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Research

Leightoniella zeylanensis belongs to the Pannariaceae

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Recent finds of *Leightoniella zeylanensis*, classified variously in the Collemataceae and Pannariaceae, enabled us to generate DNA sequence data for investigating its phylogenetic affiliation. Newly generated sequence data from the internal transcribed spacer (ITS) region and the large subunit of the nuclear ribosomal DNA (nrLSU), the small subunit of the mitochondrial ribosomal (mrSSU) DNA, and the largest subunit of the RNA polymerase II gene (RPB1) indicate that *L. zeylanensis* is a member of the Pannariaceae, belonging to a strongly supported clade together with *Physma*, *Lepidocollema*, and *Gibbosporina* (= the '*Physma* clade'). With the currently available data, however, relationships within this clade are largely impossible to reconstruct with confidence. *Leightoniella zeylanensis* was found to possess ellipsoid ascospores surrounded by a thick, gelatinous perispore with pointed ends, supporting a previously published hypothesis that such a perispore type is a synapomorphy for the *Physma* clade. A lectotype is designated for the basionym *Pterygium zeylanense* Leight.

Keywords: Pannariaceae, Collemataceae, lichen-forming ascomycetes

Introduction

The genus *Leightoniella* was described by Henssen (1965) for the single species *L. zeylanensis* based on 19th century material collected in Sri Lanka by George H. K. Thwaites and described by Leighton (1870) as *Pterygium zeylanense*. It was assigned to the *Collemataceae* by Henssen (1965) along with four additional genera with homoiomerous thallus and single-celled ascospores: *Physma* A. Massal, *Ramalodium* Nyl., *Leciophysma* Th. Fr., and *Homothecium* A. Massal. *Leightoniella* was distinguished from the other four genera on account of the periclinally arranged excipular hyphae and the presence of 'supporting tissue' at the base of the apothecium. Based on phylogenetic analysis of DNA sequence data, *Physma*, *Ramalodium* and *Leciophysma* were assigned to the Pannariaceae by Wedin et al. (2009) and Otálora et al. (2010), who found evidence that the Collemataceae proper does not include any members with single-celled ascospores. Extrapolating from these results, Ekman et al. (2014)



provisionally treated also *Leightoniella* and *Homothecium* as members of the Pannariaceae. This treatment, however, remained unconfirmed as long as DNA sequence data were unavailable.

After the original material was collected on streambank rocks in the Ambagamuwa region of the Central Province in Sri Lanka some time before December 1868, *L. zeylanensis* has only been reported a few times, once from Queensland, Australia (Verdon and Streimann 1995), and twice from New Caledonia (Aptroot and John 2015, GBIF record <https://www.gbif.org/occurrence/1324747594>). During fieldwork in Sri Lanka in 2015 by Pat Wolseley (Scientific Associate at The Natural History Museum, London) and the first author, the species was encountered on rocky boulders covered by bryophytes and small ferns under very humid conditions in lowland rainforests and submontane forests. Freshly collected samples enabled us to generate DNA sequence data and pursue the aim of this paper, namely to investigate the phylogenetic relationships of *L. zeylanensis*.

Material and methods

DNA extraction, PCR amplification, and sequencing of the entire internal transcribed spacer region of the nuclear ribosomal DNA (ITS), and part of the large subunit of the nuclear ribosomal DNA (nrLSU), small subunit of the mitochondrial ribosomal DNA (mrSSU), as well as the largest subunit of the RNA polymerase II gene (RPB1) were undertaken following the methods described by Wedin et al.

(2009) for nrLSU, mrSSU, and RPB1, and Ekman and Balaïd (2011) for the ITS.

Phylogenetic relationships were inferred in three steps. The aim of the first analysis was to test whether *Leightoniella* belongs to the Collemataceae, as traditionally treated, or to the Pannariaceae, as hypothesized by Ekman et al. (2014). The analysis started from the Peltigerales alignment by Wedin et al. (2009) before ambiguous alignment was masked. To this alignment we added newly generated nrLSU, mrSSU, and RPB1 sequences of *L. zeylanensis* (Table 1) using MAFFT 7.305 online (Kato and Standley 2013) with the 'add' option of the L-INS-i algorithm and the PAM20 scoring matrix. Subsequently, an intron in the RPB1 was excised from *L. zeylanensis*, 5' and 3' ends were trimmed (when needed) to fit the original alignment, and marker alignments concatenated. Following the rationale provided by Tan et al. (2015), we did not proceed to mask any ambiguous alignment from any of our analyses. Subsequently, we repeated the maximum likelihood bootstrap analysis of Wedin et al. (2009) using RAxML HPC version 8.2.11 (Stamatakis 2014) with search mode and likelihood models unaltered.

Although the first analysis would potentially be able to answer the question of the familial affiliation of *Leightoniella*, sparse taxon sampling prevented us from further conclusions on intrafamilial relationships. Therefore, a second analysis was conducted, this time starting from the alignment used by Ekman et al. (2014) before ambiguously aligned sites were masked (i.e. before ITS1 and ITS2 were subjected to T-Coffee masking and mrSSU to Aliscore masking). To this alignment we added a small number of recently generated sequences from GenBank representing other taxa in the

Table 1. DNA sequence data included in the BALi-Phy analysis, with details of voucher specimens and GenBank accession numbers. Newly obtained sequences in bold. NA=genes for which data were not available.

Species	Origin	GenBank accession no.			
		ITS	nrLSU	mrSSU	RPB1
<i>Gibbosporina amphorella</i>	Elvebakk et al. (2016)	KM887882	KM887882	NA	NA
<i>Gibbosporina mascarena</i>	Elvebakk et al. (2016)	KM887880	KM887880	NA	NA
<i>Leciophysma finmarkicum</i> (outgroup)	Wedin et al. (2009), Ekman et al. (2014)	KC618710	GQ258997	GQ259027	GQ259057
<i>Leightoniella zeylanensis</i> MWE206	here	MG888782	MG888780	MG920542	MG920544
<i>Leightoniella zeylanensis</i> MWE207	here	MG888783	MG888781	MG920543	MG920545
<i>Lepidocollema borbonicum</i> R1122	Magain & Sérusiaux (2014)	KF704271	NA	JX494259	NA
<i>Lepidocollema brisbanense</i> R1247	Magain & Sérusiaux (2014)	KF704262	NA	JZ494258	KF704297
<i>Lepidocollema brisbanense</i> T3	Magain & Sérusiaux (2014)	KF704277	NA	KF704281	KF704294
<i>Lepidocollema mariana</i>	Ekman et al. (2014)	NA	NA	KC608093	KC608135
<i>Lepidocollema stylophorum</i>	Ekman et al. (2014)	NA	NA	KC608097	KC608138
<i>Pannaria rubiginosa</i> (outgroup)	Ekman & Jørgensen (2002), Wiklund & Wedin (2003), Wedin et al. (2009)	AF429280	AY340558	AY340513	GQ259073
<i>Physma byrsaeum</i>	Wedin et al. (2009), Ekman et al. (2014)	NA	GQ259010	GQ259039	GQ259077
<i>Physma radians</i>	Wedin et al. (2009), Ekman et al. (2014)	KC618728	GQ259011	GQ259040	GQ259078
<i>Protopannaria pezizoides</i> I (outgroup)	Wiklund & Wedin (2003), Wedin et al. (2009), Ekman et al. (2014)	KC618729	AY340561	AY340519	GQ259081
<i>Psoroma hypnorum</i> V (outgroup)	Wiklund & Wedin (2003), Wedin et al. (2009), Ekman et al. (2014)	KC618732	AY340565	AY340523	GQ259085
<i>Staurolemma oculatum</i> (outgroup)	Wedin et al. (2009), Ekman et al. (2014)	KC618738	GQ259015	GQ259045	GQ259087
<i>Vahlia leucophaea</i> (outgroup)	Ekman & Jørgensen (2002), Wedin et al. (2007), Wedin & Wiklund (2004), Wedin et al. (2009)	AF429266	DQ900642	AY652621	GQ259090

Pannariaceae (Supplementary material Appendix 1, Table A1) as well as sequences of *L. zeylanensis* (Table 1). ITS, RPB1, and full-length mrSSU sequences were added using MAFFT as described above. The remaining shorter mrSSU sequences (*Leptogidium contortum*, *L. dendriscum* and *Steineropsis alaskana*) were added in a second step using the ‘addfragments’ and ‘multipair’ options with the rest of the parameters unchanged. Subsequently, individual markers were concatenated but no alignment sites were masked. Most taxa added to the alignment were represented by at least two markers, with the exception of *Leptogidium contortum*, *L. dendriscum*, *Steineropsis alaskana* and *Psoromidium aleuroides*, which were represented only by the mrSSU. We did not at this point add any representatives of the newly described *Gibbosporina* (Elvebakk et al. 2016), as publicly available sequences and our selection of markers only overlapped in the ITS region. Phylogenetic analysis of the concatenated alignment using PhyloBayes under a F81+ Γ +CAT model was carried out as described in Ekman et al. (2014) except that we used version 4.1c instead of 3.3b (Lartillot et al. 2009).

In the alignment of the second analysis, we observed substantial length differences between members of *Physma*, *Lepidocollema* and *Leightonella* (the ‘*Physma* clade’) and the rest of the tree (average 1907 nucleotides compared to 1769 in the rest of the tree, excluding taxa with missing data in some markers), as well as inside the *Physma* clade (standard deviation 77 nucleotides compared to 20 in the rest of the tree). This led us to question the inferred relationships in the *Physma* clade, which anyway did not include *Gibbosporina* (demonstrated to belong in the *Physma* clade by Elvebakk et al. 2016). Consequently, we performed a third analysis focussing on relationships within this clade. We carried out joint estimation of alignment and phylogeny using BALi-Phy version 2.3.8 (Suchard and Redelings 2006). We included reduced ITS, mrSSU, and RPB1 data from *Physma*, *Lepidocollema*, and *Leightonella* from the second analysis, to which we added *Gibbosporina* ITS and nrLSU sequences from GenBank, nrLSU data from *L. zeylanensis* used in the first analysis, as well as additional GenBank nrLSU data from the same specimens used to generate sequence data from other markers (Table 1). The nrLSU sequences were strikingly unequal in length at the 3’ end and were trimmed after the conserved GACCGAGGACCGCGC pattern. We partitioned the data into ITS1, 5.8S, ITS2, mrSSU, RPB1 first and second codon positions, RPB1 third codon positions, and nrLSU. We set the substitution model to a single GTR+I+ Γ . Gap models were set to RS07 (Redelings and Suchard 2007) for ITS1+ITS2, nrLSU, and mrSSU, whereas the alignment was treated as known and fixed for the 5.8S and RPB1 (i.e. taken from the PhyloBayes analysis with taxa deleted and gap-only sites stripped, except 5.8S sequences from *Gibbosporina*, which were added with MAFFT as described above). We assumed one rate for the (fast-evolving) ITS1, ITS2, and RPB1 third positions and another rate for the other (slow-evolving) subsets. The branch-length prior was assumed to follow a gamma distribution. The analysis

consisted of 4 parallel runs and included a pre-burnin of 1000 iterations followed by 100 000 cycles of Markov chain Monte Carlo (MCMC), sampling states every 20 cycles. The first 50% of each run was removed as burnin.

Morphological investigations were carried out using an Olympus SZX7 stereo microscope and an Olympus BX50 compound microscope equipped with differential interference contrast and connected to a Nikon Coolpix digital camera. Sections were mounted in water.

DNA sequence data and consensus trees from the two first analyses (the ones based on single alignments) are available from TreeBASE (<<https://treebase.org/>>), study ID S22197.

Results

The maximum likelihood bootstrap (Supplementary material Appendix 1, Fig. A1) placed *L. zeylanensis* as sister to three species of *Physma* (with 100% bootstrap support), inside a clade corresponding to clade 2 of Ekman et al. (2014) (with 100% bootstrap support), inside the Pannariaceae (with 88% bootstrap support).

The PhyloBayes analysis (Supplementary material Appendix 1, Fig. A2) placed *L. zeylanensis* in a clade together with *Physma* and *Lepidocollema* with 100% posterior probability. Relationships within the clade were uncertain. *Lepidocollema* was suggested to be paraphyletic, as all species except *L. borbonicum* formed a monophyletic group with 100% posterior probability. The mean discrepancy between runs in the PhyloBayes analysis was 0.0033, and effective sample sizes for model parameters ranged from 174 to 1393.

The BALi-Phy analysis confirmed that *Leightonella* belongs in the *Physma* clade (Fig. 1). There is strong support (100% posterior probability) for a branch uniting *Physma*, *Leightonella*, *Gibbosporina* and *Leightonella*, and for *Lepidocollema* being monophyletic. The consensus topology differs from that of previous analyses, but differences are poorly supported. PSRF-RCF values in the BALi-Phy analysis ranged from 0.978 to 1.009. The average standard deviation of split frequencies was 0.020. Effective sample sizes ranged from 104 to 10000 for model parameters.

Leightonella zeylanensis (Leight.) Henssen (1965, p. 40) (Fig. 2)

Basionym: *Pterygium zeylanense* Leight. (1870, p. 162).

Type: Sri Lanka, Central Province, “on stones upon banks of streams, Ambagamowa” (= Amabagamuwa in present-day spelling), undated, G. H. K. Thwaites (BM 001089110, lectotype, designated here, seen by MW; BM 001089111, BM 001089115, isolectotypes, seen by MW).

Thwaites collected lichens both in the Central Province and in the southern parts of Sri Lanka (Leighton 1870). Only material coming from the type locality as defined in the protologue (“on stones upon banks of streams, Ambagamowa”) are syntypes, samples annotated “south of island” or just

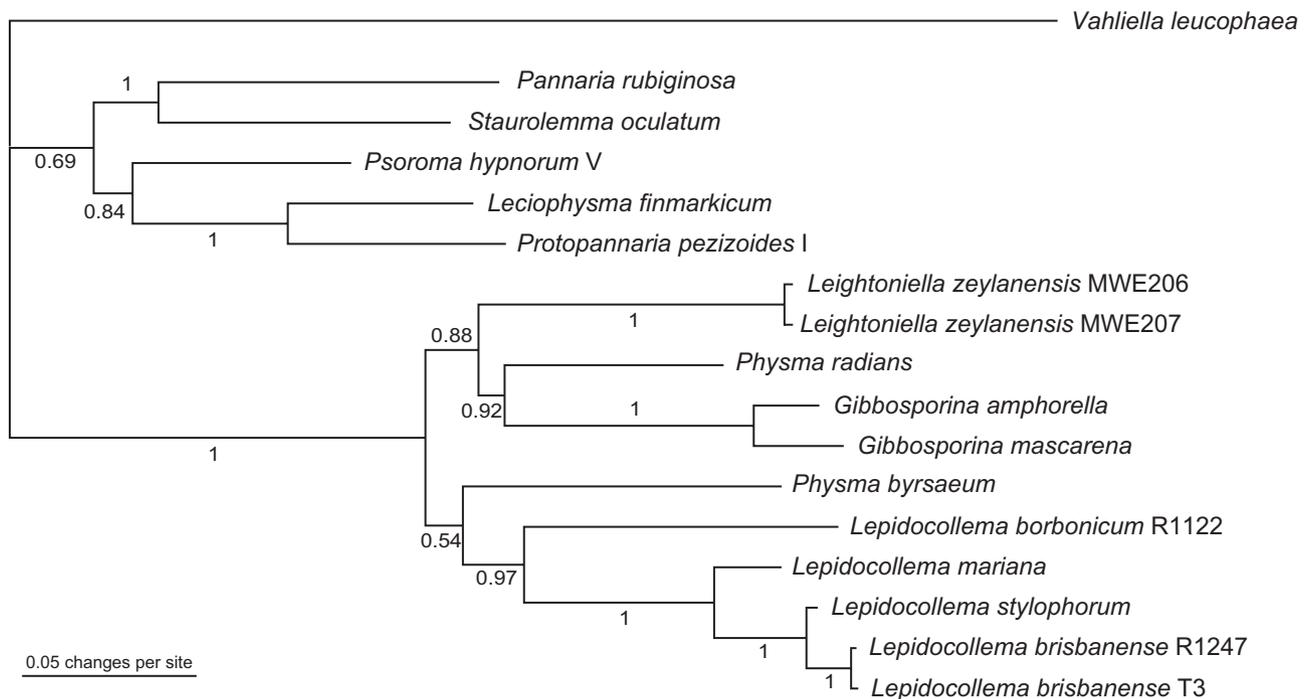


Figure 1. Bayesian majority-rule consensus tree resulting from the BAli-Phy analysis of ITS, nrLSU, mrSSU, and RPB1 sequence data from members of the Pannariaceae.

“Ceylon” are clearly not (Ambagamuwa is located in the central highlands of the island). Henssen (1965) lectotypified *Pterygium zeylanense* on syntype material in K, now housed

in BM. It was, however, clear from Henssen’s treatment that there were, at the time, at least two syntypes present in K (numbered “134” and “192”). As she did not unequivocally designate one of these specimens as the lectotype, a second-step lectotypification is required (ICN Art. 9.17). We designate the sample annotated by Henssen as lectotype (BM 001089110, corresponding to sample number “134” filed in K at the time of publication). This sample carries extensive descriptive notes in Latin, presumably by Leighton, plus drawings of the spores.

Morphology

A detailed description was provided by Henssen (1965). The fresh material from Sri Lanka (Fig. 2), however, displays an important feature not pointed out in that description: *L. zeylanensis* has single-celled, ellipsoid ascospores surrounded by a thick and gelatinous perispore with pointed ends (Fig. 2B). This perispore, although not discussed by Leighton (1870), was depicted in his Fig. 36: 1. Apart from the characteristic cupular excipulum of periclinally orientated hyphae, the genus is characterized by its complete lack of rhizines and felt on the lower thallus surface.

Additional specimens examined

New Caledonia. Sur un tronc d’Araliacée en forêt de montagne, montée du col du Dzumac vers le Mt Ouin, 1050 m a.s.l.; 17 May 1951, H. Hürlimann 4192 (M). Sri Lanka. Southern Province, Sinharaja Tropical Rainforest, 14 Feb 2015, G. Weerakoon and P. Wolseley Si111 (ABL, PDA, S). Sabaragamuwa Province, Adam’s Peak submontane forest,

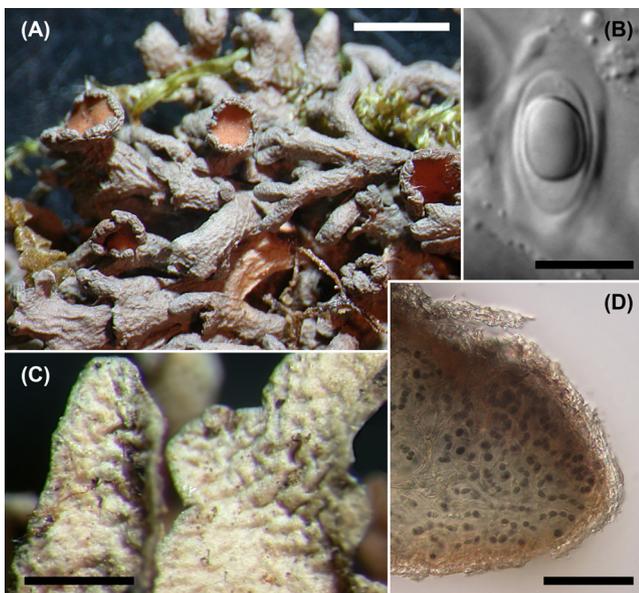


Figure 2. Morphology of *Leightoniella zeylanensis* (represented here by Weerakoon Si111 in ABL). (A) overall habit of the lichen thallus, (B) ascospore, showing the presence of a perispore, (C) lower surface of the thallus, (D) section through thallus margin, showing bark and distribution of the photobiont. Scale bars: (A)=1 mm, (B)=10 μ m, (C)=0.2 mm, (D)=25 μ m.

4 Apr 2017, G. Weerakoon AD129 (PDA). “Ceylon, south of island”, undated, G. H. K. Thwaites (BM 001089112, BM 001089113). “Ceylon”, undated, G. H. K. Thwaites (BM 001089114; S-F143561).

Discussion

Our results demonstrate that the genus *Leightoniella* is a member of the Pannariaceae, just like other genera with single-celled ascospores and a homoiomerous thallus previously classified in the Collemales (*Physma*, *Leciophysma*, *Ramalodium* and *Staurolemma*). This is in line with the prediction of Ekman et al. (2014). More precisely, *Leightoniella* is closely related to *Physma*, *Lepidocollema* and *Gibbosporina*, together with which it forms a well-supported group, the ‘*Physma* clade’. Elvebakk et al. (2016) suggested that *Xanthopsoroma* might be included in this clade, but we find no support for that hypothesis (Supplementary material Appendix 1, Fig. A2). On the other hand, we find support for their hypothesis that the presence of a gelatinous perispore is a synapomorphy of the *Physma* clade, as *Leightoniella* was found to possess a thick perispore with pointed ends. Internal relationships in the *Physma* clade are, however, largely impossible to infer with any degree of support given the currently available DNA sequence data. Even when we utilize all currently available data from the ITS, nrLSU, mrSSU, and RPB1 and integrate over alignment uncertainty (using BALi-Phy, Fig. 1), relationships remain equivocal. The contradictory topologies obtained from PhyloBayes (Supplementary material Appendix 1, Fig. A1) and BALi-Phy (Fig. 1), in which *Lepidocollema* is supported as monophyletic in the latter but not in the former, indicate either that LSU data confer a substantial amount of phylogenetic information, that alignment ambiguity is substantial, or both. Therefore, awaiting improved phylogenetic analyses based on more DNA sequence data, *Leightoniella* is retained here as a monotypic genus in the Pannariaceae.

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Author contributions – GW discovered and collected the fresh material of *L. zeylanensis*. AA identified the material, took the first initiative towards this paper, carried out morphological

investigations, and took the photos. MW provided the DNA sequence data and checked the type material in BM. SE carried out the phylogenetic analyses. All authors contributed in the writing of the paper.

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