Gaolejeunea, a new genus from China and new member of subtribe Echinolejeuneinae (Lejeuneaceae, Marchantiophyta)

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ABSTRACT. A narrowly endemic species known only from the type locality in Shangsi Co., Guangxi, as well as a peculiar member of the Chinese Lejeuneaceae, Cheilolejeunea gaoi has previously been treated as a species of Cheilolejeunea. However, the presence of Allorgella-type denticulations formed by two adjacent cells on leaf and underleaf margins, small ovate leaf lobules without distinct teeth, ental hyaline papilla, and finely segmented oil bodies all suggest that it belongs somewhere else. Maximum likelihood, maximum parsimony, and Bayesian analyses of a three marker dataset (nrITS, cpDNA rbcL and trnL/C0F) derived from 85 accessions of Lejeuneaceae and 15 outgroup species revealed that C. gaoi belongs to subtribe Echinolejeuneinae. Based on the results of phylogenetic analyses, combined with morphological data, we suggest here that C. gaoi should be established as a new genus, Gaolejeunea. We strongly recommend in situ or ex situ conservation activity for this species based on the IUCN Red List criteria.

KEYWORDS. China, conservation, endangered plant, Haplolejeunea, Hepaticae, oil bodies.

With more than 1800 species in about 70 genera (Zhu et al. 2017), Lejeuneaceae is the largest family of the liverworts. The phylogeny of Lejeuneaceae has been intensively studied in recent years. Gradstein (2013) proposed a revised classification of this family summarizing evidence from morphology and molecular phylogeny of Lejeuneaceae, recognized 68 genera, of which 49 were assigned to the subfamily Lejeuneoideae. Lejeuneoideae was further classified into three tribes, Brachiolejeuneae, Symbiezidieae and Lejeuneae. Gradstein (2013) accepted eight subtribes of Lejeuneae, but refrained from assigning seven genera to any subtribe due to the lack of molecular phylogenetic data. Since then, phylogenetic studies have introduced several changes into the classification of Gradstein (2013), especially on Lejeuneae. These treatments include the establishment of new subtribes Leiolejeuneinae (Schäfer-Verwimp et al. 2014), Leptolejeuneinae (Heinrichs et al. 2014c), Pictolejeuneinae (Bechteler et al. 2016b), Pycnolejeuneinae and Xylolejeuneinae (Heinrichs et al. 2014b); the reinstatement of Allorgella Tixier (Bechteler et al. 2016a); the broader concept of Cheilolejeunea (Spruce) Steph. with Aureolejeunea R.M.Schust., Cystolejeunea A.Evans, Cystolejeunea A.Evans, Evansiolejeunea Vanden Berghen, Omphalanthus Lindenb. et Nees and Leucolejeunea A.Evans as its synonyms (Schäfer-Verwimp et al. 2014; Ye et al. 2015); the transfer of Vitalanthus R.M.Schust. et Gianotti from Drepanolejeuneinae to Lepidolejeuneinae (Bechteler et al. 2016a), Otolejeunea Grolle et Tixier from Cyclolejeuneinae to Lepidolejeuneinae (Heinrichs et al. 2014b) and Bromeliophila R.M.Schust. from Lejeuneinae to Cyclolejeuneinae (Heinrichs et al. 2014a). While reconstructing a comprehensive phylogeny of Cheilolejeuneinae (Ye et al. 2015), two accessions of C. gaoi were found to be well separated from Cheilolejeuneinae, forming a robust clade with Anoplolejeunea (Spruce) Schiffn., a member of subtribe Echinolejeuneinae. Bechteler et al. (2016b) further indicated that C. gaoi was
resolved in Echinolejeuneinae, with a close relationship to Haplolejeunea Grolle. However, an investigation with extended sampling on the systematic position of \textit{C. gaoi} was still needed. The subtribe Echinolejeuneinae represents a rather morphologically heterogeneous assemblage of genera that share bifid underleaves and pycnolejeuneoid gynoecial innovations (Bechteler et al. 2016a). The monospecific genus \textit{Kymatolejeunea} Grolle has also been assigned to this subtribe based on morphological evidence (Gradstein 2013; Grolle 1984; Schuster 2001), which has recently been confirmed with molecular data with the establishment of a new genus \textit{Thiersianthus} R.L.Zhu et L.Shu (subtribe Lepidolejeuneinae) from Borneo (Zhu et al. 2017). Most recently a new genus (\textit{Reinerantha}) from the Western Cordillera of Ecuador has been added to subtribe Cololejeuneinae (Gradstein et al. 2018). Here, we investigate the systematic position of \textit{C. gaoi} using extensive samplings in Lejeuneeae, and propose a new genus \textit{Gaolejeunea} based on our analyses.

\textbf{Materials and Methods}

\textbf{Taxon sampling, DNA extraction, PCR amplification, sequencing and alignment.} We included three accessions of \textit{Cheilolejeunea gaoi}, three samples of \textit{Tuyamaella} S.Hatt., as well as 266 sequences downloaded from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) containing two samples of \textit{Kymatolejeunea} in this study to test the systematic position of \textit{C. gaoi}. Total DNA was extracted from fresh collected material or dried herbarium specimens using DNeasy™ Plant Mini Kit (QIAGEN) after grind pre-treatment in a multi-sample tissue-lyser (Jinxin technology, China). Two chloroplast regions (\textit{trnL}–\textit{F}, \textit{rbcL}) and the nuclear ribosomal internal transcribed spacer 1 and 2 with the intervening 5.8S ribosomal subunit (ITS1-5.8S-ITS2, collectively called \textit{nrITS}) were amplified by polymerase chain reaction (PCR), employing 35 cycles of 30 sec at 95°C, 45 sec at 50°C and 1 min at 72°C, preceded by an initial 1 min melting step at 95°C and followed by a final extension period of 7 min at 72°C. Amplification and sequencing primers followed those described in Shaw et al. (2003, \textit{trnL}–\textit{F}), Gradstein et al. (2006, \textit{rbcL}), and Hartmann et al. (2006, \textit{nrITS}). A total of 70 ITS, 58 \textit{rbcL} and 62 \textit{trnL}–\textit{F} sequences were newly generated for this study.

Sequences were aligned manually using PhyDE v.1 (Müller et al. 2010). Positions that were ambiguously aligned in the dataset were excluded before any phylogenetic analysis; gaps were coded as missing data. The ingroup consisted of 85 accessions of Lejeuneeae according to the analyses of Wilson et al. (2007). Fifteen representatives of Brachiolejeuneae and Ptychanthoideae were chosen as outgroups. Voucher information and Genbank accession numbers are listed in \textbf{Table 1} and \textbf{Supplementary Table S1}.

\textbf{Phylogenetic analysis.} Maximum parsimony (MP) analyses were performed with command files using the parsimony ratchet (Nixon 1999) generated from the program PRAP2 (Müller 2007) applying the default settings, and executed in PAUP* 4.0b10 (Swofford 2000). Heuristic bootstrap searches under parsimony were performed with 10000 replicates. The three individual data sets were first analyzed separately (results not shown here) to check for possible incongruence. Strict consensus trees from these separate analyses were compared to identify conflicting nodes supported by at least 70% (Mason-Gamer & Kellogg 1996). The three datasets were combined into a single matrix due to no evidence of incongruence. \textit{jModelTest} v.2.1.6 (Darriba et al. 2012) was used to select the best fit models of evolution for maximum likelihood (ML) analysis and Bayesian inference (BI) analysis. With the Bayesian information criterion (Posada & Buckley

\begin{table}[h]
\centering
\caption{Sequences newly generated in the study, with missing sequences indicated by a dash (−).}
\begin{tabular}{|l|l|l|l|l|}
\hline
\textbf{Taxon} & \textbf{Voucher (all deposited in HSNU)} & \textbf{\textit{rbcL}} & \textbf{\textit{trnL}–\textit{F}} & \textbf{\textit{nrITS}} \\
\hline
\textit{Cheilolejeunea gaoi} (II) & China, Ye & Wei 20090717-1 & MG847530 & – & – \\
\textit{Cheilolejeunea gaoi} (III) & China, Zhu, Wei & He 20100822-15 & MG847528 & MG847537 & MG847533 \\
\textit{Tuyamaella molischii} (Schiffn.) S.Hatt. (I) & China, Wei 20110214-13A & MG847531 & MG847538 & MG847534 \\
\textit{Tuyamaella molischii} (II) & China, Wei 20110214-19A & – & – & MG847535 \\
\textit{Tuyamaella molischii} (III) & China, Wei 20110217-2 & MG847532 & – & MG847536 \\
\hline
\end{tabular}
\end{table}
RESULTS

The aligned matrix contained 3038 nucleotide characters, including 1407 in the ITS, 1189 in the \textit{rbcl} and 442 in the \textit{trnL-F}. Of the total character sites, 1435 were constant, 1311 were parsimony-informative and 292 were variable but parsimony uninformative.

The maximum parsimony (MP) analysis resulted in 47 maximally parsimonious trees of 10095 steps, with a consistency index (CI) of 0.306 and a retention index (RI) of 0.605, RC of 0.185, HI of 0.694. A single most likely tree (lnL = –47821.8570) was found in the maximum likelihood (ML) analysis. BI analyses resulted in highly similar topologies with the most likely tree. Figure 1 shows the 50% majority-rule consensus tree from the analyses of the concatenated dataset obtained by the BI approach, with support values (≥70) from the ML and MP analyses plotted onto the Bayesian tree.

The three accessions of \textit{Cheirolejeunea gaoi} are recovered in a robust sister clade of \textit{Haplolejeunea} (MLBS=100, MPBS=100, PP=1.00). The \textit{C. gaoi}/\textit{Haplolejeunea} clade is placed sister to a well-supported clade (MLBS=94, MPBS=86.51, PP=1.00) of \textit{Anoplolejeunea conferta}. The type species of the monospecific genus \textit{Kymatolejeunea}, \textit{K. bartlettii} is found sister to a clade formed by \textit{Echinolejeunea} R.M.Schust. and \textit{Allorgella} Tixier. All the represented species from subtribe Echinolejeuneinae form a highly supported monophyletic clade (MLBS=100, MPBS=100, PP=1.00) (Fig. 1).

DISCUSSION

The present study with 33 genera of Lejeuneaceae, including all the present members of Echinolejeuneinae, is by far the most comprehensive generic sampling of this tribe. The molecular phylogeny reveals \textit{Cheirolejeunea gaoi} as a member of Echinolejeuneinae supported by bootstrap percentages and Bayesian posterior probabilities (Fig. 1). The results also support the former treatments (Bechteler et al. 2016a; Gradstein 2013; Zhu et al. 2017) classifying \textit{Allorgella}, \textit{Anoplolejeunea}, \textit{Echinolejeunea}, \textit{Haplolejeunea} and \textit{Kymatolejeunea} in Echinolejeuneinae.

\textit{Cheirolejeunea gaoi} was originally placed within \textit{Cheirolejeunea} (Zhu et al. 2000). However, researchers were puzzled by its (1) delicate plants, (2) marginal leaf denticulations of \textit{Allorgella}-type, (3) small, ovate leaf lobules without distinct teeth, (4) ental hyaline papilla, (5) finely segmented oil bodies, and (6) absence of gynoecial innovations (Zhu et al. 2000; Zhu & So 2003), which cannot be easily fitted into any existing Chinese Lejeuneaceae genus. Furthermore, a recent revised phylogeny and classification of subtribe Cheirolejeuneinae already indicated that \textit{C. gaoi} is not a member of \textit{Cheirolejeunea} (Bechteler et al. 2016b; Ye et al. 2015; Zhu et al. 2017), but much closer to \textit{Anoplolejeunea} (subtribe Echinolejeuneinae).

Morphologically, \textit{Cheirolejeunea gaoi} has an affinity with members of \textit{Piccolejeunea} Grolle by the lack of gynoecial innovation; thin-walled leaf cells without distinct trigones, reduced first tooth and indistinct second tooth of leaf apex (Zhu et al. 2000). Yet \textit{Piccolejeunea} is distinguishable by having numerous deeply pigmented ocelli in leaves, underleaves, bracts and perianths (Grolle & Reiner-Drehwald 2005). The latest phylogenetic analyses with 30 genera of Lejeuneaceae favored \textit{Piccolejeunea} as
Figure 1. A 50% majority-rule phylogram derived from Bayesian inference of the combined nrITS, rbcL and trnL-F. Support values with Bayesian posterior probabilities (PP) above 0.9, maximum likelihood bootstrap support values (MLBS) above 70, and maximum parsimony bootstrap support values (MPBS) above 70 simultaneously are indicated on the branches as follows, PP left above the branch, MLBS right above the branch, and MPBS below the branch.
a separated subtribe Pictolejeuneinae (Bechteler et al. 2016b), with no close affinity with C. gaoi, which is supported in this study.

The marginal leaf denticulations of Allorgella-type in C. gaoi also occur in Allorgella, Haplolejeunea, Kymatolejeunea, Tuyamaella, and some species of Cololejeunea (Zhu et al. 2000; Zhu & So 2001). The latter two genera resulted in a well-supported clade (PP=1, MLBS=97, MPBS=93) in our analyses. Together with Colura, Diplasiolejeunea, Macrocolura, Myriocoleopsis and Siphonolejeunea, they formed a strongly supported Cololejeuneinae, far from C. gaoi (Fig. 1).

In our analyses Cheilolejeunea gaoi was resolved in the subtribe Echinolejeuneinae clade. The subtribe Echinolejeuneinae as circumscribed by Gradstein (2013) is a small subtribe characterized by the ball-shaped lobule with strongly involute free margin and hyaline papilla distal to lobule apex. It was originally established with three monospecific genera, Anoplolejeunea, Echinolejeunea and Kymatolejeunea. Phylogenetic work (Bechteler et al. 2016a) later reinstated the genus Allorgella, and expanded Echinolejeuneinae with two more genera, Allorgella and Haplolejeunea. Our results reveal a close relationship between C. gaoi and Haplolejeunea. Haplolejeunea is a small Afro-American genus with only two species (Söderström et al. 2016) and a possible new species (H. sp.) from French Guiana (Gradstein & Ilkiu-Borges 2009), while C. gaoi is endemic to China. Morphologically, Haplolejeunea and C. gaoi share several characters including the minute size of plants, small leaf cells without intermediate thickenings, usually swollen leaf lobules without a distinct tooth at apex, ental lobular hyaline papilla, small, distant and bilobed underleaves, smooth keels of perianths, truncate apex of the leaf lobule, and lack of vegetative reproductive organs. Haplolejeunea, however, clearly differs from C. gaoi in the glistening green plants, presence of scattered ocelli in leaf lobes and underleaves, pycnolejeuneoid gynoecial innovations, male bracteoles present only at the base of androecium, and fewer oil bodies (only 1–3 per leaf cell). Haplolejeunea is readily separated from other members of Echinolejeuneinae by the presence of scattered ocelli that appear not only in leaf lobes, but also in the underleaves, bracts, and perianths. The occurrence of ocelli is common in Lejeuneaceae and Frullaniaceae (Gradstein et al. 2003). The existences and patterns of ocelli often serve as a useful character in species and generic delimitations. The separation of Lejeunea, Harpalejeunea and Microlejeunea (Czumay et al. 2013; Dong et al. 2013; Heinrichs et al. 2013) was supported by the presence of ocelli in the latter two genera. Based on the presence of ocelli and pycnolejeuneoid innovations, Lejeunea huctumalensis was transferred to Physantholejeunea R.M.Schust., a younger name of Otitonolejeunea (Spruce) Schiffn. (Wei et al. 2014). Most recently, Thiersianthus, a new genus endemic to Borneo, has been separated from the pantropical Metalejeunea Grolle mainly by the presence of scattered ocelli in leaf lobes (Zhu et al. 2017). Therefore, morphological data clearly indicate that C. gaoi does not fit in any of the five known genera of Echinolejeuneinae. Cheilolejeunea gaoi merits the recognition as a new genus, proposed in this paper as Gaolejeunea. Gaolejeunea is characterized and recognized by the delicate, green to yellowish green plants, remote bilobed underleaves, small leaf cells without intermediate thickenings, strongly inflated, usually ball-shaped leaf lobules with involute free margins, ental lobular hyaline papilla, marginal leaf denticulations of Allorgella-type, lack of ocelli in the plant, smooth keels of perianths, truncate apex of the leaf lobule without any distinct teeth, usually 3–5 oil bodies per leaf cell (Fig. 2), male bracteoles present throughout the androecium, and lack of vegetative reproductive organs.

**Taxonomy**

**Gaolejeunea** R.L.Zhu et W.Ye, gen. nov.


**Autoicous. Plant pale green to light yellowish green.**

**Leaf insertion short. Leaf surface smooth, marginal denticulations of Allorgella-type, which are formed by the projecting corners of two adjacent cells, instead of one single cell. Leaf cells without or with very small trigones. Oil bodies small, finely segmented, (2–)3–5 per cell. Ocelli absent. Lobules small, ovate, with strongly incurved free margin; first lobule tooth reduced; second tooth indistinct; hyaline papilla ental, occasionally hanging distally at the apex of the lobule. Underleaves bifid, underleaf lobes slightly diverging. Gynoecial innovations absent. Perianth inflated, with 4–5 keels.**
Androecia terminal or proliferating vegetatively from apex, with bracteoles present throughout.

Etymology. Named for the late Professor Chien Gao, Institute of Applied Ecology, Chinese Academy of Sciences. His work has contributed greatly to our knowledge of Chinese bryology. Prof. Gao also collected the type specimen of *Cheilolejeunea gaoi* in 1974.

*Gaolejeunea gaoi* (R.L.Zhu, M.L.So et Grolle) R.L.Zhu et W.Ye, comb. nov. Fig. 2


Description and illustrations. See Zhu et al. (2000 as *Cheilolejeunea gaoi*).

Selected specimens examined. CHINA. GUANGXI: Shangsi Co., Shiwandashan National Forest Park, 21°53′30′′N, 107°54′28′′E, ca. 418 m, Ye & Wei 20090716-1 (HSNU), 20090717-1 (HSNU); Ibid., 21°53′51′′N, 107°54′56′′E, 406 m, Zhu et al. 20100822-15 (HSNU); Huangpaoshan, along the rivulet of a dam, 21°57′18.11′′N, 108°02′26.05′′E, 285 m, Zhu et al. 20140414-28B (HSNU), 20140414-29 (HSNU).

Habitats. On tree trunks, sometimes on wet rocks, tree roots and tree roots in moist lowland forests at altitudes of 193–691 m. For detailed habitats, see Supplementary Table S2.

With this addition Echinolejeuneinae contains six genera that can be separated in the following key.

**Key to genera of Echinolejeuneinae**

1. Scattered ocelli present in leaf lobes and underleaves.................
   1. Scattered ocelli absent in leaf lobes and underleaves.............. 2
2. Apex of underleaves undivided.................................. *Haplolejeunea*
   2. Apex of underleaves bifid.............................................. 3

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Figure 2. *Gaolejeunea gaoi*. A. Plant (ventral view). B. Habitat. C. Tip of a branch showing the shape of the underleaves. D. Oil bodies in leaf cells. E. Marginal leaf denticulations of *Allorgella*-type. C from Ye & Wei 20090715-45A (HSNU), the others from Zhu et al. 20100822-15 (HSNU).
3. Leaf lobe apex obtuse; perianths obcordate, with 2 broad lateral keels expanded above into distinct auricles. \textit{Allorgella}

3. Leaf lobe apex rounded; perianth not obcordate, with 4–5 keels

4. Leaf lobule apex truncate; hyaline papilla ental, occasionally hanging distally at the apex of the lobule; innovations absent; endemic to China. \textit{Gaolejeunea}

4. Leaf lobule apex with a small notch; hyaline papilla inserted in the notch; innovations pycnolejeuneoid; endemic to New Zealand...

5. Leaf surface spine-toothed with sharply pointed papillae; leaf margin somewhat uniformly convex, without strong curving. \textit{Echinolejeunea}

5. Leaf surface smooth, without cellular spines; leaf margin wavy curled. \textit{Kymatolejeunea}

**CONSERVATION BIOLOGY**

As a remarkable member of Lejeuneaceae, \textit{Gaolejeunea gaoi} is surprisingly narrowly distributed in Shangsi Co., Guangxi, China. This usually epiphytic species is restricted to heavily and critically threatened lowland forests. The first national red list of Chinese endangered bryophytes classified this species as critically endangered (CR) based on its small population and habitat, which has been threatened by human activities (Cao et al. 2006; Zhu & So 2003). Its status as CR is reconfirmed (criteria A3c, B1ab(iii), IUCN Red List Categories and Criteria Version 3.1, Second edition, 2012) in the up-to-date threatened species list of China’s higher plants (Qin et al. 2017). During recent years, intensive and thorough surveys have been conducted in Guangxi and adjoining regions, as well as in the Indochinese Peninsula (Shu et al. 2017). Among these collections, 18 specimens are identified as \textit{G. gaoi}, including the type. Specimens collected and examined are listed in **Supplementary Table S2**. Despite these efforts, there are still no records of \textit{G. gaoi} outside the range of its type locality, Shangsi Co. Results show that the occurrence of \textit{G. gaoi} is restricted to two locations, one inside the Shiwanshan National Forest, the other at a nearby resort called Huapingoashan, both deeply affected by human activities. According to our data, the extent of occurrence of \textit{G. gaoi} is less than 100 km², severely fragmented, and the extent and quality of habitat is continuing to decline, which meets the IUCN red list criteria B1ab(iii). The primary threat to \textit{G. gaoi} has been habitat loss in the past owing to deforestation, and the few remaining subpopulations continue to be exposed to this threat. The second area outside Shiwanshan National Forest where this species was recently found is at a local resort, along the riverside of a rivulet of a dam, with no protected status known that would prevent further loss of habitat in that area.

Therefore we strongly recommend \textit{in situ} or \textit{ex situ} conservation activity for this species.

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**LITERATURE CITED**


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Supplementary documents online:

Supplementary Table S1. Voucher information with GenBank accession numbers. Missing sequences are indicated by a dash (—).

Supplementary Table S2. List of specimens (all deposited in HSNU) collected and examined since the discovery of Gaolejeunea gaoi.