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Molecular phylogenetic studies unmask overlooked diversity in the tropical lichenized fungal genus *Bulbothrix s.l.* (Parmeliaceae, Ascomycota)

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Species boundaries in lichen-forming fungi occurring in extra-tropical regions are relatively well studied in comparison with those in species distributed in tropical regions. Here, we aim to re-examine species boundaries in two pantropical, asexually reproducing species, *Bulbothrix isidiza* and *B. tabacina*. We generated a multi-locus DNA sequence data set from samples collected throughout the Tropics and these data were analysed in a phylogenetic framework. Our results show that *B. isidiza* and *B. tabacina*, as currently circumscribed, do not form monophyletic groups. Rather, our study supports the presence of five, independent species-level lineages in *B. isidiza s.l.* and three in *B. tabacina s.l.* Additionally, seven other species were recovered in distinct lineages. Some of the previously overlooked lineages appear to have a restricted geography, whereas others are pantropical. Morphological, chemical and ecological features were re-evaluated for each of these lineages. A new species, ***B. kenyana* sp. nov.**, is formally described from East Africa and a new combination, ***B. sublaevigatoides* comb. nov.**, is proposed. Due to limited specimen sampling, the remaining undescribed species-level lineages newly circumscribed in this study are not formally recognized here.

ADDITIONAL KEYWORDS: Africa – integrative taxonomy – molecular systematics – new species – parmelioid lichens – taxonomic re-evaluation.

INTRODUCTION

DNA sequence data coupled with empirical species delimitation methods have advanced our knowledge of species boundaries in lichen-forming fungi (reviewed in Leavitt, Moreau & Lumbsch 2015). In a number of cases, phenotype-based approaches to species recognition have been shown to underestimate the number of species in lichenized fungi. For example, in Parmeliaceae alone, Crespo & Lumbsch (2010) have estimated at least 80 cryptic lineages hidden within widely distributed or disjunct species. Based on this

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new perspective, species delimitation studies and taxonomic revisions now commonly incorporate molecular sequence data analysed with a wide range of species delimitation methods (reviewed in Crespo & Lumbsch, 2010; Lumbsch & Leavitt, 2011; Leavitt *et al.*, 2015).

In contrast to many groups of lichen-forming fungal species in temperate regions, which are relatively well studied (reviewed in Crespo & Lumbsch, 2010; Lumbsch & Leavitt, 2011), tropical species are generally less well studied (Parnmen *et al.*, 2012; Kraichak *et al.*, 2015). Indeed, some tropical regions (e.g. Africa) are biodiversity hotspots and probably harbour a large number of lichen-forming fungal species (Hawksworth, 2012). Eastern Africa is diverse, having unique ecoregions. On the one hand, it includes Afromontane ecoregions, which are generally cooler and more humid than the surrounding lowlands, while on the other it has tropical moist forest regions along the east coast. Although undescribed species of lichenized fungi are expected to be found in unexplored regions, there is a growing body of evidence through molecular phylogenetic studies indicating that distinct species-level lineages may also be hidden under a single nominal taxon (reviewed in Leavitt *et al.*, 2015).

Bulbothrix Hale is one of the tropical lichen-forming fungal genera belonging to the parmelioid core of Parmeliaceae (Crespo *et al.*, 2010; Divakar *et al.*, 2015). The genus includes *c*. 60 species, is widespread in tropical regions and reaches its highest diversity in semi-arid woodlands and secondary forests in the Neotropics (Hale, 1976; Swinscow & Krog, 1988; Elix, 1994; Benatti, 2010, 2012a). *Bulbothrix* spp. are characterized by a small foliose thallus, corticate above and below, are laciniate and are usually adnate to loosely attached to their substrate. The species have bulbate marginal cilia, an upper cortex consisting of a palisade plectenchyma with a pored epicortex and isolichenan in the cell walls and a whitish to brownish mineral grey upper cortex. The cilia and rhizinae are simple to branched. The apothecia are smooth to coronate imperforate, containing hyaline unicellular ellipsoid to bicornute ascospores, 5.0–21.0 × 4.0–12.0 μm. The conidia are bacilliform to bifusiform, 5.0–10.0 × 0.5–1.0 μm (Hale, 1976; Elix, 1993, 1994; Divakar & Upreti, 2005; Benatti, 2010). *Bulbothrix* was initially segregated from *Parmelia* Ach. on the basis of black bulbate marginal cilia (Hale, 1974).

Bulbothrix is morphologically similar to *Relicina* (Hale & Kurok.) Hale, chiefly differing in cortical chemistry, with the former containing atranorin and the latter usnic acid (Hale, 1974). However, molecular data have shown that *Bulbothrix* and *Relicina* are only distantly related, with *Bulbothrix* belonging to the *Parmelina* Hale clade and *Relicina* to the *Parmelia* clade (Crespo *et al.*, 2010). The taxonomic history of these taxa underlines many of the challenges of circumscribing natural groups and inferring evolutionary relationships based on morphological characters alone.

Bulbothrix, as currently circumscribed, does not form a monophyletic lineage, but is paraphyletic with *Parmelinella* Elix & Hale (Divakar *et al.*, 2006). Two distinct lineages are currently identified: a predominantly Neotropical clade and a predominantly Palaeotropical clade, the latter being sister to *Parmelinella* (Divakar *et al.*, 2010). Additionally, studies have shown that some species in these clades are not monophyletic (Divakar *et al.*, 2010; Kirika *et al.*, 2015). However, such studies have been limited by sparse taxonomic and specimen sampling and, because the phylogenetic position of the type species of *Bulbothrix* is currently unknown, no taxonomic conclusions have yet been drawn.

Bulbothrix isidiza (Nyl.) Hale and *B. tabacina* (Mont. & Bosch) Hale belong to the predominantly Palaeotropical clade of this genus (Divakar *et al.*, 2010). Both species are commonly found in seasonally wet and secondary forests in Africa and throughout the Tropics (Hale, 1976; Benatti, 2013). They grow on tree bark or on siliceous rocks and are characterized by the presence of asexual reproductive structures, isidia, which are cylindrical and pale. They also have sub-irregular lobes. The thallus has simple rhizines on the lower surface and contains atranorin and salazinic acid. *Bulbothrix isidiza* differs from *B. tabacina* mainly in having a pale brown lower surface with concolorous rhizines (Hale, 1976; Benatti, 2013).

Here we aim to re-examine species boundaries of these two widespread isidiate species occurring in tropical regions, *B. isidiza* and *B. tabacina*. Specifically, we ask the following questions. (1) Do the samples of *B. isidiza* and *B. tabacina* each form monophyletic groups? (2) Do populations distributed in disjunct geographical and/or ecological regions correspond to distinct lineages?

MATERIAL AND METHODS

Taxon sampling

Thirty-seven samples representing nine *Bulbothrix* spp., including newly collected samples from East Africa, Asia and South America, were compiled for this study (Table 1). We assembled a multi-locus DNA data matrix consisting of the nuclear ribosomal internal transcribed spacer region (ITS), a fragment of the nuclear ribosomal large subunit (nuLSU) and a fragment of the mitochondrial ribosomal small subunit (mtSSU). The data set included 85 sequences, 34 retrieved from previous studies (Divakar *et al.*, 2010, 2015; Buaruang *et al.*, 2015) and 51 new sequences

Table 1. Details of specimens used in this study, including: location, collector details and GenBank accession numbers. Newly obtained sequences for this study are in bold face and missing data are indicated with a dash (—)

Species	Locality	Collector(s)	Voucher specimen	GenBank accession number		
				ITS	mtSSU	nuLSU
Bulbothrix asiatica China	China: Yunnan	Li Song Wang *et al*.	14-44427	KM285403	—	—
***Bulbothrix cinerea* Brazil_2500**	**Brazil**	**s.*N*.**	**MNB3071**	**—**	**KX539200**	**KX539219**
***Bulbothrix decurtata* Kenya_9521**	**Kenya: Coast Prov., Ngangao**	**Kirika 4489**	**EA, MAF**	**KX539182**	**—**	**KX539211**
Bulbothrix decurtata South Africa_1861	South Africa: W Cape	Crespo *et al*. s/*N*	MAF-Lich 13988	DQ279483	DQ287790	EU562672
Bulbothrix hypochraea Madagascar_1374	Madagascar: Col de Tapia	Ertz 12876	BR	—	GQ919212	GQ919239
***Bulbothrix isidiza* Brazil_2504**	Brazil	**s.*N*.**	**MNB3125**	**—**	**KX539199**	**KX539218**
Bulbothrix isidiza Congo_318	Congo	Mamush s/*N*	MAF-Lich 15511	GQ919262	GQ919210	GQ919237
Bulbothrix isidiza India_15505	India: Sikkim	Divakar	MAF	KX341979	—	KX341998
***Bulbothrix isidiza* Kenya_4633**	**Kenya: Eastern Prov., Nuu Hill**	**Kirika & Lumbsch 3869**	**EA, F, MAF**	**KX539173**	**KX539189**	**KX539203**
***Bulbothrix isidiza* Kenya_4635**	**Kenya: Rift Valley Prov., Eldama Ravine**	**Kirika, Mugambi & Lumbsch 2829**	**EA, F**	**KX539177**	**KX539191**	**KX539206**
***Bulbothrix isidiza* Kenya_4636**	**Kenya: Central Prov., Mt. Kenya**	**Kirika 4363B**	**EA, F, MAF**	**KX539178**	**KX539192**	**KX539207**
***Bulbothrix isidiza* Kenya_4638**	**Kenya: Central Prov., Mt. Kenya**	**Kirika 4364**	**EA, F, MAF**	**KX539179**	**KX539193**	**KX539208**
***Bulbothrix isidiza* Kenya_4823**	**Kenya: Eastern Prov., Nuu Hill**	**Kirika & Lumbsch 4823**	**EA, F, MAF**	**KX539174**	**—**	**KX539204**
***Bulbothrix isidiza* Kenya_9297**	**Kenya: Central Prov., Mt. Kenya**	**Kirika 4363C**	**MAF**	**KX539180**	**KX539194**	**KX539209**
***Bulbothrix isidiza* Kenya_9352**	**Kenya: Eastern Prov., Makueni**	**Kirika, Malombe & Matheka 3695**	**EA, F**	**KX539175**	**KX539190**	**KX539205**
***Bulbothrix isidiza* Kenya_9386**	**Kenya: Rift Valley Prov., Kericho**	**Kirika 3214**	**EA, F**	**KX539181**	**KX539195**	**KX539210**
***Bulbothrix isidiza* Kenya_9722**	**Kenya: Eastern Prov., Nuu Hill**	**Kirika & Lumbsch 3857**	**EA, F, MAF**	**KX539176**	**—**	—
Bulbothrix isidiza Madagascar_1376	Madagascar: Col de Tapia N Ambositra	Ertz 12878	Ertz 12878 (BR)	GQ919263	GQ919238	GQ919211
***Bulbothrix isidiza* Thailand_3184**	**Thailand: Khao Yai National Park**	**Buaruang**	**RAMK27987**	—	—	**KX539215**

Table 1. *Continued*

Species	Locality	Collector(s)	Voucher specimen	GenBank accession number		
				ITS	mtSSU	nuLSU
***Bulbothrix isidiza* Thailand_3185**	**Thailand: Khao Yai National Park**	**Buaruang**	**RAMK27986**	—	—	**KX539216**
***Bulbothrix isidiza* Thailand_3186**	**Thailand: Khao Yai National Park**	**Buaruang**	**RAMK27988**	—	—	**KX539217**
***Bulbothrix isidiza* Thailand_3188**	**Thailand: Khao Yai National Park**	**Buaruang**	**RAMK27989**	**—**	**—**	**KX539223**
Bulbothrix meizospora India_351	India	Divakar	(MAF-Lich 17013)	JN943846	KR995316	JN939599
Bulbothrix meizospora India_786	India: Uttaranchal	Divakar	GPGC 02-000786	AY611068	AY611127	AY607780
***Bulbothrix sensibilis* Kenya_9383**	**Kenya: Coast Prov., Ngangao**	**Kirika, Mugambi & Lumbsch 2427**	**EA, F**	**—**	**KX539198**	**KX539214**
Bulbothrix sensibilis Rwanda_3	Rwanda: W. Province	Ertz 11025	BR	GQ919265	—	GQ919241
Bulbothrix setschwanensis China_10212	China: Yunnan	Crespo, Blanco & Arguello	MAF 10212	AY611069	—	AY607781
Bulbothrix subscortea China	China: Yunnan Prov., Nanjian Co.	Li Song Wang *et al*.	12-37673	KM249907	—	—
***Bulbothrix tabacina* Brazil_2505**	**Brazil**	**s.*N*.**	**MNB3177**	**KX539185**	—	—
***Bulbothrix tabacina* Brazil_2509**	**Brazil**	**s.*N*.**	**MNB3188**	**KX539186**	—	—
Bulbothrix tabacina Congo_317	Congo: Kahuri-Biega National	Mamush s/*N*	MAF-Lich 16111	GQ919267	—	GQ919243
Bulbothrix tabacina Kenya_1403	Kenya: Kakamega district	Crespo, Lumbsch & Divakar s/*N*	MAF-Lich16112	GQ919268	GQ919216	GQ919244
***Bulbothrix tabacina* Kenya_4822**	**Kenya: Eastern Prov., Nuu Hill**	**Kirika & Lumbsch 4822**	**EA, F, MAF**	**KX539183**	**KX539197**	**KX539212**
***Bulbothrix tabacina* Kenya_9301**	**Kenya: Eastern Prov., Makueni**	**Kirika, Malombe & Matheka 3704**	**EA, F**	**KX539184**	**—**	**KX539213**
***Bulbothrix tabacina* Thailand_3181**	**Thailand: Khao Yai National Park**	**Buaruang**	**RAMK27991**	**KX539187**	**KX539201**	**KX539220**
***Bulbothrix tabacina* Thailand_3182**	**Thailand: Khao Yai National Park**	**Buaruang**	**RAMK27990**	**KX539188**	**—**	**KX539221**
***Bulbothrix tabacina* Thailand_3183**	**Thailand: Khao Yai National Park**	**Buaruang**	**RAMK27992**	**—**	**KX539202**	**KX539222**
Parmelinella wallichiana China_250204	China: Yunnan	Crespo, Blanco & Arguello	MAF-10411	DQ279532	DQ287842	**KX341978**

Table 1. *Continued*

Species	Locality	Collector(s)	Voucher specimen	GenBank accession number		
				ITS	mtSSU	nuLSU
Parmelinella wallichiana India_322	India: Uttaranchal	Divakar	MAF	KX341980	KX341990	KX341999
Parmelinella wallichiana India_7653	India: Sikkim	Chatterjee & Divakar	MAF-7653	AY611106	AY611165	AY607819

generated for this study. Three samples of *Parmelinella wallichiana* (Taylor) Elix & Hale were used as the outgroup as *Parmelinella* has been shown to be closely related to *Bulbothrix* (Divakar *et al.*, 2006, 2010; Kirika *et al.*, 2016a).

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, USA) in accordance with the manufacturer's instructions. For sequences generated for this study, PCR amplifications were performed using Ready-To-Go PCR Beads (GE Healthcare, Pittsburgh, PA, USA). Fungal ITS rDNA was amplified using primers ITS1F (Gardes & Bruns, 1993) with ITS4 or ITS4A (White *et al.*, 1990; Larena *et al.*, 1999); nuLSU rDNA was amplified using primers LR0R and LR5 (Vilgalys & Hester, 1990); and mtSSU rDNA was amplified using the primers mrSSU1 with either mrSSU3R or mrSSU2R (Zoller *et al.*, 1999). PCR and cycle sequencing conditions were the same as in Divakar *et al.* (2015). PCR products were visualized on 1% agarose gel and cleaned using ExoSAP-IT (USB). Cycle sequencing of complementary strands was performed using BigDye v3.1 (Applied Biosystems, Foster City, CA, USA) and the same primers as 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 (http://www.geneious.com, Kearse *et al.*, 2012). Multiple sequence alignments for each locus were performed using the program MAFFT v7 (Katoh *et al.*, 2005; Katoh & Toh, 2008) and manually adjusted. 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; the remaining parameters were set to default values. For the mtSSU sequences, we used the E-INS-i alignment algorithm and '20PAM/K = 2' scoring matrix; the remaining parameters were set to default values. The program Gblocks v0.91b (Talavera & Castresana, 2007) was used to delimit and remove ambiguously aligned nucleotide positions from the MAFFT 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 conflicts and relationships were thus 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, 2014) 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, and evaluated nodal support using 1000 bootstrap pseudoreplicates with the same model settings. Exploratory analyses using alternative partitioning (e.g. ITS1, 5.8S, ITS2, nuLSU and mtSSU) schemes resulted in identical topologies and highly similar bootstrap support values. We also reconstructed phylogenetic relationships from the concatenated multi-locus data matrix under BI using the program BEAST v1.8.2 (Drummond & Rambaut, 2007). We ran two independent Markov chain Monte Carlo (MCMC) chains for 20 million generations, implementing a relaxed lognormal clock and 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 ITS, nuLSU and mtSSU as separate partitions. The first two million generations were discarded as burn-in. Chain mixing and convergence were evaluated in Tracer v1.5 (Rambaut & Drummond, 2009), considering effective sample size (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.

Alternative hypothesis testing

The phylogenetic analyses did not support the monophyly of *Bulbothrix isidiza* and *B. tabacina* as currently circumscribed. Hence we tested whether our data were sufficient to reject the monophyly of these species. The following three alternative hypotheses were tested: (1) *B. isidiza* is monophyletic, (2) *B. tabacina* is monophyletic and (3) African specimens of *B. isidiza* forming a monophyletic group. For the hypothesis testing, two different methods were employed: (1) Shimodaira-Hasegawa (SH) test (Shimodaira & Hasegawa, 1999) and (2) expected likelihood weight (ELW) test (Strimmer & Rambaut, 2002). The SH and ELW tests were performed using Tree-PUZZLE 5.2 (Schmidt *et al.*, 2002) with the combined data set on a sample of the best trees agreeing with the null hypotheses and the unconstrained ML tree. These trees were inferred in Tree-PUZZLE employing the GTR+I+G nucleotide substitution model.

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. 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 ten spores from different specimens were measured and mean values (M) and standard deviations (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 given in parentheses.

Chemical constituents were identified using high-performance thin layer chromatography (HPTLC), implementing standard methods (Arup *et al.*, 1993; Lumbsch, 2001) with a Camag horizontal developing chamber (Oleico Lab, Stockholm, Sweden) in solvent system A.

RESULTS

Fifty-one new sequences, including 16 nuclear ITS, 21 nuLSU and 14 mtSSU rDNA from 37 samples of *Bulbothrix* from Asia, eastern Africa and South America were generated in this study (Table 1). These were deposited in GenBank under accession numbers KX539173–KX539223. The aligned ITS data matrix contained 481 unambiguously aligned nucleotide positions, the nuLSU included 842 and the mtSSU 854. In the ITS alignment, 187 characters were variable and of those 147 were potentially parsimony informative; in the nuLSU, 119 were variable and 77 potentially parsimony informative; and in the mtSSU, 114 positions were variable and 56 potentially parsimony informative. The ITS sequences ranged from 600 to 800 base pairs (bp). Differences in size were due to the presence or absence of insertions of *c*. 200 bp identified as group I introns (Gutiérrez *et al.*, 2007) at the 3′ end of the SSU rDNA. We excluded group I introns and a 74-bp region of the mtSSU, a 26-bp region of the ITS1 and a 24-bp region of the ITS2 alignments from the analysis using GBlocks. SYM+G, TrNef+I+G and HKY+I+G were shown to be the best fitting models of evolution for ITS, nuLSU and mtSSU, respectively.

The ML analysis of the concatenated data matrix yielded an optimal tree with a ln likelihood value of –7676.92 (Fig. 1). In the Bayesian analysis, ESS values of all estimated parameters were well above 200, indicating that convergence among parallel runs was reached. ML and Bayesian topologies were largely similar and did not show any supported conflict [e.g. posterior probability (PP) ≥ 0.95 and ML bootstrap support (BS) ≥ 70%] and thus the ML tree topology is depicted here with the Bayesian posterior probabilities added on the internodes (Fig. 1).

Our results showed that neither *B. isidiza* nor *B. tabacina* forms a monophyletic group as currently circumscribed, although a number of distinct, well-supported clades were recovered (Fig 1). Samples representing *B. isidiza* were recovered in five well-supported, independent clades, here named ‘clade I1’, ‘clade I2’, ‘clade I3’, ‘clade I4’ and ‘clade I5’ (Fig 1). Similar to *B. isidiza s.l.*, specimens collected from pantropical populations of *B. tabacina* were not recovered in a single clade. Rather, these specimens fell into three independent, well-supported monophyletic clades, hereafter named as ‘cladeT1’, ‘cladeT2’ and ‘cladeT3’ (Fig. 1). Both the Shimodaira–Hasegawa and the Expected Likelihood Weight tests significantly rejected monophyly of *B. isidiza* and *B. tabacina*, respectively ($P \leq 0.001$).

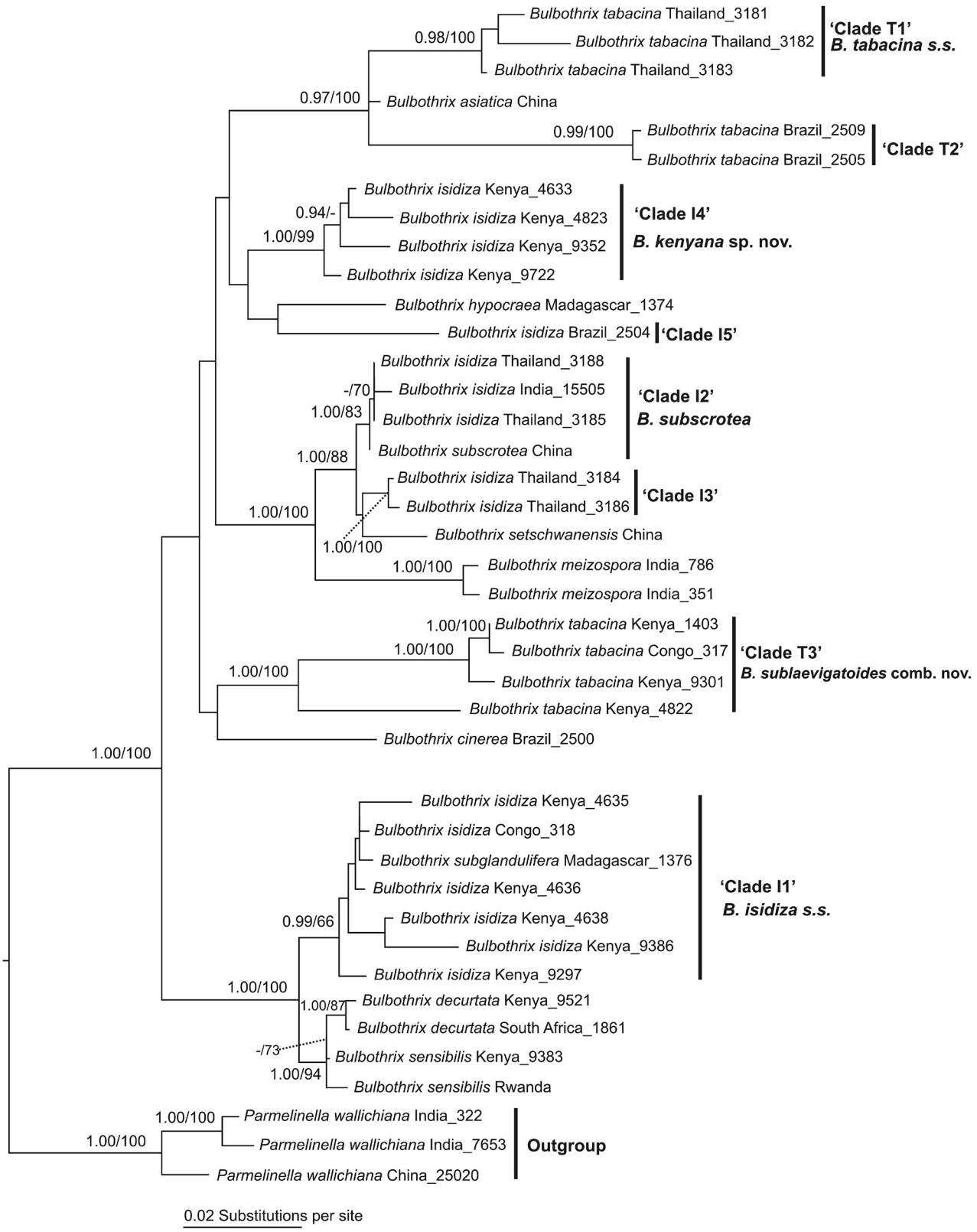

Figure 1. Phylogenetic relationships of *Bulbothrix* spp. based on a maximum-likelihood (ML) analysis of a concatenated, three-locus dataset (ITS, nuLSU and mtSSU rDNA). Bayesian posterior probabilities ≥ 0.95 and ML bootstrap values ≥ 70% are indicated above branches. *Parmelinella wallichiana* was used as the outgroup.

DISCUSSION

Bulbothrix isidiza and *B. tabacina* have traditionally been circumscribed based on isidia morphology, colour of thallus lower surface and extrolite compositions (Hale, 1976). This traditional classification is not corroborated by phylogenetic reconstructions. Species-level polyphyly is a common phenomenon in Parmeliaceae and in lichenized fungi in general. Moreover, cryptic diversity masked under widespread and or disjunct phenotypic species has repeatedly been shown in diverse groups of lichen-forming fungi (Crespo & Lumbsch, 2010; Lumbsch & Leavitt, 2011; Leavitt *et al.*, 2015; Alors *et al.*, 2016; Kirika *et al.*, 2016a,b); the occurrence of cryptic species is common in other groups of fungi as well (see, e.g., Hibbett, 2016).

Bulbothrix isidiza, which was thought to have a pantropical distribution (Hale, 1976), probably has a more restricted distributional range, probably endemic to Africa, in a predominantly African phylogenetic lineage (Fig. 1). Samples collected from montane regions of Congo, Kenya and Madagascar were recovered in 'clade I1'. Since the type of *B. isidiza* is described from Serra Chella, a mountain range in central Angola (Nylander, 1884), and 'clade I1' is widely distributed in montane regions of sub-Saharan Africa, we here interpret this clade as *B. isidiza s.s*. Samples collected in dry woodlands and dry forest in Kenya formed 'clade I4', in contrast to specimens from montane regions, which belong to *B. isidiza s.s*. 'clade I1'. *Bulbothrix isidiza s.s*. forms a well-supported (BS = 100, PP = 0.99) sistergroup relationship with a clade including samples of *B. decurtata* (Kurok.) Hale and *B. sensibilis* (J.Steiner & Zahlbr.) Hale. *Bulbothrix decurtata* is an obligate isidiate species endemic to Africa and *B. sensibilis* is an obligate apotheciate species commonly occurring in Africa (Hale, 1976; Swinscow & Krog, 1988). The saxicolous *B. decurtata* differs from *B. isidiza s.s*. ('clade I1') in having a black lower thallus surface and black-tipped isidia. Further, it differs from *B. sensibilis* in having obligate asexual reproduction, an isidiate upper surface, emaculate upper cortex and bifusiform conidia.

Bulbothrix subscortea (Asahina) Benatti was recently resurrected from *B. isidiza s.l*. based solely on morphological features (Benatti, 2012b). Benatti (2012b) characterized *B. subscortea* by having a saxicolous habitat, emaculate upper thallus surface, simple to slightly ramified isidia on the upper surface, longer cilia and rhizines with basal bulbs. In our analyses, 'clade I2' grouped samples of *B. isidiza s.l.* collected in India and Thailand with one sample of *B. subscortea* from China. Since the type specimen of *B. subscortea* is described from Taiwan (Asahina, 1957), we here consider the east Asian 'clade I2' as *B. subscortea s.s.* Samples clustered in 'clade I2' were collected from epiphytic and saxicolous habitats, indicating the substrate is not useful in distinguishing *B. subscortea* from *B. isidiza s.s*. Therefore, the only phenotypic feature that currently distinguishes both species (including samples clustered in 'clade I2') is the presence or absence of maculae on the upper surface (Benatti, 2012b; Zhang *et al.*, 2014). Our study indicates that caution should be taken when making nomenclatural changes based solely on morphological features in this group of lichen-forming fungi. Moreover, *B. isidiza s.s*. is only known from Africa, whereas *B. subscortea* is only known from Asia.

Not all Asian samples clustered in the *B. subscortea s.s* clade; two samples from Thailand grouped in 'clade I3' (Fig. 1). This clade formed an unsupported sistergroup relationship with the apotheciate species *B. setschwanensis* (Zahlbr.) Hale, endemic to the Himalayas (Divakar & Upreti, 2005). Since only one sample representing *B. setschwanensis* was included in this study and phylogenetic relationships remain unresolved, additional studies are necessary to clarify the phylogenetic position of samples grouped in 'clade I3'.

Samples of *B. isidiza s.l.* clustered in 'clade I4' (Fig. 1) are characterized by a combination of features: emaculate upper surface, papillate lower marginal zone, bulbate cilia reduced to nodules, apices lacking and ascospores of 7.5–15 × 5–7.5 µm. *Bulbothrix isidiza s.s.* ('clade I1') differs in having a maculate upper surface, bulbate cilia with long apices up to 1 mm, simple to furcate, and relatively larger ascospores 10.0–16.0 (–17.5) × 5.0–9.0 µm (Benatti, 2013). Further, our studies indicate that the species found in dry thickets, woodlands/dry forests vs. those growing in montane localities in Africa are not closely related. Alternative topology tests significantly rejected the monophyly of the African 'clade I1' and 'clade I4' ($P \leq 0.05$). However, the phylogenetic position of 'clade I4' remained unresolved. Since no name is available for this clade, below we describe a new species to accommodate African samples growing in dry, low-elevation forests clustered in it.

Finally, 'clade I5' included a single sample from Brazil, the phylogenetic placement of which remains unresolved. Given the limited number of samples, the formal description of 'clade I5' must await additional studies, especially increased sampling of specimens from Neotropical populations.

Although the resurrection of *B. subscortea* based on phenotypic features by Benatti (2012b) was in part supported by our molecular phylogenetic tree, the resurrection of *B. subglandulifera* (Hue) Hale (Hale, 1974; Benatti, 2013) was not supported. The type specimen of *B. subglandulifera* was from Madagascar (Hue,

1899). The single specimen from Madagascar included in our study, morphologically similar to *B. subglandulifera*, clustered in the *B. isidiza s.s.* clade ('clade I1', Fig. 1). Thus, our results suggest that *B. subglandulifera* should be included in *B. isidiza s.s.*, although the formal synonymization must await the study of additional material of *B. subglandulifera* for confirmation. This species was traditionally separated from *B. isidiza* by the presence of narrow lobes and granular isidia sometimes dissolving into soredia. However, in the type material, young and poorly developed true isidia and even the largest isidia always remain corticated (they never become sorediate; see Benatti, 2013). In fact, these phenotypic features are plastic in *B. isidiza s.s.*, the *B. isidiza* group and isidiate species in parmelioid lichens in general (reviewed in, e.g., Crespo & Lumbsch, 2010; Crespo, Divakar & Hawksworth, 2011) and are thus not reliable for taxon differentiation. *Bulbothrix isidiza s.s.* ('clade I1') is distinguished by a combination of features, having an isidiate upper surface, maculate upper cortex, bulbate cilia with long apices up to 1 mm, simple to furcate, and relatively larger ascospores 10.0–16.0 (–17.5) × 5.0–9.0 µm (Benatti, 2013), and is restricted in its distribution to montane regions of Africa. Indeed, vegetative phenotypic features have repeatedly been shown to be homoplasious in Parmeliaceae and in lichen-forming fungi in general (reviewed in Crespo & Lumbsch, 2010; Lumbsch & Leavitt, 2011; Divakar *et al.*, 2013; Parnmen *et al.*, 2012; Prieto *et al.*, 2013; Kraichak *et al.*, 2015; Leavitt *et al.*, 2015) and caution must be taken in this group of fungi when making any nomenclatural changes based solely on vegetative phenotypic features.

Bulbothix tabacina is another asexually reproducing, isidiate species with a supposedly pantropical distribution (Hale, 1976). Specimens collected from pantropical populations of *B. tabacina* were not recovered in a single clade, but fell into three independent, well-supported clades (Fig. 1). 'Clade T1' included samples from Thailand. Since the type of *B. tabacina* was from east Asia (Montagne, 1856), we consider this clade to represent *B. tabacina s.s.* This clade appeared closely related to *B. asiatica* Y.Y.Zhang & Li S.Wang. The latter is a recently described species from Cambodia and China, distinguished from *B. tabacina* in having an emaculate thallus upper surface and coralloid isidia and has been shown to be distinct using ITS sequences alone (Zhang *et al.*, 2014). However, in our multi-locus phylogenetic analysis, the placement of *B. asiatica* appeared unresolved, indicating that additional studies are required.

Two samples of *B. tabacina s.l.* from Brazil clustered in 'clade T2' (Fig. 1) and may belong to an undescribed species or these samples could belong to *B. tabacina* in a broader concept including *B. asiatica*. However, since only two samples were included here, a formal taxonomic conclusion must await additional sampling. Lastly, 'clade T3' included four samples from *B. tabacina s.l.* populations collected in East Africa. This clade had an unsupported relationship to another isidiate species, *B. cinerea* Marcelli & Kalb. For the East African populations ('clade T3'), the name *Parmelia sublaevigatoides* Dodge (1959) described from Uganda is available. Based on morphological features, *P. sublaevigatoides* has been considered as a synonym of *B. tabacina.* Consequently, we use this name to accommodate the samples clustered in 'clade T3', resurrecting it below and making the necessary combination into *Bulbothrix*. Unfortunately, we were unable to find any distinctive feature to characterize 'clade T3'. Nonetheless, the samples clustered in 'clade T3' are currently only known from East Africa, whereas *B. tabacina s.s.* does not occur in East Africa.

East African material of *B. decurtata* has previously been treated under *B. tabacina* (Swinscow & Krog, 1988). Saxicolous *B. decurtata* differs from *B. tabacina* in minor morphological features, such as black-tipped isidia, and therefore the distinction of the two species has not been generally accepted (see Benatti 2013). However, in our phylogenetic tree Kenyan samples representing *B. decurtata* grouped with South African material of that species and were not closely related to *B. tabacina.* Instead, specimens representing *B. decurtata* formed a well-supported sister relationship to the apotheciate *B. sensibilis* (Fig. 1), supporting *B. decurtata* as a valid, independent species. Although the relationship between the two samples of *B. sensibilis* was unresolved in the ML analysis (Fig. 1), these formed a monophyletic group in the Bayesian analysis (results not shown). This is consistent with previous findings (Divakar *et al.*, 2010). *Bulbothrix decurtata* is distinguished from *B. sensibilis* in having an isidiate upper surface, lacking apothecia, bifusiform conidia and emaculate upper cortex.

In agreement with other species complexes in parmelioid lichens (Crespo *et al.*, 2002; Molina *et al.*, 2011; Leavitt *et al.*, 2012; Alors *et al.*, 2016), the results presented here highlight that isidiate species generally thought to have wide distributional ranges may show striking phylogeographical structure. In both phenotypically circumscribed species, *B. isidiza s.l.* and *B. tabacina s.l.*, morphologically similar populations occurring in different continents or ecological habitats correspond to distinct, independent lineages and probably represent distinct species that had previously been overlooked.

In summary, our study highlights and unmasks the presence of a higher species diversity in the isidiate *Bulbothrix* spp. than previously assumed. In this

group, five species are found in Kenya, including one new species, *B. kenyana* sp. nov. In the light of our results, other species reported from East Africa, namely *B. bulbochaeta* (Hale) Hale, *B. coronata* (Fée) Hale, *B. goebelii* (Zenker) Hale, *B. hypocraea* (Vain.) Hale, *B. meizospora* (Nyl.) Hale, *B. pustulata* (Hale) Hale, *B. suffixa* (Stirt.) Hale and *B. ventricosa* (Hale & Kurok.) Hale, need to be revised to evaluate potentially hidden diversity like that detected here. Further, other ecological regions, especially woodland/dry forest areas and the coastal province of Kenya and surrounding countries in East Africa, need to be sampled to examine the species diversity in this group of lichen-forming fungi critically.

TAXONOMIC TREATMENT

BULBOTHRIX KENYANA KIRIKA, DIVAKAR & LUMBSCH, **SP. NOV.** (FIG. 2)

MycoBank Number: 817700

Diagnosis: Morphologically similar to *B. isidiza*, but differs in having emaculate upper surface, marginal cilia reduced to bulbate nodules and nucleotide differences in the ITS region ('clade I4'; Fig. 1).

Type: KENYA. Eastern Prov., Mwingi, Nuu Hill inselberg, 880–980 m 1°01′S, 38°19′E, on bark, 25 January 2015, *P. Kirika* 4823 & *H.T. Lumbsch* (holotype: EA, isotypes: F, MAF).

GenBank accession number: ITS KX539174 and nuLSU KX539204.

Etymology: The name is based on its occurrence in Kenya.

Description: Thallus foliose, adnate, 4–6 cm across. Lobes broad, irregularly to sub-irregularly branched, 2–5 mm wide, rounded crenate, with rotund apices, margins bulbate. Marginal cilia reduced to bulbate nodules in the crenae and axils. Upper surface grey, smooth, emaculate, isidiate. Isidia laminal, cylindrical, mostly simple or rarely branched, 0.2–0.4 mm high, concolorous with the upper surface and with pale brown tips. Medulla white. Lower surface pale brown, richly rhizinate, papilate margins. Rhizines pale brown, evenly distributed, simple, 0.2–1.0 mm long. Apothecia laminal, adnate to sessile, 1–6 mm in diameter, amphithecium isidiate. Disc concave, brown, imperforate. Asci eight-spored. Ascospores oval to ellipsoid, 7.5–15.0 × 5.0–7.5 µm (M = 6.00–7.00 × 10.30–11.75 µm, ±SD = 1.3–4.0 × 1.8–1.7 µm, $N = 40$). Pycnidia absent.

Figure 2. *Bulbothrix kenyana* sp. nov., habit (holotype *Kirika & Lumbsch, 4823* [EA])

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

Remarks: Bulbotrhix kenyana can easily be confused with *B. isidiza* in the field, but the former differs in having an emaculate upper surface, papilate to naked marginal zone and marginal cilia reduced to bulbate nodules. Ascospore size in both species largely overlaps, and both contain atranorin and salazinic acid. Despite morphological similarties, *B. kenyana* did not form a sister relationship with *B. isidiza s.s.* in our multi-locus molecular phylogenetic reconstruction (Fig. 1). The new species is also highly similar morphologically to *B. subscortea*, which is endemic to Asia. However, the latter has relatively larger ascopores (16 × 9 µm) and cilia with generally longer apices, up to 0.8 mm. In our molecular phylogenetic reconstruction, *B. subscortea* formed a sistergroup relationship with an apotheciate species, *B. setschwanensis*, endemic to Asia. *Bulbothrix kenyana* occurs as an epiphyte on species of *Acacia* Mill. and *Commiphora* Jacq. and also on rocks at lower elevations (800–1850 m) in dry thickets and woodland/dry forest areas of Kenya. At present it is only known from six localities in Eastern and Rift valley provinces of Kenya.

Additional specimens examined: KENYA. Eastern Prov., Makueni Wote, Ngutwa village, Matooi hill Dry woodland, 1400 m, 01°49′S, 37°56′E, on rocks, 12 December 2013, P. Kirika 3695, *I. Malombe & K. Matheka* (EA, F, MAF); Mwingi, Nuu Hill, inselberg with dry woodland dominated by *Terminalia*, *Combretum* and *Acacia* spp., *c*. 1000 m, 01°02′S, 38°20′E, on bark, 12 March 2014, P. *Kirika 3857 & H.T. Lumbsch* (EA, F, MAF), 4633 (EA, F, MAF); Sultan Hamud, Emali Hill, degraded *Acacia–Commiphora* woodland, 1352 m, 02°04′S, 37°24′E, on bark, 22 September 2014, *P. Kirika* 4586 (EA, F, MAF); Makueni Co.,Utu, Chyulu hills National Reserve, dry rocky woodland, 1121 m, 02°42′S, 37°58′E, on bark, 23 September 2014, *P. Kirika* 4620 (EA, MAF); Rift Valley, Nakuru, Mt. Suswa Conservancy, rocky wooded bushland, 1846 m, 01°07′S, 36°24′E, on bark, 22 June 2015, *P. Kirika* 4888 (EA, F, MAF); 4889 (EA, F, MAF); Ngoina, Kipsonoi river, Unilever estate along Ikonge–Ngoina road, degraded dry riparian forest on *Eucalyptus*, 1633 m, 00°30′S, 35°04′E, on bark, 23 August 2015, *P. Kirika* 4937 (EA, F).

BULBOTHRIX SUBLAEVIGATOIDES (DODGE) KIRIKA, DIVAKAR & LUMBSCH, **COMB. NOV.**

MycoBank Number: MB 817715

Basionym: Parmelia sublaevigatoides Dodge, Annals of the Missouri Botanical Garden **46**: 88. (1959).

Type: UGANDA. Mount Elgon (BM, lectotype).

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