Figure 1. Phylogenetic relationships among *Parmelinella* taxa based on a maximum-likelihood (ML) analysis of a concatenated, three locus dataset (ITS, nuLSU & mtSSU rDNA). Since the ML and Bayesian inference topologies were identical, only the ML topology is shown here. Posterior probabilities ≥ 0.95/ ML bootstrap values ≥ 70% are given above the branches.

Specimens representing *Parmelinella wallichiana* did not form a monophyletic lineage (Fig. 1). This is inconsistent with currently hypothesized species boundaries based on phenotypical features (Divakar and Upreti 2015; Benatti 2014). Species-level polyphylies are commonly found in Parmeliaceae and other groups of lichen-forming fungi (see reviews by Crespo and Lumbsch 2010; Lumbsch and Leavitt 2011).

Specimens representing *P. wallichiana* s. lat. fell into four distinct well-supported clades. Clade 'A' included samples from Kenya, Cameroon, and a single sample from South India. Clade 'B' included a single sample from coastal region (Coast Province) of Kenya. Clade 'C' included most samples from Asia; and clade 'D' was represented by a single sample from South America (Brazil). Specimens in clade 'A' are characterized in having smaller ascospores (5–10 × 5–7.5 μm), whereas they are larger (15–20 × 9–14 μm) in clade 'C'. Further, the same strongly supported monophyletic clades – 'A' and 'C' – were recovered in reciprocally monophyletic clades in the independent gene trees (data not shown) (Hudson and Coyne 2002). Presence of the same clades in different single-locus genealogies can be taken as strong evidence that the clades are reproductively and evolutionarily isolated lineages representing distinct species-level lineages (Dettman et al. 2003; Pringle et al. 2005; de Quieroz 2007). The relationships among the clades were well supported (Fig. 1). Clade 'B' formed sister-group relationship with clade 'A', whereas clade 'D' was sister to *P. lindmanii*, and clade 'C' sister to a clade including clade 'D' and *P. lindmanii*. The type material of *Parmelinella wallichiana* is from Nepal in the Himalayas (Hale 1976a; Divakar and Upreti 2005). Since all samples sequenced by us from the Himalayan regions (China and India) clustered in clade 'C', we consider this clade as *P. wallichiana* s. str.

For clade 'A' there are a few potential names available that we studied. For example, *Parmelia junodi* was described from the Cape Province in South Africa (Steiner 1907) and *Parmelia tiliacea* var. *eximia* has been described from Tanzania (Steiner 1888). These taxa have previously been considered synonyms of *P. wallichiana* (Hale 1976a). However, according to a recent study by Benatti (2014), *Parmelia tiliacea* var. *eximia* is a synonym of *Parmelinella cinerascens* and the type material of *Parmelia junodi* contained mixture of different species, such as *Parmelinopsis minarum* or *P. horrescens* and a fragment to belonged *Parmelinella cinerascens*. Thus we conclude that those two names are synonyms of *Parmelinella cinerascens*. The latter is a rare species occurring in South America and until recently was classified in the genus *Canoparmelia* (Elix et al. 1986). Recently, based on morphological data, *Canoparmelia cinerascens* was transferred to the genus *Parmelinella* (see Benatti 2014). Unfortunately, we were unable to sequence this species and hence cannot confirm the phylogenetic position of *C. cinerascens*. Samples clustered in clade 'A' collected from Africa and South India are morphologically similar to *Parmelinella wallichiana* s. lat. Since there is no name available for this clade, a new species is described below to accommodate samples from Africa and South India (clade 'A'). Further, the segregation of this new taxon from *P. wallichiana* s.str. is corroborated by morphological data, discussed below. The new species has a disjunct distribution occurring in Africa and South India. There are

abundant examples of this disjunct distribution pattern in flowering plants (see e.g. Mani 1974; Kadereit 2004).

Clades 'B' and 'D' were each represented by a single specimen from Kenya and Brazil, respectively. The sample from the coastal region of Kenya (clade 'B') has a deviating morphology, i.e. very narrow, sublinear and dichotomous lobes, although the specimen from coastal Brazil (clade 'D') was more similar to *P. wallichiana* s. lat. In both cases, study of additional samples will be required before a formal description of these putative species.

Our results add a further example to a growing body of evidence of the existence of distinct lineages hidden under currently circumscribed species (reviewed in Bickford et al. 2007; Crespo and Lumbsch 2010; Lumbsch and Leavitt 2011). Whereas, some studies found no obvious phenotypical differences and interpreted the discovered additional species diversity as cryptic (reviewed in Crespo and Lumbsch 2010; Lumbsch and Leavitt 2011; Hibbett 2016), re-examination of material falling into different clades uncovered previously unrecognized morphological differences. This has been shown in other cases as well (see e.g. *Parmelia barrenoae*, Divakar et al. (2005); *Physconia thorstenii*, Divakar et al. (2007); *Caloplaca citrina* group Vondrák et al. (2009); *Melanelixia californica*, Divakar et al. (2010); *Parmelia mayi*, Molina et al. (2011a); *Cladia aggregata* group, Parnmen et al. (2012); *Parmotrema perforatum* group, Widhelm et al. in press) and demonstrates the importance of careful re-analysis of morphological and chemical characters in order to phenotypically circumscribe species. Further, the species-level lineages uncovered in this widely distributed isidiate taxon showed biogeographic structure in what was previously believed to be a pantropical species. Although geographical structure of species detection using molecular data has recently been shown to be a common phenomenon in lichenized fungi (Argüello et al. 2007; Divakar et al. 2010a; Otalara et al. 2010; Amo de Paz et al. 2012; Parnmen et al. 2012; Moncada et al. 2014; Leaviit et al. 2015b; Alors et al. 2016); caution must be taken to generalizing for all isidate lichen taxa (Leavitt et al. 2013a; Roca-Valiente et al. 2013; Divakar et al. 2016).

Taxonomic treatment

Parmelinella schimperiana **Kirika & Divakar, sp. nov.**
MycoBank No. MB 817294
Figure 2

Type. KENYA, Eastern Province, Makueni Co., Wote, Ngutwa village, Matooi hill, dry woodland, 01°49'S, 37°66'E, 1400m, on bark, 12 December 2013, P. Kirika, I. Malombe & K. Matheka, 3703 (holotype: EA, isotype: F). **GenBank accession number.** ITS KX341985, nu LSU KX342003

Diagnosis. Morphologically similar to *P. wallichiana* but differs in having smaller ascospores (5–10 × 5–7.5 μm), being restricted in distribution to Africa and South India, and molecular phylogenetic position (Clade 'A'; Fig. 1).

Figure 2. Morphology of the new species; *Parmelinella schimperiana* (holotype [EA]).

Etymology. The taxon name is in the honor of W.G. Schimper, the first botanist to collect lichens in East Africa.

Description. Thallus foliose, adnate to loosely adnate, 3–7 cm across. Lobes broad, irregularly to subirregularly branched, 3–8 mm wide, rounded crenate, with rotund apices, margins ciliate. Cilia simple, frequent in the lobe axils, 0.1–0.6 mm long. Upper surface grey, grey-green smooth, emaculate, usually pruinose, thallus irregularly cracked towards the centre on older parts, isidiate. Isidia laminal, cylindrical, mostly simple or branched 0.1–0.5 mm high, concolorous with the upper surface. Medulla white. Lower surface black with more than 2 mm broad, brown papillate margins, rhizinate. Rhizines black, evenly distributed, simple, 0.2–1 mm long. Apothecia laminal, adnate to sessile, 1–5 mm in diameter. Disc concave, brown, imperforate. Asci 8-spored. Ascospores ellipsoid to subglobose, 5–10 × 5–7.5 µm (M = 5.5–6.4 × 7.6–8.5 µm, ±SD = 0.7–1.0 × 1.0–2.3 µm, n = 100). Pycnidia absent.

Secondary chemistry – Cortex K+ yellow, UV–; medulla K+ yellow turning red, C–, KC–, P+ orange-red, UV–; upper cortex with secalonic acid A and atranorin, medulla with salazinic acid.

Distribution and ecology. At present the new species is known from Kenya, Cameroon and South India. It occurs in montane regions and in dry woodland areas. It is predominantly corticolous and sometimes saxicolous rarely terricolous, found corticolous on *Mangifera indica*, *Juniperus procera*, *Podocarpus* spp., *Lannaea* spp. and on *Eucalyptus* in artificial habitats.

Additional specimens examined. KENYA. Eastern Prov.: Marsabit Co., Marsabit National Park, Lake Paradise, disturbed forest on ridge, 2°16'N 37°56'E, 1434m, on bark, P. Kirika 4678 & H.T. Lumbsch (EA, F, MAF). Eastern Prov.: Marsabit National Park at roadside between Marsabit Lodge and Lake Paradise, forest on slope, 2°18'N 37°57'E, 1513m, on bark, *P. Kirika* 4715 & H.T. Lumbsch (EA, F, MAF). Eastern Prov.: Tharaka South, Chiakariga, Kijege Hill, *Acacia-Commiphora* woodland, 00°16'S, 37°50'E, 1160m, on bark, P. Kirika 3432 (EA, F). Eastern Prov.: Mwingi Co., Mumoni Hill, *Eucalyptus* plantation, 00°31'S, 38°00'E, 1620–1695m, on bark, P. Kirika 3487 & G. Mugambi (EA, F). Eastern Prov.: Tharaka South, Chiakariga, Kijege Hill, *Acacia-Commiphora-Encephalartos* woodland, 00°16'S, 37°50'E, 1160m, P. Kirika 3436 (EA, F). Rift Valley: Eldama Ravine, Lembus Forest off Eldama Ravine-Eldoret Road, remnant montane forest, 0°13'N, 35°69'E, 2275m, on bark, P. Kirika 2870, G. Mugambi & H.T. Lumbsch (EA, F); 0°16'N, 35°75'E, 2137m, on rock, P. Kirika 2815, G. Mugambi & H.T. Lumbsch (EA, F). Rift Valley: Kericho, James Finlay Tea Estate, Chomogondy, secondary forest, 00°23'S, 35°18'E, 2056m, on bark, P. Kirika 3145 (EA, F). Rift Valley: small disturbed remnant forest in tea plantation, 0°44'S, 35°31'E, 2049m, on bark, P. Kirika 2974 G. Mugambi & H.T. Lumbsch (EA, F). Rift Valley: Bomet , Koiwa, Unilever riparian forest, 00°35S, 35°17'E, 2030m, on bark, P.Kirika 4900 (EA). Rift Valley: Kajiado Co., Ngong Hills, Upland grassland with rocky outcrops, 01°24'S, 36°38'E, 2430m, on soil, P. Kirika 3334 (EA, F). CAMEROON. E of Mount Cameroon, vic. of Ekona, E.A. Orock 56009 (BRY-C). INDIA. S India: Tamil Nadu, Vellore distr., Yellagiri hills, 13°30'N, 79°05'E, 1393m, on *Mangifera indica* tree trunk, hill side with teak and eucalyptus vegetation, H.T., Lumbsch, P.K. Divakar, D.K. Upreti, J. Tandon 19705a (MAF).

Remarks. *Parmelinella schimperiana* is morphologically most similar to *P. wallichiana*, but differs in having smaller ascospores (5–10 × 5–7.5 μm), whereas the ascospore size in *P wallichiana* is: 15–20 × 9–14 μm.

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RESEARCH ARTICLE

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Oomycete-specific ITS primers for identification and metabarcoding

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Abstract

Microbial metabarcoding studies using high throughput sequencing technologies generate unprecedented amounts of DNA sequence data and make it possible to determine not only the composition of the communities but also the underlying factors powering the evolution of these communities. Despite the potential of community level studies in helping to better understand the ecology of pathogens and to manage the losses caused by them, very few oomycete addressing metabarcoding studies have been carried out and with highly variable results. The aim of this study was to develop new oomycete-specific ITS region PCR primers with improved specificity for metabarcoding and identification of oomycetes. The modified ITS1oo and the newly developed ITS3oo primers show improved *in silico* specificity for oomycetes and when paired with the universal ITS4 successfully amplified the DNA from all eleven tested oomycete species from six genera. High throughput sequencing of 20 soil samples from forest nurseries and bordering areas, using the primer pair ITS1oo/ITS4, recovered more than 400 oomycete OTUs, which is a significant increase over previous studies, and indicates the ability of the new method to detect various oomycete groups from complex substrates. The average fraction of oomycete reads per soil samples was 32–36%, with a maximum of 69%. The recovered oomycete OTUs represented the groups *Lagenidiales*, *Peronosporales*, *Pythiales* and *Saprolegniales*, with *Pythiales* dominating in all samples. In addition, the new primers were successfully used in identifying pathogens directly from infected plant tissues with Sanger sequencing. The pathogen was identified to the species or genus level in four samples out of six. In conclusion, the developed oomycete-specific primers provide a reliable method for the identification and metabarcoding of oomycetes.

Key words
oomycete, community barcoding, next generation sequencing, ITS, community analysis, soil community

Introduction

Oomycetes are microscopic stramenopiles that are found in both aquatic and terrestrial environments (Sparrow 1960, 1976, Karling 1981, Dick 2001). Many oomycete species are important pathogens, causing serious economic losses by infecting vegetables, berries, trees, arthropods and vertebrate animals (Kamoun 2003, Herrero et al. 2011). Molecular methods enable rapid identification of pathogens in environmental samples and infected tissues by using specific PCR primers and rapidly evolving high-throughput sequencing (HTS) technologies. For oomycetes, the cytochrome c oxidase subunit 1 (cox1), the internal transcribed spacer (ITS) (Robideau et al. 2011, Vettraino et al. 2012) and the cytochrome c oxidase subunit 2 (cox2) (Choi et al. 2015) have been identified as suitable barcodes. The choice of metabarcoding primers that cover all known oomycete taxa and discriminate other groups, however, is still limited by the inconsistent performance of some existing oomycete-specific primers.

Robideau et al. (2011) evaluated the cox1, the ITS and the large ribosomal subunit (LSU) for use in DNA barcoding of oomycetes and suggested using cox1 and ITS in parallel due to their similar performance in resolving oomycete species and with both having superior performance in certain groups. Choi et al. (2015) compared the perfomance of cox1 and cox2 and found the latter to be more easily amplified across a wide range of oomycetes with existing primer sets. They also determined that the cox2 was more efficiently amplified from historic herbarium specimens and noted that in case of cox2 there is existing sequence data for several historic type specimens. As a result, Choi et al. (2015) suggested using the cox2 in addition to the ITS for oomycete barcoding. Additionally, Choi et al. (2015) proposed that for below species-level resolution the cox2-1 spacer could be used. For the cox2, there are also internal primers that can be used to amplify a 350 bp fragment suitable for barcoding (Hudspeth et al. 2000).

Of oomycete-specific ITS primers, ITS6 and ITS7 (Cooke et al. 2000) have been used for community studies, but with notable difficulties, as Coince et al. (2013) recovered only a small percentage of oomycete sequences using these primers. Sapkota and Nicolaisen (2015) optimized the ITS6/ITS7 assay by raising the annealing temperature and as a result improved the specificity of the primers. Other studies, however, have suggested that taxon recovery could be increased by using lower annealing temperatures (Ishii and Fukui 2001, Acinas et al. 2005) or multiple annealing temperatures (Schmidt et al. 2013). It is also advisable to re-optimize the PCR reaction whenever the reaction mixture is altered (Innis et al. 1990).

Another oomycete-specific ITS primer, the ITS-O, has been published by Bachofer (2004). Whereas this primer has so far not found use in oomycete community studies, it has been used successfully to amplify the DNA of a wide range of oomycetes in phylogenetic research (Spring et al. 2006, Thines 2007).

The aim of the current study was to develop new oomycete ITS primers with improved taxon coverage and specificity for use in community-level studies. In order to reduce material costs, we decided to develop two oomycete specific forward primers that can be combined with various universal reverse primers. The new and existing primers were analyzed *in silico* to evaluate the coverage and specificity of the primers and the primers selected as suitable for oomycete ITS barcoding were tested *in vitro* on cultures, infected plant tissues and soil samples.

Methods and materials

Pure cultures of oomycetes and fungi

DNA extracts from the pure cultures of eleven oomycete species from six genera were used in testing of the primers. Additionally, DNA from the cultures of five fungal species was used to test the specificity of the primers (Table 1).

Sampling and DNA extraction

A total of 20 soil samples were collected from beds of forest nurseries and bordering control areas (Table 2). Each sample consisted of 40 subsamples, which were taken

Table 1. Cultures of oomycetes and fungi that were used in testing the specificity of the new primers.

Species	Isolation year	Strain/culture code	Host
Achlya oligochanta	2010	HJ33C	*Astacus astacus*
Aphanomyces astaci	2008	KTY3-4	*Astacus astacus*
Aphanomyces astaci	2003	UEF8866-2	*Pacifastacus leniusculus*
Aphanomyces astaci	2014	AT1D	*Austropotamobius torrentium*
Aphanomyces laevis	2008	KTY5-2	*Astacus astacus*
Aphanomyces stellatus	2010	HJ38C	*Astacus astacus*
Aphanomyces salsuginosus	2014	NJM0912	*Salangichithys microdon*
Phytophthora infestans	2014	An2-13	n/a
Phytophthora infestans	2014	HiPa1-13	n/a
Phytophthora infestans	2014	Ti17-13	n/a
Phytophthora cactorum	2012	145714	*Betula pendula*
Pythium sp.	2007	T4B	*Astacus astacus*
Saprolegnia australis	2007	S23	*Astacus astacus*
Saprolegnia parasitica	2007	S14	*Astacus astacus*
Scoliolegnia sp.	2007	S16	*Astacus astacus*
Alternaria infectoria	2014	TU-3 TFC 2013-46	n/a
Armillaria cepistipes	2013	EPS 110	*Fraxinus excelsior*
Fusarium culmorum	2014	KV-6 TFC 2013-54	n/a
Neonectria radicicola	2013	EPS 82	n/a
Ulocladium castanea	2014	CBS 124390	n/a

Table 2. Soil samples used in evaluating the performance of the new oomycete specific primer pair ITS1oo/ITS4 in high-throughput sequencing.

Sample	Geocode	Time of sampling	Sampling plot description
1.1	58°48.00'N, 24°30.03'E	10/16/2014	1 y/o *Picea abies* nursery bed
1.2	58°48.02'N, 24°30.00'E	10/16/2014	Area bordering the nursery
2.1	58°20.51'N, 24°36.58'E	17/10/2014	Former *Picea abies* nursery bed
2.2	58°20.53'N, 24°36.42'E	17/10/2014	Area bordering the former nursery
3.1	59°9.49'N, 26°16.94'E	14/10/2014	2 y/o *Betula pendula* nursery bed
3.2	59°9.48'N, 26°16.67'E	14/10/2014	Area bordering the nursery
4.1	59°20.14'N, 26°51.37'E	9/26/2014	1 y/o *Picea abies* nursery bed
4.2	59°20.14'N, 26°51.36'E	9/26/2014	Area bordering the nursery
5.1	59°33.77'N, 26°5.60'E	9/25/2014	3 y/o *Picea abies* nursery bed
5.2	59°33.76'N, 26°5.60'E	9/25/2014	Area bordering the nursery
6.1	59°32.29'N, 26°16.40'E	9/25/2014	1 y/o *Picea abies* nursery bed
6.2	59°32.29'N, 26°16.36'E	9/25/2014	Area bordering the nursery
7.1	59°12.08'N, 26°13.67'E	11/5/2014	1 y/o *Betula pendula* nursery bed
7.2	59°11.89'N, 26°14.40'E	11/5/2014	Area bordering the nursery
8.1	56°6.22'N, 27°17.89'E	11/6/2014	1 y/o *Picea abies* greenhouse nursery bed
8.2	56°6.16'N, 27°17.98'E	11/6/2014	Area bordering the nursery
9.1	58°2.18'N, 25°58.17'E	11/7/2014	2 y/o *Picea abies* nursery bed
9.2	58°2.08'N, 25°57.97' E	11/7/2014	Area bordering the nursery
10.1	58°10.46'N, 26°11.18'E	11/7/2014	1 y/o *Betula pendula* nursery bed
10.2	58°10.44'N, 26°11.16'E	11/7/2014	Area bordering the nursery

Table 3. Symptomatic plant samples with oomycete infection that were used in testing of the new primers ability to identify pathogens directly from infected plant tissues.

Plant sample	Sampling location	Time of sampling	Host species / symptoms	Identified pathogen / sequence similarity
1.	Tartu county	06/11/2014	*Alnus incana*/ bark discolouration	*Phytophthora* sp./ 99%
2.	Tartu county	07/09/2014	*Solanum tuberosum*/ leaf rot	*Phytophthora infestans*/ 100%
3.	Tartu county	07/17/2014	*Aegopodium podagraria*/ leaf discolouration	*Plasmopara nivea*/ 99%
4.	Tartu county	09/14/2014	*Solanum lycopersicum*/ leaf rot	*Phytophthora infestans*/ 99%
5.	Tartu county	09/14/2014	*Vitis vinifera*/ leaf discolouration	No result
6.	Tartu county	n/a	*Cucurbita pepo*/ leaf discolouration	No result

with a 5 cm diameter sterile plastic pipe from the top 5 cm soil layer of a 50x50 m plot. The subsamples were pooled, dried and thoroughly mixed following Tedersoo et al. (2014). In addition, six samples of plant tissues with signs of oomycete infection were collected by excising a part of the symptomatic tissues (Table 3).

DNA was isolated from 2 g of soil with the MO BIO PowerMax Soil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA). DNA from symptomatic

plant samples was isolated with the MO BIO PowerSoil DNA Isolation Kit from up to 0.2 g of material. Prior to DNA extraction, soil and tissue samples were crushed using bead beating with two 3.2 mm diameter stainless steel balls (BioSpec Products, Bartlesville, OK, USA). The 2 ml tubes containing the samples and balls were shaken for 5 min at 30 Hz with the Retsch Mixer Mill MM400 (Retsch, Haan, Germany). Pure culture DNA was extracted using a proteinase K-based method (100 µl 0.2 M $(NH_4)_2SO_4$ and 2.5 µl proteinase K; incubation at 56 °C for 24 h and at 98 °C for 15 min).

Primer design

The new oomycete-specific forward primers ITS1oo and ITS3oo were selected by aligning all oomycete and other stramenopile ITS sequences present in the International Nucleotide Sequence Databases (www.insdc.org). ITS sequences were aligned with MAFFT (http://mafft.cbrc.jp/alignment/software/) and checked by using SEAVIEW software (http://doua.prabi.fr/software/seaview). Primer sequences were then selected within the desired regions by screening for segments that are conserved across all oomycetes. Specificity of the primers was analysed by running BLASTn comparisons against the INSDc and manually by comparing against a custom ITS database containing sequences of nine major eukaryotic groups (Bengtsson-Palme et al. 2013).

PCR conditions and validation

PCR amplification was carried out using a reaction mixture consisting of 18 µl of PCR grade water, 5 µl of 5x HOT FIREPol Blend Mastermix (10 mM $MgCl_2$) (OÜ Solis Biodyne, Tartu, Estonia), 0.5 µl of both primers (20 µM) and 1 µl of DNA sample. Amplifications were done with Eppendorf 5341and Eppendorf 6321 thermal cyclers (Eppendorf AG, Hamburg, Germany) by running the following programme: 15 min at 95 °C, 35 cycles of 30 s at 95 °C, 30 s at 55 °C, 1 min at 72 °C and a final cycle of 10 min at 72 °C.

The new forward primers were optimized for use with the universal reverse primer ITS4 (White et al. 1990) using OLIGOANALYZER 3.1 (https://eu.idtdna.com/calc/analyzer) to compare their calculated melting temperatures and GC content. The stability of possible homo- and heterodimers as well as hairpin structures was evaluated to avoid reduced amplification efficiency. All amplification tests were done at a 55 °C annealing temperature. Validation tests were carried out with DNA from various oomycete pure cultures as well as with DNA extracted from plants supposedly infected with pathogenic oomycetes and from 20 soil samples (Table 1). DNA from the pure cultures of five fungal species was used as negative control to test the specificity of the primers. The quality of all DNA samples used in specificity checks was tested by running PCR amplifications with universal ITS primers ITS1 and ITS4 (White et al. 1990).

Sequencing of infected plant samples

PCR products obtained from the six symptomatic plant samples were purified using to the ExoSAP method (Bell 2008) and Sanger-sequenced in Macrogen (Macrogen Europe, Amsterdam, The Netherlands). Sequencing was done with the oomycete-specific ITS1oo or ITS3oo primers as well as with the universal ITS4 primer. The obtained sequences were compared against the INSDc to confirm the identification.

High-throughput sequencing of soil samples

In total, 20 soil samples were sequenced using Illumina Miseq 2x300 PE HTS technology in the Estonian Biocentre (Tartu, Estonia). Amplicons were prepared with the primers ITS1oo and ITS4ngs (Tedersoo et al. 2014), both of which were tagged with one of the MID identifiers (cf. Tedersoo et al. 2014). The ITS1oo was used as the forward primer in order to sequence both ITS1 and ITS2 regions. PCR was performed as described above but in four replicates. PCR products were pooled and 5 µl of each product was resolved on 1% agarose gel to confirm amplification. Negative controls without template and positive controls containing DNA of *Aphanomyces astaci* were used in the sequencing process. The quantity of the products was normalized with the SequalPrep Normalization Plate Kit (Invitrogen, Carlsbad, CA).

Analysis of Illumina sequencing data

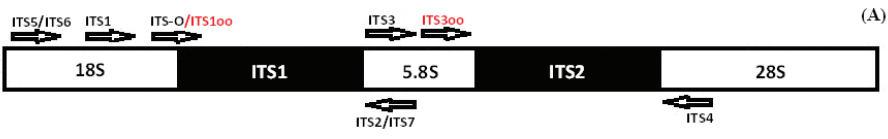
Based on sequencing primers, read 1 and read 2 were shuffled to contain regions of ITS1 and ITS2, respectively (using FQGREP (https://github.com/indraniel/fqgrep)). These paired-end reads were analysed separately, because in most cases the amplified full-length ITS region exceeded 600 bp and could not be merged. Sequencing reads were quality filtered and assigned to samples using MOTHUR (Schloss et al. 2009) (average quality over 15 bases ≥ 30). Potential chimeras were detected and removed using USEARCH 7.0.1090 (Edgar 2010). Sequences shorter than 150 bases were discarded and longer sequences were trimmed to 150 bases for clustering. The quality filtered ITS1 and ITS2 sequences were separately clustered to Operational Taxonomic Units (OTUs) based on 97% sequence similarity using CD-HIT (Li and Godzik 2006). The most abundant sequence was selected as a representative (using mothur) for BLASTn searches against a custom oomycete nucleotide database combined from the reference collections of Hyde et al. (2014) and Robideau et al. (2011) and INSDc. For each OTU, 10 best-matching references were determined for precise annotation. We considered OTUs to belong to oomycetes if they best matched known oomycetes. Oomycete OTUs with e-values < e^{-20} and identities above 80% were considered reliable enough to assign sequences to an order. OTUs with best matches other than oomycetes were assigned at the class level if e-value was < e^{-20} and identity above 75%.

Results

Primer selection and in silico analyses

As a result of aligning all oomycete ITS sequences present in the INSDc, it was possible to choose two short regions which are conserved across the majority of oomycetes and allow for the discrimination of other taxonomic groups. The primer ITS1oo overlaps with the primer ITS-O (Bachofer 2004) across 17 positions out of a total of 18 and is therefore not an original primer but a modification of ITS-O. This modification comes from a one bp shift which results in the deletion of a cytosine at the 5' end and the addition of an adenine at 3' end. The position of the added 3' adenine is polymorphic in other groups such as fungi and plants and should therefore make the modified ITS1oo more specific than the original ITS-O (Bachofer 2004). Primer coverage analysis of ITS1oo and ITS3oo shows that the primer sequences are conserved in nearly all known oomycete taxa. In case of ITS3oo, mismatches can be seen in some accessions of *Hyaloperonospora* and *Perofascia lepidii*. Both primers have significant mismatches in comparison to most other stramenopiles, fungi and plants (Figure 1).

The location of the 18 bp long ITS1oo, modified from the ITS-O (Bachofer 2004), covers 13 nucleotides at end of the ribosomal 18S gene and 5 nucleotides in the beginning of ITS1. The similarly 18 bp long ITS3oo is located at the end of the 5.8S gene, ending 7 nucleotides before the beginning of ITS2 (Figure 1). Both of the new primers were used as forward primers in combination with the universal reverse primer ITS4. The ITS4 was chosen due to its position at the beginning of the 28S gene, which allows for the amplification of both ITS1 and/or ITS2 when used together with ITS1oo or ITS3oo.



ITS1oo (5'-3') ITS-O (Bachofer 2004)	Mismatching taxon (% accessions)	ITS3oo (5'-3')	Mismatching taxon (% accessions) (B)
-GGAAGGATCATTACCACA		AGTATGYYTGTATCAGTG	
CGGAAGGATCATTACCAC-			
-	-	********M********	*Hyaloperonospora* (100%)
-	-	G**********G******	*Perofascia* (100%)
WD*****RNNNNN	*Fungi*	R*C*Y***Y**TYG**Y*	*Fungi*
************RNNNNN	*Plantae*	G*C*C*****CC*GG*Y*	*Plantae*
**********Y*RH*	Other *Stramenopiles*	**C********CKG****	Other *Stramenopiles*
ITS6 (Cooke et al. 2000)		**ITS7 (Cooke et al. 2000)**	
GAAGGTGAAGTCGTAACAAGG		AGCGTTCTTCATCGATGTGC	
-	-	**********G*****	*Saprolegnia* (90%)
-	-	Y**Y*S**********	*Aphanomyces* (40%)
-	-	*****C**********	*Halophytophthora* (85%)
****TAA**********	*Fungi*	T**********CSA	*Fungi*
*****A**********	*Plantae*	TR**********CNA	*Plantae*
**********	Other *Stramenopiles*	T**********T****G	Other *Stramenopiles*

Figure 1. A Map of universal and oomycete-specific ITS region primers **B** Taxa with mismatches in the binding sites of primers ITS1oo and ITS3oo. Only taxa with 10% or more mismatching accessions are shown.

Analyses of pure culture and infected plant material

The primer pairs ITS1oo/ITS4 and ITS3oo/ITS4 produced a single amplification band of the expected length from all 15 tested oomycete strains, representing six genera (*Achlya*, *Aphanomyces*, *Phytophthora*, *Pythium*, *Saprolegnia*, *Scoliolegnia*) and eleven species. No visible bands were obtained in gel with DNA from five fungal species.

- Four samples, extracted directly from the symptomatic tissues of a grey alder (*Alnus incana*), a potato (*Solanum tuberosum*), a tomato (*Solanum lycopersicum*) and a goutweed (*Aegopodium podagraria*), produced a single amplification band with both primer pairs ITS1oo/ITS4 and ITS3oo/ITS4 and were sequenced. Sequencing of the grey alder sample was successful with the primer ITS3oo, whereas the other three samples were successfully sequenced with both ITS1oo and ITS3oo. Comparisons against the NCBI GenBank nucleotide database showed that the sequence from the first sample belongs to *Phytophthora* sp. (99% similarity), the sequences from the second and third samples belong to *Phytophthora infestans* (100% and 99% similarity) and the sequence from the goutweed sample belongs to *Plasmopara nivea* (99% similarity). One sample from a zucchini plant (*Cucurbita pepo*) and one from a grape vine (*Vitis vinifera*) produced multiple amplification bands of different sizes with both primer pairs and were not sequenced.

Soil sample oomycete diversity

Altogether 67133 quality filtered ITS1 reads were recovered from the 20 soil samples. In all, 281 singletons were discarded from further analyses. Nearly 66% of all reads belonged to unknown taxa, 25% to oomycetes and 9% to other taxonomic groups (Figure 2). The quality filtered ITS1 sequences were clustered into 1820 OTUs based on 97% similarity threshold, 30% of which were assigned to a known class or order. Out of the 554 assigned OTUs, nearly 73% belonged to oomycetes, 16% to fungi and 9% to plants. Of 404 oomycete OTUs, 307 were assigned to a known order. On average, oomycetes comprised 61 OTUs (range, 13-94) represented by 32% (range, 1-66%) of reads in soil samples (Figure 3).

For the ITS2 subregion, 77734 quality filtered reads comprised 1720 OTUs and 241 singletons. Out of all ITS2 reads, 30% were assigned to oomycetes and 8% to fungi, whereas 60% belonged to unknown taxa (Figure 2). Oomycetes comprised 493 of the 672 identified taxa (73%). In total, 333 of these taxa were assigned to a known order. The number of oomycete OTUs averaged 86 (range, 42–148) per soil sample (Figure 3). On average, oomycetes contributed to 36% (range, 12–69%) in soil samples.

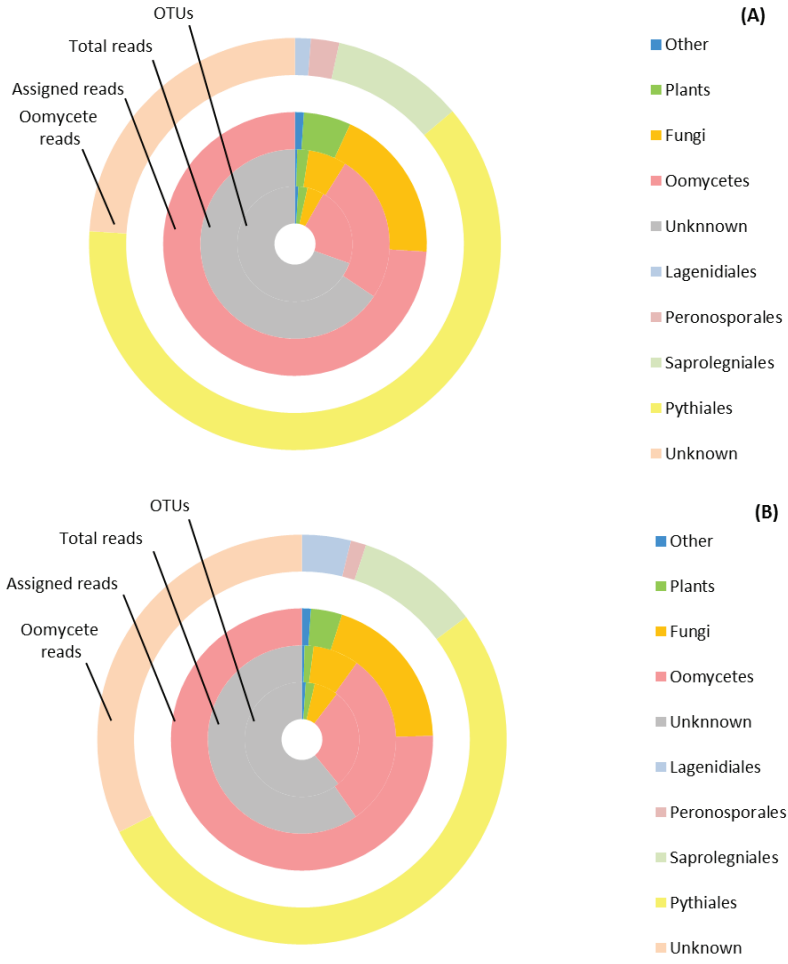


Figure 2. OTU and read distributions of ITS1 (**A**) and ITS2 (**B**) reads. Panels starting from outermost: **1** Oomycete read distribution between orders **2** Read distribution between classes, excluding reads of unknown origin **3** Read distribution between classes, including reads of unknown origin **4** OTU distribution between classes.

Discussion

The ultimate aim of this study was to validate an alternative method for metabarcoding oomycetes in complex substrates such as soil. We developed a novel taxon-specific PCR assay for the ITS region-based identification of oomycetes. When compared with the previously developed ITS-O, ITS6 and ITS7 primers, the ITS1oo, modified from the original ITS-O (Bachofer 2004), and the newly designed ITS3oo exhibit somewhat greater *in silico* specificity for oomycetes. In comparison to the ITS-O, the modified ITS1oo includes an additional 3' terminal adenine, a position that is polymorphic in fungi and plants and should therefor add to the specificity of the primer. Based on our

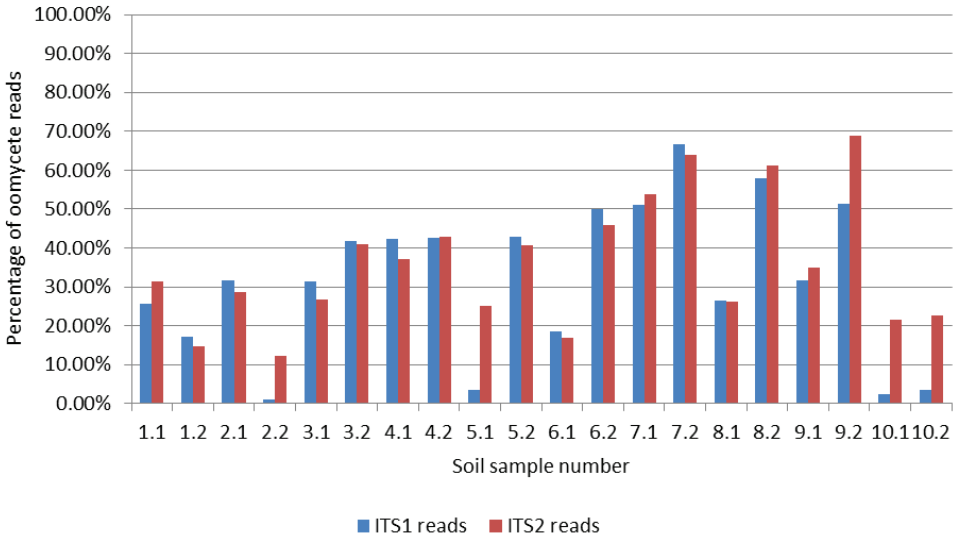


Figure 3. Fraction of oomycete reads in individual soil samples.

analyses and in contrast to Sapkota and Nicolaisen (2015), the primer ITS6 has only one mismatching position in comparison to the majority of corresponding plant accessions in the INSDc, whereas the ITS1oo has several mismatches in the 3′ end. This may significantly lower the specificity of the ITS6, as a single internal mismatch does not reduce the amplification efficiency markedly (Kwok et al. 1990). In addition, the ITS6 has no mismatches compared to the majority of non-oomycete stramenopile accessions, while the ITS1oo has 3′ mismatches against several non-oomycete stramenopile groups. Both ITS1oo and ITS6 show complete coverage of all oomycete groups present in the INSDc, whereas the ITS7 has one mismatch in comparison to the accessions of *Saprolegnia* (Sapkota and Nicolaisen 2015) and *Halophytophthora* and 2-3 mismatches against four species of the known pathogenic genus *Aphanomyces* (Sapkota and Nicolaisen 2015). The presence of two or more mismatches can limit the usability of ITS7 in detecting these taxa, especially when using relatively high annealing temperatures (Sipos et al. 2007) as suggested by Sapkota and Nicolaisen (2015). In comparison, the primer ITS3oo has a single mismatch compared to the accessions of genus *Hyaloperonospora* and two mismatches against the single known species of *Perofascia*.

Furthermore, the modified and newly developed forward primers are located in the very end of the conserved fragments that reduce the size of amplicons by 10-20% compared with the ITS6 forward primer, which is of great importance for HTS platforms producing short fragments such as Illumina and Ion Torrent. When combined with universal reverse primers, these oomycete-specific primers could be used in multiplex with other specific forward primers to address several taxonomic groups of pathogens simultaneously, without adding the cost of multiple barcoded reverse primers (Tedersoo et al. 2015).

Previous studies have used oomycete-specific primers ITS6 and ITS7 to amplify the ITS1 region with highly variable success. For example, Vannini et al. (2013) recovered only 23 oomycete OTUs from 10 forest soil samples, where oomycetes contributed to 79% of all reads. More recently, Coince et al. (2013) recovered a total of 10 oomycete OTUs from 20 samples of forest soil that contributed to 15% of all reads. Sapkota and Nicolaisen (2015) improved the ITS6/ITS7 based method by optimizing the annealing temperature and as a result recovered 67 oomycete OTUs (95% of all reads) from 26 agricultural soil samples, but may have missed multiple taxa due to overly strict PCR conditions. Furthermore, it should be noted that fine tuning of PCR conditions is only possible in-house, because PCR buffer including salts ($MgCl_2$) and stabilizers (BSA), the type of polymerase and concentration of primers and templates all affect primer specificity (Innis et al. 1990, Cha and Thilly 1993).

In this study, we recovered 404 ITS1-based and 493 ITS2-based oomycete OTUs from 20 soil samples from forest nurseries and bordering control areas. The number of recovered oomycete OTUs is considerably higher than in previous studies, which could be due to higher diversity in the analysed soil samples or a result of some properties of the new assay. Oomycete reads comprised on average 32% and 36% of the total reads of individual soil samples for ITS1 and ITS2, respectively. The assigned oomycete OTUs belonged to the orders of *Lagenidiales*, *Peronosporales*, *Pythiales* and *Saprolegniales*, confirming the ability of the proposed new assay to detect various oomycete groups from complex samples. *Pythiales* were found to be dominating in the soil samples, making up nearly 50% of the total oomycete reads, a result that is in line with previous oomycete community studies (Arcate et al. 2006, Sapkota and Nicolaisen 2015).

The new primers were also used to identify oomycete pathogens from infected plant samples by using Sanger sequencing. The pathogens were successfully determined in four samples out of six. Sequencing was successful with both ITS1oo and ITS3oo from a goutweed (*Aegopodium podagraria*), a potato (*Solanum tuberosum*) and a tomato (*Solanum lycopersicum*) sample, whereas in the case of a grey alder (*Alnus* incana) sample only ITS3oo produced an identifiable sequence. This could indicate a somewhat higher specificity of ITS3oo in comparison to ITS1oo in some cases when identifying pathogens from infected plant material. Two samples out of six produced multiple amplification bands, possibly indicating the presence of several oomycete species in the infected sample. This result shows that the new primers can be used to detects oomycete pathogen species directly from infected plant samples in cases where the infected tissue in dominated by one pathogen, without co-amplification of plant and fungal DNA.

Taken together, we provide highly oomycete-specific forward primers that can be used in combination with previously developed oomycete-specific or universal reverse primers. Considering the rapid evolution of high-throughput sequencing, the full ITS sequence is certainly preferable over ITS1 or ITS2 used alone, because these subregions may differ in the taxonomic resolution across genera.

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Characterization of microsatellite markers in the cosmopolitan lichen-forming fungus *Rhizoplaca melanophthalma* (Lecanoraceae)

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Abstract

Rhizoplaca melanophthalma s.l. is a group of morphologically distinct and chemically diverse species that commonly occur in desert, steppe and montane habitats worldwide. In this study, we developed microsatellite markers to facilitate studies of genetic diversity, population structure, and gene flow in the nominal taxon of this group, *Rhizoplaca melanophthalma*. We characterized 10 microsatellite markers using a draft genome of *R. melanophthalma* s. str. assembled from Illumina reads. These loci were tested for 21 *R. melanophthalma* s. str. specimens and also with a subset of 18 specimens representing six additional species in the *R. melanophthalma* complex. The number of alleles per locus in *R. melanophthalma* s. str. ranged from 3 to 11 with an average of 6.7. Nei's unbiased gene diversity ranged from 0.35 to 0.91. Amplifications of the microsatellite loci were largely successful in the other six species, although only three markers were found to be polymorphic. The new markers will provide an additional resource for studying genetic, population- and landscape-level processes in the cosmopolitan taxon *Rhizoplaca melanophthalma* s. str.

Key words

Ascomycetes, gene flow, landscape genetics, lichen-forming fungi, microsatellites, *Rhizoplaca melanophthalma*

Introduction

Rhizoplaca melanophthalma (DC.) Leuckert & Poelt s.l. represents a group of morphologically distinct and chemically diverse species of lichen-forming fungi with broad ecological and geographical distributions. Species in this group occur all over the world in disjunct populations in continental climates, although species in this complex are notably absent from Australia. *Rhizoplaca melanophthalma* s.l. commonly grows on siliceous or calcareous rock in arid climates, but can also be found in montane coniferous forests, alpine tundra habitats, and bi-polar populations in the Arctic and Antarctica (McCune 1987). Members of this group are commonly used in air-quality biomonitoring research, making it an important species for conservation (Aslan et al. 2004; Dillman 1996). The species complex belongs to the recently re-circumscribed monophyletic genus *Rhizoplaca* in Lecanoraceae (Zhao et al. 2016).

Previous multi-locus and phylogenomic studies support the circumscription of multiple species within *R. melanophthalma* s.l. (Leavitt et al. 2011, 2013, 2016b), many of which occur in sympatry in Western North America. In Western North America the distribution area of these species extends from the northern boreal zone to Mexico along the Rocky Mountains with a center of diversity in the Great Basin region (Leavitt et al. 2011). *Rhizoplaca melanophthalma* s. str. has the broadest ecological and geographic distribution of all known species within this complex, with populations occurring in desert, montane and steppe ecosystems in Antarctica, Central Asia, Europe, and North and South America (Leavitt et al. 2013).

The *Rhizoplaca melanophthalma* group provides an interesting system for assessing genetic diversity, population structure and gene flow in symbiotic fungal species with broad ecological and geographic distributions. To facilitate additional research into population- and landscape-level processes, 10 microsatellite markers were developed for *R. melanophthalma* s.str.

Materials and methods

A total of 42 specimens representing seven different species in the *Rhizoplaca melanophthalma* species complex were included in this study. Twenty-one of these represented *R. melanophthalma* s. str., three *R. haydenii*, four *R. novomexicana*, two *R. parilis*, four *R. polymorpha*, six *R. porteri* and two *R. shushanii* (Table 1). DNA was extracted from these specimens as described previously (Leavitt et al. 2011).

A draft genome of an axenic culture of *R. melanophthalma* was obtained from a previous study (Leavitt et al. 2016a). The program MSATCOMMANDER 1.0.8 (Faircloth 2008) was used to search for di-, tri-, tetra-, penta-, and hexanucleotide microsatellite repeats in contigs >5 kb from the draft assembly. Only repeats with a minimum length of 8 bp for dinucleotide repeats and 6 bp for the rest were accepted. A total of 244 scaffolds contained microsatellite repeats (87 di-, 127 tri-, 11 tetra-, 5 penta-, and 14 hexanucleotides). For 25 of these repeats, primers were designed with

Table 1. Voucher information for *Rhizoplaca* specimens used in this study. Herbaria codes are provided for each specimen in parentheses following voucher number.

Species	DNA No.	Voucher	Locality
R. melanophthalma	8639c	Leavitt 2013-CO-CP-8639C (F)	USA, CO
R. melanophthalma	8639d	Leavitt 2013-CO-CP-8639D (F)	USA, CO
R. melanophthalma	8654a	Leavitt 2013-CO-RM-8654A (F)	USA, CO
R. melanophthalma	8654b	Leavitt 2013-CO-RM-8654B (F)	USA, CO
R. melanophthalma	8663B	Leavitt 8663 (F)	USA, UT
R. melanophthalma	8663j	Leavitt-8663 (F)	USA, UT
R. melanophthalma	8665b	Leavitt-8665 (F)	USA, NV
R. melanophthalma	8665e	Leavitt-8665 (F)	USA, NV
R. melanophthalma	8665i	Leavitt-8665 (F)	USA, NV
R. melanophthalma	8665M	Leavitt-8665 (F)	USA, NV
R. melanophthalma	8668b	Leavitt-8668 (F)	USA, NV
R. melanophthalma	8668f	Leavitt-8668 (F)	USA, NV
R. melanophthalma	8668q	Leavitt-8668 (F)	USA, NV
R. melanophthalma	8668s	Leavitt-8668 (F)	USA, NV
R. melanophthalma	8668w	Leavitt-8668 (F)	USA, NV
R. melanophthalma	6026	H9203303 (F)	Kyrgyzstan, Ala-Buka
R. melanophthalma	6029	H9203135 (F)	Kyrgyzstan, Panfilov District
R. melanophthalma	6030	H9203327 (F)	Kyrgyzstan, Chatkal
R. melanophthalma	6435	Vondrak 9409 (PRA)	Russia, Chelyabinsk
R. melanophthalma	6604	MAF-Lich 16805 (MAF)	Spain, Teruel
R. melanophthalma	6605	MAF-Lich 16778 (MAF)	Spain, Teruel
R. haydenii	8683	Leavitt 8683 (F)	USA, ID
R. haydenii	8935p	Leavitt 8935 (F)	USA, ID
R. haydenii	8935s	Leavitt 8935 (F)	USA, ID
R. novomexicana	8684a	Leavitt 8684A (F)	USA, NM
R. novomexicana	8684b	Leavitt 8684B (F)	USA, NM
R. novomexicana	8684c	Leavitt 8684C (F)	USA, NM
R. novomexicana	8684d	Leavitt 8684D (F)	USA, NM
R. parilis	8665N	Leavitt-8665 (F)	USA, NV
R. parilis	8665u	Leavitt-8665 (F)	USA, NV
R. polymorpha	8668g	Leavitt-8668 (F)	USA, NV
R. polymorpha	8668l	Leavitt-8668 (F)	USA, NV
R. polymorpha	8668p	Leavitt-8668 (F)	USA, NV
R. polymorpha	8668r	Leavitt-8668 (F)	USA, NV
R. aff. *porteri*	8665x	Leavitt-8665 (F)	USA, NV
R. aff. *porteri*	8668j	Leavitt-8668 (F)	USA, NV
R. aff. *porteri*	8668m	Leavitt-8668 (F)	USA, NV
R. porteri	8665t	Leavitt-8665 (F)	USA, NV
R. porteri	8668e	Leavitt-8668 (F)	USA, NV
R. porteri	8668h	Leavitt-8668 (F)	USA, NV
R. shushanii	8664A	Leavitt 13-TLM-001 (BRY-C)	USA, UT
R. shushanii	8664B	Leavitt 13-TLM-001 (BRY-C)	USA, UT

Table 2. Microsatellite loci developed for *Rhizoplaca melanophthalma* s. str.

Locus	Primer sequences (5'–3')	Repeat motif	Allele size range (bp)	GenBank accession no.
Rmel1	F: *GGCTGGGTGTTGTAGTGTTG R: ATACCTGGCGCTCAAGAATG	$(GTT)_{18}$	179–203	KX755412
Rmel2	F: *TGGTGGATCTGAGAGCGTAC R: ACTTCAACCTTCACAACGCC	$(CAGGCT)_{10}$	328–382	KX755413
Rmel3	F: *CAAAGGTCAGGAGAGGAGGG R: TGGACGCGTGGCAATTTATC	$(AC)_{10}$	380–412	KX755414
Rmel4	F: *ATCGAGACTTACTTCCCGCC R: AATCGTATCTCCAGACCCGC	$(GT)_{11}$	353–385	KX755415
Rmel5	F: *TTAGCCCGAGACCACATACG R: TGGAGAGATGAAGCTGGCTC	$(CT)_{12}$	311–321	KX755416
Rmel6	F: *ACACCAGATCTCACTCAGGC R: CCGGGAGTAGGTGTAGATGC	$(AC)_{10}$	184–192	KX755417
Rmel7	F: *TCCGGAACTGGCTTGATAGG R: CTGAAGTCGATGTTGGGAGC	$(CCTT)_{11}$	314–362	KX755418
Rmel8	F: *TTTGCCCGACGTGCAATATC R: CTGCAGCACTCTAACCATGC	$(AG)_{11}$	420–438	KX755419
Rmel9	F: *ATCTCCTGCATCTTCTCCGC R: AACGTCACATTCGCAGTCAC	$(AC)_{10}$	309–331	KX755420
Rmel10	F: *TCATCACACCAGACACAGGG R: ACCTTAGGCCCAGACACATG	$(AG)_{10}$	464–468	KX755421

* M13 tail: TGTAAAACGACGGCCAGT.

Primer3 (Rozen and Skaletsky 2000) as implemented in MSATCOMMANDER. An M13 tag (5'-TGTAAAACGACGGCCAGT-3') was appended to forward primers and 5' ends of the reverse primers were tailed with 5'-GTGTCTT-3' tag.

Singleplex PCR reactions were performed in 10 µl reaction volumes consisting of 5.89 µl H_2O, 1 µl 10x buffer (Roche Diagnostics, Indianapolis, USA), 0.6 µl 8 mM dNTP, 1 µl BSA, 0.15 µl Taq (Roche Diagnostics, Indianapolis, USA), 0.16 µl 6-FAM labeled M13 primer, 0.04 µl 10 µM M13 tailed forward primer, 0.16 µl 10 µM reverse primer, and 1 µl of genomic DNA. DNA amplification was performed using a touchdown PCR with initial denaturation at 95 °C for 5 min; followed by first 11 cycles of 30 s at 95 °C, 30 s at 60–50 °C, 1 min. at 72 °C, and then 35 cycles of 30 s at 95 °C, 30 s at 50 °C, 1.5 min. at 72 °C, and a final extension of 10 min. at 72 °C.

Fragment analysis was performed on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, California, USA) using GeneScan-500 LIZ (Life Technologies, Warrington, UK) as an internal size standard. Genotyping was performed utilizing the microsatellite plugin in Geneious 9.1.2 (Biomatters Limited). Polymorphism within the microsatellites was tested in GenAlEx 6.5 (Peakall and Smouse 2012) by calculating Nei's unbiased genetic diversity.

Table 3. Sample size, number of alleles (*A*) and Nei's unbiased genetic diversity (H_e) of ten microsatellite loci developed for *Rhizoplaca melanophthalma* s. str.

	Total		
Locus	***n***	***A***	H_e
Rmel1	21	8	0.886
Rmel2	20	6	0.858
Rmel3	21	9	0.866
Rmel4	21	11	0.919
Rmel5	20	5	0.679
Rmel6	21	3	0.643
Rmel7	20	6	0.763
Rmel8	20	7	0.779
Rmel9	21	9	0.881
Rmel10	20	3	0.353
Average		6.7	0.765

Results and discussion

Of the 25 microsatellites assessed, 18 amplified successfully and 10 were polymorphic in all 21 *R. melanophthalma* s. str. specimens (Table 2). The number of alleles per locus ranged from three to 11 with an average of 6.7. Nei's unbiased genetic diversity varied between 0.353 and 0.919 with the average genetic diversity being 0.765 (Table 3). The same 18 microsatellites that amplified successfully with *R. melanophthalma* s. str. also amplified in *R. haydenii*, *R. novomexicana*, *R. parilis*, *R. polymorpha*, *R. porteri*, and *R. shushanii*, but only three loci were polymorphic in all these species. For these three loci (Rmel1, Rmel, 4, and Rmel8) the number of alleles ranged from 11 to 15 with the average of 13 and Nei's unbiased genetic diversity varied between 0.892 and 0.905 with average of 0.900.

The 10 polymorphic microsatellite markers for the lichen-forming fungus *R. melanophthalma* will help elucidate population processes that have led to the observed distribution patterns in this widespread species.

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RESEARCH ARTICLE

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The first ITS phylogeny of the genus *Cantharocybe* (Agaricales, Hygrophoraceae) with a new record of *C. virosa* from Bangladesh

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Abstract

This is the first internal transcribed spacer (ITS) phylogeny of the enigmatic genus *Cantharocybe* and includes ITS sequences from two out of the three holotype collections. Two species are reported from the Americas and only a single species from Asia. Additionally, a collection of *Cantharocybe virosa* collected from tropical Bangladesh was included in this study. This species is a new record for Bangladesh, and is characterized by its tawny gray or grayish brown pileus and stipe surface, smooth ellipsoid basidiospores, elongated necked lecythiform cystidia, a trichoderm pileipellis, and abundant clamp connections. Molecular phylogenetic analysis using ITS, and combined analyses of ITS with the large subunit of nuclear ribosomal RNA (nrLSU) showed that the collection from Bangladesh is conspecific with the Indian *C. virosa*. A large, previously unknown intron was found in the ITS of *C. brunneovelutina* and *C. virosa*, while the *C. gruberi* sequence was found to be truncated where the intron would have been inserted. The intron was not identical between *Cantharocybe* species, and may be phylogenetically informative. Morphological description, color photographs and line drawings are provided for Bangladesh collection *C. virosa*. A key to the genus *Cantharocybe* is provided.

Key words

Biogeographic distribution, tropical mushroom, molecular phylogeny, taxonomy

* Md. Iqbal Hosen and Deborah Jean Lodge contributed equally to this work.

Introduction

The genus *Cantharocybe* was introduced by Bigelow and Smith in 1973 to accommodate *Clitocybe gruberi* Smith, based on the large yellow basidiomata, oblong to subcylindrical to elongated basidiospores and the presence of lageniform to lecythiform cheilocystidia. However, other taxa in this genus do not have large-sized yellow basidiomata and oblong to elongated basidiospores. Therefore, Lodge et al. (2014) extended the generic circumscription of the genus to include taxa with large, clitocyboid, yellow, dark brown to brownish gray basidiomata with long decurrent or adnate with decurrent tooth lamellae; abundant cheilocystidia which are usually lecythiform, sometimes with a mucronate apex, with or without a rounded capitulum; smooth, inamyloid, oblong, elongate, ellipsoid to broadly ellipsoid or rarely subglobose, basidiospores; a trichoderm or cutis pileipellis; and caulocystidia similar to cheilocystidia. To date, *Cantharocybe* unites only three known species (http://www.indexfungorum.org), *C. gruberi* (Smith) Bigelow & Smith, *C. brunneovelutina* Lodge, Ovrebo & Aime and *C. virosa* (Manim. & K.B. Vrinda) T.K.A. Kumar, found in North America and Spain, Belize in Central America and India, respectively (Bigelow and Smith 1973, Justo et al. 2010, Esteves-Raventós et al. 2011, Ovrebo et al. 2011, Kumar and Manimohan 2013). Although *C. gruberi* was reported from China by Bi et al. (1993), the voucher specimen was re-identified as *Oudemansiella bii* Zhu L. Yang & Li F. Zhang (Yang and Zhang 2003). Recent molecular phylogenetic studies show that *Cantharocybe* is at the base of the hygrophoroid clade and is sister to *Ampulloclitocybe* (Pers.) Redhead, Lutzoni, Moncalvo & Vilgalys, but it is not clear if *Cantharocybe* and *Cuphophyllus* Donk (Bon) are members of Hygrophoraceae s.s. (Matheny et al. 2006, Binder et al. 2010, Lodge et al. 2014).

The phylogenetic relationships among the taxa of *Cantharocybe* are well resolved, based on partial nrLSU sequence analyses (Ovrebo et al. 2011, Kumar and Manimohan 2013). Except for *C. gruberi* (non-holotype sequences from Esteves-Raventós et al. 2011), ITS sequences for other taxa of *Cantharocybe* are unavailable. Since the ITS region is an informative genetic region for species recognition in many groups of fungi (Schoch et al. 2012), we generated ITS sequences from holotype specimens of *C. brunneovelutina* and *C. virosa*. This is the first publication with ITS sequences from all holotype collections of *Cantharocybe* except *C. gruberi*, which we used to elucidate their phylogenetic relationships.

The first author has recently collected *Cantharocybe* material from tropical Bangladesh that is morphologically similar to *C. virosa* to some extent. Careful microscopic observation of the material from Bangladesh indicates that it could be conspecific with the Indian *C. virosa*, but the nrLSU sequence analysis suggested that it could be a new species or perhaps a variety of *C. virosa*. Therefore we attempted to obtain the holotype material of *C. virosa* from TENN in order to generate additional sequences and compare with the collection from Bangladesh. Fortunately, we received cloned ITS sequence of the holotype *C. virosa* (TENN 63483) from K.W. Hughes (Tennessee, USA) that we included in our further phylogenetic studies. The goal of this study is to

elucidate the taxonomic position of the Bangladeshi collection of *Cantharocybe*, and clarify the confusion with an Indian collection of *C. virosa* using morphological and molecular evidence.

Materials and methods

Collection and deposition

Cantharocybe specimens (Iqbal568 and 693) were collected from Madhupur upazila of Bangladesh on ground near to or associated with the roots of *Cocos nucifera*, a tree of the plant family Arecaceae during the monsoon (June to August) of 2012–2013. Specimens examined are deposited in the Cryptogamic Herbarium of Kunming Institute of Botany of the Chinese Academy of Sciences (KUN), China; and in the private herbarium of Iqbal (PHI). *Cantharocybe brunneovelutina* was previously deposited at BRH and CFMR (Ovrebo et al. 2011).

Morphological studies

The morphological description of the basidiomata is based on field notes and documented by photographs. Color codes are according to Kornerup and Wanscher (1978). A small fragment of dried specimen was revived in H_2O, 5% KOH, and Congo red. The notation [n/m/p] is used in the descriptions of basidiospores measurements, which means *n* basidiospores from *m* basidiomata of *p* collections were measured; 20 basidiospores were measured from each voucher specimen. Dimension for basidiospores are given as (a–)b–c(–d), in which 'b–c' contains a minimum of 90% of the measured values and extreme values 'a' and 'd' are given in parentheses. Q_m = Q ± SD: Q indicates the length/width ratio of a measured basidiospore, Q_m indicates to the average of Q basidiospores and SD is the standard deviation. For the pileipellis and stipitipellis observations radial-vertical section were made halfway of the pileus and stipe, respectively. Line drawings were done free hand.

Molecular studies

The protocol for DNA extraction followed that of Doyle and Doyle (1987). ITS1/ITS4 or ITS1/ITS5 (White et al. 1090) and LROR/LR5 (Vilgalys and Hester 1990) primer pairs were used for the amplification of the internal transcribed spacer region (ITS) and the large subunit nuclear ribosomal RNA (nrLSU), respectively. PCR amplification was carried out following the protocol of Hosen et al. (2013). PCR confirmation was confirmed on 1% agarose electrophoresis gels stained with ethidium bromide. The amplified PCR products were sent to a commercial sequencing provider company (BGI, China) for sequencing.

Three sequences (nrLSU: KF303143 and KX452406, ITS: KX452403) were generated from the Bangladeshi *Cantharocybe*. Additionally, two ITS sequences were also obtained from the type materials of *C. brunneovelutina* (BZ-1883: KX452404) and *C. virosa* (TENN-63483: KX452405). ITS sequences generated for this study were cloned in pMD18-T following manufacturer's instructions. The newly generated sequences were deposited in GenBank. An initial BLASTn search of the nrLSU sequence obtained from the Bangladeshi material against the NCBI database (http://www.ncbi.nlm.nih.gov/) gave *C. virosa* (=*Megacollybia virosa* Manim. & K.B. Vrinda), *C. brunneovelutina* and *C. gruberi* as closest hits, with maximum similarities of 96%, 96% and 95%, respectively. The closest nrLSU sequences including *Ampulloclitocybe* and *Cuphophyllus* were retrieved from GenBank and additional taxa were chosen after consulting Lodge et al. (2014) and then combined with nrLSU sequence from Bangladesh materials. Two additional datasets were constructed: ITS and ITS+nrLSU to clarify relationships between Indian and Bangladesh collections of *Cantharocybe*. All datasets were aligned with Mafft v.6.8 (Katoh et al. 2005) and manually adjusted with BioEdit v.7.0.9 (Hall 1999) using default settings. Maximum Likelihood (ML) and Bayesian Inference (BI) methods followed those in Hosen et al. (2013). *Phyllotopsis nidulans* (Pers.) Singer was served as outgroup for all dataset analyses as inferred from other phylogenetic studies (Ovrebo et al. 2011, Kumar and Manimohan 2013, Lodge et al. 2014).

Both ML and BI analyses were conducted using RAxML v.7.2.6 (Stamatakis 2006) and MrBayes v.3.1.2 (Ronquist and Huelsenbeck 2003) with default settings. For ML analyses, the General Time Reversible Model of evolution with estimated gamma distribution was selected, and statistical support values were obtained using nonparametric bootstrapping (BS) with 1000 replicates. For BI analysis, the substitution model suitable for ITS and nrLSU datasets were determined using the Akaike Information Criterion (AIC) implemented in MrModeltest v.2.3 (Nylander 2004), and the models were SYM+G and GTR+I+G, respectively. Bayesian Inference analyses were conducted using the selected evolutionary model with four chains and generations set to 0.5 million for the ITS and nrLSU datasets, and four million for the combined dataset (ITS+nrLSU). Runs were terminated once the average standard deviation of split frequencies went below 0.01. Trees were sampled every 100 generations, with the first 25% of trees discarded as burn-in. Posterior probabilities (PP) were calculated using the "sump" and "sumt" commands implemented in MrBayes.

Results

Molecular results

Three datasets (ITS, nrLUS and ITS+nrLSU) are constructed and analysed separately. The ITS dataset includes 17 sequences of fungal taxa (Table 1) and consisting of 1052 nucleotide sites (gaps included) of which 417 are parsimony informative. The nrLSU

Table 1. Species of fungal taxa used in the molecular phylogenetic analyses.

Name of the species	Voucher/isolate or collection number	Origin	GenBank accession number	
			ITS, 5.8S	nrLSU
Ampulloclitocybe clavipes	AFTOL-ID 542	USA	AY789080	AY639881
Cantharocybe brunneovelutina	BZ-1883*	Belize	**KX452404**	HM588721
Cantharocybe gruberi	AH24539	Spain	JN006422	JN006420
Cantharocybe gruberi	DED6609	USA	-	AF261530
Cantharocybe gruberi	AFTOL-ID 1017	USA	DQ200927	DQ234540
Cantharocybe virosa	Iqbal-568	Bangladesh	**KX452403**	**KF303143**
Cantharocybe virosa	Iqbal-693	Bangladesh	-	**KX452406**
Cantharocybe virosa	TENN63483*	India	**KX452405**	JX101471
Cuphophyllus acutoides var. *pallidus*	CFMR TN-257	USA	KF291096	KF291097
Cuphophyllus adonis	CFMR CHIL-1	Chile	KF291035	KF291036
Cuphophyllus aff. *pratensis*	AFTOL-ID 1682	USA	DQ486683	DQ457650
Cuphophyllus aurantius	CFMR PR-6601	Puerto Rica	KF291099	KF291100
Cuphophyllus basidiosus	AFTOL-ID 1759	USA	DQ486684	DQ457651
Cuphophyllus borealis	BHS2009-104	-	HM020684	HM026552
Cuphophyllus canescens	AFTOL-ID 1800	USA	DQ486685	DQ457652
Cuphophyllus flavipes	Hattori-JP-6	Japan	KF291044	KF291045
Cuphophyllus fornicatus	Boertmann 2009/94 (CFMR)	Denmark	KF291123	KF291124
Cuphophyllus griseorufescens	PDD:27230	New Zealand	GU233328	GU233423
Phyllotopsis nidulans	HMJAU7272	China	GQ142019	GQ142039

Note: Newly generated sequences are highlighted in bold. An asterisk (*) at the isolate indicates holotype material.

dataset contains 19 sequences of the same taxa (Table 1) as well as two additional sequences of *Cantharocybe* with 934 aligned nucleotide sites (gaps included) of which 131 are parsimony informative. The combined (ITS+nrLSU) dataset includes 17 sequences of fungal taxa (Table 1) and consists of 1986 nucleotide (first 1052 for ITS and the next 934 for LSU, gaps included) sites including 541 that are parsimony informative. The aligned datasets are deposited in TreeBASE (S19556).

The ITS sequences of *C. brunneovelutina* and *C. virosa* are longer than normal because a ca. 210 bp intron is inserted about 70 bp after the ITS1 primer. This intron was not previously known as Blast searches turned up no matches, but the intron sequences were similar between *Cantharocybe* species. The GenBank ITS sequence DQ200927 of *C. gruberi* was found to be truncated on the 3' end, where the intron was likely inserted. We infer that the partial ITS sequence of *C. gruberi* deposited in GenBank as part of the Assembling the Fungal Tree of Life (AFTOL) project was truncated because the first 70 bp of the ITS are missing beginning at a point which coincides with the position of the intron insertion in *C. brunneovelutina* and *C. virosa*. Lodge et al. (2014) found that introns which were inserted within 100–150 bp of a primer

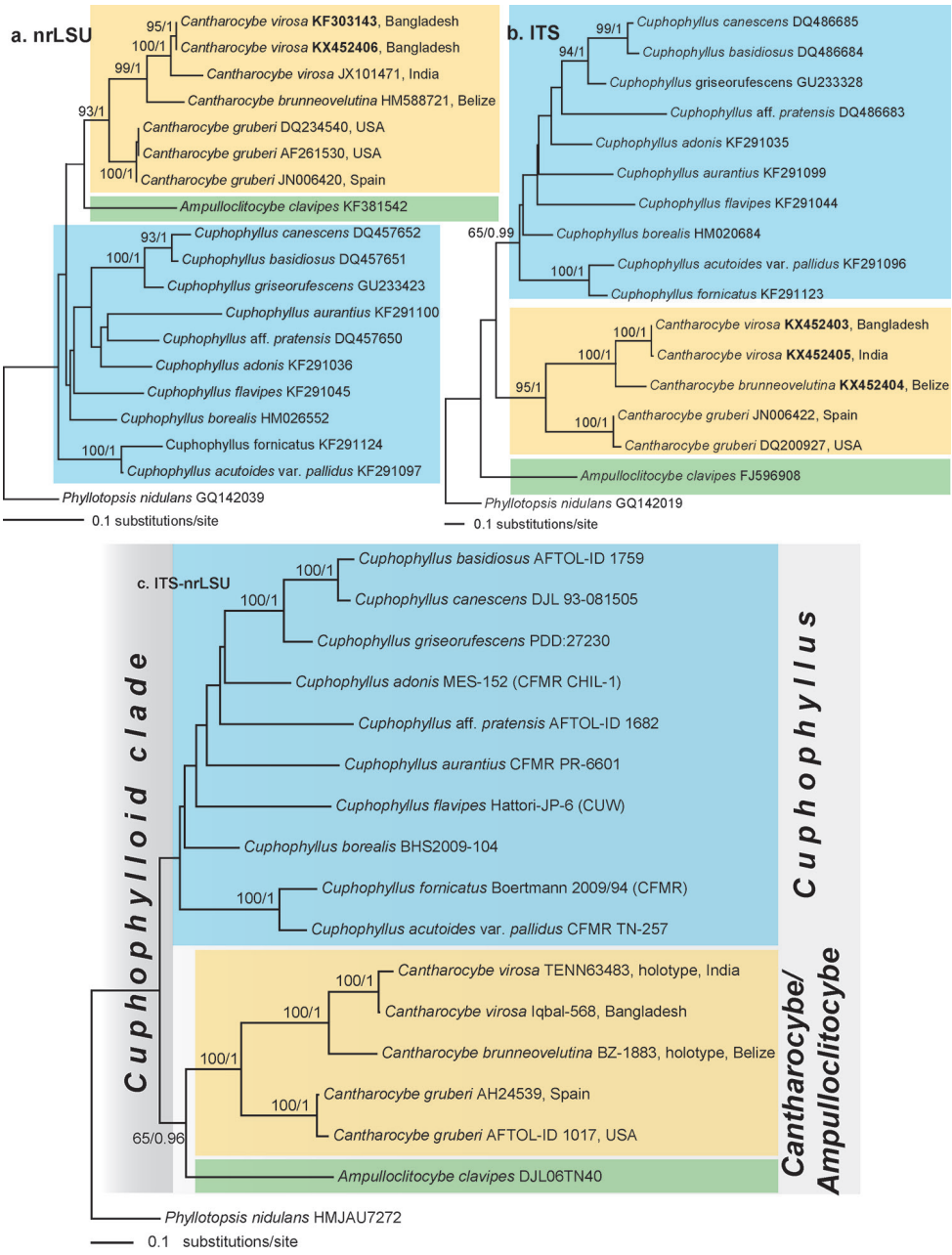

Figure 1. Phylogenetic relationships of the genus *Cantharocybe* inferred from nrLSU (**a**), ITS (**b**) and ITS+nrLSU (**c**) sequences using maximum likelihood (ML). RAxML bootstrap supports values (>50%) and Bayesian posterior probabilities (>0.95) are indicated on the branches at nodes (ML/PP). Newly generated sequences are highlighted in bold. GenBank accession or voucher numbers are provided after the species name.

disrupted replication unless the DNA was cloned using a vector such as the method we used to obtain our ITS sequences. As there is no notation in GenBank that the AFTOL ITS sequence of *C. gruberi* was cloned, we infer that forward reads were disrupted by the same intron that was found in *C. brunneovelutina* and *C. virosa*. Thus, it is likely that Matheny and Hibbett's AFTOL program only obtained back-reads from the 5' end using the ITS4 primer. The authors of the *C. gruberi* DQ200927 sequence may or may not have obtained a partial read of an intron, but if so, it would have been of diminishing quality with distance from the ITS4 primer, it would not have matched any known ITS sequences, and it would have been impossible to correct or corroborate without a forward read from the 3' end. We therefore infer that if a partial read of an intron was obtained by Matheny and Hibbett for *C. gruberi*, that it was trimmed from the GenBank submission because it did not match ITS1 sequences and it could not be corrected or corroborated. Blast searches of GenBank using the ITS sequence of *C. virosa*, after removing the intron, retrieved *C. gruberi* sequences with highest similarity.

Tree topologies obtained from both ML and BI methods of phylogenetic analyses are congruent, the ML trees are shown in Fig. 1. The Bangladesh sample of *Cantharocybe* clusters in a strongly supported clade with the Indian sample of *C. virosa* in all three datasets, indicating that they are conspecific (Fig. 1).

Taxonomy

***Cantharocybe virosa* (Manim. & K.B. Vrinda) T.K.A. Kumar**
Figs 2–3

Cantharocybe virosa (Manim. & K.B. Vrinda) T.K.A. Kumar, Mycotaxon 124: 235 (2013).
≡ *Megacollybia virosa* Manim. & K.B. Vrinda, Mycotaxon 111: 364 (2010).

Description. Basidiomata medium-sized to large. Pileus 50–80 mm diam., convex at first then applanate, sometimes uplifted with cracked margin, tawny gray, dark brown (6E4–5) to grayish brown (5E3–5E4, 6E3–6F4), dry, pruinose or with fine appressed scales under lens, margin without striation. Hymenophore lamellate; lamellae adnate to decurrent, subdistant to crowded, white to pallid white (5A1, 6A1); lamellulae numerous, concolorous with lamellae. Stipe 50–80 × 10–15 mm, central, slightly curved, cylindrical, gradually thickening towards the base, at the apex ribbed by the subdeccurent lines of the hymenophore, upper half pale gray or brownish gray (5D2) to grayish brown (5E3) pruina or squamules and the remaining half nearly concolorous with the pileus, with cottony mycelium at the base, interior solid, milky white to white. Context 12 mm thick in the center of the pileus, milky white to white (6A1), firm, solid, unchanging when cut or bruised.

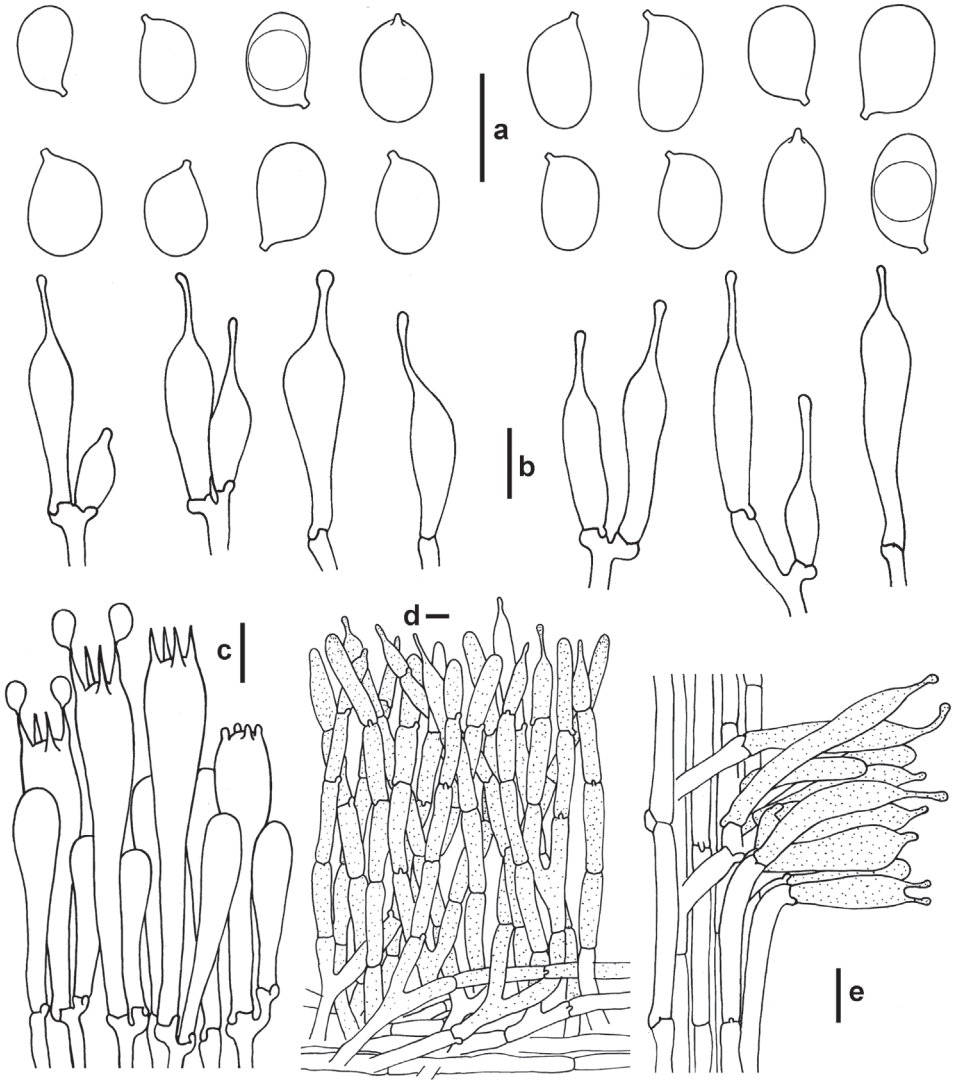

Figure 2. Microscopic features of *Cantharocybe virosa* (HKAS 79012, Iqbal 568). **a** Basidiospores **b** Cheilocystidia **c** Basidia at different stages of development **d** Trichoderm pileipellis **e** Surface of stipe in longitudinal section showing caulocystidia. Scale bars: 10 μm.

Basidiospores [40/2/2] (8–) 8.5–10(–11.5) × 5–6.2(–7.0) μm, [Q = 1.54–1.62, Q_m = 1.58 ± 0.12] ellipsoid to broadly ellipsoid, hyaline, thin-walled, smooth, inamyloid, apiculus conspicuous. Basidia 45–60 × 8–11 μm, clavate, hyaline, thin-walled, 4-spored, sterigmata up to 8 μm long; basal septum usually clamped. Cheilocystidia 25–45 × 5–9 μm, abundant, lecythiform to lageniform, sometimes with a mucronate apex, basal portion usually clavate, the upper portion extending into an elongated neck up to 15 μm long with or without a rounded capitulum; basal septa often clamped.

Figure 3. Basidiomata of *Cantharocybe virosa*. **a, c** Adnate-decurrent lamellae and pileus-stipe surface **b, d** Longitudinal section illustrating unchanged solid context **a, b** from HKAS 79012, Iqbal 568 **c, d** from Iqbal 693. Scale bars: 2 cm.

Pleurocystidia absent. Lamellar trama parallel to sub-regular composed of branching filamentous hyphae 4–10 µm wide, hyaline to pale yellow, thin-walled. Pileipellis a trichoderm, slightly interwoven, composed of 6–10 µm wide hyphae with often pale brown vacuolar to plasmatic pigments; terminal cells 20–65 × 6–10 µm, usually cylindrical to somewhat narrowly clavate, sometimes mucronate; pileocystidia with or without extending neck, occasionally with one or two short rounded capitula, elongated neck up to 15 µm long, clamp connection frequently present at septa. Stipitipellis composed of vertically arranged, branching, 7–10 µm wide filamentous hyphae, outer surface more or less covered with cylindrical to narrowly clavate cells (35–100 × 6–11 µm) with or without a rounded capitulum, mostly similar to cheilocystidia but sometimes double necked with an extending capitulum head, pale brown vacuolar to plasmatic pigments. Clamp connections common at septa.

Habitat. Solitary or in clusters, associated with roots of *Cocos nucifera* (collection Iqbal 568) or along the roadside on ground (collection Iqbal 693) near *C. nucifera*.

Distribution. Known from tropical South Asia, Bangladesh and India.

Specimens examined. Bangladesh, Dhaka division: Tangail, Madhupur, Bangladesh Agricultural Development Corporation (BADC) campus, 24°37'35"N,

90°03'33"E, 05 Aug 2012, 20–25 m, Iqbal 568 (HKAS 79012, PHI-12); same location, 18 Jun 2013, Iqbal 693 (PHI-13). Belize, Orange walk district: La Milpa Field State, La Milpa Archaeological Site, 17°50'30"N, 89°1'0"W, 100 m (CFMR), 25 Oct 2002, DJL-BZ-85 (BZ-1883).

Discussion

Morphology and phylogenetic relationships of *Cantharocybe*

The Bangladeshi *C. virosa* is characterized by its gray to grayish brown basidiomata, moderately crowded lamellae, fine squamules on stipe surface formed from clusters of lecythiform caulocystidia, ellipsoid to broadly ellipsoid basidiospores, and a trichoderm pileipellis.

Based on molecular analyses, *C. virosa*, a species recently described from India is conspecific with the Bangladeshi collection (Fig. 1). However, the Indian *C. virosa* has a pale grayish brown pileus, long cheilocystidia which can be up to 63 µm with a long neck up to 35 µm, a cutis pileipellis or occasionally disrupted with trichodermal patches (Kumar and Manimohan 2013). In comparison, the Bangladeshi collection has a grayish brown to dark brown pileus, cheilocystida with short neck up to 15 µm long, and clearly defined trichoderm pileipellis. Geographically, *C. virosa* is distributed in the Kerala state (South-West region) of India, while the new collection was collected from Tangail district of the Dhaka division of Bangladesh. Moreover, the nrLSU sequence obtained from the Bangladeshi collection does not perfectly match (96%) with the holotype *C. virosa* retrieved from GenBank, and the genetic distance between them is 0.96% (8 bases differences and 23 deletions in the Indian collection) of 831 nucleotide sites. These morphological variations, geographic distance and nrLSU sequence inferred suggest that they may have diverged recently from each other due to its allopatric speciation.

Surprisingly, when we blasted the newly generated ITS sequences from the holotypes of *C. virosa* (TENN 63483) and *C. brunneovelutina* (BZ-1883) individually against the NCBI database, we did not find *C. gruberi* as the closest sister species among the first 100 matched species, even 80–88% matched only with some taxa of *Tricholoma*, *Lepista*, *Macrolepiota*, *Lepiota*, etc. The ITS sequence from the newly collected material from Bangladesh also gave a similar result. These results are caused by the presence of a large intron in *C. brunneovelutina* and *C. virosa*, and the truncated sequence of *C. gruberi* deposited in GenBank (missing the intron and 3' end). Subsequently, we retrieved the closest ITS sequences of those taxa including *C. gruberi* from GenBank and reconstructed an ITS phylogenetic tree where all *Cantharocybe* taxa were nested together within the same clade with strong BS value (99% ML BS, 1.0 PP) and apart from *Tricholoma* and *Lepista* clades (data not shown). In the ITS sequence analyses, the Indian entity *C. virosa* showed only 5 base pair difference with 4 deletions out of 742 nucleotides (genetic distance 0.68%) from the Bangladeshi material. Fur-

ther extended combined dataset (ITS+nrLSU) also showed little divergence (genetic distance 0.83%) between them (Fig. 1c). This small variation in ITS sequences, with negligible differences in the color of the basidioma, size of basidia and cheilocystidia which were possibly due to environmental variations, do not warrant a new variety or species, suggesting that both south Asian entities are conspecific. Furthermore, both collections were the same ecology and associated with the roots of *C. nucifera* in a tropical region. Neither coconut trees nor palms in general have been shown to associate with ectomycorrhizal fungi. Halbwachs et al. (2013) however, found hyphae of a *Cuphophyllus* species (a genus near *Cantharocybe*) and several species of *Hygrocybe* as endophytes in plant roots including those of a monocot (*Plantago major*) so another type of root symbiosis with *C. nucifera* is possible. The considerable variation in the nrLSU sequence (96% match, 8 bases differences with 23 deletion) of the Indian *C. virosa* may be explained by the fact that the nrLSU sequence obtained from that collection was not clean, showing evidence of a contaminating sequence or minor indel (K.W. Hughes, pers. comm.).

Cantharocybe brunneovelutina and *C. gruberi* can also be separated from *C. virosa* morphologically. *Cantharocybe brunneovelutina* differs from *C. virosa* by its velutinous basidioma, unusual cheilocystidia with multiple prong-like appendages at the apex resembling a basidia-like structure, and a trichoderm pileipellis (Ovrebo et al. 2011). The type species of this genus, *C. gruberi*, has a pale yellow to lemon yellow pileus and narrowly elliptical to oblong basidiospores measuring 11–16(–17) × (4.5–) 6–7.5 µm (Bigelow and Smith 1973).

Molecular phylogenetic analyses indicated that the genus *Cantharocybe* is monophyletic, with strong bootstrap values (Fig. 1). Likewise, Ovrebo et al. (2011) and Lodge et al. (2014) showed that the monophyletic clade of *Cantharocybe* has strong BS value comprising *C. gruberi* and *C. brunneovelutina* using single locus or multi-locus sequence analyses. Although recent phylogenetic studies (Ovrebo et al. 2011, Kumar and Manimohan 2013, Lodge et al. 2014) showed the monophyly of *Cantharocybe*, the sister relationship with other genera remains unclear. *Cantharocybe* nests at the base of the hygrophoroid clade together with *Ampulloclitocybe* and *Cuphophyllus* (Binder et al. 2010, Matheny et al. 2006, Ovrebo et al. 2011, Lodge et al. 2014), but their relationships were not confidently resolved. In our combined dataset (ITS+nrLSU) analysis, *Ampulloclitocybe* is only weakly supported (65% ML BS, PP = 0.96) as sister to the *Cuphophylloid* clade (Fig. 1c). This is accordance with the recent phylogenetic study of Lodge et al. (2014).

Distribution and ecology of *Cantharocybe*

Cantharocybe is an uncommon genus that only consists of three species. *Cantharocybe gruberi* has wide distribution from America (New Mexico, western North America and British Columbia) to Europe (Spain). *Cantharocybe brunneovelutina* is reported from tropical Central America (Belize) whereas *C. virosa* is from tropical South Asia

(Bangladesh and India). Based on the branching order with strong bootstrap support at all nodes in our phylogeny in which *C. gruberi* is basal, we infer that the genus *Cantharocybe* may have originated in America or Europe and then migrated independently to Central America and South Asia.

The south Asian species usually occurs with the roots of *Cocos nucifera* (Manimohan et al. 2010, Kumar and Manimohan 2013, this study as well), the North American and European collections were found on needle beds or ground under conifers and *Pinus nigra*, respectively (Bigelow and Smith 1973, Esteves-Raventós et al. 2011), and the Central American species was found in humus around dead palm trees (Ovrebo et al. 2011). However, their symbiotic association with trees is still unknown. To facilitate identification of *Cantharocybe* taxa worldwide, a key to the species is given bellow.

Key to the taxa of Cantharocybe

1 Basidiomata medium (up to 75 mm broad), with dark brown pileus, cheilocystidia resembling basidia with apical appendages, basidiospores elliptical to oblong or subcylindrical, Belize (Central America, tropical)..... ***C. brunneovelutina***

– Basidiomata medium to large (45–200 mm broad), yellow or pale grayish brown to grayish brown, basidiospores oblong to cylindrical or ellipsoid to broadly ellipsoid or rarely subglobose, cheilocystidia lecythiform..... **2**

2 Basidiomata large (up to 200 mm broad), yellow, basidiospores 11–16(–17.5) × (4.5–) 6–7.5 µm, elliptical, oblong to cylindrical, western North America (temperate zone) and Spain (Europe)..... ***C. gruberi***

– Basidiomata medium (up to 100 mm broad), pale grayish brown to tawny gray to grayish brown, basidiospores 6.5–11(–12) × 5.5–6.5(–7) µm, ellipsoid to broadly ellipsoid or rarely subglobose, Bangladesh and India (South Asia, tropical)..... ***C. virosa***

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