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## PHYLOGENETIC ANALYSIS OF THE FAMILY HYPNACEAE BASED ON *rbcL* GENE SEQUENCES

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### SUMMARY

The molecular phylogenetic analysis of 365 OTUs was carried out using nucleotide sequences of the chloroplast gene *rbcL*, focusing on the most diversified moss family Hypnaceae. Nucleotide sequences of *rbcL* were newly determined in 71 samples. Neither the family Hypnaceae nor the type genus *Hypnum* came together in a monophyletic group. Most of the relationships in the clade of hypnalean mosses corresponding especially to families remain obscure. However, some new findings shown in the following were obtained: (1) *Hypnum plumaeforme*, *H. sakuraii*, and *H. oldhamii* make a phylogenetic group with *Ectropothecium* spp. and *Vesicularia* spp., (2) *Gollania taxiphylloides* makes a monophyletic clade with *Taxiphyllum* spp. and *Hondaella brachytheciella*, and *G. splendens* makes a clade with *Homomallium japonico-adnatum*, while other species of *Gollania* come together in a monophyletic group.

KEYWORDS: Hypnaceae, Pylaisiaceae, *Hypnum*, *Stereodon*, *Gollania*

### INTRODUCTION

Since Schimper (1856) established the family Hypnaceae, some characteristic members have been isolated from the Hypnaceae and grouped into new families. Nishimura (1999) reviewed the history of the Hypnaceae. Several authors who revised neighboring families have transferred many genera to the Hypnaceae without due discussions

(e.g., Andrews, 1954; Seki, 1969; Noguchi, 1972; Buck, 1980; Nishimura *et al.*, 1984). Recently described hypnalean genera with double costa and smooth leaf laminal cells were placed in the Hypnaceae; *Horridohypnum* (Buck, 1981), *Andoa* (Ochyra, 1982), *Mahua* (Buck, 1983), *Caribaeohypnum* (Ando & Higuchi, 1984), *Irelandia* (Buck, 1984), *Podperaea*

(Iwatsuki & Glime, 1984), *Elharveya* (Crum, 1986), *Pseudotaxiphyllum* (Iwatsuki, 1987), *Taxiphyloopsis* (Higuchi & Deguchi, 1987), *Fallaciella* (Crum, 1991), etc. The Hypnaceae has thus been enlarged and heterogeneous, comprising more than 60 genera and 1000 species. Although the Hypnaceae have been variously circumscribed (e.g., Nishimura *et al.*, 1984), they remain as a rather heterogeneous group. Hedenäs (1989) mentioned that the Hypnaceae is a kind of catchall for genera taxonomically ill defined.

Delimitation of families of hypnalean mosses and their arrangement in phylogenetic sequence are extremely challenging. These families often received moderate support by molecular phylogenetic analyses, but inferences regarding their mutual relationships are rather weak (e.g., Tsubota *et al.*, 1999, 2002a; Buck *et al.*, 2000; Maeda *et al.*, 2000; Arikawa & Higuchi, 2002; Goffinet & Buck, 2004). The Hypnaceae are consistently resolved as a polyphyletic entity from molecular phylogenetic analyses (Arikawa & Higuchi, 1999, 2003; Tsubota *et al.*, 1999, 2002a, b; Buck *et al.*, 2000). Even the type genus *Hypnum* is shown to be polyphyletic.

The present paper is the first step toward the refinement of the family Hypnaceae to be a natural group. In order to redefine the Hypnaceae, we need to construct a reliable tree covering all genera in the order Hypnales. The ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit gene (*rbcL*) is a good molecular marker for constructing phylogenetic tree, because so many sequences are already available. The aim of this paper is to construct a preliminary phylogenetic tree of the Hypnaceae and related families using a large number of *rbcL* gene sequences, to delimit the Hypnaceae, and to make clear the relationship among hypnaceous taxa.

## MATERIALS AND METHODS

This study consists of two steps: (1) obtaining sequence data (DNA extraction, PCR amplification, DNA sequencing, and download from DNA database), and (2) data analysis (construction of phylogenetic trees). A total of 71 hypnalean samples and one sample of *Racomitrium aristatum* Mitt. were obtained from the fields, and the voucher specimens are deposited in the herbaria of the National Museum of Nature and Science, Japan (TNS) or Hiroshima University (HIRO). Methods of obtaining *rbcL* sequence data were essentially the same as our previous studies (Arikawa & Higuchi, 1999, 2002, 2003; Tsubota *et al.*, 1999, 2000, 2001a, b, 2002; Arikawa *et al.*, 2006). In addition to the newly obtained sequences, 288 *rbcL* sequences of hypnalean species and five sequences of *Racomitrium* spp. were downloaded from the DNA database of the DDBJ/EMBL/GenBank International Nucleotide Sequence Database Collaborations. A total of 365 sequences treated in this study were aligned manually. The undetermined sites were excluded from the analysis and determined sites were concatenated to a single data matrix. Bootstrap probabilities (BPs) were calculated with neighbor-joining (NJ) method using Clustal W 1.83 (Thompson *et al.*, 1994). A maximum-likelihood (ML) tree were searched with the local rearrangement method from the neighbor-joining (NJ) tree generated by the MOLPHY version 2.3b3 package (Adachi & Hasegawa, 1996; NucML 2.3b3 and NJdist 1.2.5).

## RESULTS

A total of 72 sequences of *rbcL* were newly sequenced and the sequences were deposited in the DDBJ/EMBL/GenBank International Nucleotide Sequence Database Collaborations. Voucher information and accession numbers of

the sequences were listed in Appendix A. A total length of 1,161 sites was used for the following phylogenetic analyses. Some identical sequences over the 1,161 sites were integrated into one sequence each. A total of 333 sequences among 365 sequences were then analyzed for constructing a phylogenetic tree. TN93 model was used for the analysis. The log-likelihood of the tree searched with the local rearrangement method from the neighbor-joining (NJ) tree was  $-22478.17 \pm 1180.69$  (Fig. 1). The nodes supported with both local bootstrap probabilities (LBPs; in %) 70% with ML method or higher and bootstrap probabilities (BPs; in %) calculated with NJ method 50% or higher are shown as solid lines in Fig. 1; the other nodes are shown as broken lines. Most of the relationships in the clade of hypnalean mosses corresponding especially to families remain obscure. As also shown in the recent studies (e.g., Arikawa & Higuchi, 1999; Goffinet *et al.*, 2001; Tsubota *et al.*, 2002), neither the family Hypnaceae nor the type genus *Hypnum* came together in a monophyletic group. However, some new findings were obtained: (1) *Hypnum plumaforme* Wilson, *H. sakuraii* (Sakurai) Ando, and *H. oldhamii* (Mitt.) A. Jaeger & Sauerb. made a phylogenetic group with *Ectropothecium* spp. and *Vesicularia* spp., (2) *Gollania taxiphylloides* Ando & Higuchi made a monophyletic clade with *Taxiphyllum* spp. and *Hondaella brachytheciella* (Broth. & Paris) Ando, and *G. splendens* (Lisiba) Nog. made a clade with *Homomallium japonico-adnatum* (Broth.) Broth., while other species of *Gollania* came together in a monophyletic group near the clade of *Ctenidium* and the Pylaisiaceae.

## DISCUSSION

Hedenäs (1989) pointed out that *Hypnum cupressiforme* Hedw., the type species of the genus *Hypnum*, is not closely related to most of the other

species commonly included in the genus. This conclusion was supported by all molecular phylogenetic analyses that involved at least several species of *Hypnum sensu lato* (e.g., Goffinet *et al.*, 2001; Tsubota *et al.*, 2002a; Gardiner *et al.*, 2005). The present analysis also confirmed this result. Gardiner *et al.* (2005) resurrected the family Pylaisiaceae based on the phylogenetic analysis deduced from ITS, *trnL-trnF* region and morphological data. The Pylaisiaceae *sensu* Gardiner *et al.* (2005) consists of *Homomallium*, *Pseudohygrohypnum*, *Pylaisia*, and *Stereodon* (the main part of *Hypnum sensu lato* except its type species; see also Ignatov & Ignatova, 2004). The Pylaisiaceae clade is recognizable in the present result (Fig. 1). Although the BP calculated with NJ method is not high (46%), *Hypnum plumaforme*, *H. sakuraii*, and *H. oldhamii* make a phylogenetic group with *Ectropothecium* spp. and *Vesicularia* spp. This group makes a weak clade with *Pylaisia*, *Homomallium*, *Foreauella*, *Hypnum fauriei* Cardot, and *H. calcicolum* Ando. This clade is recognizable as the Pylaisiaceae clade. Gardiner *et al.* (2005) did not include any species of *Ectropothecium*, *Vesicularia*, and *Foreauella* in their analyses. In order to delimit the ranges of the family Pylaisiaceae and the genera *Hypnum* and *Stereodon* respectively, further samplings of additional taxa would be necessary.

The genus *Gollania* is one of the members of the Hypnaceae and is most closely related to *Hypnum* (Higuchi, 1985). Most species of *Gollania* examined came together in a monophyletic group with strong statistical support (100% LBP and 98% BP with NJ), while *G. taxiphylloides* made a monophyletic clade with *Taxiphyllum* spp. and *Hondaella brachytheciella*, and *G. splendens* made a clade with *Homomallium japonico-adnatum*. *Gollania taxiphylloides* has been known to be a species endemic to Japan long before, but it has puzzled Japanese

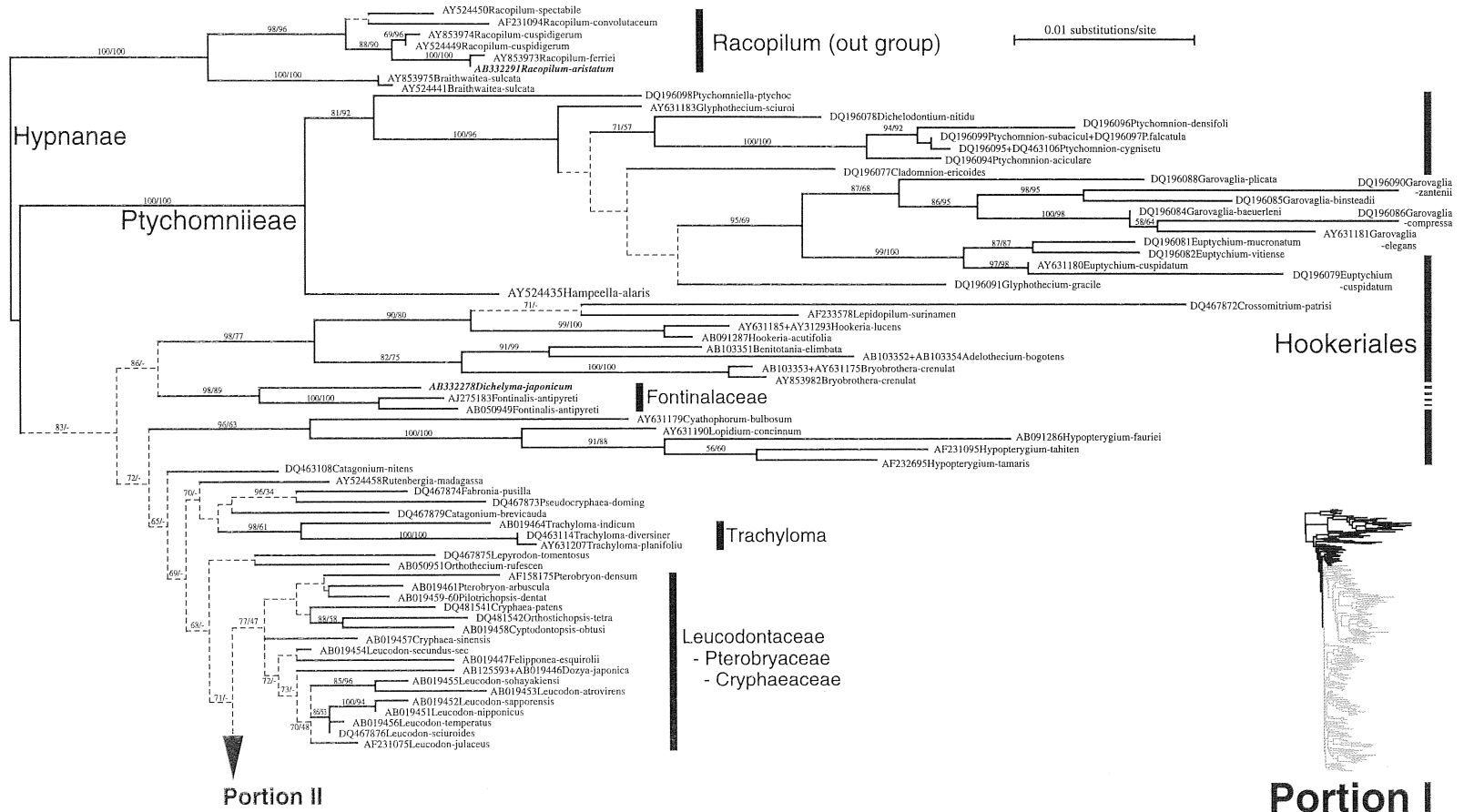
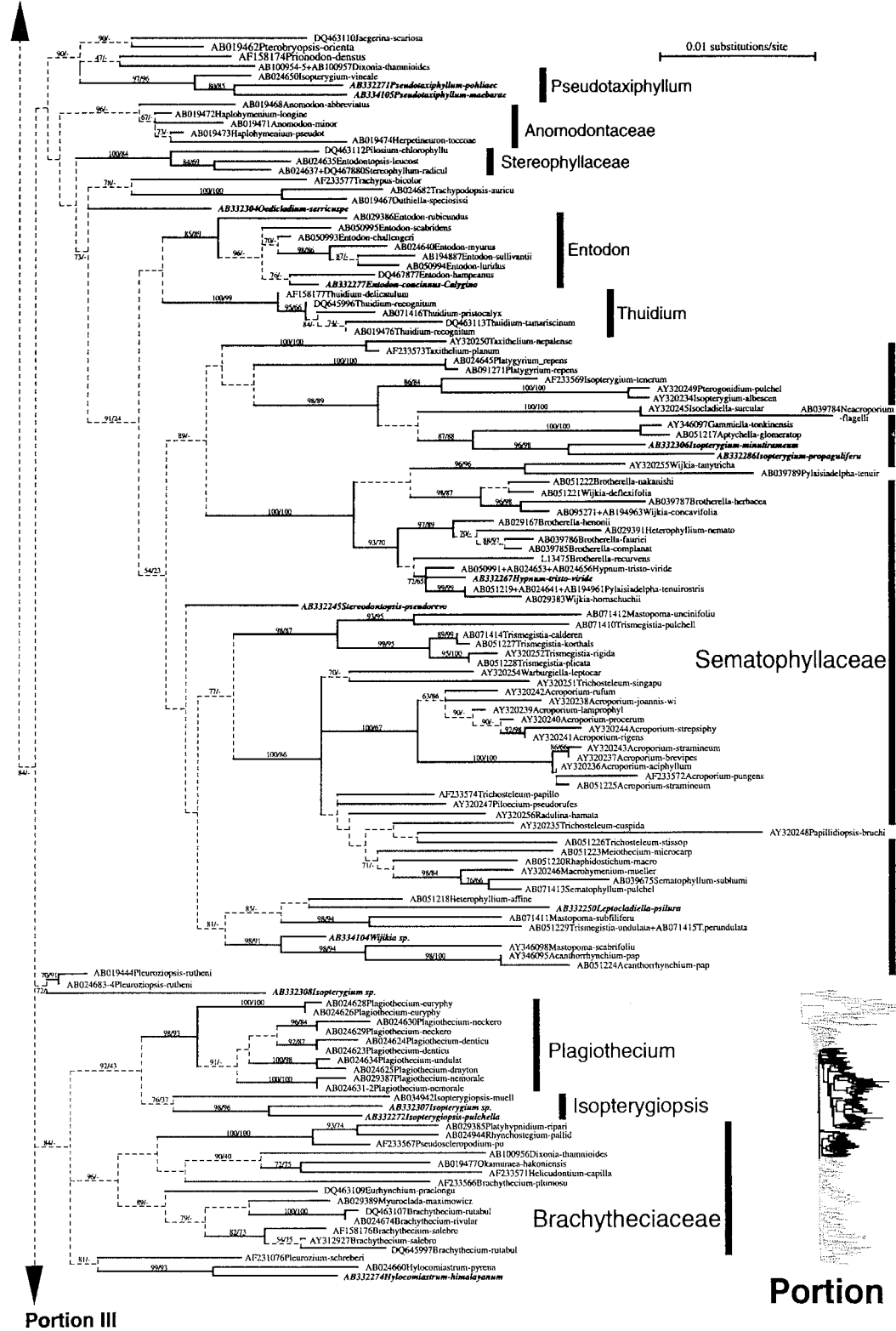


Fig. 1. The tree searched with the local rearrangement method from the neighbor-joining (NJ) tree of the aligned *rbcL* gene sequences (1,161 bp; TN93 model;  $2\alpha/\beta = 5.969$ ;  $\ln L = -22478.17 \pm 1180.69$  by NucML). The horizontal length of each branch is proportional to the estimated number of nucleotide substitutions. The root is arbitrarily placed on the branch leading to *Racopilum* spp. and *Braithwaitea* spp. The numbers above the nodes are the local bootstrap probabilities (LBPs; in %) and bootstrap probabilities calculated with NJ method (BPs; in %). LBPs 70% or higher and BPs 50% or higher are surely shown. The nodes supported with both LBPs 70% or higher and BPs 50% or higher are shown as solid lines; the other nodes are shown as broken lines. Sequences newly determined in this study are in *Bold-italic*.

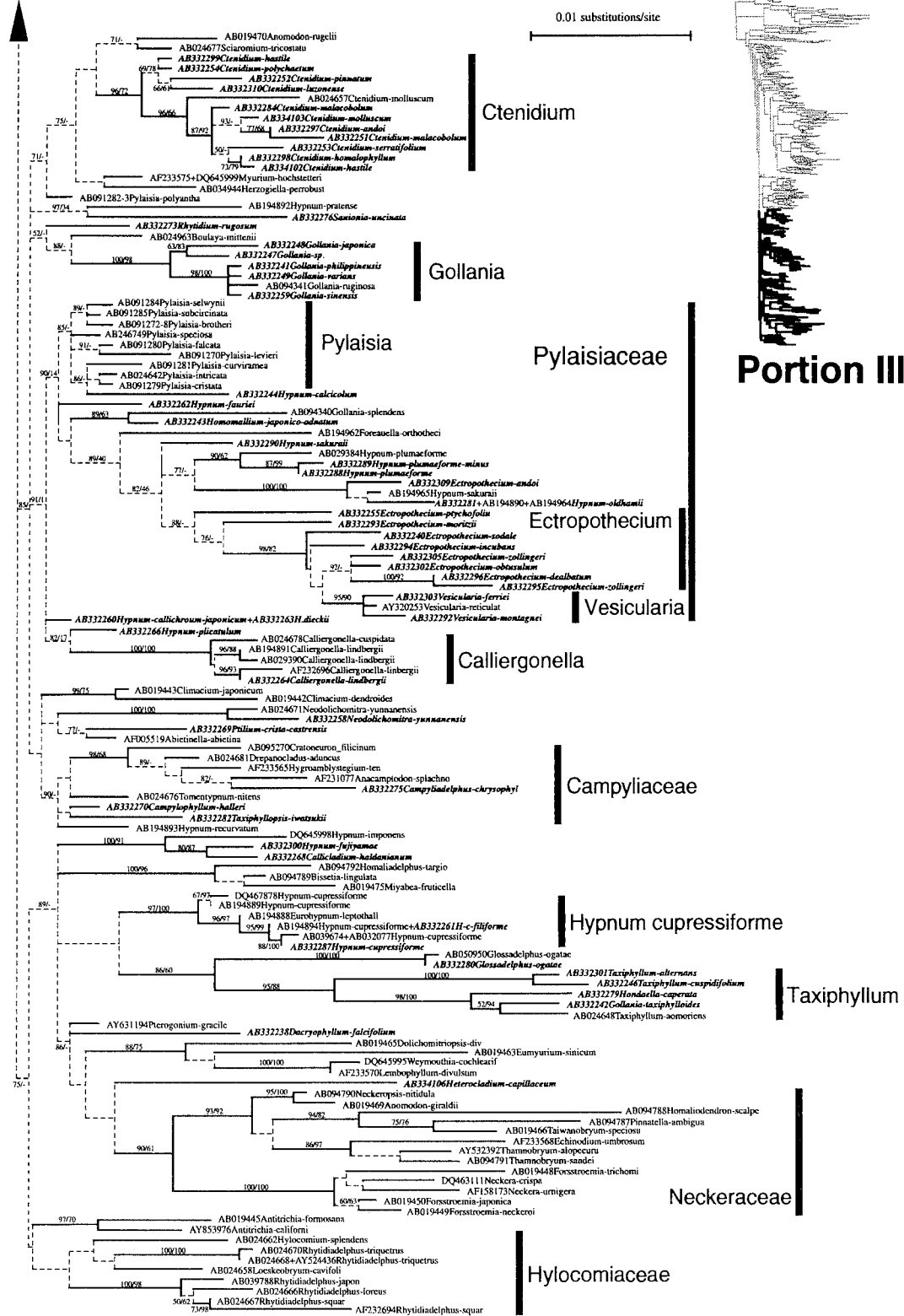
# Phylogenetic Analysis of Hynpnaceae Based on *rbcL*

Portion I



# Bryology in the New Millennium

Portion II



bryologists for the solution of its generic identity since it has been found only in sterile condition and has several characters overlapping with these allied genera: *Gollania*, *Hypnum*, and *Taxiphyllum* (Ando & Higuchi, 1981). The sporophytes of *G. splendens* also have not been found yet either. Further morphological studies and taxonomic treatments are needed for stabilizing their taxonomic positions.

Subgeneric taxa have been proposed in the genus *Hypnum* (e.g., Ando, 1973). Although all subgeneric taxa were included in this study, their segregation is not supported and the relationship remained obscure.

Buck (2007) pointed out that a combination of multiple genes with carefully observed morphology should provide us with a stable classification. He also mentioned, "to date, though, the largest group among the mosses lacking phylogenetic resolution is the Hypnales." Not only exhaustive taxon sampling but also application of more suitable genes than *rbcL* would be needed for constructing a phylogenetic tree with higher resolution. In order to circumscribe the family Hypnaceae, further molecular phylogenetic analysis based on multiple genes and careful reexaminations of morphological characters are needed.

#### ACKNOWLEDGEMENT

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## APPENDIX A

Taxon sampling and DDBJ/EMBL/GenBank accession numbers.

The sequence of information is: species, accession number, locality, and specimen number (herbarium). *Callicladium haldanianum* (Grev.) H.A. Crum, AB332268, Japan: Nagano, M. Higuchi 42196 (TNS); *Calliergonella lindbergii* (Mitt.) Hedenäs, AB332264, Japan: Iwate, N. Nishimura 11954 (TNS); *Campylocladus chrysophyllus* (Brid.) R.S. Chopra, AB332275, Japan: Nagano, M. Higuchi 37073 (TNS); *Campylophyllum halleri* (Sw. ex Hedw.) M. Fleisch., AB332270, Japan: Shizuoka, M. Higuchi 39601 (TNS); *Ctenidium andoi* N. Nishim., AB332297, Taiwan, N. Nishimura 12099 (TNS); *Ctenidium hastile* (Mitt.) Lindb., AB334102, Japan: Kochi, N. Nishimura 11145 (OKAY, TNS, HIRO); *Ctenidium homalophyllum* Broth. & Yasuda ex Iisiba, AB332298, Japan: Kochi, K. Kawai 2913 (TNS); *Ctenidium luzonense* Broth., AB332310, Japan: Kagoshima, Y. Tateishi 22288 (TNS); *Ctenidium malacobolum* (Müll.Hal.) Broth., AB332251, Taiwan, M. Higuchi 43795 (TNS); *Ctenidium malacobolum* (Müll.Hal.) Broth., AB332284, Japan: Miyazaki, N. Nishimura 12039 (TNS); *Ctenidium molluscum* (Hedw.) Mitt., AB334103, Japan: Kochi, N. Nishimura 11139 (OKAY, TNS, HIRO); *Ctenidium precrassum* Sakurai, AB332299, Japan: Okayama, N. Nishimura 12109 (TNS); *Ctenidium pinnatum* (Broth. & Paris) Broth., AB332252, Taiwan, M. Higuchi 43853 (TNS); *Ctenidium polychaetum* (Bosch & Sande Lac.) Broth., AB332254, Malaysia, Borneo, M. Higuchi 45075 (TNS); *Ctenidium serratifolium* Cardot (Broth.), AB332253, Malaysia, Borneo, M. Higuchi 44960 (TNS); *Dacryophyllum falcifolium* Ireland, AB332238, USA: California, K. M. Kellman 4674 (TNS); *Dichelyma japonicum* Cardot, AB332278, Japan: Iwate, N. Nishimura 11971 (TNS); *Ectropothecium andoi* N. Nishim., AB332309, Japan: Kagoshima, Y. Tateishi 22286 (TNS); *Ectropothecium dealbatum* (Reinw. & Hornsch.) A. Jaeger, AB332296, Taiwan, N. Nishimura

12085 (TNS); *Ectropothecium incubans* (Reinw. & Hornsch.) A. Jaeger, AB332294, Taiwan, N. Nishimura 12087 (TNS); *Ectropothecium moritzii* A. Jaeger, AB332293, Taiwan, N. Nishimura 12075 (TNS); *Ectropothecium obtusulum* (Cardot) Z. Iwats., AB332302, Japan: Ehime, N. Nishimura 12055 (TNS); *Ectropothecium ptychophyllum* N. Nishim., AB332255, Malaysia, Borneo, M. Higuchi 45055 (TNS); *Ectropothecium sodale* (Sull.) Mitt., AB332240, Vanuatu, M. Higuchi 39817 (TNS); *Ectropothecium zollingeri* (Müll.Hal.) A. Jaeger, AB332295, Taiwan, N. Nishimura 12108 (TNS); *Ectropothecium zollingeri* (Müll. Hal.) A. Jaeger, AB332305, Japan: Ogasawara Isls., Y. Tateishi 20556 (TNS); *Entodon concinnus* ssp. *calyginosus* (Mitt.) Mizush., AB332277, Japan: Kochi, K. Kawai 2844 (TNS); *Glossadelphus ogatae* Broth. & Yasuda, AB332280, Japan: Miyazaki, M. Higuchi 47056 (TNS); *Gollania japonica* (Cardot) Ando & Higuchi, AB332248, Taiwan, M. Higuchi 42036 (TNS); *Gollania philippinensis* (Broth.) Nog., AB332241, Taiwan, M. Higuchi 40681 (TNS); *Gollania sinensis* Broth. & Paris, AB332259, China, Sichuan, M. Higuchi 29776 (TNS); *Gollania taxiphyllodes* Ando & Higuchi, AB332242, Japan: Tokyo, M. Higuchi 47030 (TNS); *Gollania varians* (Mitt.) Broth., AB332249, Japan: Kagoshima, M. Higuchi 44796 (TNS); *Gollania* sp., AB332247, Taiwan, M. Higuchi 43851 (TNS); *Heterocladium capillaceum* Broth. ex Iisiba, AB334106, Japan: Hiroshima, H. Tsubota 4977 (HIRO); *Homomallium japonico-adnatum* (Broth.) Broth., AB332243, Japan: Tokyo, M. Higuchi 47039 (TNS); *Hondaella caperata* (Mitt.) B.C.Tan & Z.Iwats., AB332279, Japan: Tokyo, M. Higuchi 47036 (TNS); *Hylocomiastrum himalayanum* (Mitt.) Broth., AB332274, Japan: Nagano, M. Higuchi 43016 (TNS); *Hypnum calcicolum* Ando, AB332244, Japan: Tokyo, M. Higuchi 47019 (TNS); *Hypnum callichroum* ssp. *japonicum* Ando, AB332260, Japan: Nagano, M. Higuchi 43042 (TNS); *Hypnum cupressiforme* Hedw., AB332287, Japan: Fukui, N. Nishimura 12008 (TNS);

- Hypnum cupressiforme* var. *filiforme* Brid., AB332261, Russia, Saint Petersburg, T. Yoshida s. n. (TNS); *Hypnum dieckii* Renauld & Cardot, AB332263, Japan: Iwate, N. Nishimura 11987 (TNS); *Hypnum fauriei* Cardot, AB332262, Japan: Tokyo, M. Higuchi 47011 (TNS); *Hypnum fujiyamae* (Broth.) Paris, AB332300, Japan: Iwate, K. Kawai 3541 (TNS); *Hypnum oldhamii* (Mitt.) A. Jaeger & Sauerb., AB332281, Japan: Miyazaki, M. Higuchi 47065 (TNS); *Hypnum plicatulum* (Lindb.) A. Jaeger & Sauerb., AB332266, Japan: Nagano, M. Higuchi 43097 (TNS); *Hypnum plumaeforme* Wilson, AB332288, Japan: Miyazaki, N. Nishimura 12029 (TNS); *Hypnum plumaeforme* var. *minus* Broth. ex Ando, AB332289, Japan: Miyazaki, N. Nishimura 12026 (TNS); *Hypnum sakurarii* (Sakurai) Ando, AB332290, Japan: Miyazaki, N. Nishimura 12030 (TNS); *Hypnum tristo-viride* (Broth.) Paris, AB332267, Japan: Nagano, M. Higuchi 43159 (TNS); *Isopterygiopsis pulchella* (Hedw.) Z. Iwats., AB332272, Japan: Nagano, M. Higuchi 41549 (TNS); *Isopterygiopsis pulchella* (Hedw.) Z. Iwats. AB332307, New Zealand, South Isl., M. Higuchi 46764 (TNS); *Isopterygium minutirameum* (Müll.Hal.) A. Jaeger, AB332306, Taiwan, N. Nishimura 12095 (TNS); *Isopterygium propaguliferum* Toyama, AB332286, Japan: Miyazaki, N. Nishimura 12037 (TNS); *Isopterygium* sp., AB332308, New Zealand, South Isl., M. Higuchi 46988 (TNS); *Leptocradiella psilura* (Mitt.) M. Fleisch., AB332250, Taiwan, M. Higuchi 40848 (TNS); *Neodolichomitra yunnanensis* (Besch.) T.J. Kop., AB332258, China, Sichuan, M. Higuchi 29749 (TNS); *Oedocladium serricuspe* (Broth.) Nog. & Z. Iwats., AB332304, Japan: Kagoshima, N. Nishimura 11929 (TNS); *Pseudotaxiphyllum maebarae* (Sakurai) Z. Iwats., AB334105, Japan: Hiroshima, H. Tsubota 4956 (HIRO); *Pseudotaxiphyllum pohliaecarpum* (Sull. & Lesq.) Z. Iwats., AB332271, Japan: Ibaraki, M. Higuchi 47050 (TNS); *Ptilium crista-castrensis* (Hedw.) De Not., AB332269, Japan: Nagano, M. Higuchi 41606 (TNS); *Racopilum aristatum* Mitt., AB332291, Japan: Miyazaki, N. Nishimura 12025 (TNS); *Rhytidium rugosum* (Ehrh. ex Hedw.) Kindb., AB332273, Japan: Nagano, M. Higuchi 43103 (TNS); *Sanionia uncinata* (Hedw.) Loeske, AB332276, Japan: Nagano, M. Higuchi 42944 (TNS); *Stereodontopsis pseudorevoluta* (Reimers) Ando, AB332245, Japan: Kochi, K. Kawai 2810 (TNS); *Taxiphyllopsis iwatsukii* Higuchi & Deguchi, AB332282, Japan: Okayama, H. Kiguchi s. n. (TNS); *Taxiphyllum alternans* (Cardot) Z. Iwats., AB332301, Japan: Okayama, M. Chishiki 4900 (TNS); *Taxiphyllum cuspidifolium* (Cardot) Z. Iwats., AB332246, Japan: Tokyo, M. Higuchi 46994 (TNS); *Vesicularia ferriei* (Cardot & Thér.) Broth., AB332303, Japan: Ehime, N. Nishimura 12045 (TNS); *Vesicularia montagnei* (Schimp.) Broth., AB332292, Taiwan, N. Nishimura 12064 (TNS); *Wijkia* sp., AB334104, Australia: Tasmania, H. Deguchi 36184 (HIRO).