Research

Leightoniella zeylanensis belongs to the Pannariaceae

Gothamie Weerakoon, André Aptroot, Mats Wedin and Stefan Ekman

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 Homotheicum A. Massal. Leightoniella was distinguished from the other four genera on account of the periclinaly 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 sequences of *L. zeylanensis* (Table 1) using MAFFT 7.305 online (Katoh 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 were concatenated. Following the rationale provided by Tăn 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 affilitation 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 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.

<table>
<thead>
<tr>
<th>Species</th>
<th>Origin</th>
<th>ITS GenBank accession no.</th>
<th>mrLSU GenBank accession no.</th>
<th>mrSSU GenBank accession no.</th>
<th>RPB1 GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Gibbosporina amphorella</em></td>
<td>Elvebakk et al. (2016)</td>
<td>KM887882</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td><em>Gibbosporina mascarena</em></td>
<td>Elvebakk et al. (2016)</td>
<td>KM887880</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><em>Lepidocollema broronicum</em> (outgroup)</td>
<td>Wedin et al. (2009), Ekman et al. (2014)</td>
<td>KC618710 GQ258997 GQ259027 GQ259057</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><em>Leightonella zeylanensis</em> MWE206</td>
<td>here</td>
<td>MG888782 MG888780 MG920542 MG920544</td>
<td>MG920545</td>
<td>MG920545</td>
<td>MG920545</td>
</tr>
<tr>
<td><em>Leightonella zeylanensis</em> MWE207</td>
<td>here</td>
<td>MG888783 MG888781 MG920543 MG920545</td>
<td>MG920545</td>
<td>MG920545</td>
<td>MG920545</td>
</tr>
<tr>
<td><em>Lepidocollema brisbanense</em> R1122</td>
<td>Magain &amp; Sérusiaux (2014)</td>
<td>NA</td>
<td>JX494259</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><em>Lepidocollema brisbanense</em> R1247</td>
<td>Magain &amp; Sérusiaux (2014)</td>
<td>NA</td>
<td>JX494258</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><em>Lepidocollema brisbanense</em> T3</td>
<td>Magain &amp; Sérusiaux (2014)</td>
<td>NA</td>
<td>JX494257</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><em>Lepidocollema mariana</em></td>
<td>Ekman et al. (2014)</td>
<td>NA</td>
<td>KC608093</td>
<td>KC608135</td>
<td>KC608138</td>
</tr>
<tr>
<td><em>Lepidocollema stylophorum</em></td>
<td>Ekman et al. (2014)</td>
<td>NA</td>
<td>KC608097</td>
<td>KC608135</td>
<td>KC608138</td>
</tr>
<tr>
<td><em>Physma byrsaeum</em></td>
<td>Wedin et al. (2009), Ekman et al. (2014)</td>
<td>NA</td>
<td>GQ259010 GQ259039 GQ259077</td>
<td>GQ259077</td>
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</tr>
<tr>
<td><em>Physma radians</em></td>
<td>Wedin et al. (2009), Ekman et al. (2014)</td>
<td>NA</td>
<td>GQ259011 GQ259040 GQ259078</td>
<td>GQ259078</td>
<td>GQ259078</td>
</tr>
<tr>
<td><em>Protopannaria pezizoides</em> 1 (outgroup)</td>
<td>Wiklund &amp; Wedin (2003), Wedin et al. (2009), Ekman et al. (2014)</td>
<td>KC618728 AY340561 AY340519</td>
<td>GQ259081</td>
<td>GQ259081</td>
<td>GQ259081</td>
</tr>
<tr>
<td><em>Psoroma hypnorum</em> V (outgroup)</td>
<td>Wiklund &amp; Wedin (2003), Wedin et al. (2009), Ekman et al. (2014)</td>
<td>KC618732 AY340565 AY340523</td>
<td>GQ259085</td>
<td>GQ259085</td>
<td>GQ259085</td>
</tr>
<tr>
<td><em>Staurolemma oculatum</em> (outgroup)</td>
<td>Wedin et al. (2009), Ekman et al. (2014)</td>
<td>KC618738 AY340565 AY340523</td>
<td>GQ259085</td>
<td>GQ259085</td>
<td>GQ259085</td>
</tr>
</tbody>
</table>
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+G+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 *Leightoniella* (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 *Leightoniella* 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 *Leightoniella* belongs in the *Physma* clade (Fig. 1). There is strong support (100% posterior probability) for a branch uniting *Physma*, *Leightoniella*, *Gibbosporina* and *Leightoniella*, 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.

*Leightoniella 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” (= Ambagamuwa in present-day spelling), undated, G. H. K. Thwaites (BM 001089110, lectotype, designated here, seen by MW; BM 001089111, BM 001089115, isolecotypes, 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
“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,

**Discussion**

Our results demonstrate that the genus *Leightoniella* is a member of the Pannariaceae, just like other genera with single-celled ascospores and a homoiomorous thallus previously classified in the Collemataceae (*Physma, Leciodphysma, Ramalodium and Staurolema*). 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 *Xanthosporoma* 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 RPBI 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.

**Acknowledgements** – We are grateful to Pat Wolseley, who played an important role in the excursion during which *L. zeylanensis* was rediscovered in Sri Lanka, and to Bodil Cronholm, the Molecular Systematics Laboratory at the Swedish Museum of Natural History, for skilful lab assistance. The first author is grateful to Prof. Siril Wijesundara, Research Professor at the National Institute of Fundamental Studies, for facilitating field studies in Sri Lanka and for support and advice and to Omal Arachchige and Dushantha Wasala for field assistance.

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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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**References**


