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Systematic position of the enigmatic liverwort *Mizutania* (Mizutaniaceae, Marchantiophyta) inferred from molecular phylogenetic analyses

Hiroaki Masuzaki,[1] Masaki Shimamura,[1] Tatsuwo Furuki,[2] Hiromi Tsubota,[3] Tomio Yamaguchi,[1] Haji Mohamed Abdul Majid [4] & Hironori Deguchi[1]

1 *Department of Biological Science, Graduate School of Science, Hiroshima University, Kagamiyama 1-3-1, Higashi-Hiroshima 739-8526, Japan*

2 *Natural History Museum and Institute, Chiba, Aoba-cho 955-2, Chuo-ku, Chiba-shi, Chiba 260-8682, Japan*

3 *Miyajima Natural Botanical Garden, Graduate School of Science, Hiroshima University, Mitsumaruko-yama 1156-2, Miyajima-cho, Hatsukaichi 739-0543, Japan*

4 *Biology Department, University Brunei Darussalam, Jalan Tungku Link, Gadong, Brunei Darussalam BE1410*

Author for correspondence: *Hiroaki Masuzaki, masuhiro@hiroshima-u.ac.jp*

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4 *Biology Department, University Brunei Darussalam, Jalan Tungku Link, Gadong, Brunei Darussalam BE1410*

Author for correspondence: *Hiroaki Masuzaki, masuhiro@hiroshima-u.ac.jp*

Abstract Phylogenetic analyses of liverworts using chloroplast *rbcL* and *rps4* sequences were performed with special reference to the enigmatic thalloid liverwort, *Mizutania riccardioides*. The results showed that *Mizutania* is nested within leafy liverwort family Calypogeiaceae (subclass Jungermanniidae), thereby refuting the traditional interpretation that *Mizutania* is a member of the simple thalloid liverworts (subclass Metzgeriidae) related to Aneuraceae. Male plants with three ranked bracts, which are newly discovered in this study, also show a close morphological affinity to those of leafy liverworts. Parallel evolution of the flattened gametophyte seems to have occurred sporadically in the Jungermanniidae in association with adaptation to prostrate growth on the ground or on living leaves. The unistratose thalloid features of *Mizutania* are interpreted as an extremely reduced or highly specialized form of a creeping leafy liverwort. The rudimentary sexual branches which develop on the margins of the thallus are similar in many characteristics to those of Calypogeiaceae. We reduce the monotypic family Mizutaniaceae to a synonym of Calypogeiaceae.

Keywords *Mizutania*; molecular phylogeny; *rbcL*; *rps4*

■ INTRODUCTION

Based on morphology, liverworts (Marchantiophyta) have traditionally been subdivided into the marchantioid group and jungermannioid group, the latter of which comprises two subgroups: the simple thalloid liverworts and the leafy ones (Schuster, 1966). Recent molecular phylogenetic analyses of liverworts (e.g., Heinrichs & al., 2005; Forrest & al., 2006; He-Nygrén & al., 2006; Crandall-Stotler & al., 2008) have brought unexpected results. For example the Treubiales, previously treated as members of the simple thalloids, were resolved as the earliest diverging group along with the leafy Haplomitriales. The Blasiales, previously treated as simple thalloid liverworts, were resolved in the complex thalloid liverworts. And the Pleuroziales, possessing leaves with water sacs, were resolved as sister to the Metzgeriales, comprising typical simple thalloid liverworts. Only in a single decade, the analyses led to significant alteration of the conventional liverwort classification (compare Crandall-Stotler & Stotler, 2000; Crandall-Stotler & al., 2008). However, several taxonomically enigmatic taxa still remain to be studied by modern analysis, in order to resolve their classification. The family Mizutaniaceae Furuki & Z. Iwats. is one of these enigmatic taxa.

Mizutania riccardioides Furuki & Z. Iwats. was described as a new species of liverwort based on specimens collected by M. Mizutani from Mt. Kinabalu, Borneo in 1963. Since then, it has been collected from several other localities in Borneo and also in Malay Peninsular Malaysia (Furuki & Iwatsuki, 1989). This taxon is characterized by ribbon-like, unistratose thalli. Developing young thalli and regenerant buds on the thallus margins have a wedge-shaped apical cell with two cutting faces (Inoue & al., 2008) but apical protective structures such as slime papillae or scales are absent. Short female branches, rhizoids and exogenous gemmae are often found on the thallus margins. The surface of the thallus and gemmae are covered with massive verrucae. *Mizutania* plants show a close morphological affinity with the prothalli of ferns, particularly those belonging to Hymenophyllaceae Mart. and Vittariaceae Ching. *Mizutania* had long been misidentified as a fern prothallius for over a quarter of a century and specimens had been neglected and stored away amongst unidentified bryophyte specimens in herbaria. However, the presence of perichaetial leaves surrounding the archegonia proved that *Mizutania* belongs to the bryophytes, because fern archegonia are always embedded in the prothallus and have no protective leafy structures (Furuki & Iwatsuki, 1989). In addition, branching of the thallus of *Mizutania* originates from an apical cell, which is characteristic of a typical bryophyte. Furthermore, marginal meristems of fern prothalli typically develop into branch thalli, a character not found in *Mizutania* (Inoue & al., 2008). Finally, the unicellular rhizoids and presence of oil bodies in the cells strongly support the genus as a member of the liverworts. Therefore, the thalli

of *Mizutania* are not similar to fern prothalli. The chromosome number, $n = 9$, is also an additional character suggesting that the taxon is a member of the liverworts (Inoue & Furuki, 1992); in liverworts, $n = 9$ is the most common number.

With a strong emphasis on the morphological characters mentioned above, *Mizutania* has been placed in its own family Mizutaniaceae (Furuki & Iwatsuki, 1989). Schuster (1992) and Crandall-Stotler & al. (1994) considered *Mizutania* to be a member of the simple thalloid liverworts and placed the Mizutaniaceae in the suborder Metzgeriineae. Recent classification studies have treated the families Mizutaniaceae, Aneuraceae, Metzgeriaceae and Vandiemeniaceae as closely related to each other and have retained these families in the Metzgeriales (Crandall-Stotler & al., 2008). Although *Mizutania* superficially resembles members of Aneuraceae, particularly *Riccardia*, there are many morphological differences. The thallus of *Riccardia*, one of the simplest forms in the Metzgeriales, is composed of multiple cell layers in cross-section, whereas *Mizutania* is consistently unistratose. *Riccardia* may form endogenous gemmae (Furuki, 1991; Schuster, 1992) and ventral or occasionally dorsal rhizoids on the thallus, while *Mizutania* produces exogenous gemmae and marginal rhizoids, and perichaetia originating from marginal cells (Furuki & Iwatsuki, 1989). The chromosome number of *Mizutania* of $n = 9$ (Inoue & Furuki, 1992) is also in contrast to the basic number of $n = 10$ in *Riccardia* (Hewson, 1970).

The systematic position of *Mizutania* is still enigmatic, because fertile plants with sporophytes have not yet been collected. In the present study, the ribulose bisphosphate carboxylase large subunit (*rbcL*) and small ribosomal protein 4 (*rps4*) chloroplast genes of *Mizutania* were newly sequenced. Phylogenetic analyses across Marchantiopyhta were carried out based on the *rbcL* and *rps4* sequences, focusing particularly on Mizutaniaceae. Based on the known and newly obtained morphological, molecular and ecological data, the systematic position of *Mizutania* and the evolutionary origin of its unique unistratose thallus are assessed.

■ MATERIALS AND METHODS

Taxon sampling. — We generated *rbcL* sequence data from 147 exemplars belonging to 111 genera in 58 families. Sequences for 55 exemplars, comprising 42 with complete and 13 with partial sequences, were newly obtained and for 92 exemplars were downloaded from GenBank. The full length of *rbcL* was 1428 bp in most of the studied taxa, except in *Blasia pusilla* L. where it was 1437 bp and *Cavicularia densa* Steph. where it was 1455 bp. Sequences for four mosses and three algal species were added to the dataset as outgroups. The full-length *rbcL* gene was newly sequenced for *Andreaea rupestris* Hedw. var. *fauriei* Besch., *Takakia lepidozioides* S. Hatt. & Inoue and *Tetraphis geniculata* Girg. Sequences for *Sphagnum cuspidatum* Ehrh., *Chara vulgaris* L., *Coleochaete orbicularis* Pringsh. and *Nitella translucens* (Persoon) Agardh were obtained from the DNA database. The *rps4* (609 bp) sequence data of leafy liverworts were generated from 56 exemplars belonging to 42 genera in 22 families. Six exemplars with partial sequences were newly obtained, and 50 were downloaded from GenBank. Sequences for *Marchantia polymorpha* L. and *Riccardia capensis* Arnell were added to the dataset as outgroups based on the result of phylogenetic analyses of *rbcL* sequences. For accession numbers and voucher information of the datasets, see Appendices S1 and S2 in the Electronic Supplement to this article. Liverwort taxonomy and nomenclature basically follows that of Crandall-Stotler & al. (2008). Fresh materials of *Mizutania* were collected from three localities in West Malaysia (Peninsular Malaya); Cameron Highlands (Aug. 2000, 2000 m alt., *T. Yamaguchi 18890*, *T. Furuki 16385*, *16391*; March 1989, 1440 m alt, *T. Furuki & T. Matsui 7659*), Fraser's Hill (Sep. 2005, 1200 m alt., *H. Masuzaki 147*, *170*, *T. Furuki 20448*, *T. Yamaguchi 25802*) and Genting Highlands (July 2007, 1600 m alt., *H. Masuzaki 894*). Voucher specimens are deposited in HIRO with duplicates in CBM.

DNA extraction and PCR amplification. — The protocol of the DNA-glass binding interaction method for extraction of total DNA and direct sequencing by PCR was following the method of Tsubota & al. (2005). For the denaturation of proteins in the DNA extraction, a half volume of chloroform was added to the tube and mixed by vigorous shaking for 5 min. The design of the PCR and DNA sequencing primers follows Tsubota & al. (1999). The list of the newly designed primers in this study is shown in Table 1.

Phylogenetic analysis. — Homology searches of the DNA databases using BLAST (Altschul & al., 1997) were carried out based on the *rbcL* and *rps4* sequences of *Marchantia polymorpha* (Ohyama & al., 1986, X04465). DNA sequences of the regions were pre-aligned using the program ClustalW v.1.82 (Thompson & al., 1994), and the alignment was refined manually against complete sequences with an editor program. Based on the DNA sequences, phylogenetic analyses were performed following the methods of Tsubota & al. (2004) and Ozeki & al. (2007). Prior to the construction of the phylogenetic tree, MrModeltest v.2.2 (Nylander, 2004) was used in hierarchical likelihood ratio tests and the Akaike information criterion (AIC; Akaike, 1974) to make a rational decision regarding the nucleotide-based substitution model that best fits our data. The best model was used for each analysis and for the approximately unbiased (AU) test (Shimodaira, 2002) in the final stage of the analysis scheme. Trees were constructed using the following six program packages to obtain the candidate topologies: (1) MEGA 4 (Tamura & al., 2007) with the neighbor-joining (NJ) method (Saito & Nei, 1987) using the TN93 model (Tamura & Nei, 1993) with a gamma distribution for rates among sites; (2) PAUP* (Swofford, 2002) with the MP method (Fitch, 1971) using a heuristic search of 1000 random addition analyses with tree bisection-reconnection (TBR) branch-swapping under the assumption of weighting transversions at 2; (3) PAUPRat (Sikes & Lewis, 2001) over PAUP* with the MP method to implement Parsimony Ratchet searches (Nixon, 1999) using the strategy with random weighting of each character in fifty runs of 200 iterations; (4) MOLPHY v.2.3b3 (Adachi & Hasegawa, 1996) with the maximum likelihood (ML) method using HKY85 model (Hasegawa & al., 1985) and TN93 model; (5) PHYML

v.2.4.4 (Guindon & Gascuel, 2003) with the ML method using GTR (Lanave & al., 1984; Tavaré, 1986; Rodriguez & al., 1990) + proportion invariant + gamma (GTR + I + G) model; and (6) MrBayes v.3 (Ronquist & Huelsenbeck, 2003) with the Bayesian inference (BI) method using the GTR + I + G model with 10,000,000 generations. The burn-in value was 100,000 and the value of sample frequency was 100. Based on the ML criteria (Felsenstein, 1981), a likelihood value was re-calculated for each topology obtained by NJ, MP, ML and BI methods using the program package PAML v.3.15 (Yang, 1997; updated March, 2006) with the GTR + G model and CONSEL v.0.1i (Shimodaira & Hasegawa, 2001; updated Sept. 26, 2005) in calculation of the *p*-value. The value relates to the reliability of the candidate topologies based on the AU test using the multiscale bootstrap technique to assess the significance of the difference between the likelihood values of the best and the other topologies. A 50% majority-rule condensed tree for the topologies with high ranking log-likelihood values that passed the AU test was also computed by MEGA 4.

Supporting values (calculated probabilities or the consensus of the resulting topologies) of more than 50% were overlaid to assess the robustness of each branch of the condensed topology: local bootstrap probabilities (LBP; Adachi & Hasegawa, 1996) with the ML method using the HKY85 model by MOLPHY; classical bootstrap probabilities (BP; Efron, 1979; Felsenstein, 1985) based on 10,000 replications using the NJ method by MEGA; Bayesian posterior probabilities by MrBayes (PPB); and the values of percentage of supported topologies with high ranking log-likelihood values that passed the AU test are shown on or near each branch (LBP/BP/PPB/AU; in %).

Table 1. Newly designed primer sequences used for PCR amplification and sequencing of the *rbcL* (5′ to 3′) and *rps4* (3′ to 5′) genes. The primer numbers correspond to the rough positions in the sequence of *Marchantia polymorpha* (Ohyama & al., 1986).

rbcL gene (5′ to 3′)	Sequences
Forward primers	
rbcL-26Fmas	AAGTCCCTCC CTACGAATCA A
HrL1	ATGTCACCAC AAACGGAGAC TAAAGCAGG
rbcL39Fmas	AAAGCTGGTG TTAAAGATTA
rbcL111Fmas	GCAGCATTTC GTATGAC
rbcL634Fmas	ATGCGTTGGA GAGA
rbcL920Fmas	CATGGTATGC ATTTCCGTGT
Reverse primers	
rbcL128Rmas	GTCATACGAA ATGCTGC
rbcL302Rmas	ACATAAGCAA TATATTGATT
rbcL650Rmas	CGATCTCTCC AACGCA
rps4 gene (3′ to 5′)	
Forward primers	
rps4 2Fmas	TGTCCCGTTA TCGAGGACC
rps4 275Fmas	AGATAATGTG ATCTTTCGAT TAGGTATGGC
rps4 347Fmas	CAATTGGTTA ATCATAGACA
rps4 553Fmas	CAAACGATTA ATCGTGAATGGA
Reverse primers	
rps4 305Rmas	GCCATACCTA ATCGAAAGAT CACATTATCT
rps4 531Rmas	TCCATTCACG ATTAATCGTT TG
rps4 605Rmas	ACTTGGCGAG AATAATATTC AAC
rps4 609RL	TTAAGCTTGA CGAGAATAAT ATTC

■ RESULTS

Morphological and ecological features of *Mizutania*. — In several localities in Peninsular Malaysia, *Mizutania riccardioides* inhabits the sides of mountain trails in rain forests at 100–2000 m alt. The species grows on various substrates such as tree bases, shaded moist banks, and moist, humic soil under slightly overhanging banks and away from direct sunlight (Fig. 1A–B), often intermixed with other liverworts such as *Arachniopsis major* Herzog, *Bazzania vitata* (Gottsche) Trev., *Psiloclada clandestina* Mitt., *Schiffneria hyalina* Steph. and *Zoopsis liukiuensis* Horik.

Thalli are prostrate on the substrate, pale bluish-green due to the massive verrucae on the surface (Fig. 1C–D), 5–20 mm long, 1.0–1.5 mm wide, unistratose (Fig. 1F), without costae and any apical protective structures such as slime papillae or scales. Irregular branches arise from the margins of the thallus (Fig. 1E). Young lateral branches and thalli possess an apical cell, however the mature thalli lack an apical cell. Cells of the thallus vary in shape and size, from rectangular to polyangular, 80–150 × 30–70 µm, with small or large trigones. Oil bodies are 10–30 per cell and consist of minute granules, 5–15 × 5–30 µm (Fig. 1G). Rhizoids are unicellular, scattered on the margins of the thallus, 0.5–1.5 mm long and 10 µm wide, and also arise from the basal cells of the perichaetium. Oval to oblong gemmae develop exogenously on the margins and surface of thallus; they are pale bluish-green and consist of 1–4 cells, 50–100 × 25–30 µm (Fig. 1H).

Sexuality is apparently dioicous. Male branches were newly discovered in a specimen from Cameron Highlands, Malaysia (*T. Furuki & T. Matsui 7659*). The male branches are short, with loosely triseriate bracts and develop on the margins of the thallus (Fig. 1I–J). In the six male branches that the authors investigated, no dorsiventrality was observed (Fig. 2). Outer bracts are ligulate, entire or bifid, consisting of 10–30 cells, and sometimes coalescent. Cells of the bracts are slightly smaller than those of the thallus, 25–60 × 20–50 µm. Short female branches with loosely triseriate bracts are also developed on the margins of the thallus (Fig. 1L–M). The archegonium is composed of a short stalk, an indistinct venter and a very short neck. The neck jacket is composed at most of 5 cells in perimeter, and 8–10 tiers of cells (Fig. 3). No morphological difference is recognizable between male and female bracts. Sporophytes are unknown.

Phylogenetic relationship of *Mizutania*. — A total of 664 distinct topologies based on the dataset of *rbcL* sequences were obtained in the NJ, MP, ML and BI trees, and 309 topologies passed the AU test. The best ML tree with supporting values (LBP/BP/PPB/AU) is shown in Fig. 4.

Mizutania was nested within the family Calypogeiaceae (including *Calypogeia*, *Eocalypogeia*, *Metacalypogeia*, *Mnioloma* and *Gongylanthus*) in the leafy liverworts. In our analysis using *rbcL* sequences, the phylogenetic position of *Gongylanthus* (Arnelliaceae) was controversial since the genus was positioned in its own clade according to the different trees obtained by BI, NJ, ML and MP. Moreover *Pleurozia*, an enigmatic leafy liverwort possessing the lenticular apical cell like that of metzgerialean liverworts, was resolved as sister to *Radula* (Fig. 4).

Relationships of several families in the Jungermannineae were not well resolved with *rbcL*. The authors therefore

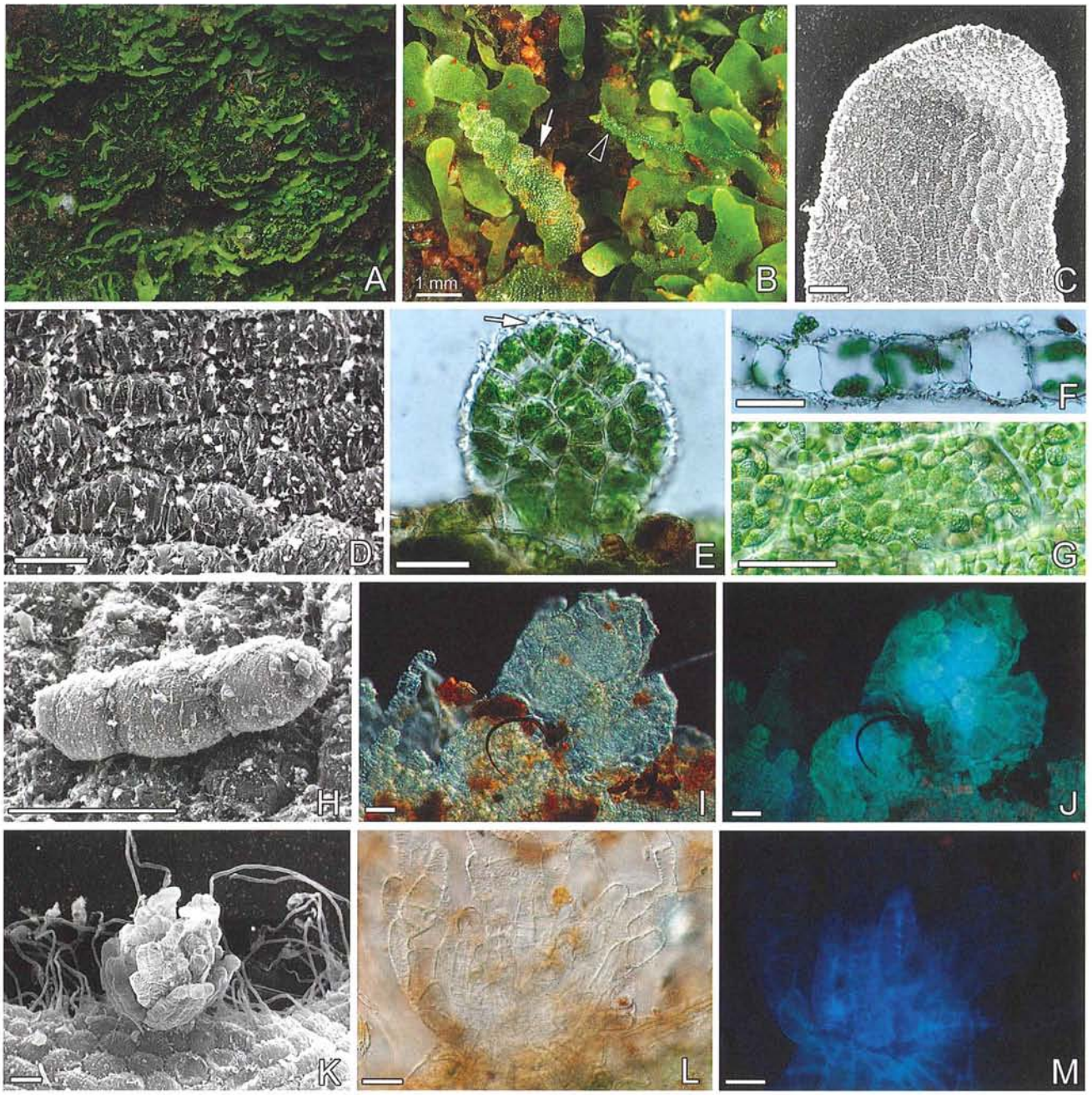

Fig. 1. *Mizutania riccardioides* Furuki & Z. Iwats. **A,** colony on the shaded moist banks (in Fraser's Hill, Peninsula Malaya, Malaysia); **B,** *M. riccardioides* sometimes intermixed with other flattened-form leafy liverworts such as *Schiffneria hyalina* Steph. (arrowhead) and *Zoopsis liukiuensis* Horik. (arrow) on humic soil along mountain trails or trails in forest or valley; **C,** the apex of the mature thallus lacking an apical cell and any apical protective structures; **D,** thallus dorsal surface covered with verrucae; **E,** young thallus developing from the margin of mature thallus as a regenerate bud; young thalli have an apical cell (arrowhead); **F,** transverse section of thallus; **G,** oil bodies consist of minute granules; **H,** exogenous gemmae covered with verrucae; **I,** male inflorescence on the thallus margin; **J,** globular antheridia clearly visualized by its autofluorescence under fluorescence microscope (FM); **K,** female inflorescence; **L,** archegonia surrounded with bracts; **M,** autofluorescence of archegonia under FM. Bars: C, 100 μm; D–M, 50 μm. I and J: the curved line in central is an insect foot.

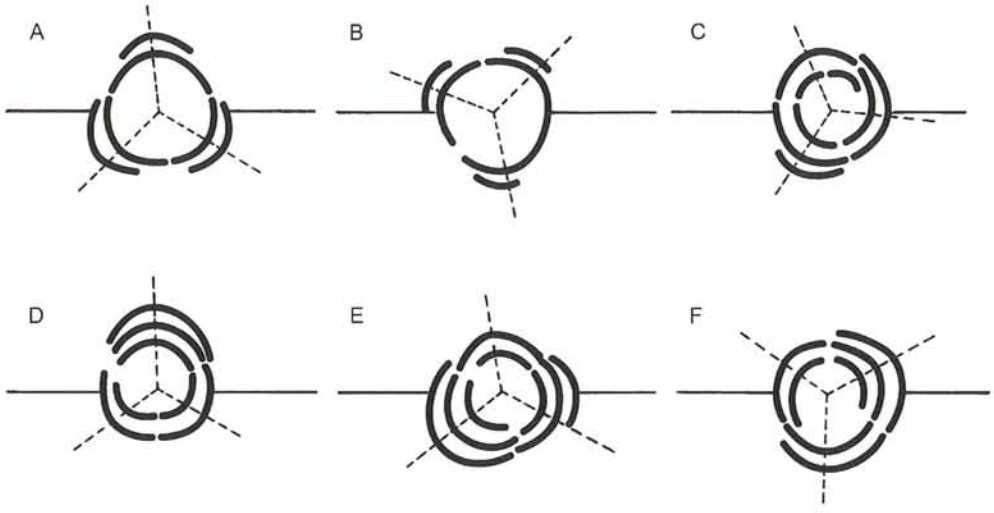

Fig. 2. Schematic illustration of the arrangement of bracts in transverse section based on the six male inflorescences of *Mizutania*. The antheridia surrounded with three ranked bracts, which have no dorsiventrality. The horizontal lines, curves and dash lines represent thalli, cross section of bracts and axes of bracts, respectively.

undertook additional analyses within the Jungermanniineae using the chloroplast *rps4* gene. A total of 95 distinct topologies were obtained in the BI, NJ, ML and MP trees, and 61 topologies passed the AU test. The best ML tree with supporting values (LBP/BP/PPB/AU) is shown in Fig. 5. *Mizutania* was nested within the well-supported family Calypogeiaceae. *Gongylanthus* was excluded from the Calypogeiaceae clade and the monophyly of Arnelliaceae including *Gongylanthus* and *Southbya* was well supported.

■ DISCUSSION

Re-evaluation of the morphology and phylogenetic position of *Mizutania*. — Based on thallus morphology, *Mizutania* was formally placed in the Metzgeriales as a monotypic simple thalloid liverwort related to the Aneuraceae. However, this classification is not supported by the morphology of its sexual reproductive structures. The spherical antheridia of *Mizutania* are protected by loosely arranged triseriate bracts. Protective structures of archegonia and antheridia in *Mizutania* resemble those of leafy liverworts (Jungermanniidae) rather than those of simple thalloid liverworts. Antheridia of Aneuraceae are enclosed within the thallus chamber and there are no leafy protective structures around the antheridia (Renzaglia, 1982). The archegonial neck of *Mizutania* consists of five rows of cells in the perimeter (Fig. 3) and this is typical of Jungermanniidae and Metzgeriaceae (this study). In Aneuraceae it is composed of six to seven rows of cells (Renzaglia, 1982). Jungermanniidae are distinguished from the thalloid Metzgeriidae in having tetrahedral apical cells with three cutting faces except for the Pleuroziaceae which have a lenticular apical cell with two cutting faces. Triseriate foliation on the sexual branches of *Mizutania* should have originated from a tetrahedral apical cell and the unistratose thallus from a two-sided apical cell with two cutting faces. Co-existence of a thalloid vegetative system with leafy sexual branches, as found in *Mizutania*, is also found in other 'thalloid' leafy liverworts, like *Cololejeunea metzgeriopsis* (K.I. Goebel) Gradst. & al. and *Radula yanoella* R.M. Schust. (Goebel, 1905; Schuster, 1984; Gradstein & al., 2006).

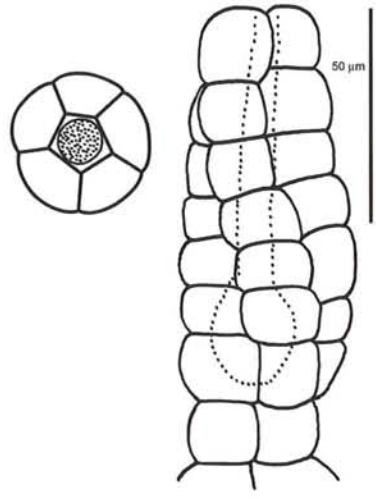

Fig. 3. Schematic illustration of the archegonium and its cross section. Neck jacket is composed of mostly five cells in perimeter.

Phylogenetic relationships among liverworts resulting from our analyses based on the *rbcL* and *rps4* sequences basically agree with other recent molecular phylogenetic studies (Forrest & Crandall-Stotler, 2004, 2005; Crandall-Stotler & al., 2005; Heinrichs & al., 2005, 2007; Forrest & al., 2006; He-Nygrén & al., 2006; Qiu & al., 2006; Hendry & al., 2007; Roo & al., 2007). Our phylogenetic analyses also revealed that *Mizutania* nests within the family Calypogeiaceae in the leafy liverworts (Figs. 4–5), thereby refuting the interpretation of *Mizutania* as a member of the simple thalloids (Fig. 4). The phylogenetic position of *Mizutania* as sister to the clade comprising *Mnioloma* and *Calypogeia* was shown in every analysis using *rbcL* and *rps4*. *Mizutania* should therefore be considered as a member of leafy liverworts, specifically the Calypogeiaceae (Jungermanniales suborder Jungermanniineae). Crandall-Stotler & al. (2009) suggested that the anomalous taxonomic placement of *Mizutania*

Fig. 4. The ML tree based on the analysis of the *rbcL* sequences. Numbers above the branches are LBP/BP/PPB/AU; in %. ▶