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New molecular data on *Pyrenulaceae* from Sri Lanka reveal two well-supported groups within this family

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Abstract: *Pyrenulaceae* is one of the dominant lichen families in the tropics, especially in lowland evergreen forests. Although very species-rich, phylogenetic relationships within *Pyrenulaceae* have not been extensively studied using molecular data, and its morphology-based generic delimitation remains untested. A recent lichenological survey carried out in the Knuckles Mountain Range in Sri Lanka allowed the first author to collect fresh specimens of different species of *Pyrenula*, and to investigate the phylogenetic relationships within the family *Pyrenulaceae* using a multigene analysis (ITS, nuLSU and mtSSU) and a preliminary dataset of 21 taxa. This data shows that the family *Pyrenulaceae* can be divided into two well-supported groups and suggests that the genus *Pyrenula* is not monophyletic. Characters usually used for generic classification in this family (ascospore colour and septation, structure of the ascospore locules, secondary chemistry, hamathecium structure, ostiole position) do not correlate with these two groups. However, the presence of pseudocyphellae is restricted to species of *Pyrenula* from one group.

Key words: Ascomycota, lichens, multigene phylogeny, pseudocyphellae, *Pyrenulales*, systematics

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Introduction

The lichen family *Pyrenulaceae* Rabenh. is an important element of the epiphytic lichen flora in tropical rainforests (Sipman & Harris 1989; Komposch & Hafellner 2002; Rivas Plata *et al*. 2008; Aptroot 2009). It is most abundant in montane, sub-montane, semi-evergreen and lowland evergreen rainforests, where together with *Graphidaceae* Dumort., it constitutes the dominant component of the crustose epiphytic lichen flora. The *Pyrenulaceae* mainly comprises corticolous species and they are nearly all associated with the green-algal genus *Trentepohlia* Mart., the most common photobiont for crustose species with a mainly tropical distribution. This family belongs to *Pyrenulales* (Chaetothyriomycetidae, Eurotiomycetes), an order characterized by perithecial ascomata with an ascohymenial development and fissitunicate asci, and septate to muriform ascospores (Parguey-Leduc 1973; Parguey-Leduc & Janex-Favre 1981; Harris 1989; Aptroot *et al*. 2008). The circumscription of this order has undergone many recent changes (Lutzoni *et al*. 2004; del Prado *et al*. 2006; Lumbsch & Huhndorf 2007; Nelsen *et al*. 2009), because morphological characters used to circumscribe families and genera in this order were also found in members of another fungal class, Dothideomycetes. The use of molecular data has therefore helped disentangle the classification at the family and order levels for the two fungal classes Dothideomycetes and Eurotiomycetes, and *Pyrenulales* now includes four families: *Celotheliaceae* Lücking, Aptroot & Sipman, *Pyrenulaceae*, *Requienellaceae* Boise and *Monoblastiaceae* Walt. Watson (Lumbsch & Huhndorf 2010).

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Pyrenulaceae is a large family with about 10 genera and 224 currently accepted species (Kirk *et al*. 2008; Aptroot 2012). The main genus is *Pyrenula* Ach., which so far includes 169 accepted species (Aptroot 2012). As for many other crustose, corticolous tropical groups of lichens, molecular data is lacking for most species, mainly due to difficulties in recovering good quality genomic DNA from herbarium specimens, even when recently collected. Most of the existing studies with molecular data on *Pyrenulaceae* were based on a limited number of species from this family and have focused more on relationships between the main classes and orders in ascomycetes (Lutzoni *et al*. 2004; James *et al*. 2006; Lumbsch & Huhndorf 2007; Gueidan *et al*. 2008; Schoch *et al*. 2009). The study by del Prado *et al*. (2006) was the first focusing on the order *Pyrenulales* and examining the phylogenetic placement of families and genera traditionally classified within this order. However, no study so far has focused on phylogenetic relationships within the family *Pyrenulaceae*. The current generic delimitation in this family has therefore not yet been tested using molecular data.

In Sri Lanka, the diversity of *Pyrenulaceae* is poorly known, with only a few studies referring to these crustose lichens (Nayanakantha & Gajameragedara 2003; Wijeyaratne 2003; Attanayaka 2006). Moreover, only a handful of species have been recorded in these inventories, although the diversity of *Pyrenulaceae* in neighbouring India suggests that many more species should also be present in Sri Lanka (Singh & Sinha 2010). During a recent lichenological survey carried out in the Knuckles mountain range in Sri Lanka (Weerakoon 2010), specimens of *Pyrenulaceae* were collected from different habitats. The sampling of these fresh specimens allowed us to obtain DNA sequences and assemble a molecular dataset in order to carry out a preliminary investigation of the relationships within the family *Pyrenulaceae*.

Material and Methods

Taxon sampling

As part of a lichen survey in the Knuckles mountain range in Sri Lanka, several specimens of *Pyrenulaceae* were collected, air dried and stored in labelled packets in 2010. These specimens were identified using a key by Aptroot (2012). Some of the voucher specimens of *Pyrenulaceae* for which sequence data was already available in GenBank were borrowed from DUKE and F and re-identified using the same key (Aptroot 2012). Identifications were carried out using an OLYMPUS SZX12 dissecting microscope and a ZEISS Axioscope 2 plus compound microscope. Photographs were taken in the Sackler Biodiversity Imaging Laboratory at the Natural History Museum using a Zeiss Stemi SV11 stereomicroscope coupled with a Canon EOS imaging system.

The specimens of *Pyrenula* from Sri Lanka were more than a year old and, therefore, potentially already too old to obtain good genomic DNA extracts. Therefore, only the largest and healthiest were selected for molecular analysis. Two other species of *Pyrenula* collected in the UK (*P. chlorospila* and *P. macrospora*) were also added to the taxon sampling. Amplifications worked relatively well for eight specimens (*Pyrenula aspistea* GW1042 and GW1044, *P. chlorospila* CG1520b, *P. fetivica* GW835 and GW307A, *P. macrospora* CG1520a, *P. mamillana* GW818A and *P. massariospora* GW1028; Table 1). The molecular dataset was completed using sequences available in GenBank for a total of 21 taxa of *Pyrenulaceae*, including *Anthracothecium australiense*, *A. prasinum*, *Pyrgillus javanicus* and 18 specimens of *Pyrenula* (Table 2). Additional ITS sequences were obtained in this study for specimens with other molecular data available in GenBank. Two species of *Verrucariales* (*Endocarpon pusillum* and *Staurothele areolata*) and two species of *Chaetothyriales* (*Exophiala xenobiotica* and *Phialophora europaea*) were also included because they belong to the sister subclass Chaetothyriomycetidae (Gueidan *et al.* 2008) and two species of Eurotiomycetidae (*Byssochlamys nivea* and *Xeromyces bisporus*) were used as outgroups.

Molecular data

Perithecia and, when possible, thallus fragments were collected from herbarium specimens with a sterile razor blade and transferred to an Eppendorf tube. Genomic DNA was then obtained using a protocol modified from Zolan & Pukkila (1986), as described in Gueidan *et al.* (2007). DNA extracts were checked with a gel electrophoresis and for each sample the band intensity was used to choose the appropriate genomic DNA dilution for amplification. A dataset of three markers was assembled: the large subunit of the nuclear ribosomal RNA gene (nuLSU), the small subunit of the mitochondrial ribosomal RNA gene (mtSSU), and the region including the internal transcribed spacers 1 and 2 and the 5.8S subunit of the nuclear ribosomal RNA gene (ITS). These markers were amplified using primers and PCR programs described in Table 3. For the three gene regions, 1 µl of a 1/10 or 1/100 dilution of genomic DNA was added to the following PCR mix: 2·5 µl PCR buffer $10 \times NH_4$ (Bioline, London, UK), 1·5 µl of $MgCl_2$ (50 mM), 0·5 µl dNTP (100 mM), 1 µl primers (10 µM), 0·5 µl DNA polymerase Bioline BioTaq (5 U μl^{-1}), and water to a total volume of 25 µl. PCR was performed using a Techne TC-4000 PCR machine (Bibby Scientific Ltd, Stone, UK). Cloning was conducted on

TABLE 1. *Locality and voucher information for specimens for which molecular data has not previously been published*

Species	Voucher no.	Herbaria*	Locality and voucher information
Pyrenula aspistea (Ach.) Ach.	GW1042	BM, SJ	Sri Lanka, Knuckles Mountain Range, Kalupahana, alt. 1245 m., *G. Weerakoon & S. C. Wijeyaratne* 1042, 30 March 2010
P. aspistea (Ach.) Ach.	GW1044	BM, SJ	Sri Lanka, Knuckles Mountain Range, Kalupahana, alt. 1245 m., *G. Weerakoon & S. C. Wijeyaratne* 1044, 30 March 2010
P. chlorospila (Nyl.) Arnold	CG1520b	BM	Great Britain, Devon, Slapton, Slapton Ley Nature Reserve, alt. 10 m., *C. Gueidan* 1520, 24 August 2010
P. fetivica (Kremp.) Müll. Arg.	GW307A	BM, SJ	Sri Lanka, Knuckles Mountain Range, Lakegala, alt. 811 m., *G.Weerakoon & S. C. Wijeyaratne* 307A, 30 March 2009
P. fetivica (Kremp.) Müll. Arg.	GW835	BM, SJ	Sri Lanka, Knuckles Mountain Range, Illukkubura, alt. 1500 m., *G.Weerakoon & S. C. Wijeyaratne* 835, 15 February 2010
P. macrospora (Degel.) Coppins & P. James	CG1520a	BM	United Kingdom, Devon, Slapton, Slapton Ley Nature Reserve, alt. 10 m., *C. Gueidan* 1520, 24 August 2010
P. mamillana (Ach.) Trevis.	GW818A	BM, SJ	Sri Lanka, Knuckles Mountain Range, Illukkubura, alt. 1500 m., *G. Weerakoon & S. C. Wijeyaratne* 818A, 17 February 2010
P. massariospora (Starbäck) R. C. Harris	GW1028	BM, SJ	Sri Lanka, Knuckles Mountain Range, Kalupahana, alt. 1245 m., *G. Weerakoon & S. C. Wijeyaratne* 1028, 30 March 2010

* BM = Natural History Museum, London (UK); SJ = University of Sri Jayewardenepura, Nugegoda (Sri Lanka)

PCR products with multiple bands using a Topo TA cloning kit (Invitrogen, Carlsbad, CA). PCR product clean-up and sequencing were carried out by the sequencing facility of the Natural History Museum in London, using PCR Clean-up Filter Plates (Millipore, Billerica, MA), BigDye chemistry and an ABI 3730xl sequencing machine (Applied Biosystems, Carlsbad, CA, USA).

Alignments and phylogenetic analyses

DNA sequences were edited and assembled using Sequencher 4.8 (Gene Codes Corporation, Ann Arbor, MI). Sequences were manually aligned in MacClade 4.08 (Maddison & Maddison 2003). Ambiguous regions were delimited according to Lutzoni *et al.* (2000) and excluded from the alignments. Two species of Eurotiomycetidae (*Byssochlamys nivea* and *Xeromyces bisporus*) were selected as outgroups. The congruence of the three datasets was tested using a 70% reciprocal bootstrap criterion (Mason-Gamer & Kellogg 1996): the three matrices (nuLSU, mtSSU, ITS) were analyzed separately using 500 bootstrap pseudoreplicates with RAxML VI-HPC (Stamatakis *et al.* 2005, 2008) on the Cipres Web Portal (http://www.phylo.org). No conflicts were detected and the three datasets were combined. Phylogenetic relationships were investigated using a Maximum Likelihood (ML) approach with the software RAxML VI-HPC as implemented on the Cipres Web Portal. The combined dataset was analyzed using a GTRMIX model applied to three partitions (nuLSU, mtSSU and ITS). Support values were obtained using a bootstrap analysis of 1000 pseudoreplicates. Additional support values were obtained using weighted Maximum Parsimony (wMP) and a Bayesian approach (MB). The wMP bootstrap analysis was conducted in PAUP* version 4.0b10 (Swofford 1999). Constant sites were excluded and gaps were treated as fifth characters. Step matrices were obtained for each of the three previously mentioned partitions by using StMatrix 4.2 (Lutzoni & Zoller, Duke University, www.lutzonilab.net/downloads/). A tree search was carried out using 1000 random addition sequences (RAS). The same most parsimonious tree was recovered for 388 of the 1000 RAS. A bootstrap analysis of 1000 replicates and ten RAS was then conducted using PAUP*. For the Bayesian approach, the Akaike Information Criterion as implemented in Modeltest 3.7 was used to estimate the model of molecular evolution. A GTR+I+G model was used for the three partitions (nuLSU, mtSSU and ITS). Two analyses of four chains were run for 5 million generations using MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003), and trees were sampled every 500 generations. All runs converged on the same average likelihood score and topology. A burn-in sample of 5000 trees was discarded for each run. The remaining 10 000 trees were used to estimate the posterior probabilities with the 'compute consensus' command in PAUP*.

TABLE 2. *Specimen data and sequences used in this study*

Species	Collection number*	Classification	Geographic origin	mtSSU†	nuLSU†	ITS†
Byssochlamys nivea L.R. Fraser	CBS 100.11	*Eurotiales*	–	FJ225775	FJ358279	FJ389934
Xeromyces bisporus Westling	CBS 236.71	*Eurotiales*	–	FJ225783	FJ358291	–
Endocarpon pusillum Hedw.	CG470 (DUKE)	*Verrucariales*	–	FJ225677	EF643754	**JQ927447**
Staurothele areolata (Ach.) Lettau	CG378 (DUKE)	*Verrucariales*	–	FJ225699	EF643772	**JQ927448**
Exophiala xenobiotica de Hoog *et al.*	CBS 115831	*Chaetothyriales*	–	FJ225746	FJ358246	–
Phialophora europaea de Hoog *et al.*	CBS 129.96	*Chaetothyriales*	–	FJ225750	FJ358248	EF551553
Anthracothecium australiense (Müll. Arg.) Aptroot	AFTOL 1649	*Pyrenulales*	USA, NC	FJ225773	FJ358271	–
A. prasinum (Eschw.) R.C. Harris	F-19113-n (F)	*Pyrenulales*	Australia	DQ329002	DQ329027	**JQ927449**
Pyrenula aspistea (Ach.) Ach.	GW1042 (BM)	*Pyrenulales*	Sri Lanka	–	**JQ927469**	**JQ927450**
P. aspistea (Ach.) Ach.	GW1044 (BM)	*Pyrenulales*	Sri Lanka	**JQ927462**	**JQ927470**	**JQ927451**
P. aspistea (Ach.) Ach.	AFTOL 2012	*Pyrenulales*	Hong Kong	–	EF411063	–
P. chlorospila (Nyl.) Arnold	CG1520b (BM)	*Pyrenulales*	England	**JQ927463**	**JQ927471**	**JQ927452**
P. cruenta (Mont.) Vain.	AFTOL 386	*Pyrenulales*	Puerto Rico	AY584719	AF279407	–
P. fetivica (Kremp.) Müll. Arg.	GW307A (BM)	*Pyrenulales*	Sri Lanka	**JQ927464**	**JQ927472**	**JQ927453**
P. fetivica (Kremp.) Müll. Arg.	GW835 (BM)	*Pyrenulales*	Sri Lanka	**JQ927465**	–	**JQ927454**
P. laevigata (Pers.) Arnold	*Palice* 5608	*Pyrenulales*	Slovakia	AY568029	AY607736	–
P. macrospora (Degel.) Coppins & P. James	CG1520a (BM)	*Pyrenulales*	England	**JQ927466**	**JQ927473**	**JQ927455**
P. mamillana (Ach.) Trevis.	GW818A (BM)	*Pyrenulales*	Sri Lanka	**JQ927467**	**JQ927474**	**JQ927456**
P. massariospora (Starbäck) R.C. Harris	GW1028 (BM)	*Pyrenulales*	Sri Lanka	**JQ927468**	**JQ927475**	**JQ927457**
P. nitida (Weigel) Ach.	F 5929 (F)	*Pyrenulales*	Czech Republic	DQ328998	DQ329023	**JQ927458**
P. nitida (Weigel) Ach.	s. n.	*Pyrenulales*	Germany	AY568030	AY607737	–
P. quassiaecola Fée	F-19092-b (F)	*Pyrenulales*	Australia	DQ329001	DQ329026	**JQ927459**
P. subpraelucida Müll. Arg.	F-17550-f	*Pyrenulales*	Costa Rica	DQ328986	DQ329015	–
P. thelomorpha Tuck.	F-19082-a (F)	*Pyrenulales*	Australia	DQ328999	DQ329024	**JQ927460**
Pyrenula sp.	F-19082-r (F)	*Pyrenulales*	Australia	DQ329000	DQ329025	**JQ927461**
Pyrenula sp.	AFTOL 387	*Pyrenulales*	USA, NC	AY584720	AY640962	DQ782845
Pyrgillus javanicus Nyl.	AFTOL 342	*Pyrenulales*	Costa Rica	FJ225774	DQ823103	DQ826741

* Herbaria are indicated in parenthesis after the collection number for specimens for which new sequences were produced.
† Missing sequences are indicated by dashes and GenBank accession numbers of newly obtained sequences are shown in bold.

TABLE 3. *List of primers for the three loci (nuLSU, mtSSU and ITS) used in this study and PCR programs used for their amplification*

Gene regions	PCR primers	Additional primers for sequencing	PCR programs
nuLSU	LR0R (Rehner & Samuels 1994) LR7 (Vilgalys & Hester 1990)	LR3, LR3R, LR5, LR5R, LR6, LR6R (Vilgalys & Hester 1990)	1 min at 95°C, 35 times (45 s at 95°C, 40 s at 52°C, 2:30 min at 72°C), 10 min at 72°C
mtSSU	mtSSU1 (Zoller *et al.* 1999) mtSSU3R (Zoller *et al.* 1999)	mtSSU2, mtSSU2R (Zoller *et al.* 1999)	3 min at 94°C, 35 times (1 min at 94°C, 1 min at 52°C, 1:30 min at 72°C), 7 min at 72°C
ITS	ITS1F (Gardes & Bruns 1993) ITS4 (White *et al.* 1990)	ITS2, ITS3 (White *et al.* 1990)	5 min at 94°C, 35 times (1 min at 94°C, 1 min at 53°C, 1 min at 72°C), 7 min at 72°C

Results

New sequences recovered in this study are 15 for ITS, seven for mtSSU and seven for nuLSU. The combined dataset included 2302 characters (413 for ITS, 716 for mtSSU and 1173 for nuLSU). The amplification of some markers failed for a number of species and some sequences were not available in GenBank. As a result, the combined dataset included missing data for two mtSSU, one nuLSU and eight ITS (see Table 2). Among these 2302 characters, 562 were parsimony-informative. The most likely tree obtained with RAxML is presented in Figure 1, with ML and wMP bootstrap values and posterior probabilities. As in previous studies (Lutzoni *et al.* 2004; del Prado *et al.* 2006; James *et al.* 2006; Lumbsch & Huhndorf 2007; Gueidan *et al.* 2008; Schoch *et al.* 2009), *Pyrenulaceae* (*Pyrenulales*) forms a sister group to the lineage including *Verrucariales* and *Chaetothyriales* (all 100% bootstrap; Fig. 1). The family *Pyrenulaceae* is divided into two well-supported groups, group 1 and group 2, both supported by 100% bootstrap values (Fig. 1). Group 1 includes two species of *Anthracothecium* (*A. australiense* and *A. prasinum*) and 7 species of *Pyrenula* (*P. chlorospila*, *P. macrospora*, *P. nitida*, *P. thelomorpha*, *P. quassiaecola*, and two *Pyrenula* spp.). All the specimens collected in Sri Lanka belong to group 2. This group includes the mazaediate species *Pyrgillus javanicus* and seven species of *Pyrenula* (*P. aspistea*, *P. cruenta*, *P. fetivica*, *P. laevigata*, *P. mamillana*, *P. massariospora* and *P. subpraelucida*).

Discussion

The generic delimitation within the family *Pyrenulaceae* has never been tested with molecular data, mostly due to technical difficulties as discussed above. Specimens of *Pyrenulaceae* collected in Sri Lanka also proved difficult to work with using molecular techniques, but the molecular results that we obtained from this limited taxon sampling were sufficient to show the presence of two strongly supported groups within *Pyrenulaceae*. The members of these two groups do not differ greatly morphologically or anatomically, and the division in the two groups does not seem to correlate with the usual, mostly ascomatal characters used for generic classification in this family: ascospore colour and septation, structure of the ascospore locules, secondary chemistry, hamathecium inspersion and chemistry, and ostiole position. However, one morphological feature is present in one group and absent in the other: the pseudocyphellae (Fig. 2). Except for the two species of *Anthracothecium* A. Massal., all species of *Pyrenula* in group 1 have pseudocyphellae, whereas they are absent in group 2 for all species of *Pyrenula* and for *Pyrgillus javanicus*. The species sampling is, however, still too limited (only 12 out of 169

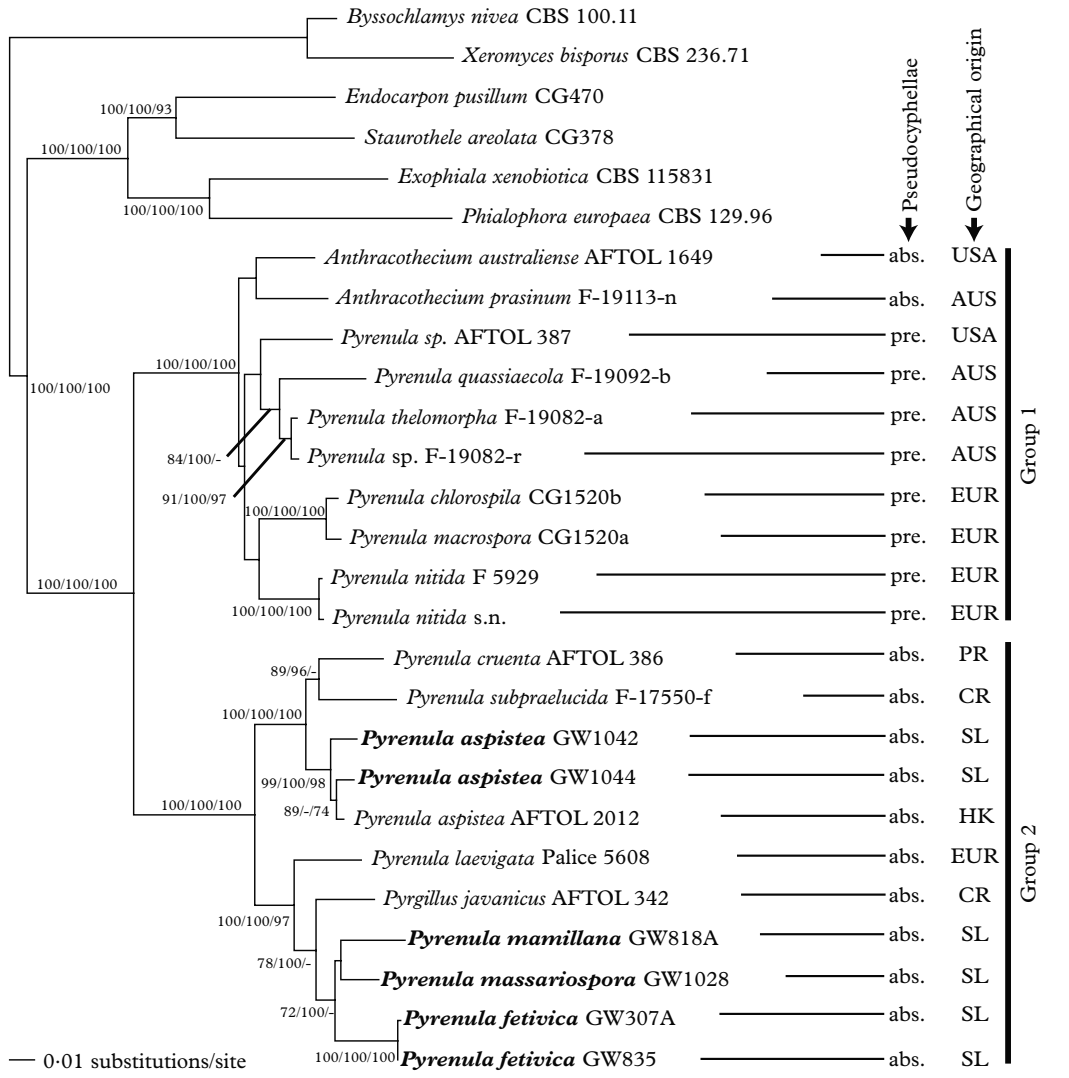

FIG. 1. Phylogenetic relationships within *Pyrenulaceae* based on a maximum likelihood approach using three gene regions (ITS, nuLSU, mtSSU). Most likely tree obtained with RAxML. Support values are indicated below or above the branches, with ML bootstrap/posterior probabilities/wMP bootstrap. Only bootstrap values superior or equal to 70% and posterior probabilities superior or equal to 95% are shown (dashes show non-significant values). The presence (pre.) or absence (abs.) of pseudocyphellae and the geographic origin are mapped on the tree for members of the family *Pyrenulaceae* (AUS = Australia, CR = Costa Rica, EUR = Europe, HK = Hong Kong, PR = Puerto Rico, SL = Sri Lanka, USA = United States of America). Specimens from Sri Lanka are highlighted in bold.

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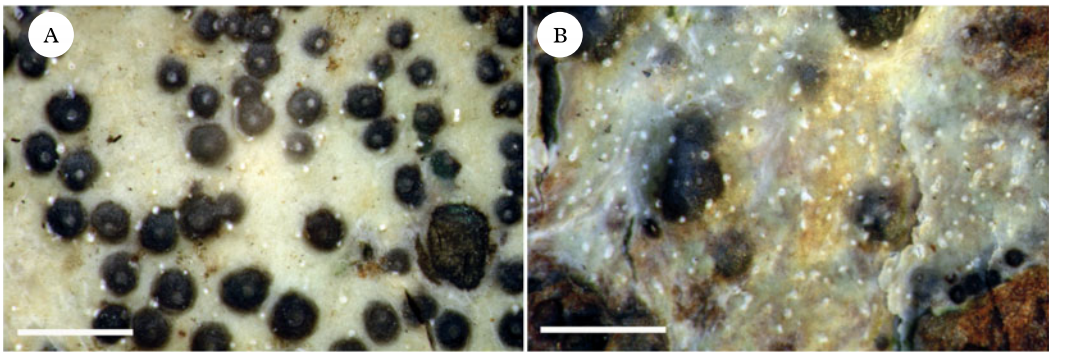

FIG. 2. Pseudocyphellae or small white pores visible on the upper surface of the thallus in *Pyrenula*. A, *Pyrenula chlorospila* CG1520b; B, *Pyrenula macrospora* CG1520a. Scale = 1 mm.

Pyrenula species and 15 out of 224 *Pyrenulaceae* species) to draw definitive conclusions, and further taxon sampling will be required to evaluate the informativeness of this character and other morphological, anatomical or chemical features.

Pyrenulaceae is most diverse in the tropics, but a few species are more commonly found in temperate climates. Among these temperate taxa, the two species collected in Great Britain, *P. chlorospila* and *P. macrospora*, are well supported as sister taxa. They are commonly found growing side by side on the bark of trees, and differ morphologically only by the size of their perithecia (0·2–0·4 mm for *P. chlorospila* and 0·4–1·2 mm for *P. macrospora*). Because the genetic variation between these two species is rather low and comparable for example, to that found among the three specimens of *P. aspistea*, further taxon and gene sampling will be necessary to investigate the delimitation between these two species. Also of interest are the phylogenetic placements of all taxa collected in Sri Lanka into group 2 and all taxa collected in Australia into group 1. Members of the family *Pyrenulaceae* are found worldwide but our results on a preliminary taxon sampling seem to suggest that phylogenetic groupings are correlated with geographic origin in this family.

Three genera of *Pyrenulaceae* have been included in our study: *Anthracothecium*, *Pyrenula* and *Pyrgillus* Nyl. *Anthracothecium* is characterized by large black perithecia and large brown muriform ascospores. Although these characters are shared with some species of *Pyrenula*, the type of ascospore septation is different in *Anthracothecium*. More particularly, the presence of a thick ascospore wall in the young ascospores of species of *Anthracothecium* separates this genus from *Pyrenula*. Moreover, species of *Anthracothecium* form a small group largely confined to the rainforest. *Anthracothecium* and *Pyrenula* differ morphologically from *Pyrgillus*, which is characterized by its perithecioid mazaedia and transversally septate ascospores. In our phylogeny, the two species of *Anthracothecium* belong to group 1 and cluster together. However, their relationship to other members of group 1 is not well supported and only two species of *Anthracothecium* have been sampled so far, so no conclusion can be reached as yet concerning the placement of this genus. Similarly, the morphologically well-characterized species *Pyrgillus javanicus* is nested within a group including species of *Pyrenula*. The genus *Pyrenula* is therefore not monophyletic according to our molecular results, but further work will be necessary before a revision of the generic delimitation within the family *Pyrenulaceae* can be carried out.

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to work on the material from Sri Lanka at the Natural History Museum (NHM) in London. Molecular work on material from Sri Lanka and the UK was supported by NHM funds to CG. Sequencing of other samples was done at the Pritzker Laboratory for Molecular Systematics at The Field Museum (Chicago) and this work was supported by a NSF grant (DEB-0717476). Holger Thüs and other staff members of the NHM Botany Department are specially thanked for their support, as well as two anonymous reviewers for their helpful comments.

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