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Molecular contribution to the systematic position of *Mastopoma scabrifolium* (Broth. *in* Moell.) B.C. Tan & Tran Ninh (Sematophyllaceae, Bryopsida)

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Abstract – A new molecular cladistic study of 42 species (including *Mastopoma scabrifolium*) in 25 genera of Sematophyllaceae in Peninsular Malaysia and Singapore has revealed the non-monophyly of the genus *Mastopoma*. *Mastopoma scabrifolium* was shown in a MP phylogenetic tree based on *rbc*L sequences to be forming a separate clade with *Acanthorrhynchium papillatum*. A transfer of this species to *Acanthorrhynchium* is proposed. *Acanthorrhynchium scabrifolium* (Broth. *in* Moell.) B.C. Tan & Chang Ying is a new combination.

Musci / Sematophyllaceae / *rbc*L / cladistic / *Mastopoma* / *Acanthorrhynchium*

INTRODUCTION

Molecular evidence, such as *rbc*L and *trn*L sequences, is gaining importance in the taxonomy of Bryopsida these days. Species are transferred, new genera get created, and family grouping are re-organized on the basis of cladograms produced by molecular sequence alignments (Akiyama & Tsubota, 2001; Arikawa & Higuchi, 1999, 2003; Stech, 1999; Stech & Frahm, 1999a, 1999b, 2000, 2001; Tsubota *et al*., 2001a). Here we report the taxonomic implication revealed in the case of *Mastopoma scabrifolium* (Broth. in Moell.) B.C. Tan & Tran Ninh based on *rbc*L data obtained in connection with a morphological and molecular cladistic study of the Sematophyllaceae in Peninsular Malaysia and Singapore (Chang Ying, 2004).

Among the various genes used in plant phylogenetics, the *rbc*L gene was the one of the first plant genes sequenced and studied by systematists (Soltis and Soltis, 1998). Over the decades, it has been extensively applied in phylogenetic studies among different plant groups at different taxonomic levels. In bryophytes, the *rbc*L gene has been mainly used to reconstruct phylogenetic relationships at the generic and familial levels (e.g., Goffinet, Bayer and Vitt, 1998; De Luna *et al.,* 1999 and 2000). In the recent works by Arikawa and Higuchi (2003) and Tsubota *et al.* (2000, 2001a and 2001b), *rbc*L gene sequences were shown to be useful to address the relationships among closely related genera, mainly within the families

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Sematophyllaceae and Hypnaceae. Therefore, we chose *rbc*L gene as the molecular target in our investigation of the systematic position of *Mastopoma scabrifolium*.

On the basis of morphology, the generic placement of this species has been unsatisfactorily, resulting in its shifting into a number of genera, such as *Acanthocladium, Trichosteleum, Trismegistia* and *Mastopoma* (see Tan and Tran Ninh, 1998).

MATERIAL AND METHODS

Representatives of 42 species (including *M. scabrifolium*) in 25 genera of the families Sematophyllaceae, Hypnaceae, Myuriaceae and Thuidiaceae were included in a *rbc*L sequence study (Table 1). Twenty-six taxa were collected and sequenced by the authors, with the *rbc*L sequences of the remaining taxa downloaded from GenBank. The voucher specimens are deposited in SINU.

Total DNA was extracted from fresh specimens or herbarium specimens by a modified CTAB method (Doyle and Doyle, 1990). PCR reactions (2 min. at 94°C, 35 cycles of a regime of 30-45 sec. at 94°C, 30-45 sec. at 45-50°C, and 90-150 sec. at 72°C) were carried out to amplify the *rbc*L fragments. The primers are listed in Table 2.

PCR products were purified using the QIAquick purification kit (Qiagen). Cycle sequencing reactions (35 cycles of a regime of 30 sec. at 96°C, 15 sec. at 50°C, and 4 min. at 60°C) were carried out using the ABI PRISM kit (Applied Biosystems). The sequencing primers are listed in Table 2. Sequencing products were then analyzed on an ABI 3100 machine (Applied Biosystems).

An alignment of all the 41 sequences was created using CLUSTAL X (1.81) (Thompson *et al.*, 1997). To reconstruct the phylogenetic trees, PAUP* 4.0b10 (Swofford, 2002) was used in MP and ML analyses, while MrBayes v. 2.01 (Huelsenbeck and Ronquist, 2001) was employed in the BI method.

In the MP analyses, all the data were equally weighted. Heuristic searches were implemented with 1000 replicates of random taxon addition and TBR branch swapping. The confidence of branching was assessed by bootstrap analyses with 1,000 replicates using simple taxon addition.

In ML and BI analyses, the five commonly used models (JC69, K80, F81, HKY and GTR) were tested to compare the results. In ML analyses, site-specific rate variation was set according to the different codon positions. The analyses were carried out using heuristic searches with as-is addition and TBR branch swapping. In BI analyses, the number of Markov chains was four. The number of generation was 3,000,000 and the sampling frequency was one tree per 100 generations. The substitution rate was also set as site-specific.

RESULTS

Of the 42 moss species investigated in the study, 26 *rbc*L gene sequences were newly obtained by us. The complete coding region of *rbc*L gene for all 26 taxa is 1,428 bp, with no insertions or deletions. The overall result is congruent

Table 1. List of species and families included in this study (with GenBank accession numbers). (*) marks the taxa newly sequenced in this study. Voucher data are provided in the Appendix.

	Accession No.
Hypnaceae	
Hypnum cupressiforme Hedw.	AB 032077
**Isopterygium albescens* (Hook.) Jaeg.	AY 320234
**Vesicularia reticulata* (Doz. & Molk.) Broth.	AY 320253
Myuriaceae	
**Piloecium pseudorufescens* (Hampe) Müll. Hal.	AY 320247
Thuidiaceae	
Boulaya mittenii (Broth.) Card.	AB 024963
Sematophyllaceae	
Acanthorrhynchium papillatum (Harv.) M. Fleisch.	AB 051224
**Acroporium aciphyllum* Dixon	AY 320236
**Acroporium brevipes* (Broth.) Broth.	AY 320237
**Acroporium johannis-winkleri* Broth.	AY 320238
**Acroporium lamprophyllum* Mitt.	AY 320239
**Acroporium procerum* (Müll. Hal.) M. Fleisch.	AY 320240
**Acroporium rigens* (Broth. ex Dixon) Dixon	AY 320241
**Acroporium rufum* (Reinw. & Hornsch.) M. Fleisch.	AY 320242
**Acroporium stramineum* (Reinw. & Hornsch.) M. Fleisch.	AY 320243
**Acroporium strepsiphyllum* (Mont.) B.C. Tan	AY 320244
Brotherella henonii (Duby) Fleisch.	AB 029167
**Clastobryum cuculligerum* (Lac.) Tix.	AY 346096
**Gammiella tonkinensis* (Broth. & Par.) B.C. Tan.	AY 346097
Heterophyllium affine (Hook. ex Kunth) M. Fleisch.	AB 051218
**Isocladiella surcularis* (Dixon) B.C. Tan & H. Mohamed.	AY 320245
**Macrohymenium muelleri* Dozy & Molk.	AY 320246
**Mastopoma scabrifolium* (Broth.) B.C. Tan & Tran Ninh	AY 346098
Mastopoma subfiliferum Horik. & Ando	AB 071411
Mastopoma uncinifolium (Broth.) Card.	AB 071412
Meiothecium microcarpum (Harv.) Mitt.	AB 051223
**Papillidiopsis bruchii* (Dozy & Molk.) W.R. Buck & B.C. Tan	AY 320248
Papillidiopsis macrostica (Broth. & Par.) W.R. Buck & B.C. Tan	AB 051220
**Pterogonidium pulchellum* (Hook.) Müll. Hal.	AY 320249
Pylaisiadelpha tenuirostris (Bruch & Schimp. ex Sull.) W.R. Buck bbBuckBucBuckBuck	AB 051219
**Radulina hamata* (Dozy & Molk.) W.R. Buck & B.C. Tan	AY 320256
Sematophyllum subhumile ssp. japonicum (Broth.) Seki	AB 039675
**Taxithelium nepalense* (Schwäegr.) Broth.	AY 320250
**Trichosteleum fleischeri* B.C. Tan, Ho B.C. & B. Seah	AY 320235
**Trichosteleum singapurense* Fleisch.	AY 320251
Trichosteleum stissophyllum (Hampe & Müll. Hal.) Jaeg.	AB 051226
Trismegistia korthalsii (Dozy & Molk.) Broth.	AB 051228
**Trismegistia rigida* (Mitt.) Broth.	AY 320252
Trismegistia undulata Broth. & M.Yasuda	AB 051229
**Warburgiella leptocarpa* (Schwäegr.) M. Fleisch.	AY 320254
Wijkia deflexifolia (Ren. & Card.) H. Crum	AB 051221
Wijkia hornschuchii (Dozy & Molk.) H. Crum	AB 029383
**Wijkia tanytricha* (Mont.) H. Crum	AY 320255

Table 2. Primers used for PCR amplification and sequencing. Nucleotide sequences displayed from 5' to 3'. [(*) indicates the primers designed by the first author; the others were designed by Tsubota *et al.* (1999)]. *rbcL*-130f and *trnL*24r are the two primers used in normal PCR amplification.

Forward Primers	*Sequence*	*Tm (°C)*
rbcL-130f	ACA ATG ATA CTG TTT GTT ATA G (22)	50.9
*rbcL*152f	GAA TCC TCC ACT GGT ACA TG (20)	57.3
*rbcL*384f	AAG CTT TAC GAG CTT TAC G (19)	52.4
*rbcL*415f*	ACG TAT TCC TCC AGC TTA TTC C (22)	58.4
*rbcL*940f*	GGT GGA GAC CAT ATT CAC GC (20)	59.4
*rbcL*600r	GTG AAA TCA AGT CCA CCA CG (20)	57.3
*rbcL*957r	GCG TGA ATA TGG TCT CCA CC (20)	59.4
*rbcL*1160r*	CCA AAG ATT TCA GTT AAT GC (20)	51.2
*rbcL*1346hr	GCA GCT AAT TCA GGA CTC C (19)	56.7
*trnR*24r	CTC TAA TCC ACT GAG CTA CA (20)	55.3

with the result of the previous molecular study of Sematophyllaceae done by Tsubota *et al.* (1999). All the newly obtained sequences were submitted to GenBank. The accession numbers are listed in Table 1.

The MP analysis (heuristic search) yielded only one most parsimonious tree (length 685 steps, CI = 0.499, RI = 0.500). The most parsimonious tree is shown in Fig. 1. Bootstrap values (> 50%) are given above the branches.

In the single most parsimonious tree, the three *Mastopoma* species do not form one monophyletic clade (Fig. 1). Instead, *Mastopoma scabrifolium* shows a close relationship to *Acanthorrhynchium papillatum*. These two species form one clade with a high bootstrap value of 99%. *Mastopoma subfiliferum* forms a clade with *Trismegistia undulata.* The branch support for this clade is a high 98%. The other species of *Mastopoma, M. uncinifolium,* joins the two *Trismegistia* species forming another clade. This clade is supported by a bootstrap value of 91%.

Similarly, in the phylogenetic trees reconstructed using ML and BI methods, the same three clades mentioned above are present in all the cladograms generated.

DISCUSSION

Based on the MP cladogram (Fig. 1), *Mastopoma* is not a monophyletic genus. The three species of *Mastopoma* form three separate clades in association with species from other genera, all with high bootstrap values. In all other molecular trees generated by ML and BI analytical methods (trees not shown), *M. scabrifolium* consistently stands apart from the other two species of *Mastopoma* and forms a separate clade with *Acanthorrhynchium papillatum.*

This relatively isolated position of *M. scabrifolium* can also be seen in the pairwise comparision of the DNA sequences. There are a total of 25 nucleotides differences between *M. scabrifolium* and *Acanthorrhynchium papillatum* (20 transitions and 5 transversions). A difference of 1-30 nucleotide sequences between

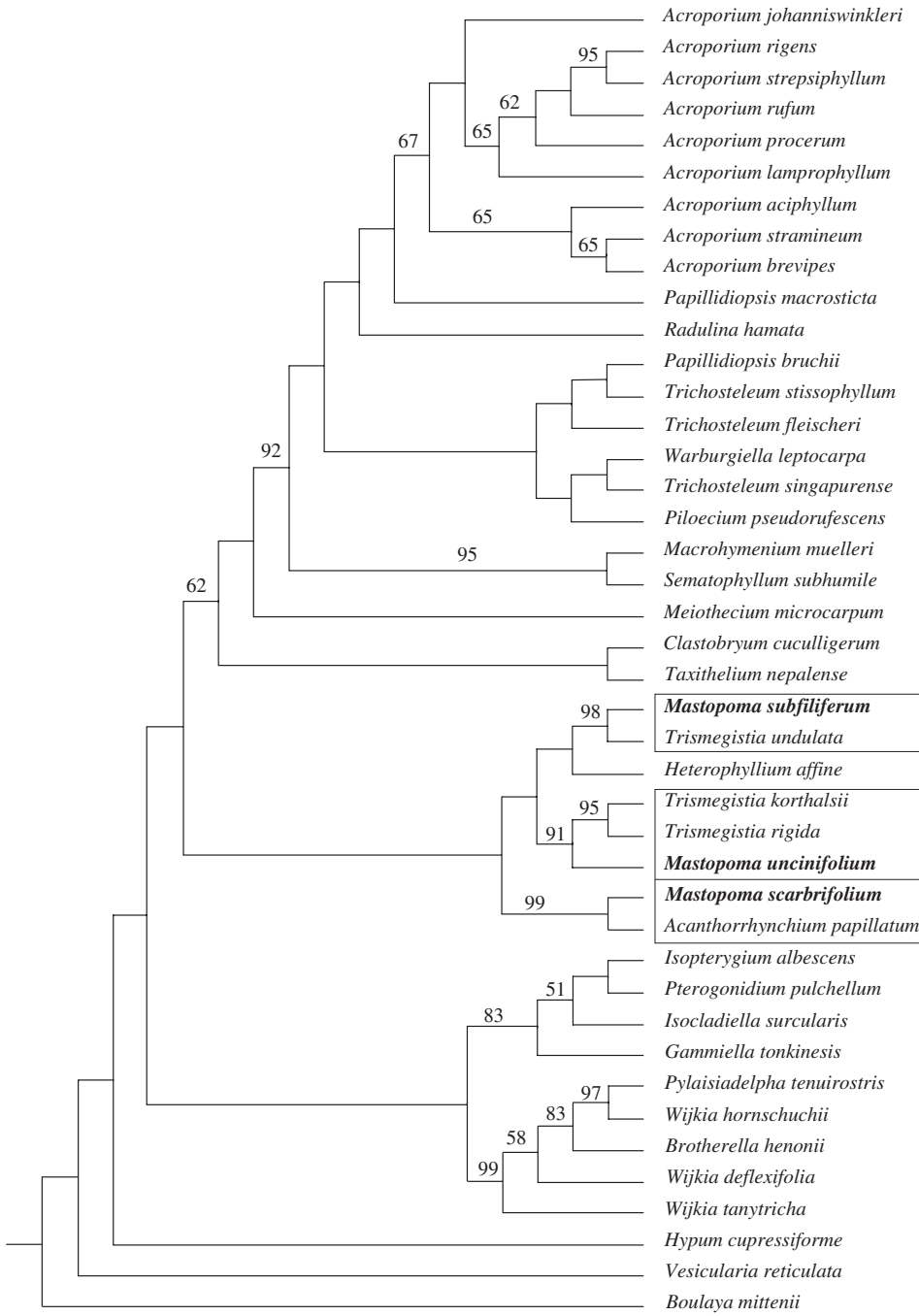

Fig. 1. The single most parsimonious tree based on MP analysis of *rbc*L gene sequences. The designated outgroup is *Boulaya mittenii*. Numbers above the branch are the bootstrap values (> 50%).

Appendix 1. Information on the specimens from which DNA was extracted for this study. All the voucher specimens are kept in SINU.

Scientific Name	*Voucher Number*	*Collection Locality*
Acroporium aciphyllum	sc8827	Antique, Philippines
Acroporium brevipes	cy0117	Gn.Beremban, Cameron Highland, Malaysia
Acroporium johannis-winkleri	cy0118	Gn.Beremban, Cameron Highland, Malaysia
Acroporium lamprophyllum	sc6409	Lake Kasudsuran, Ormoc city, Philippines
Acroporium procerum	cy0115	Gn.Brinchang, Cameron Highland, Malaysia
Acroporium rigens	cy0122	Gn.Brinchang, Cameron Highland, Malaysia
Acroporium rufum	cy0107	Gn.Brinchang, Cameron Highland, Malaysia
Acroporium stramenium	cy0119	Gn.Bunga Buah, Genting Highland, Malaysia
Acroporium strepsiphyllum	cy0101	Gn.Brinchang, Cameron Highland, Malaysia
Clastobryum cuculligerum	B02-0680	Maxwell Hill, Taiping, Malaysia
Gammiella tonkinensis	B02-0980	Fraser Hill, Malaysia
Isocladiella surcularis	cy0111	Gn.Brinchang, Cameron Highland, Malaysia
Isopterygium albescens	cy0255	Hillview Ave, Singapore
Macrohymenium muelleri	cy0103	Gn.Brinchang, Cameron Highland, Malaysia
Mastopoma scabrifolium	B02-0210	Gn. Panti, Malaysia
Papillidiopsis bruchii	cy0149	Endau Rompin, Malaysia
Piloecium pseudorefescens	cy0135	Endao Rompin, Malaysia
Pterogonidium pulchellum	cy0142	DBS botanical garden, NUS, Singapore
Radulina hamata	Tan01-956	Lake shore,Lake Duminagat, Malindang, Philippines
Taxithelium nepalense	cy0159	Bukit Timah, Singapore
Trichosteleum fleischeri	cy0251	MacRitchie Reservoir, Singapore
Trichosteleum singapurense	cy0252	MacRitchie Reservoir, Singapore
Trismegistia rigida	cy0132	Endau Rompin, Malaysia
Vesicularia reticulata	cy0140	DBS Botanical Garden, NUS, Singapore
Warburgiella leptocarpa	cy0106	Gn.Brinchang, Cameron Highland, Malaysia.
Wijkia tanytricha	cy0112	Gn.Brinchang, Cameron Highland, Malaysia

species of the same genus has been observed in *Acroporium* (unpublished data, Chang Ying, 2004). On the other hand, the differences between *M. scabrifolium* and the two *Mastopoma* species, *M. uncinifolium* and *M subfiliferum*, were 50 (40 transitions and 10 transversions) and 48 nucleotides (38 transitions and 10 transversions). Though there is no definite conclusion on how many nucleotides differences can be used to define a genus, this result provides the first molecular evidence that *M. scabrifolium* is a doubtful member of *Mastopoma.*

Based on the morphology, *Mastopoma scabrifolium* was first described as a species of *Acanthocladium*. The type collection came from Java (Moeller, 1919). Fleischer (1923) reduced it incorrectly to a form of *Trismegistia brauniana* (Bosch & Lac.) Flesich. Because of its strongly unipapillose leaf cells, the taxon was placed in the genus of *Trichosteleum* by Buck and Tan (1989). Later, Tan and Tran Ninh (1998), reporting on the similarity of its unipapillose leaf areolation with that of *M. papillosum* Broth., transferred it to *Mastopoma*. However, the two authors commented in the same publication that *M. scabrifolium* differs from *M. papillosum* and other species of the genus in having only a weakly toothed and differ-

entiated leaf border (Tan and Tran Ninh, 1998). Furthermore, *M. scabrifolium* exhibits a marked dimorphism between the stem and branch leaves, a feature not seen in *Mastopoma,* but present in *Acanthorrhynchium*. The stem leaves of *M. scabrifolium* are broadly ovate and cuspidate, while the branch leaves are lanceolate with acute to short acuminate apices (Tan & Tran Ninh, 1998). More importantly, the species was illustrated by Tan & Tran Ninh (1998) to have a clearly constricted leaf base where the much enlarged and vesicular alar cells are formed, again, a feature commonly seen in *Acanthorrhynchium papillatum* (Harv.) Fleisch.

With the new insight gained from molecular evidence and renewed analysis of morphological characters, we are convinced that *M. scabrifolium* is appropriately a species of *Acanthorrhynchium*. To effect a change of the species classification, the following new combination is proposed:

Acanthorrhynchium scabrifolium (Broth.) B.C. Tan & Chang Ying, *comb. nov*.

Basionym: *Acanthocladium scabrifolium* Broth. *in* Moell., *Hedwigia* 60: 326. 1919.

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