

The systematic position of *Microlejeunea ocellata* (Marchantiophyta: Lejeuneaceae), an extraordinary species endemic to Australia and New Zealand

Rui-Liang Zhu^{1,2,3} and Lei Shu^{1,2}

¹ Bryology Laboratory, School of Life Sciences, East China Normal University, 500 Dongchuan Road, Shanghai 200241, China; ² Shanghai Key Lab for Urban Ecological Processes and Eco-Restoration, Tiantong National Station of Forest Ecosystem, East China Normal University, 500 Dongchuan Road, Shanghai 200241, China

ABSTRACT. *Microlejeunea ocellata* (Herzog) Grolle was originally recognized as *Rectolejeunea ocellata* Herzog, a very enigmatic species known only from Australia and New Zealand. The systematic position of this species has been controversial owing to a number of interesting characters. A monospecific subgenus, *Rectolejeunea* subg. *Notholejeunea* R.M.Schust. was previously established to accommodate this species. Our molecular phylogenetic and morphological evidence reveals that this species represents a separate genus. Here we propose a new generic name, *Cumulolejeunea*, because recombining subg. *Notholejeunea* R.M.Schust. at generic rank is blocked by the earlier *Notholejeunea* Kuntze of 1903.

KEYWORDS. *Hattoriolejeunea*, Hepaticae, liverworts, lowland rain forests, *Notholejeunea*, *Rectolejeunea*.



The liverworts, one of three early diverging extant land plant lineages, are represented by 7271 species in 386 genera (Söderström et al. 2016). Lejeuneaceae, the largest family of liverworts with over 1800 species in about 70 genera (Zhu et al. 2017; Zhu & Shu 2017), is considered taxonomically most difficult. Although much progress in molecular phylogenetics has been made in recent years, the division of Lejeuneaceae into natural subunits has not yet been satisfactorily solved (Bechteler 2016a,b; Gradstein 2013; Zhu & Shu 2017). A number of rare and interesting taxa such as *Aphanotropis saxicola* Herzog, *Calatholejeunea paradoxa* (Schiffn.) K.I.Goebel, *Cephalantholejeunea temnanthoides* (R.M.Schust.) R.M.Schust., *Cephalolejeunea parvilibula* Mizut., *Dactylophorella muricata* (Gottsche) R.M.Schust., *Hattoriolejeunea akiyamae* Mizut., *Lep- tolejeunea spinistipula* (Mizut.) Xiao L.He and *Schusterolejeunea inundata* (Spruce) Grolle have not been studied by molecular-phylogenetic methods (Gradstein et al. 2018; Zhu & Shu 2017). *Rectolejeunea ocellata*, first described by Herzog (1949) based on E.A. Hodgson's collection from

New Zealand, is considered to be an extraordinary plant (Schuster 2000). Its systematic position was considered to be a mystery owing to a number of interesting characters, including exceedingly small cells without distinct trigones and intermediate thickenings, basal cluster of very enlarged ocelli, persistent leaves, scattered laminar ocelli, short and blunt tooth of the leaf lobule, possible lack of distinct oil bodies in chlorophyllose cells in leaves, and lejeuneoid gynoecial innovations (Schuster 2000). Grolle (1995) insisted that *Rectolejeunea* is restricted to the Neotropics and he transferred *R. ocellata* to the pantropical *Microlejeunea* (Spruce) Steph. mainly based on the lejeuneoid gynoecial innovation which is totally absent in *Rectolejeunea* s.str. (Grolle 2001). Schuster (2000), however, still placed it within *Rectolejeunea* by erecting a new monospecific subgenus in *Rectolejeunea* to accommodate *R. ocellata*: *Rectolejeunea* subgen. *Notholejeunea* R.M.Schust. Renner & de Lange (2011) believed that “maintenance of *Rectolejeunea ocellata* R.M.Schust. within *Rectolejeunea* is appropriate.” In the recent most comprehensive treatment of *Rectolejeunea*, the subgenus *Notholejeunea* was reduced to a synonym of *Microlejeunea* (Reiner-Drehwald & Grolle 2012)

³ Corresponding author's e-mail: rlzhu@bio.ecnu.edu.cn
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and *Rectolejeunea ocellata* was excluded from *Rectolejeunea* and placed in *Microlejeunea* as in Grolle (2001). Such a treatment also occurs in the most recent world checklist of liverworts and hornworts (Söderström et al. 2016). Recent molecular evidence also confirmed that both *Microlejeunea* and *Rectolejeunea* belong to tribe Lejeuneae Dumort., but *Microlejeunea* in subtribe Lejeuneinae Gradst. and *Rectolejeunea* in subtribe Lepidolejeuneinae Gradst. (Bechteler et al. 2016a; Gradstein 2013; Zhu et al. 2017).

During our studies on generic revisions of Lejeuneaceae, we examined a number of collections of *Rectolejeunea* and *Microlejeunea*, and found that *Microlejeunea ocellata* differs from the other members of *Microlejeunea* in the (4–)5–10 rows of medullary cells of the stem (always 3 rows in *Microlejeunea* s.str. (Wei & Zhu 2013)) and never reduced leaf lobules. The systematic position of *Rectolejeunea ocellata*, therefore, is still open to question. The present study aims to reassess its systematic position using morphological and new molecular evidence.

MATERIALS AND METHODS

Morphological observation. All morphological and anatomical characters were examined using an Olympus BX43 microscope equipped with a DP71 digital camera. Habitat data were gathered based on the study of collections of selected herbarium specimens. Oil body data were from D. Glenny's observation on fresh samples from New Zealand.

Taxon sampling. Our sampling covered all subtribes of tribe Lejeuneae. One hundred and one species in 34 genera from tribe Lejeuneae were included in our phylogenetic studies. Phylogenetic analyses indicated that *Microlejeunea ocellata* is nested within subtribe Lejeuneinae of subfamily Lejeuneoideae. At present Lejeuneinae contains four genera: *Harpalejeunea* (Spruce) Schiffn., *Hattoriolejeunea* Mizut., *Lejeunea* Lib., and *Microlejeunea* (Spruce) Steph. We were unable to analyze the monospecific *Hattoriolejeunea* Mizut. owing to the lack of samples for DNA extraction. Twenty-five accessions of the remaining genera in Lejeuneinae were included in our analyses, including four species of *Harpalejeunea*, five of *Microlejeunea* and 14 of *Lejeunea*. This sampling represents both morphological and geographic variations in the subtribe. The sequences of two accessions of *Microlejeunea ocellata*

were newly generated in the present study, and the remaining sequences were downloaded from GenBank. Outgroups consisted of 13 species from subfamily Ptychanthoideae. The accession of *Microlejeunea ocellata* II was originally determined as *Rectolejeunea* sp. in GenBank. Species names, voucher information, and GenBank accession numbers for all sequences are presented in **Table 1** and **Supplementary Table S1**.

DNA extraction and sequencing. Total DNA were extracted from dried herbarium specimens using DNeasy Plant Mini Kits (Qiagen, Hilden, Germany) after grind pre-treatment in a multi-sample tissuelyser (Jinxin Technology, China). Sequence data from three molecular markers (*rbcL*, *trnL–trnF* and nrITS) were used in our study as in previous publications (Gradstein et al. 2006; Hartmann et al. 2006; Zhu et al. 2017). Bidirectional sequencing was generated by Jie Li Biology Inc., China (<http://www.genebioseq.com>).

Phylogenetic analyses. Sequences were aligned using MAFFT version 7 (Katoh & Standley 2013) and then manually aligned in PhyDE v.0.997. The program Gblocks v.0.91b (Castresana 2000; Talavera & Castresana 2007) was used to delimit and remove regions of alignment uncertainty with options for a “less stringent” selection on Gblocks web server (http://molevol.cmima.csic.es/castresana/Gblocks_server.html).

To test for phylogenetic congruence among loci, comparisons were made among individual gene topologies to identify potential well-supported conflicts among clades in single-gene maximum likelihood (ML) topologies. Each locus was subjected to a ML analysis and nodal support was assessed using 1000 bootstrapping pseudoreplicates with RAxML-HPC V.8.2.6 (Stamatakis 2014) on the Cipres Science Gateway (Miller et al. 2010, <http://www.phylo.org>). Results were visualized by FigTree v1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree/>). Individual single-locus topologies were assessed for well-supported (bootstrap support >75%) conflict compared to the other single-locus ML trees and combined if no supported conflict was observed. Since no supported conflicts were detected among the single-gene trees, phylogenetic analyses of the concatenated three-locus dataset were performed.

Phylogenetic analyses of the concatenated data matrix were performed using RAxML V.8.2.6, PAUP* V4.0 (Swofford 2002) and MrBayes v.3.2.6

Table 1. Sequences newly generated in the study, including taxa, vouchers and GenBank accession numbers. “—” missing sequences.

Taxon	Voucher	<i>rbcl</i>	<i>trnL-trnF</i>	nrITS
<i>Microlejeunea ocellata</i> (Herzog) Grolle I	New Zealand, Glenlynn 8578 (CHR)	—	MH063889	MH063887
<i>Microlejeunea ocellata</i> (Herzog) Grolle III	Australia, Curnow 5661 (CANB)	—	MH063888	MH063886

(Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003) on the Cipres Science Gateway. In the ML analysis, the GTR+G+I substitution model was used with 1000 pseudoreplicates, with the data partitioned by loci. In MP analysis, branch swapping was performed using TBR and 500 random sequence addition replicates, and the strict consensus of most parsimonious trees was computed. Autapomorphic and constant characters were excluded prior to the analysis. The clade support was assessed by conducting 10000 bootstrap replicates with ten random taxon additions and heuristic search options. For the Bayesian analysis, the models of evolution selected by MrModeltest 2.3 (Nylander 2004) were set for each partition. Default priors of model parameters were also defined for each partition. Two parallel MCMC runs of 50000000 generations were conducted, each run containing three heated chains and one cold chain. The default value of temperature coefficient was used for all analyses. Trees and estimated parameter values were sampled every 1000 generations, thus obtaining a total of 50000 samples from which the first 12500 were discarded as burn-in. A 50% majority-rule consensus tree, with each branch lengths and posterior probabilities of clades was generated to summarize the results of the post burn-in posterior distribution of trees.

RESULTS

Our phylogenetic analyses using MP, ML and BI show highly congruent topologies that differed in the degree of resolution for some clades and do not produce well-supported incongruent clades among them. Out of 2611 base pairs analysed, 1190 are constant and 224 variable characters are parsimony-uninformative while 1197 are parsimony-informative. The MP analyses result in 15 equally parsimonious trees with a length of 12622 steps, a consistency index of 0.2236 and a retention index of 0.7764.

Phylogenetic relationships of *Microlejeunea ocellata* based on concatenated data matrix, with bootstrap support (BS) from the ML analysis and

MP analysis, and posterior probabilities (PP) values from the MrBayes analysis, are shown in **Fig. 1**. In all analyses *Microlejeunea ocellata* formed a well-supported monophyletic group and was recovered as a separate lineage from *Microlejeunea*, but sister to *Lejeunea* (BS_{MP}=92, BS_{ML}=96, PP_{BI}=1.00). Our phylogenetic results agree with the previous molecular studies on the classification of Lejeuneaceae (Bechteler et al. 2016b; Heinrichs et al. 2014a,b; Ye et al. 2015; Ye & Zhu 2018; Zhu et al. 2017). The topology shows that accessions of *Microlejeunea ocellata*, *Harpalejeunea*, *Microlejeunea*, and *Lejeunea* are nested within the strongly supported lineage of Lejeuneinae (BS_{MP}=100, BS_{ML}=100, PP_{BI}=1.00).

Morphological observations are presented in the following sections.

DISCUSSION

Our molecular analyses based on a representative sampling of Lejeuneaceae show that *Microlejeunea ocellata* known from Australia and New Zealand is not nested in the clade of *Microlejeunea* or *Rectolejeunea*. *Microlejeunea ocellata* is placed sister to *Lejeunea*, and remotely related to *Rectolejeunea* and *Microlejeunea* (**Fig. 1**). *Microlejeunea ocellata* resembles *Lejeunea* in the lejeuneoid gynoecial innovations, over four rows of medullary cells in transverse section of stem, proximal hyaline papilla of the leaf lobule, bilobed underleaves, and 4–5-keeled perianths without horns. *Lejeunea*, however, is immediately separated from *Microlejeunea ocellata* by the lack of ocelli in leaf lobes and perianths, larger leaf cells, presence of oil bodies in all leaf cells.

Out of three genera in subtribe Lejeuneinae listed in the “World Checklist of Liverworts and Hornworts” (Söderström et al. 2016) (excluding *Taxilejeunea* (Spruce) Steph., a synonym of *Lejeunea*), only monospecific *Hattoriolejeunea* could not be sequenced in the present study. *Hattoriolejeunea* was originally described based on the single collection made by H. Akiyama from Seram in 1985 (Mizutani 1987). This taxon is known only from the type specimen. Although we attempted to recover the species in the type locality in Seram in May 2017,

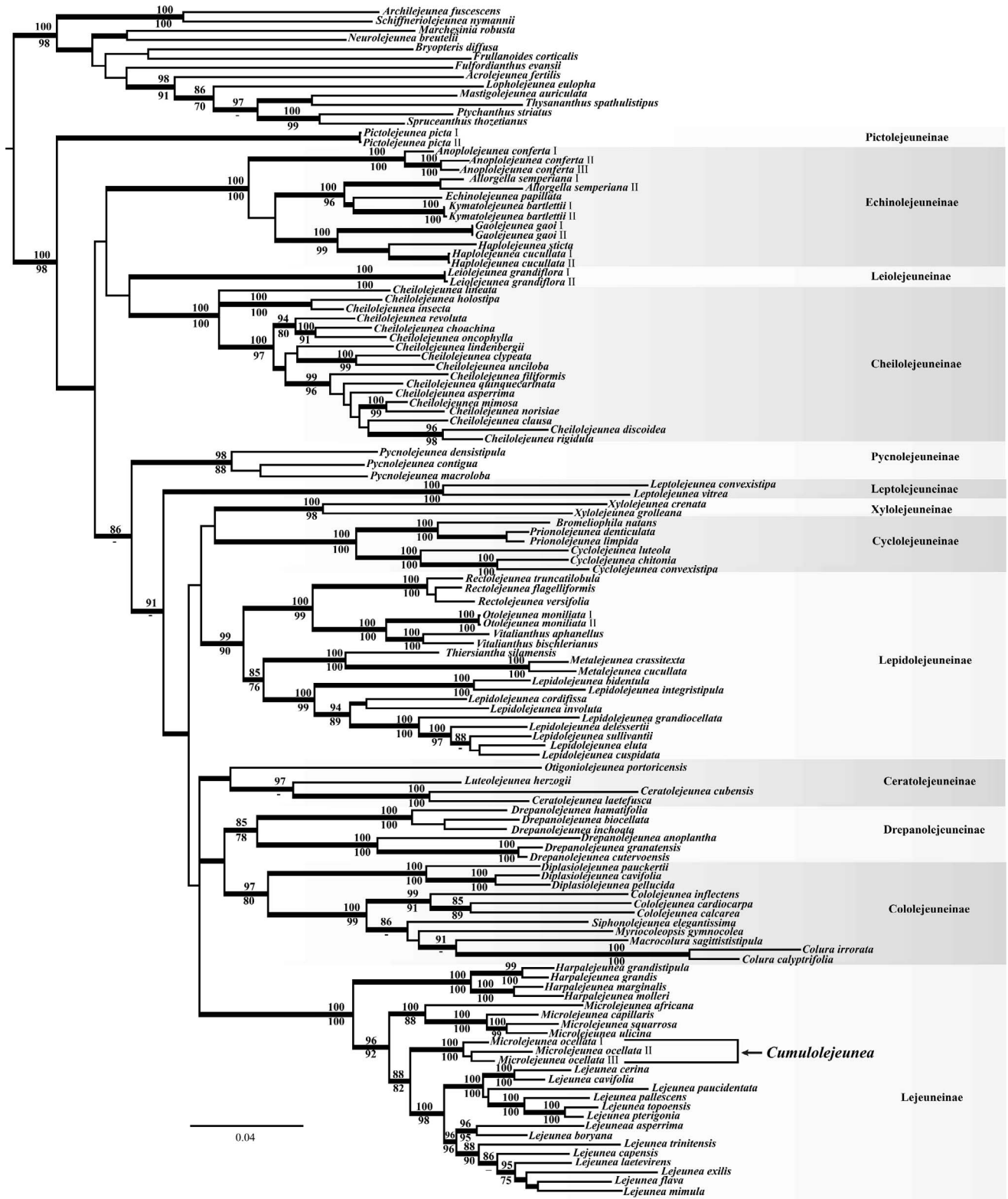


Figure 1. Phylogeny of Lejeuneaceae illustrating position of *Microlejeunea ocellata*, inferred from combined dataset (*rbcL*, *trnL-trnF* and nrITS) and topology displayed as majority rule consensus tree of trees recovered in stationary phase of Bayesian search with branches thickened when $PP_{BI} \geq 0.98$. $BS_{ML} \geq 80$ and $BS_{MP} \geq 70$ are displayed besides the nodes with the former above the branch and the later below the branch.

no samples were successfully available for our molecular study. *Hattoriolejeunea* has a habit of *Trichocolea* Dumort. (Mizutani 1987). The most striking characters of this plant include the densely ciliate teeth at margins of leaves and underleaves, robust stems with over seven rows of cortical cells in transverse section, and the lack of ocelli (Mizutani 1987). *Hattoriolejeunea* is most similar to *Lejeunea*, and may even be a member of *Lejeunea* based on our morphological studies. It is a great pity no molecular data could be included for it in the present analyses, but in morphology *Hattoriolejeunea* is immediately distinguished from *Microlejeunea* (including *M. ocellata*) by the lack of ocelli. The existence of ocelli and patterns of ocelli often serve as one of most useful characters in species and generic delimitation in recent phylogenetic studies on Lejeuneaceae (Bechteler et al. 2016a; Czumay et al. 2013; Dong et al. 2013; Schäfer-Verwimp et al. 2017; Ye & Zhu 2018; Zhu et al. 2017, 2018).

The phytogeographical isolation, distinct morphological character states and the isolated phylogenetic position of *Microlejeunea ocellata* within subtribe Lejeuneinae support elevating *Rectolejeunea* subgen. *Notholejeunea* R.M.Schust. to generic status. Owing to the presence of *Notholejeunea* Kuntze (Kuntze 1903), a new generic name *Cumulolejeunea* is proposed. *Cumulolejeunea* is well characterized by the lejeuneoid gynoecial innovations, basal cluster of very enlarged ocelli, persistent leaves, and extremely small leaf cells, 1(–3) granular oil bodies absent in few to ca. 50% of cells of leaf lobe (based on examination of fresh samples from New Zealand by D. Glenn). In morphology *Cumulolejeunea* is most analogous to the pantropical *Microlejeunea* in the presence of ocelli in leaf lobes, lejeuneoid gynoecial innovation, bilobed underleaves, and 4–5-keeled perianths without horns. Several most striking features such as stem structure, oil body, and basal cluster of very enlarged ocelli, however, are rather different in the two genera. In *Microlejeunea*, the stem has only three rows of medullary cells in transverse section, the basal ocelli not of cluster of very enlarged ocelli, and oil bodies present in all leaf cells, and keel of female bract usually winged (Wei & Zhu 2013). *Cumulolejeunea* is similar to *Rectolejeunea* and *Thiersianthus* R.L.Zhu et L.Shu of subtribe Lepidolejeuneinae especially in the smaller leaf cells without distinct trigones and intermediate thickenings, presence of basal ocelli in leaf lobes, bilobed underleaves, 4–5-keeled perianths without horns,

and lack of ocelli in underleaves. The latter two genera, however, are readily recognized by the pycnolejeuneoid gynoecial innovations, which is one of most striking features of subtribe Lepidolejeuneinae (Zhu et al. 2017).

TAXONOMY

Cumulolejeunea R.L.Zhu et L.Shu, nom. nov.

≡ *Rectolejeunea* subgen. *Notholejeunea* R.M.Schust., J. Hattori Bot. Lab. 89: 128. 2000 (non *Notholejeunea* Kuntze, Lexicon Generum Phanerogamarum 609. 1903).

TYPE SPECIES: *Cumulolejeunea ocellata* (Herzog) R.L.Zhu et L.Shu

Etymology. The generic name *Cumulo-* refers to cluster of basal ocelli.

Cumulolejeunea ocellata (Herzog) R.L.Zhu et L.Shu, *comb. nov.*

Fig. 2

≡ *Rectolejeunea ocellata* Herzog, Trans. & Proc. Roy. Soc. New Zealand 77: 255. 1949. ≡ *Microlejeunea ocellata* (Herzog) Grolle, Haussknechtia 8: 60. 2001.

TYPE: NEW ZEALAND. NORTH ISLAND: Hawke's Bay Land District, Morere Bush near Wairoa, on bark, 21 Jan. 1934, E. A. Hodgson 293 (lectotype designated by Grolle (2001): JE!).

Description. *Cumulolejeunea ocellata* was fully described and illustrated by Schuster (2000 as *Rectolejeunea ocellata*). It is characterized by the lejeuneoid gynoecial innovations (if present), basal cluster of very enlarged ocelli, presence of scattered ocelli usually larger than non-ocellate cells in leaf lobes, bilobed underleaves, persistent leaves, 4–10 rows of medullary cells of the stem, proximal hyaline papilla of the leaf lobule, and extremely small leaf cells, 1(–3) granular oil bodies absent in few to ca. 50% of cells of leaf lobe (Glenny 2017, pers. comm.). *Cumulolejeunea ocellata* also shows some variations. The outer lateral margins of underleaf lobes are entire to weakly dentate (Fig. 2F–G). In the shoots with well developed leaf lobules, the keel of the leaf is arched, but usually almost straight when the leaf lobules are reduced (Fig. 2A–B). The gynoecial innovations are sometimes absent in Australian samples.

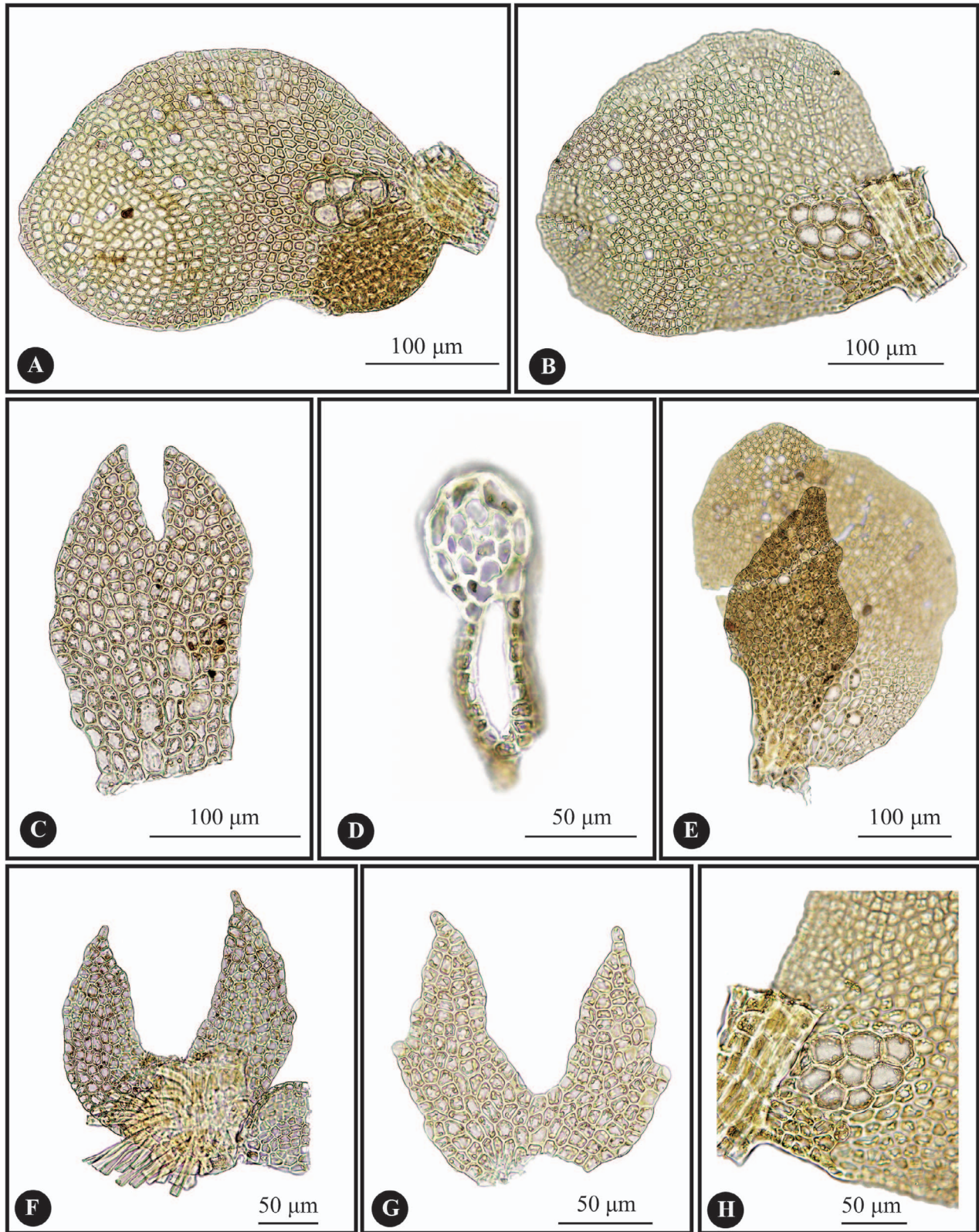


Figure 2. *Cumulejeunea ocellata* (Herzog) R.L.Zhu et L.Shu. A–B. Leaves, B, ventral view. C. Female bracteole. D. Transverse section of stem. E. Female bract, ventral view. F–G. Underleaves. H. Leaf base showing basal cluster of enlarged ocelli and reduced leaf lobule, ventral view. All from *J.A. Curnow 5661* (CANB as *Microlejeunea ocellata*).

Habitats and distribution. The single species, *Cumulolejeunea ocellata*, is known on rocks, stones, decaying logs, tree roots, tree bases and tree trunks in well shaded depression in open rain forests at an altitude of 10–1100 m in Australia (New South Wales, Queensland) and New Zealand (North Island, South Island).

Representative specimens examined. AUSTRALIA. QUEENSLAND: Burnett District, Bunya Mountains National Park, ca. 70 km NE of Dalby, Paradise Falls, 26°52'23"S, 151°34'49"E, 1010 m, on rock beside track just above falls, 6 May 2005, J. A. Curnow 6370 (CANB as *Rectolejeunea queenslandica* (B.M.Thiers) Xiao L.He); *ibid.*, on dry rock in watercourse, between pools of trickling stream, below the falls, 6 May 2005, J. A. Curnow 6369A (CANB as *R. queenslandica*); NEW SOUTH WALES: Central Coast, Robertson Nature Reserve, corner of South and Missingham roads, Robertson, 34°35'40"S, 150°30'45"E, 760 m, mixed temperate rainforest, on rock, 4 May 2002, J. A. Curnow 5662 (CANB as *Microlejeunea ocellata*), *ibid.*, on rotten wood, 4 May 2002, J. A. Curnow 5661 (CANB as *M. ocellata*). NEW ZEALAND. NORTH ISLAND: Wellington Land District, Lake Wairarapa, Western Lake Road, on sandstone, 10 m, 23 Nov. 2001, D. Glenny 8578 (CHR 542471 as *M. ocellata*).

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Supplementary document online:

Supplementary Table S1. Sequences from GenBank used in this study, including taxa, locations, vouchers, and GenBank accession numbers.