

ON THE PHYLOGENETIC POSITION OF THE GENUS *CLAPODIUM*:
A REVIVAL OF A 19TH CENTURY IDEA

О ПОЛОЖЕНИИ РОДА *CLAPODIUM* В ФИЛОГЕНИИ МХОВ:
ВОЗВРАЩЕНИЕ К ИДЕЕ 19 ВЕКА

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Abstract

Molecular phylogenetic analysis of nuclear ITS and plastid *rps4-trnS* found species of the genus *Claopodium* in the clade formed by species of Brachytheciaceae and Meteoriaceae, including Trachypodaceae. *Claopodium* is resolved as sister to Brachytheciaceae, thus we suggest its placement in this family, despite it will be the only taxon of the family with pluripapillose laminal cells. The taxonomic value of the papillose leaf cells in pleurocarpous mosses is discussed.

Резюме

Молекулярно-филогенетический анализ последовательностей ДНК ядерного (ITS) и хлоропластного (*rps4-trnS*) участков выявляет положение рода *Claopodium* в кладе, включающей Brachytheciaceae, Meteoriaceae и Trachypodaceae. Большинство анализов показывает сестринское положение *Claopodium* к Brachytheciaceae, и, соответственно, мы относим его к этому семейству, несмотря на то, что он, таким образом, оказывается единственным родом семейства, в котором клетки листа имеют папиллы, одиночные или множественные. Обсуждается таксономическая значимость признаков папиллозности клеток листа у бокоплодных мхов.

KEYWORDS: Brachytheciaceae, Meteoriaceae, nrITS, *rps4-trnS*, papillose cells, branch initials

INTRODUCTION

Pleurocarpous mosses of the order Hypnales with 4400 species include more than one third of the current species diversity of bryophytes (Huttunen *et al.*, 2012a). The order forms a terminal clade in moss phylogeny and its lineages were diversified much more rapidly as compared to acrocarpous mosses (Shaw *et al.*, 2003; Laenen *et al.*, 2014). The classification of the order Hypnales at the family level is especially difficult, because peristomial characters used in the Fleischer–Brotherus system of pleurocarps appeared to be highly homoplasious. Molecular phylogenetic data showed that they are largely associated with epiphytism (Huttunen *et al.*, 2004, 2012b; Hedenäs, 2012).

Molecular phylogenetic approach has resulted in changes of the genera affiliation in many families, and these corrections are still continuing for some genera that were least considered in the course of general revisions. The genus *Claopodium* (Lesq. & James) Renaud & Carot is one of such ‘hanging’ taxa.

Claopodium was originally described as a subgenus of the genus *Hypnum*, which included at that time a vast

majority of pleurocarpous moss species. While “Bryologia Europaea” introduced many narrower conceived genera for European species (Bruch *et al.*, 1836–1855), in the other regions of the world *Hypnum sensu lato* persisted (Müller, 1851; Mitten, 1859; Dozy & Molkenboer, 1855–1870). Lesquereux & James (1884) followed the latter tradition, accepting *Heterocladium*, *Thuidium*, *Eloidium* and other taxa at the subgeneric rank.

In the original description of *Hypnum* subgen. *Claopodium*, Lesquereux & James (1884) underlined its similarity with *Thuidium* due to papillose leaf cells and leaf shape, and also with *Eurhynchium* in the absence of paraphyllia and in the peristome structure, although they did not explain which details of the latter structure they meant. They also noted that *Hypnum* subgen. *Claopodium* differs from both of these genera in the form of capsule and lid. Grout (1928) selected *C. whippleanum* as a type of the genus.

The presence of papillae was considered to be an important character state in the Fleischer–Brotherus system of bryophytes which dominated in the 20th century. Relying on the importance of papillae, Brotherus (1925)

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defined, e.g. Theliaceae with *Thelia*, *Myurella* and *Fauriella* (now placed in Theliaceae, Plagiotheciaceae, and Pylaisiadelphaceae), and Thuidiaceae, that included, among others, *Anomodon* (now Anomodontaceae), *Heterocladium* (polyphyletic, but the type of the genus resolved in Neckeraceae; Enroth *et al.*, 2019), *Leptopterigynandrum* (Taxiphyllaceae), *Miyabea* (Miyabeaceae), and *Claopodium*. Crum & Anderson (1981) included in the Thuidiaceae also *Myurella*, *Pseudoleskea*, and *Pseudoleskeella*, obviously because of the presence of papillae in some species.

The doubts about so high importance of papillae appeared since the transfer of *Myurella* to the Plagiotheciaceae, first by placing it in the group of genera with axillary rhizoids (Hedenäs, 1987) and then more formally, with support of molecular data (Hedenäs & Pedersen, 2002).

Then Anomodontaceae were excluded from the Thuidiaceae, because of strongly differentiated peristomes (Buck & Crum, 1990). Later one of species of *Anomodon*, *A. giralddii*, was found to be closely related to *Homalia* and even placed in that genus (Olsson *et al.*, 2010). Alternative suggestion for placement of this species and also a closely related *A. attenuatus* was suggested by Ignatov *et al.* (2019), who revived an idea of Limpricht to segregate subgenus *Pseudanomodon*, which is very close to *Homalia* and thus obviously belongs to Neckeraceae.

Claopodium is currently accepted in Leskeaceae (Goffinet *et al.*, 2009; Frey & Stech, 2009), following the revision of the limit between Thuidiaceae and Leskeaceae by Buck & Crum (1990), when many genera of Thuidiaceae were transferred to Leskeaceae.

The genus *Claopodium* was included in a number of molecular phylogenetic analyses (Gardiner *et al.*, 2005; Ignatov *et al.*, 2007; Huttunen *et al.*, 2012a) and these analyses found it in a position sister to Brachytheciaceae. However, no taxonomic resolutions were made, mainly because of limited sampling, thus *Claopodium* remained in Leskeaceae (Goffinet *et al.*, 2009; Frey & Stech, 2009) or Thuidiaceae (e.g. Tropicos, <https://www.tropicos.org/nameSearch>, accessed 10 June 2020; http://www.theplantlist.org/tpl/search?q=Claopodium&_csv=on, accessed 10 June 2020) despite it was already clear that the genus does not relate to these families.

The present study aims to resolve the familial position of *Claopodium*. The original hypothesis was that the genus needs segregation in a separate family.

MATERIAL AND METHODS

Sampling and molecular markers. Nuclear ITS was used in phylogeny reconstruction, as it is most variable of widely used molecular markers in pleurocarpus mosses and therefore usually suits the resolving of relationships even among terminals, and there is more sequences available in GenBank than for any other marker. We tested variation in several plastid regions and found that *rps4-trnS*, if combined with ITS, is variable enough for resolving deep nodes in our study group. Additionally

sequences were available in GenBank for several species in our study groups. The dataset was formed by selecting most similar groups by BLAST and by previously published results. Only a limited number of new sequences were added for Meteoriaceae, for many of which only ITS2 region was studied previously (Huttunen *et al.*, 2007; Huttunen & Quandt, 2007).

DNA isolation, PCR-amplification and sequencing.

Extraction, PCR and sequencing protocols for sequences generated in earlier projects are described in Huttunen *et al.* (2008) and Huttunen & Ignatov (2010). For PCR-amplification of chloroplast region *rps4-trnS* primers *trnS-F* and *rps5'* from Hernández-Maqueda *et al.* (2008) were used. Laboratory work for newly sequenced samples was done in the molecular laboratory in the Turku University Herbarium (TUR), University of Turku, and in the molecular laboratory in N.V. Tsitsin Main Botanical Garden, Moscow. DNA was extracted using the Nucleospin Plant II DNA Extraction Kit (Machery-Nagel) following the respective manufacturer's protocol. Uncleaned PCR products were sent to Macrogen Inc., South Korea (www.macrogen.com) for purification and sequencing. Sequences were edited manually with PhyDE® v0.9971 (Müller *et al.*, 2005). All sequences are deposited in EMBL (European Molecular Biology Laboratory) or NCBI (The National Center for Biotechnology Information) GenBank. The sequencing protocol in the molecular laboratory of the N.V. Tsitsin Main Botanical Garden differed so that amplification products were separated on a 1% agarose gel in 1x TAE with ethidium bromide staining and purified using MinElute © Gel Extraction Kit (Qiagen, Germany). Purified PCR products were sequenced using the ABI PRISM © BigDye™ Terminator v.3. kit (Applied Biosystems) and further analyzed on an ABI PRISM 3730 automated sequencer (Applied Biosystems) at the "Genom" Common Facilities Centre, Moscow, Russia. Data on sequences generated de novo are in Appendix, while for others the Genbank number is shown in trees.

Sequence editing and phylogenetic analyses. Alignment of the sequence data was performed in Bioedit using alignment from Huttunen & Ignatov (2010) as scaffold. Bayesian Analyses were performed in MrBayes 3.2.6 (Ronquist *et al.*, 2012), with 20,000,000 generations, four runs, 25% trees burn-in, and chain temperature 0.02. Convergence of each analysis was evaluated using Tracer1.4.1 (Rambaut & Drummond, 2007). Maximum Likelihood (ML) trees were estimated using RAXML 8.2.12 (Stamatakis, 2014) from 1000 independent searches each starting from distinct random trees. Analyses were performed on the Cipres Science Gateway (<http://www.phylo.org/portal2>) on XSEDE (Miller *et al.*, 2010).

Myurium was selected as the outgroup for rooting the trees due to its position in the sister clade to Brachytheciaceae and Meteoriaceae in a broader analysis of the pleurocarpus mosses (Huttunen *et al.*, 2012a).

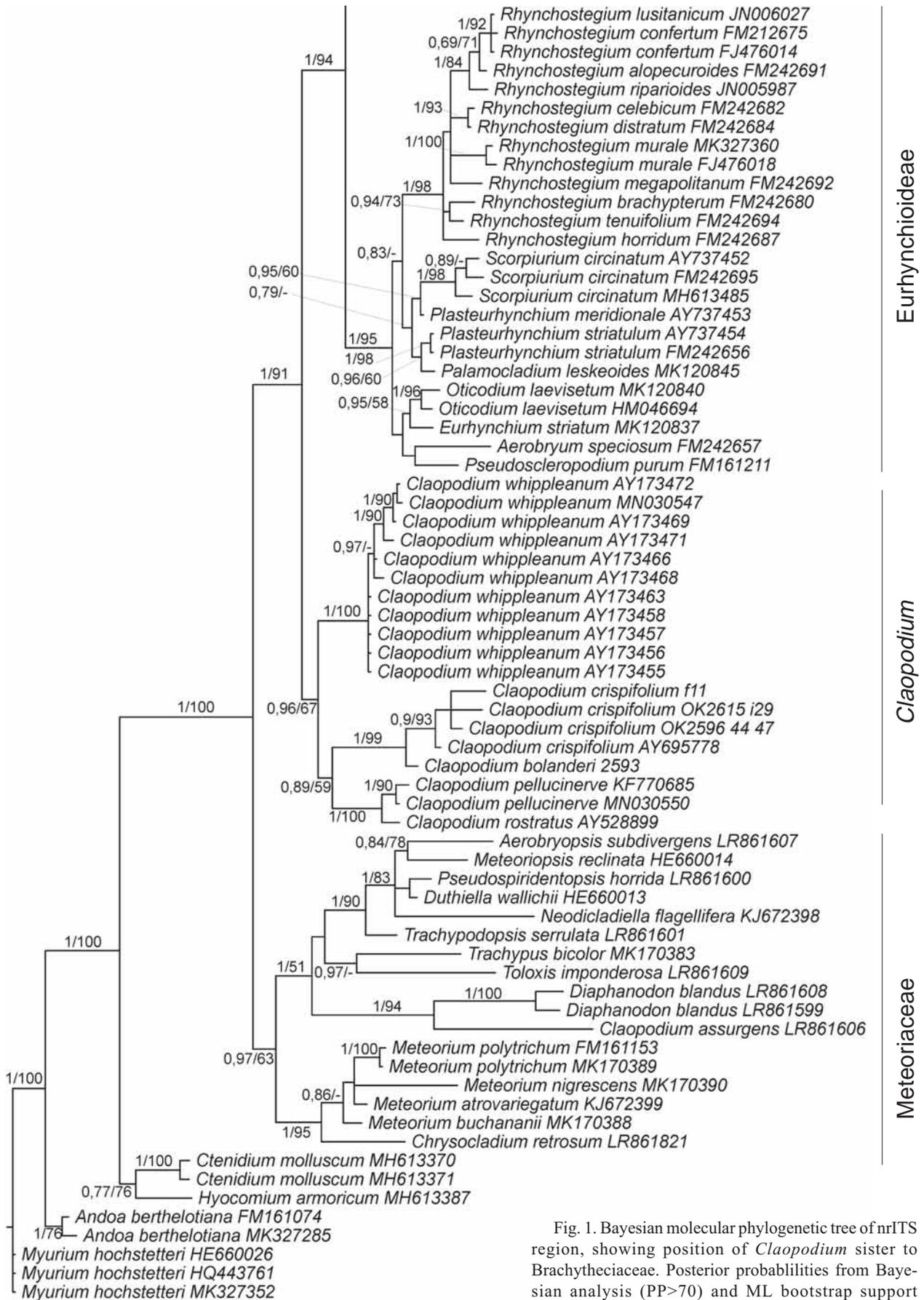
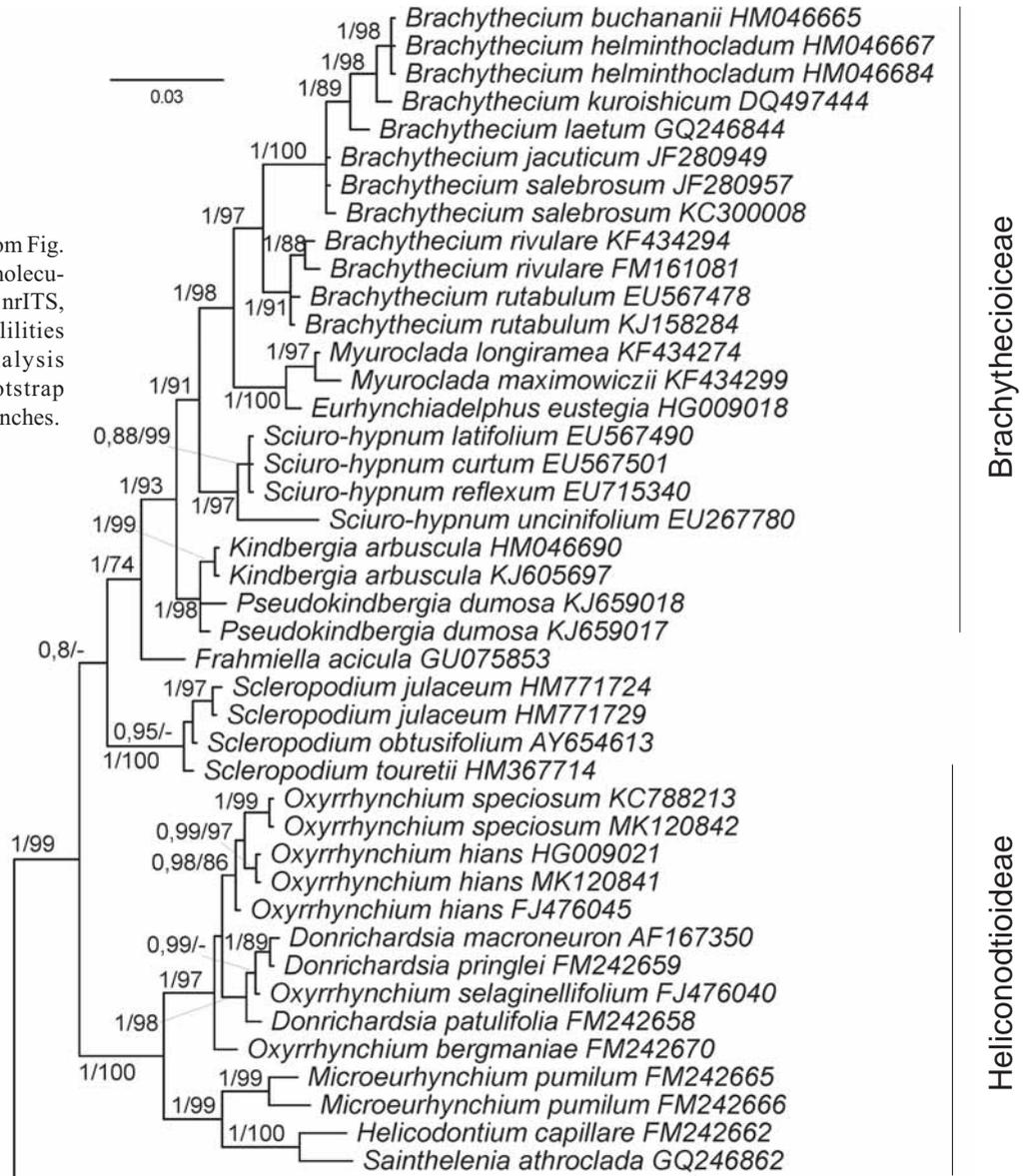


Fig. 1. Bayesian molecular phylogenetic tree of nrITS region, showing position of *Claopodium* sister to Brachytheciaceae. Posterior probabilities from Bayesian analysis (PP>70) and ML bootstrap support (BS>50) are shown at branches (continued on page 4).

Fig. 1a (continued from Fig. 1 in page 3): Bayesian molecular phylogenetic tree of nrITS, with Posterior probabilities from Bayesian analysis (PP>70) and ML bootstrap support (BS>50) at branches.



Morphological observations. Material for Laser Confocal Scanning Microscopy was taken from herbarium and studied with preparation similar to ordinary light microscopy material. Shoots without fixation were stained by 0,1mM DAPI and berberin and investigated under Olympus FV-1000, with 405 and 473 nm lasers; series of 5-15 optical obtained with 40x objective lens and up to 6x digital zoom were Z-stacked by the microscope software.

RESULTS

Both methods of the analysis reveal the same tree topology (Fig. 1), resolving the terminal clade of *Ctenidium*+*Hyocomium*+*Meteoriaceae*+*Claopodium*+*Brachytheciaceae* with maximal support (PP=1, BS=100). The clade of *Meteoriaceae*+*Claopodium*+*Brachytheciaceae* is also resolved with maximal support, and contains two subclades: *Meteoriaceae*, with moderate support (PP=0.97, BS=63), and *Claopodium*+*Brachytheciaceae*, with high support (PP=1, BS=91). The only exception was *Claopodium assurgens*, which was resolved in a clade with *Di-*

aphanodon within *Meteoriaceae*. Hereafter in the text the generic name *Claopodium* will refer to the *Claopodium* clade including *C. bolanderi*, *C. crispifolium*, *C. pellucinerve*, *C. rostratum*, and *C. whippleanum*, and excluding *C. assurgens*.

The *Claopodium*+*Brachytheciaceae* clade is subdivided into *Claopodium*, with moderate support (PP=0.96, BS=67), and *Brachytheciaceae*, with high support (PP=1, BS=94). Within the *Meteoriaceae*, the genus *Meteorium* was resolved as monophyletic, while other genera represented by a single species each formed assemblages similar to previous analyses (Huttunen *et al.*, 2004). *Claopodium assurgens* is clustered with *Diaphanodon blandus* with substantial support (PP=1, BS=94).

Within *Brachytheciaceae*, subfamilies *Eurhynchioidae* and *Helicodontioideae* are resolved monophyletic with high support: PP=1 & BS=95 and PP=1 & BS=100 respectively, and core *Brachythecioideae* is also strongly supported (PP=1 & BS=93), excluding genera *Frahmiella*

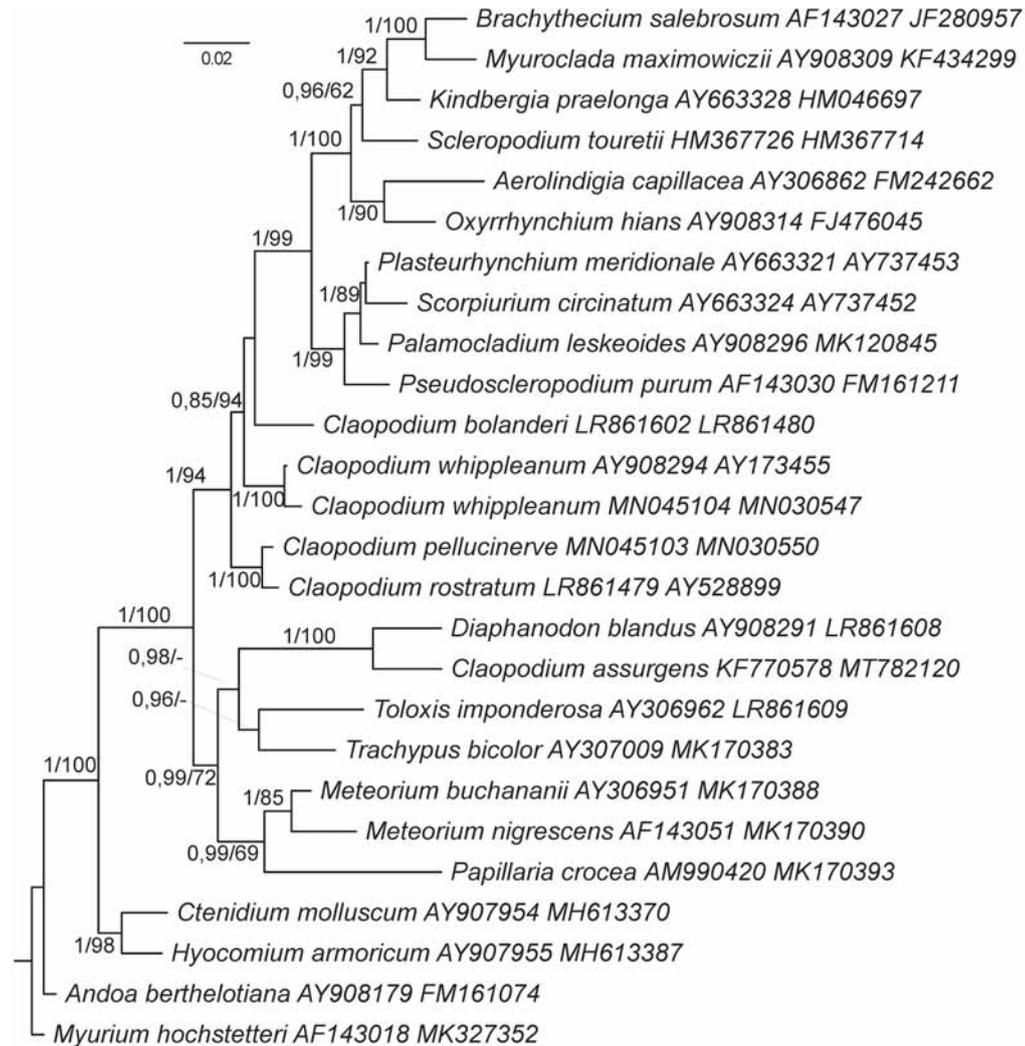


Fig. 2 Bayesian molecular phylogenetic tree based on concatenated dataset of nrITS and *rps4-trnS*, with Posterior probabilities from Bayesian analysis (PP>70) and ML bootstrap support (BS>50) at branches.

and *Scleropodium*, which subfamilial positions vary between different analyses (Huttunen & Ignatov, 2004). Genera of the Brachytheciaceae are monophyletic in tree in Fig. 1 as well.

The Bayesian tree based of *rps4-trnS* plastid region (Supplementary materials 1) results in a similar topology, although the clade supports are generally quite low due to overall lower variation than in ITS region, and the genus *Claopodium* itself is not resolved as monophyletic, as well as e.g. *Meteorium*.

However the concatenated tree of ITS and *rps4-trnS* region for a smaller selection showed a slightly higher support for clades as compare with ITS tree: e.g. for Meteoriaceae PP=0.99 & BS=80 vs. PP=0.97 & BS=63, for *Claopodium*+Brachytheciaceae BS=94 vs. BS=91, for Brachytheciaceae BS=99 vs. BS=94.

Morphological observations. Studies of the proximal branch leaf arrangement around branch primordia in *C. whippleanum*, *C. bolanderi* and *C. rostratum* (Fig. 3) revealed them having the same pattern as in all other Brachytheciaceae and Meteoriaceae.

However, arrangement of proximal branch leaf in *Claopodium* is not easy to observe. First, the outermost foliose structures around branch primordia are spaced after bud becomes larger, so it is difficult to say which is the outermost (in most Brachytheciaceae the triangular leaves cover next leaves by their basal angle, but this is not the case in *Claopodium*). The second reason is that outermost structures are quite fragile and easily fall down, although in this case the order of the proximal branch leaves is becoming more apparent (e.g. Fig. 3B). Observation by light microscope often do not help to understand this arrangement, as the stem is slightly flattened in *Claopodium* and buds are seen only in profile (Fig. 3C), and only in rare lucky cases the position of the outermost leaf is clearly seen with LCSM (Fig. 3G–H). Some of LCSM photos unequivocally show that the arrangement of foliose structures around branch primordia in *Claopodium* is the same as in Brachytheciaceae and Meteoriaceae (Fig. 3D–F).

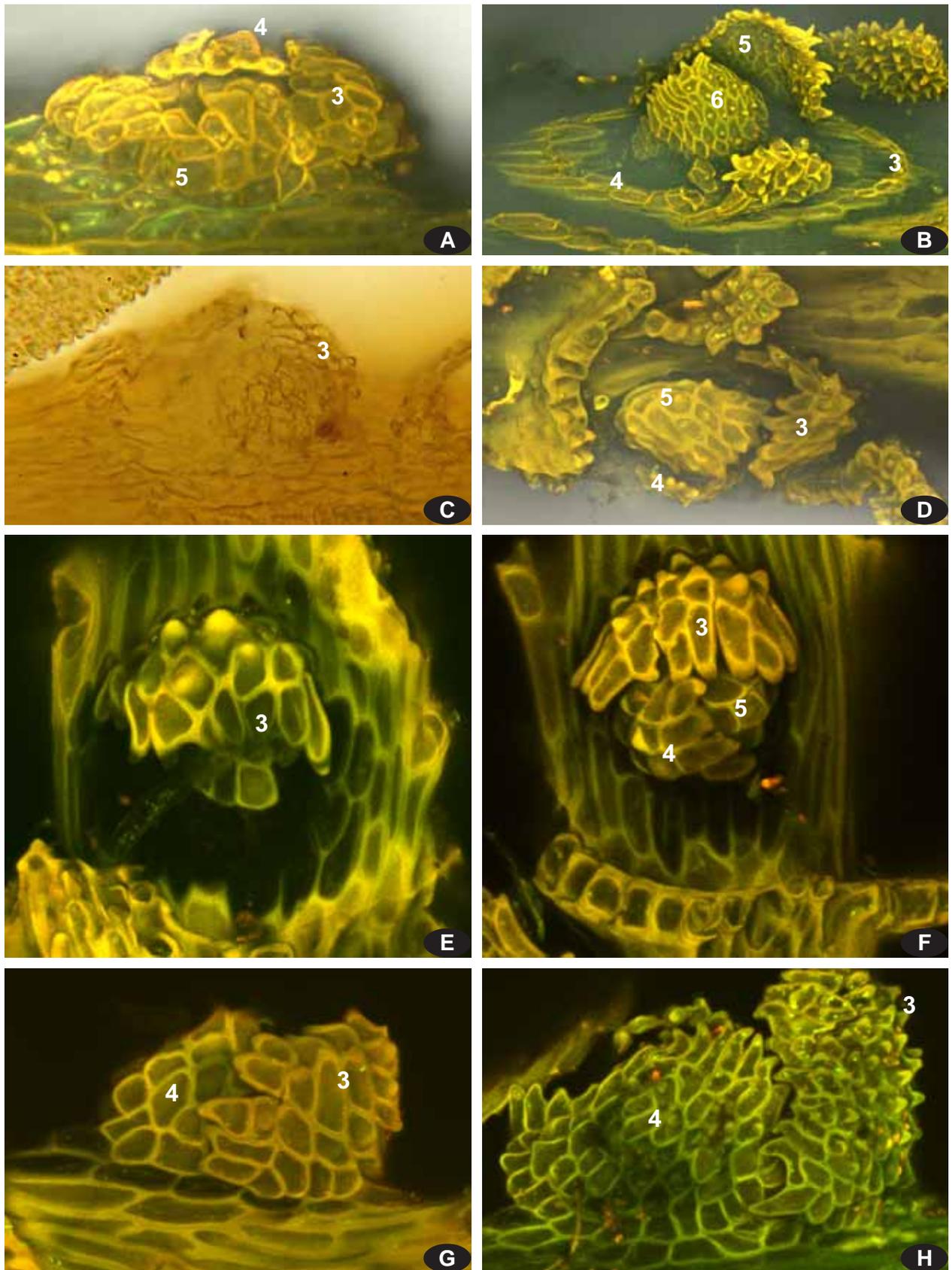


Fig. 3. Branch primordia in *Claopodium*: A, C: *C. rostratum* (Russia, Adygeya, Ignatov, MHA9001806); B, D: *C. bolanderi* (USA, California, Shevock 33066, MHA9060731), E–H: *C. whippleanum* (U.S.A., California, Ignatov, MHA 9060747). Numbering of proximal branch leaves follows Spirina & Ignatov (2005): the outermost, pointed downwards leaf is morphologically the third one, while the first and second branch leaves are reduced. In Fig. B third leaf is broken, however its insertion cells show its identity.

DISCUSSION

The overall topology of ITS tree agrees with the previously obtained subdivisions into subfamilies and grouping of genera in the family Brachytheciaceae (Ignatov & Huttunen, 2002; Huttunen & Ignatov, 2004; Huttunen *et al.*, 2007) and, in general, also Meteoriaceae (Huttunen & Quandt, 2007), which ensures that the dataset of ITS is comprehensive for taxonomic conclusions.

Plastid data are less variable, thus the tree based solely on *rps4-trnS* has a similar topology but lacks significant support (shown in Supplementary Materials only). Concatenated tree based on a smaller subset of taxa with the ITS plus plastid data results in moderate branch support, additionally supporting monogeneous ITS tree. *Claopodium* was found in a sister position to Brachytheciaceae, albeit not in as a clade, but in a grade.

The position of *Claopodium* is not that surprising in light of historical fluctuation in morphological delimitation of the family Brachytheciaceae. In 20th century the family included a number of genera, placed in it because of similar habit, e.g. *Tomentypnum* was placed in the family because of its similarity with *Homalothecium* (cf. Corley *et al.*, 1981; Crum & Anderson, 1981), or sometimes was included in *Homalothecium* (Robinson, 1962). Another approach was taken by Noguchi (1991), who transferred *Duthiella* from Meteoriaceae to Brachytheciaceae because of the perfect peristome, which he considered as an important key character for differentiation between these two families.

The molecular phylogenetic analysis of Ignatov & Huttunen (2002) and Huttunen & Ignatov (2004) determined the generic content of the family Brachytheciaceae. It has not changed since that with the exception of adding a monospecific South American genus *Stenocarpidiopsis*. At the same time, molecular definition of the family made the general morphological circumscription of the family Brachytheciaceae more vague due to inclusion of ecostate plants (*Unclejackia*), specialized tropical epiphytes with short seta (*Squamidium*, *Zelometorium*), as well as other epiphytic plants like *Helicodontium* and *Okamuraea*, with so different peristome that they were never placed in Brachytheciaceae in 'pre-molecular era'.

Fortunately, the specific arrangement of juvenile, proximal branch leaves around branch primordia (Ignatov, 1999), previously called pseudoparaphyllia, helped to distinguish representatives of the Brachytheciaceae and Meteoriaceae from other pleurocarps: in these families the outermost leaf is pointed downwards, covering the most part of bud. Later it was shown that such specific pattern of leaf arrangement can be assumed as the reduction of the first and second branch leaves (Spirina & Ignatov, 2005; Ignatov & Spirina, 2012), thus the third leaf appears to be outermost. This explains its position different from other pleurocarps where the outermost leaves are in lateral, 'four o'clock position' (Ignatov & Hedenäs, 2007). Partial reduction of outermost leaves was

subsequently found in some other families, but this pattern was either unstable, represented only in some buds, as in Fontinalaceae (Spirina & Ignatov, 2011) and Lembohyllaceae (Spirina & Ignatov, 2015), while in Leucodontaceae it is stable, but the outermost (morphologically the third) branch leaf is commonly subdivided into narrow lobes (Spirina & Ignatov, 2010). Thus, Brachytheciaceae and Meteoriaceae remain the only families where such pattern is stable and clearly performed. Position of *Claopodium* in a clade with Brachytheciaceae and Meteoriaceae is thus additionally supported by the similar pattern. The remaining question is its familial position, whether it should be placed in the Meteoriaceae, Brachytheciaceae or its own family.

The placement in the Meteoriaceae, though contradicting the results of molecular phylogenetic analysis, may be attractive from the morphological basis: almost all species of Meteoriaceae have papillose cells, while none of the Brachytheciaceae have real papillae, if one does not consider strongly prorate cells ("with papillae in distal cell corners") in some *Brachythecium* segregated formally to the genus *Bryhnia*. The presence of papillae was even considered as the main distinction between Brachytheciaceae and Meteoriaceae (Huttunen & Ignatov, 2004).

However, combination of two characters, structure and arrangement of papillae in leaf laminal cells and cell shape differ between Meteoriaceae and *Claopodium*. Both taxa may have uni- and pluripapillose cells, but in most pluripapillose Meteoriaceae papillae are in row and cells are elongate and narrow, while in pluripapillose *Claopodium* cells are rhombic and simple papillae are scattered over cell lumen. There are some exceptional cases in Meteoriaceae, such as: *Papillaria* s.str. (de Oliveira *et al.*, 2020) with rhombic cells and papillae scattered over cell lumen, but papillae are branching and complex in structure; *Floribundaria walkeri* with scattered simple papillae over cell lumen but elongate cells; and *Duthiella flaccida* with rhombic pluripapillose cells but papillae in row.

Claopodium assurgens has unipapillose leaf cells similar to *Diaphanodon blandus* with simple papilla at center of cell lumen. The two species also share irregularly to regularly pinnately branching stems with dimorphic leaves: stem leaves in both species are larger in size, rather abruptly tapering from ovate basal part to acuminate acumen, while branch leaves are smaller, more gradually tapering to acuminate acumen. The anomalous position of *C. assurgens* is also supported by a very different sporophyte structure: its capsule is almost straight vs. curved in other *Claopodium* species; operculum high-conic and attenuate to rostrum vs. low conic; exostome teeth are narrow vs. broad; and endostome basal membrane is low (Fleischer, 1923) vs. high. *Diaphanodon* shares with *C. assurgens* erect capsule, rostrate lid, narrow lanceolate exostome teeth and low endostome basal membrane, while unlike *C. assurgens*, *Diaphanodon* has subglobose to ovoid capsules and several specialized characters in its sporophyte: seta is short, 2 mm, vs. 10–15 mm in *C.*

assurgens; endostomial cilia are absent vs. 2 reduced, and spores are large, > 30 µm vs. < 20 µm. Although it is likely that *C. assurgens* can be placed directly to *Diaphanodon*, we are pending such a taxonomic resolution until a more comprehensive analysis of this group and hopefully addition of few *Claopodium* taxa that might be closely related to *C. assurgens*, and *Diaphanodon procumbens*. The later species has an interesting combination of gametophytic character states that is intermediate between *D. blandus* and *C. assurgens*. In addition, testing the phylogenetic position of the newly delimited *Diaphanodon* would require denser sampling of closely related Meteoriaceae species.

The obtained phylogenetic trees support two possible solutions for taxonomic placement of the *Claopodium*: it can be segregated in a separate monogeneric family or included in the Brachytheciaceae. Although the former solution will leave Brachytheciaceae less heterogeneous morphologically, we prefer the latter one. Monogeneric families are necessary if taxon lacks obvious close relatives, but close relationship between *Claopodium* and Brachytheciaceae is well-supported from phylogenetic analyses, present and previous (e.g. Huttunen *et al.*, 2012a). It is also worthy to remember the original description of *Claopodium*, where it was compared not only with Thuidiaceae, but also with *Eurhynchium* (at that time including *Kindbergia*, *Eurynchiastrum*, and *Oxyrrhynchium*). The combination of short, slightly curved, horizontal and usually dark brown capsules does not look unique among pleurocarpous mosses, but in temperate flora is not common either, being rather different from longer and gradually curved capsules characteristic of Amblystegiaceae and some Thuidiaceae+Leskeaceae, as was outlined by Hedenäs (1989, 1997), calling Pylaisiaceae that time temperate Hypnaceae. These differences were also likely implied by Lesquereux & James (1884), and considered important, albeit not explicitly formulated.

Recent changes in systematics of pleurocarps are mostly related to the earlier overestimation of sporophytic characters (especially peristome modifications), while gametophytic characters (e.g. proximal branch leaves arrangement around branch primordia) appeared to be more conservative and informative in taxonomy. However in the case of *Claopodium* it is the opposite: papillose leaf laminal cells appeared to be less important for taxonomy than the capsule shape.

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Appendix. Specimen vouchers and EMBL or Genbank accession numbers

Species	Isolate	Provenance	Voucher	ITS	rps4–trnS
<i>Aerobryopsis subdivergens</i>	SH178	China, Hunan	Koponen et al. 51514 (H)	LR861607	
<i>Chrysocladium retrorsum</i>	SH5	China, Hunan	Koponen et al. 55572 (H)	LR861821	
<i>Claopodium assurgens</i>	OK2595	Australia, Queensland	Streimann 46523 (MHA 9060729)	LR861606	MT782120
<i>Claopodium bolanderi</i>	OK2593	USA, California	Shevock 33066 (MHA9060731)	LR861602	LR861480
<i>Claopodium crispifolium</i>	OK2596	USA California	Norris 76870 (MHA9060736)	LR861605	LR861481
<i>Claopodium crispifolium</i>	OK2615	USA, California	Ignatov sn (MHA9060726)	LR861604	LR861482
<i>Claopodium crispifolium</i>	OK2616	USA, California	Ignatov sn (MHA9060724)	LR861603	LR861483
<i>Claopodium rostratum</i>	OK2614	USA, Chicago,	Ignatov 13-2028 (MHA9060744)		LR861479
<i>Diaphanodon blandus</i>	SH1660	China, Xizang	G. Mische & U. Wundisch (TUR114184)	LR861608	
<i>Diaphanodon blandus</i>	SH104	Philippines, Mt. St. Tomas	B.-C. Tan sn 29-30.XI.1986 (H)	LR861599	
<i>Pseudospiridentopsis horrida</i>	SH11	China, Hunan	Koponen et al. 55834 (H)	LR861600	
<i>Toloxis imponderosa</i>	ABAGAM56	Ecuador, Quito to Santo Domingo	J.-P. Frahm 107 (BONN)	LR861609	
<i>Trachypodopsis serrulata</i>	SH211	China, Hunan	Koponen et al. 54101 (H)	LR861601	