# A reconsideration of the systematic position of *Goniomitrium* (Funariaceae) based on chloroplast sequence markers

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**Abstract.** According to new *rps*4 and *trn*L-*trn*F sequences, *Goniomitrium* should be removed from the Pottiaceae and placed again in the Funariaceae. *Goniomitrium* and *Pyramidula* form a sister clade to the rest of the Funariaceae, and are accommodated in their own subfamily, the Pyramiduloideae *subfam. nov. Clavitheca nom. nov.* is proposed to replace the illegitimate *Corynothec*a Ochyra. The exclusion of the Ephemeraceae from the Funariales is here corroborated.

**Keywords.** *Goniomitrium*, Funariaceae, subfamilial division, phylogeny, *Clavitheca.*

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The genus *Goniomitrium* Hook. & Wilson has been largely accepted as a member of the Funariaceae by the taxonomic revisions of Fife (1985) and Fife and Seppelt (2001). It shows the morphological features characterizing the family: autoicous or paroicous sexuality, usually obovate leaves with thin-walled, non-ornamented cells, perigonial paraphyses with inflated terminal cells, stomata enclosed by a single guard cell and inflated calyptrae (Fife & Seppelt 2001). The protonematal morphology also points in the direction of a close relationship between *Goniomitrium* and the Funariaceae (Duckett, pers. comm.). Therefore it was a major surprise when Goffinet and Cox (2000) studied the phylogenetic relationships among the basal most arthrodontous mosses using nucleotide sequence data from one nuclear and two chloroplast loci of the type species and came to the conclusion that *Goniomitrium acuminatum* Hook. & Wilson, should be transferred to the Pottiaceae. Werner et al. (2004) examined the phylogenetic relationships in the Pottiaceae. They included the *rps*4 sequence given by Goffinet and Cox (2000) in their data-set with a large number of Pottiaceae species and confirmed the opinion of these authors.

Because the strong incongruence between morphological and molecular data we decided to use the opportunity to test the ordinal affinities of *Goniomitrium* when fresh material of *G. seroi* Casas and *G. acuminatum* was made available to us.

## Material and Methods

***Plant material.*** The chloroplast DNA sequences published by Goffinet and Cox (2000) were used to test their conclusions based on new sequences. Details on voucher or reference data as well as GenBank accession numbers are given there. Additionally we


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**TABLE 1.** Collection identification and GenBank accession numbers for the taxa included in the molecular analysis.

| Species | Geographic origin | Voucher specimen | GenBank Accession No. | |
|---|---|---|---|---|
| | | | *rps*4 | *trn*L-*trn*F |
| *Ephemerum sessile* (Bruch) Müll. Hal. | Spain, Sevilla | MUB 11417 | DQ337183 | DQ337177 |
| *Goniomitrium seroi* Casas | Spain, Valencia | MUB 18068 | DQ337186 | DQ337178 |
| *Goniomitrium seroi* Casas | Spain, Canary Islands, Tenerife | HERB. DIRKSE 012829 | DQ337187 | DQ337179 |
| *Goniomitrium acuminatum* Hook & Wils. | Australia, Australian Capital Territory | CANB 669855; MUB 19624 | DQ337185 | DQ337181 |
| *Pyramidula tetragona* (Brid.) Brid. | Morocco, Rif | MUB 19626 | DQ337184 | DQ337180 |

sequenced the *trn*L-*trn*F region and the partial *rps*4 sequences of two samples of *Goniomitrium seroi* from Spain (Canary Islands and Valencia), one specimen of *Goniomitrium acuminatum* from Australia, one specimen of *Pyramidula tetragona* (Brid.) Brid. from Morocco, and one specimen of *Ephemerum sessile* (Bruch) Müll. Hal. from Spain. The voucher data and GenBank accesion numbers of these additional samples are given in **Table 1.**

***DNA extraction.*** Total DNA was extracted by the NaOH extraction method described by Werner et al. (2002). 5 µl of the crude NaOH extract were diluted by the addition of 45 µl of 100 mM Tris-1 mM EDTA (pH 8.3) and stored frozen at −18°C until the PCR reaction was carried out.

***DNA sequencing.*** PCR reactions were performed in an Eppendorf Mastercycler using 4 µl of the DNA solution in a 50 µl final volume. The reaction mix contained the primers trnC and trnF (Taberlet et al. 1991) for the amplification of the chloroplast *trn*L-*trn*F region, and the primers rps5 (Nadot et al. 1994) and trnas (Buck et al. 2000) for the amplification of the chloroplast *rps*4 gene. The final concentration of the primers was 400 µM, in the presence of 200 µM each of dNTP, 2 mM MgCl2, 2 units Taq polymerase (Oncor Appligene), 1µl BLOTTO (10% skimmed milk powder, 0.2% $NaN_3$ in water) and the buffer provided by the supplier of the enzyme. BLOTTO attenuates the inhibition of PCR by plant compounds (De Boer et al. 1995). Amplification started with 3 min denaturation at 94°C, followed by 35 cycles of 15 s at 94°C, 30 s at 50°C, and 1 min at 72°C. A final extension step of 7 min at 72°C completed the PCR. 5 µl of the amplification products were visualized on a 1% agarose gels and successful amplifications were cleaned with the QIAquick purification kit (Qiagen). The amplification primers were used in the sequencing reactions with the Big Dye sequencing kit and separated on a ABI-Prism 3700 sequencing machine using standard protocols.

The sequences were aligned using CLUSTALX (Thompson et al. 1997) with the gap open penalty set to 10 and the gap extension penalty set to 1. BioEdit (Hall 1999) was used for minor manual adjustment of the alignment. An aligned matrix is available on request from the first author. Portions of the sequences that could not be aligned unambiguously were excluded.

Phylogenetic inferences were made under the optimality criteria of maximum parsimony (MP) and maximum likelihood (ML) using PAUP*4.0b10 (Swofford 2003). The following search strategy was implemented to uncover the most parsimonious trees: a) 200 random addition replicates with the steepest descent and the tree-bisection-reconstruction (TBR) options selected, and with only 10 trees saved per replicate; b) all saved trees would be swapped under TBR until completion with steepest descent turned on, and no limit to the number of trees saved. Support for branches was estimated using the bootstrap method (Felsenstein 1985) with 10 random addition searches performed on 1000 pseudoreplicated matrices. For ML analyses, the optimal model of sequence evolution for each partition was identified using MrModeltest 2.2 (Nylander 2004) based on the Akaike Information Criterion. The GTR+I+G model was selected with the following settings for base frequencies (A:0.4252, C:0.1224, G:0.1304 & T:0.3220), relative substitution rates (A-C: 1.0389, A-G:4.7864, A-T:0.3004, C-G: 1.5484, C-T: 4.8780 & G-

T:1.0000), proportion of invariable sites (I=0.2377) and gamma distribution shape parameter (G=0.6463). This model was implemented in PAUP*4.0b10 (Swofford 2003) when searching the most likely tree (using the same approach as described for the MP analysis), and in MrBayes 3.0 (Huelsenbeck & Ronquist 2002) when exploring the tree space to recover posterior probabilities. The data were treated as a single partition (homogeneous Bayesian). The Bayesian analysis was replicated four times, each run with three heated and one cold chains, to test for the presence of multiple local optima, and each time with single tree was saved to a tree file every 50 generations for a total of $10^6$ generations. Of the 20 001 trees saved, the first 1001 (the "burnin") were ignored for determining posterior probabilities and confidence intervals for model parameters. Ultimately all four sets of 19,000 trees were merged, and posterior probabilities obtained from the consensus of 76,000 trees. Posterior probability support for bipartitions was considered statistically significant when $P \geq 0.95$.

## Results

Sequences for the *rps*4 and the *trn*L-*trn*F loci were obtained for *Ephemerum sessile*, two exemplars of *Goniomitrium seroi*, one of *G. acuminatum* and one of *Pyramidula tetragona* (**Table 1**). The aligned matrix compiled for 44 exemplars, comprised 1402 sites (*rps*4: 600 and *trn*L-*trn*F: 802), of which 546 were excluded. Inferences under maximum parsimony were made from 235 potentially parsimony informative characters, of which corresponded to the partial *rps*4 gene and to the *trn*L-*trn*F region. The analysis with maximum parsimony as optimality criterion resulted in 10 most parsimonious trees (**Fig. 1**), of length 938, with a consistency index (CI) of 0.56 (0.46 when autapomorphic characters were removed) and a retention index (RI) of 0.71. The heuristic search under the criterion of ML led to three optimal trees (**Fig. 1**) characterized by a score of –Ln = 6028.5920. The topological ambiguity between the three trees lay in the affinities of *Physcomitrella patens* (Hedw.) Bruch & Schimp. to either of the exemplars of *Aphanorrhegma serratum* (Hook. & Wilson) Sull., and in the relationship of the outgroup taxon, *Tayloria scabriseta* (Hook.) Mitt. (**Fig. 1**). The optimal trees obtained under the MP and ML criteria are topologically congruent, and not surprisingly they both are nearly identical to the tree presented by Goffinet and Cox (2000) with the exception of some minor deviations that affected nodes with low bootstrap support. Overall support from individual nodes was higher in terms of posterior probabilities. The relationships within the Funariideae sensu Goffinet and Buck (2004) are better resolved and supported under likelihood (Bayesian) compared to the inferences under maximum parsimony: the Gigaspermaceae are sister to Encalyptales/Funariales sensu stricto, with the affinities of the Disceliaceae remaining ambiguous. The monophyly of the Haplolepideae is well (i.e., PP=1.00) or poorly (BP=57%) supported, and the position of the Timmiaceae is clearly unresolved. The diplolepideous mosses, here represented by members of the Bartramiales, Orthotrichales and Splachnales form a well-supported clade (BP=97% and PP=1.00).

The new sequences of *rps*4 and *trn*L-*trn*F of *Ephemerum sessile* included here are very close to those of *Ephemerum spinulosum* Bruch & Schimp. (Goffinet & Cox 2000). *Ephemerum* is here resolved as sister to *Splachnobryum obtusum* (BS=93%, PP=1.00), with both sharing a common ancestor with *Syntrichia ruralis* (BP=100%, PP=1.00).

The newly generated sequences of *Goniomitrium acuminatum* differ from those presented by Goffinet and Cox (2000), and are similar to those obtained here for *G. seroi*. These two species form a monophyletic lineage (BP=97% & PP=1.00; **Fig. 1**) that is sister to *Pyramidula tetragona* (BP=100%, P=1.00; **Fig. 1**). This highly supported clade is sister to the "core" Funariaceae, which is also defined by a robust branch (i.e., BP=100%, PP=1.00; **Fig. 1**). The *Goniomitrium* and *Pyramidula* clade does not share the deletion of one codon of the *rps*4 gene considered characteristic of the Funariaceae (Goffinet & Cox 2000; Goffinet et al. 2001). Nevertheless, the monophyly of the Funariaceae including *Goniomitrium* and *Pyramidula* is highly supported (BP=97%, PP=1.00; **Fig. 1**). The incongruence between our results and those presented by Goffinet and Cox (2000) led us to reëxamine the voucher used by these authors. The specimen which is deposited at H (*Streimann 48855*) has been reidentified as *Acaulon integrifolium* Müll. Hal., a member of the Pottiaceae. It has been this

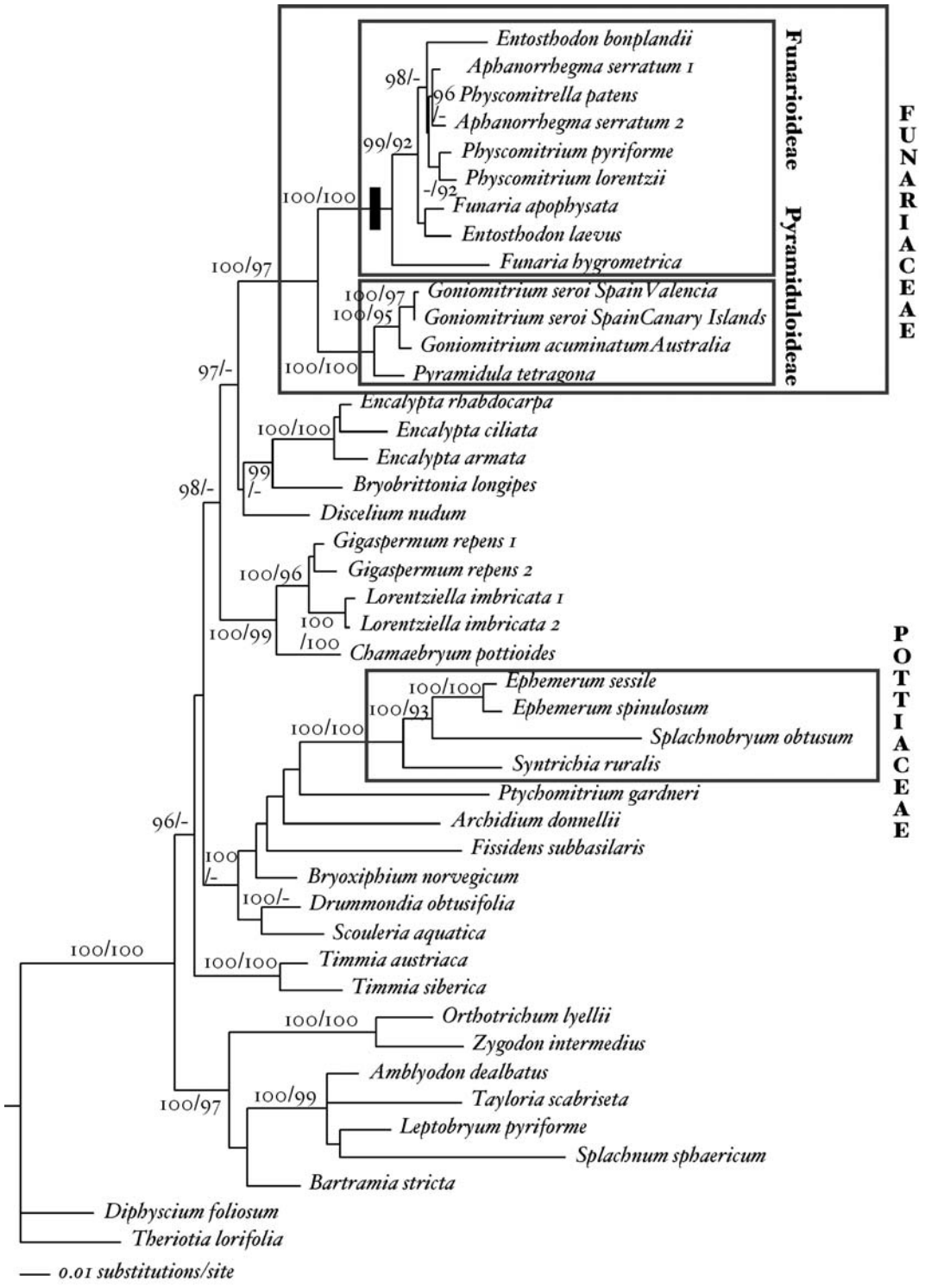


**Figure 1.** Phylogenetic circumscription of the Funariaceae and the relationships within the family based on *trn*L-*trn*F and *rps*4 sequences. One of three most likely trees (–Ln = 6028.5920). Numbers above branches correspond to posterior probabilities estimated from the consensus of 76,000 trees obtained by Bayesian analysis based on 4 times 1,000,000 generations. Posterior probabilities are followed by bootstrap percentages based on 1,000 (pseudo)replicates. The black box marks the loss of one codon in the *rps*4 gene shared by members of the Funarioideae.

mistake in the identification that led to the confusion in the systematic position of *Goniomitrium*.

## Discussion

Morphological reduction, that is the loss of synapomorphies, is considered to be rampant in mosses (e.g., Buck et al. 2000) and is the main challenge to identifying and defining monophyletic groups in the Bryopsida (e.g., Goffinet et al. 2004). When Goffinet and Cox (2000) and Goffinet et al. (2001) reconstructed the Ephemeraceae and *Goniomitrium* to be members of the Pottiales, rather than the Funariales, both taxa were interpreted as highly reduced lineages. *Ephemerum* and *Goniomitrium* both lack a peristome, and their unicostate leaves are composed of smooth or more rarely of prorulose cells. Both character-states are known to occur in the Pottiaceae (Zander 1993). The affinities of *Ephemerum* to the Pottiaceae are here confirmed based on data obtained for another species, *E. sessile*. By contrast, *Goniomitrium* is resolved as part of the Funariaceae, forming a well-supported clade with *Pyramidula*. The results obtained previously (Goffinet & Cox 2000) were based on a misidentified voucher, which is in fact a member of the Pottiaceae. Consequently, *Goniomitrium* should be returned to the Funariaceae.

Fife (1980) considered *Goniomitrium* and *Pyramidula* to be closely related and strongly isolated in the Funariaceae, exhibiting many morphological features that could mark the transition between this family and the Gigaspermaceae. Based on a phenetic analysis of morphological characters, wherein the two genera formed one of four generic clusters, Fife (1980) suggested that the two genera could be accommodated in their own subfamily. *Goniomitrium* and *Pyramidula* differ from other Funariaceae by the golden to pale brown spores, ellipsoid or weakly subreniform, 50–90 μm in greater diameter (rarely as small as 40 μm), minutely verrucate or appearing smooth, becoming reticulate; persistent calyptra, either mitrate and bearing eight conspicuous radial plicae, or cucullate-rostrate, clasping at base, and distinctly 4-angled. The other members of the Funariaceae have reddish-brown spores, subreniform to nearly spherical, less than 50 μm diameter (usually less than 40 μm, very rarely greater than 50 μm), verrucate, lirate, baculate, spinate, finely verrucate or nearly smooth, very rarely reticulate; deciduous calyptra, neither angled nor conspicuously plicate.

Phylogenetic inferences from variation in nucleotide sequences of two chloroplast loci support the hypothesis that *Goniomitrium* and *Pyramidula* are closely related and phylogenetically distinct from other members of the family (**Fig. 1**): the two genera form a well-supported sister group to the remainder of the Funariaceae sampled here. The alternative hypothesis, whereby *Goniomitrium* is closely related to *Funaria* (Stone 1981) finds no support from the sequence data. The early divergence of the two lineages within the Funariaceae is marked by the lack of a codon in the *rps*4 gene in all members of the *Funaria*-clade and the plesiomorphic presence of this triplet in the sequences of *Goniomitrium* and *Pyramidula*. A common ancestry of all Funariaceae is, however, unambiguous, and accommodating *Goniomitrium* and *Pyramidula* in their own family seems inappropriate at this point. As suggested by Fife (1985), recognition of the *Goniomitrium-Pyramidula* clade at the subfamilial level may be adequate.

The other three clusters of genera considered by Fife (1985) as possible subfamilies are not supported as monophyletic entities by the molecular data. Consequently and according to our results, we propose to formally recognize two subfamilies within the Funariaceae:

**Pyramiduloideae** O. Werner, Ros & Goffinet, *subfam. nov.*

*Capsulae immersae ad exsertas, symmetricae, operculatae. Peristomium absens. Calyptra persistens, alteruter mitriformis, 8 conspicuas plicas radiales plicas ferens, basique 8-lobata, vel cucullata-rostrata, basi amplectens et distincte 4-angulata (tetrangulata). Sporae aureae ad dilute brunas, ovoideae, ellipsoideae vel leviter subreniformes, 40–90 μm majore diametro, minute verrucatae vel laeves, reticulescentes.*

***Diagnosis.*** Capsules immersed to exserted, symmetric, operculate. Peristome absent. Calyptra persistent, either mitrate and bearing 8 conspicuous radial plicae, 8-lobed at base or cucullate-rostrate, clasping at based, and distinctly 4-angled. Spores golden to pale brown, ovoid, ellipsoid or weakly subreniform, 40–90 μm in greatest diameter, mi-

nutely verrucate or appearing smooth, becoming reticulate. TYPE: *Pyramidula* Brid.

***Circumscription.*** *Pyramidula* Brid. and *Goniomitrium* Hook. & Wilson

**Funarioideae**

***Diagnosis.*** Capsules immersed to long-exserted, symmetric or strongly asymmetric, operculate or inoperculate. Peristome double, single, rudimentary or absent. Calyptra deciduous, mitrate or cucullate, usually strongly rostrate and inflated at base, neither angled nor conspicuously plicate. Spores reddish-brown, subreniform to nearly spherical, usually less than 40 μm in greatest diameter, verrucate, lirate, baculate, finely verrucate or nearly smooth, rarely reticulate. TYPE: *Funaria* Hedw.

***Circumscription.*** The Funarioideae include the remainder of the Funariaceae as defined by Goffinet and Buck (2004): *Aphanorrhegma* Sull., *Brachymeniopsis* Broth., *Bryobeckettia* Fife, *Clavitheca* O. Werner, Ros & Goffinet, *Cygnicollum* Fife & Magill, *Entosthodon* Schwägr., *Funaria* Hedw., *Funariella* Sérgio, ×*Funariophyscomitrella* Wettst., *Loiseaubryum* Bizot, *Nanomitriella* E. B. Bartram, *Physcomitrella* Bruch & Schimp., *Physcomitrellopsis* Broth. & Wager and *Physcomitrium* (Brid.) Brid.

The genus *Corynotheca* is illegitimate, because this name has been previously applied to a vascular plant in the Liliaceae (*Corynotheca* F. Muell. ex Benth., Fl. Austral. 7: 49. 1878). With the aim of legitimizing the genus, we propose here a new name.

**Clavitheca** O. Werner, Ros & Goffinet, *nom. nov.*
*Corynotheca* Ochyra, Polish Bot. Stud. 1: 60. 1990, *hom. illegit.* (Art. 53.1 St. Louis Code).
TYPE: **Clavitheca poeltii** (Ochyra) O. Werner, Ros & Goffinet, *comb. nov.* (*Corynotheca poeltii* Ochyra, Polish Bot. Stud. 1: 60, f. 1–32. 1990).

At least one generic representative of each of the four phenetic clusters recognized by Fife (1985) is included in the cladogram presented here (**Fig. 1**). Consequently his phenetic groups can be included in the subfamilies proposed in this paper, with the exception of that composed exclusively of *Loiseaubryum*. Only the subfamilial position of this and another two genera, for which no sequence data are available, remain uncertain. Consequently their phylogenetic position remains provisional. *Loiseaubryum* is the most phenetically distinctive genus in the family and *Nanomitriella* is considered allied to it based on sporophyte features (Fife 1985). Ochyra (1983) found that *L. ephemeroides* Bizot, the only species of the genus, shows morphological affinities with *Physcomitrium* subgenus *Cryptopyxis* (Müll. Hal.) Broth., and the genus *Micropoma* Lindb., which Fife (1982) placed in synonymy with the former. By contrast, Fife and Magill (1982) suggested accommodating *Loiseaubryum* in the Ephemeraceae on the basis of the two-celled stomata and the persistent protonema. *Loiseaubryum* and *Nanomitriella* are patristically highly derived within the Funariaceae and their correct systematic position must await further study. The genus *Clavitheca*, although showing a very peculiar sporophyte, seems to show affinities with *Entosthodon* (Ochyra 1990) and is therefore included in the subfamily Funarioideae.

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