Chloroplast data reveal two conflicting hypotheses for the positions of *Campylostelium* and *Grimmia pitardii* (Bryophyta)

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Due to conflicting morphological characters, the systematic placement of the Mediterranean-Central Asian *Grimmia pitardii*, lately considered a member of *Campylostelium* (Ptychomitriaceae), has been controversial. Phylogenetic inferences based on the chloroplast gene *rps4* and the *trnL-F* region were performed to clarify its systematic affinities. *Grimmia pitardii* is consistently resolved as a member of a maximally supported clade together with the genus *Campylostelium*. This combined clade forms the sister group to a similarly high supported clade comprising *Grimmia*, *Racomitrium, Coscinodon,* and *Schistidium*. Consequently, *G. pitardii* must be treated as *Campylostelium pitardii* (Corb.) E. Maier. Analysis of the systematic position of *Campylostelium* and *Ptychomitrium*, traditionally placed in the family Ptychomitriaceae, yielded two conflicting topologies: one groups *Ptychomitrium* and *Campylostelium,* whilst the second branches *Campylostelium* first, grouping *Ptychomitrium* with Grimmiaceae.

KEYWORDS: Bryophyta, *Campylostelium*, *Campylostelium pitardii,* chloroplast sequences, *Grimmia*, Grimmiaceae, Ptychomitriaceae, *Ptychomitrium,* systematics, *trnL-F, trnS-rps4*

INTRODUCTION

Grimmia is one of the most complex and species-rich genera within Grimmiaceae. Without morphological synapomorphies to characterize it, and with an inordinate number of taxa described without critically evaluating existing ones, the genus challenges the use of only classical taxonomic methodologies for its study. Recent studies have reduced the number of accepted taxa to about 80 (Muñoz & Pando, 2000; Greven, 2003) and clarified many aspects of its taxonomy. However, many questions still remain to be solved around the classical *Grimmia* concept, as the treatment by Ochyra & al. (2003) lets suspect.

One example of the complexity within *Grimmia* is represented by the scarce and idiosyncratic *G. pitardii* Corb., originally described from Tunisia (Pitard & Corbière, 1909), and afterwards from Tajikistan (as *Usmania campylopoda* Laz., cf. Lazarenko, 1970) and Irak (as *G. gibbosa* S. Agnew, cf. Agnew, 1973). It is indeed a rare species growing directly on the ground and not on rocks as it is the rule in *Grimmia*. *Grimmia pitardii* is distributed across southern Europe (Crete, Cyprus, France, Greece, Italy, and Spain), Canary Islands and Maghreb (Morocco and Tunisia), Turkey, and Central Asia (Tajikistan and Uzbekistan). Besides its habitat, this taxon differs from any other *Grimmia* in habit, leaf morphology, costa anatomy, and peristome features (Maier, 1998).

Maier (1998) was the first to note its oddness in *Grimmia* (as defined by Limpricht, 1890), and compared *G. pitardii* with *G. plagiopodia* Hedw. (type of *Grimmia*), *Campylostelium saxicola* (F. Weber & D. Mohr) Bruch & Schimp. and *C. strictum* Solms. On the basis of the plurilobed mitrate calyptra, costa anatomy, peristome teeth with basal membrane, and the outer peristome layer as thick as the inner peristome layer, she concluded that *Grimmia pitardii* was indeed a *Campylostelium* (Ptychomitriaceae), and proposed the new combination *C. pitardii* (Corb.) E. Maier.

Neither Muñoz & Pando (2000) nor Greven (2003) adopted Maier's views, considering that although similar to *Campylostelium*, *G. pitardii* also shared important characters with members of *Grimmia* subg. *Grimmia* (e.g., cygneous seta and ventricose capsule), leaving the question open to future studies.

The goal of this study is, therefore, to clarify the systematic position of *Grimmia pitardii* using a molecular approach based on the plastid *rps4* gene and *trnL-F* region (cpDNA). According to recent molecular studies (La Farge & al., 2000; Tsubota & al., 2003; Hedderson & al., 2004) as well as unpublished data from the authors we included representatives of the genera treated within

Grimmiaceae and Ptychomitriaceae by Buck & Goffinet (2000). A secondary aim was to explore the relationships between *Ptychomitrium* and *Campylostelium*, and to determine whether they should be considered members of an integrative Ptychomitriaceae or, whether *Campylostelium* should be segregated in an independent Campylosteliaceae (cf. Limpricht, 1890).

MATERIAL AND METHODS

Plant Material. — Vouchers are deposited in BCB, MA, MO, MUB and S. GenBank accession numbers, voucher numbers of the herbaria as well as the origin of specimens are listed in Table 1. Three sequences were downloaded from GenBank (italics in Table 1), and the remaining fifty three were obtained for this study.

DNA isolation amplifications and sequencing. — Total DNA of gametophore tips from dried herbarium specimens or recent collections was isolated using the CTAB method described by Doyle & Doyle (1987), modified for bryophytes as described in Shaw (2000). PCR amplifications of the *rps4* gene, including the *trnS-rps4* spacer as well as the *trnL-F* region were performed in 50 µl-reactions containing 1.5 U *Taq* DNA polymerase, 1 mM dNTPs-Mix each 0.25 mM, 1× buffer, 1.5 mM $MgCl_2$, 10 pmol of each amplification primer and 1 µl of DNA. The *trnS-rps4* region was amplified using the primers trnS-R and rps4-5′ described in Nadot & al. (1994), whereas the *trnL-F* region was amplified using the original Taberlet & al. (1991) primers, C and F. Amplification cycles were as follows: 2 min at 94ºC, 30 cycles with 2 min 94ºC, 1 minute 55ºC and 1 min 72ºC, and a final 7 min extension step at 72ºC. Amplified products were cleaned using spin filter columns (PCR Clean-up DNA Purification Kit, MoBIO Laboratories, California) following the manufacturers protocols. Cleaned products were directly sequenced using dye terminators (Big Dye Terminator v2.0, Applied Biosystems, California).

Data analysis. — Sequences were edited and manually aligned using PhyDE® (Müller & al., 2005) following alignment rules described in Kelchner (2000) and Quandt & Stech (2004, 2005). Following the approach in Quandt & al. (2003) and Quandt & Stech (2004, 2005), the data matrix was screened for inversions using secondary structure models calculated with RNAstructure (Matthews & al., 2004). As discussed in Quandt & al. (2003) and Quandt & Stech (2004), presence or absence

Table 1. List of investigated specimens, with GenBank accession numbers for the regions sequenced, including voucher numbers and the herbaria where the specimens are kept. Fifty-three sequences were obtained for this study, and three (in italics) were downloaded from GenBank.

Species	GenBank accession no. *rps4*	*trnL-F*	Origin	Herbarium & voucher no.
Campylostelium strictum (Solms) Kindb.	DQ399604	DQ399631	Portugal. Marvao	BCB 43791
Coscinodon calyptratus (Drumm.) C.E.O. Jensen	DQ399614	DQ399641	U.S.A. South Dakota	MO 5126877
Coscinodon cribrosus (Hedw.) Spruce	DQ399615	DQ399642	U.S.A. Maine	MO 4441357
Crossidium davidai Catches.	DQ399626	DQ399627	Spain. Canary Islands	MUB 5349
Grimmia anodon Bruch & Schimp.	DQ399619	DQ399646	U.S.A. Nevada	MA 25617
Grimmia crinita Brid.	DQ399620	DQ399647	Spain. Huesca	MA 22641
Grimmia funalis (Schwägr.) Bruch & Schimp.	DQ399625	DQ399652	Norway. Finmark	S B64173
Grimmia hartmanii Schimp.	DQ399623	DQ399650	Sweden. Värmlands Lan	S B30709
Grimmia incurva Schwägr.	DQ399622	DQ399649	Sweden. Jamtlands Lan	S B70022
Grimmia ovalis (Hedw.) Lindb.	DQ399618	DQ399645	U.S.A. Nevada	MO 5217105
Grimmia pitardii Corb. 1	DQ399605	DQ399632	Spain. Almeria	MA 19752
Grimmia pitardii Corb. 2	DQ399606	DQ399633	Spain. Almeria	MA 19751
Grimmia pitardii Corb. 3	DQ399607	DQ399634	Spain. Murcia	MUB 15032
Grimmia plagiopodia Hedw.	DQ399616	DQ399643	Sweden. Torne Lappmark	S B70024
Grimmia pulvinata (Hedw.) Sm.	DQ399617	DQ399644	U.S.A. California	MA 25026
Grimmia trichophylla Grev.	DQ399624	DQ399651	U.S.A. California	MA 25700
Grimmia ungeri Jur.	DQ399621	DQ399648	U.S.A. Nevada	MA 25618
Ptychomitrium formosicum Broth. & Yosuda	DQ399601	DQ399628	Taiwan. Taichung Co	MO 5219650
Ptychomitrium gardneri Lesq.	DQ399602	DQ399629	U.S.A. Idaho	MO 5135689
Ptychomitrium sellowianum (Müll. Hal.) A. Jaeger	DQ399603	DQ399630	Paraguay. Paraguarí	MO 5215787
Racomitrium aciculare (Hedw.) Brid.	DQ399609	DQ399636	Spain. Cantabria	MA 22069
Racomitrium carinatum Cardot	DQ399610	DQ399637	South Korea. Kyonggi-do	MA 21356
Racomitrium heterostichum (Hedw.) Brid.	DQ399608	DQ399635	U.S.A. California	MO 5125302
Schistidium apocarpum (Hedw.) Bruch & Schimp .	DQ399611	DQ399638	Spain. León	MA 13294
Schistidium rivulare (Brid.) Podp.	DQ399613	DQ399640	Spain. Palencia	MA 20932
Schistidium trichodon (Brid.) Poelt	DQ399612	DQ399639	Austria. Totes Gebirge	MA 7455
Syntrichia rigescens (Broth. & Geh.) Ochyra	*AF481037*	DQ400972	Morocco. High Atlas	MUB 11378
Tortula atrovirens (Sm.) Lindb.	*AF480990*	*AY651833*	Spain. Sevilla	MUB 11352

of detected inversions was not coded for the phylogenetic analyses. However, in order to gain information from substitutions within detected inversions the latter were reverse-complemented and included in the analysis. Incomplete and ambiguous data at the beginning or end of the sequences were excluded from subsequent analyses. The data matrix has been deposited in TreeBASE (http://www.treebase.org/treebase/).

For phylogenetic inference, all characters were given equal weight, and gaps were treated as missing data. Parsimony analyses were conducted using *win*PAUP*4b10 (Swofford, 2002) and PRAP (Müller, 2004a). The latter program (available at http://www.botanik.uni-bonn.de/system/downloads/) generates command files for PAUP*4b10 that allow parsimony ratchet searches as designed by Nixon (1999) for analysis of large data sets. In the present study, 10 random addition cycles of 200 ratchet iterations each were used. Each iteration comprised two rounds of TBR branch swapping, one on a randomly re-weighted data set (25% of the positions), and the other on the original matrix saving one shortest tree. Since each random addition cycle rapidly converged to the same tree score, cycles were not extended to more than 200 iterations, nor were further cycles added. Shortest trees collected from the different tree islands were used to compute a strict consensus tree. Furthermore, the data set was analysed employing a simple indel coding approach as advocated by Simmons & Ochoterena (2000) using the PAUP command file generated by Seqstate (Müller, 2004b) and the same options in effect.

Internal branch support was estimated by heuristic bootstrap searches with 1000 replicates and 10 addition sequence replicates per bootstrap replicate. Decay values as further measurement of support for the individual clades were obtained using PRAP in combination with PAUP and the same options in effect as in the ratchet.

Maximum likelihood analyses were executed assuming a general time reversible model (GTR+G), and a rate variation among sites following a gamma distribution (four categories represented by mean). GTR+G was chosen as the model that best fits the data by Modeltest v3.6 (Posada & Crandall 1998) employing the interface MTgui (Nuin, 2005). The settings proposed by Modeltest v3.6 (BaseFreq = [0.3792 0.1178 0.1229], Nst = 6, Rmatrix = [0.5689 2.3709 0.1546 0.1819 2.3709], Shape = 0. 2102) were executed in PAUP. Maximum likelihood bootstrap searches were performed as "fast" stepwise-addition searches with 1000 replicates.

For further measurement of support, posterior probabilities were calculated using MrBayes v3.1 (Huelsenbeck & Ronquist, 2001). As in the maximum likelihood analysis, the GTR model of nucleotide substitution was employed, assuming site-specific rate categories following a gamma distribution. In addition an independent analysis with an appended indel matrix was performed employing the binary model for the indel partition. The a priori probabilities supplied were those specified in the default settings of the program. Posterior probability (PP) distributions of trees were created using the Metropolis-coupled Markov chain Monte Carlo (MCMCMC) method and the following search strategies suggested by Huelsenbeck & al. (2001, 2002). Two runs with four chains each were run simultaneously for 10^6 generations each run, with the temperature of the heated chains set to 0.2. Chains were sampled every 10 generations and the respective trees were written to a tree file. Calculation of the consensus tree and of the posterior probability of clades was done based upon the trees sampled after the burn-in (25%). Consensus topologies and support values from the different methodological approaches were compiled and drawn using TreeGraph (Müller & Müller, 2004).

RESULTS

Four inversions were detected in the combined data set, of which 3 are situated in the P8 stem loop region of the *trnL* intron (compare Quandt & Stech, 2005) and one 8–11 bp downstream of the 3′ *trnL* exon (alignment positions: 800–823; 858–907; 954–964; 1130–1139). The combined aligned data set (*rps4* and *trnL-F*) corrected for inversions is 1125 position long. 594 positons correspond to the *trnS-rps4* region and the remaining positions correspond to the *trnL-F* region. Primer sequences were trimmed from the sequences. Of 236 variable characters (118 each region) 169 were parsimony informative (84 from the *rps4* and 85 from the *trnL-F* region).

The MP ratchet analysis retained 18 most parsimonious trees (MPT, length = 385, CI = 0.735, RI = 0.839, RC = 0.617). With regard to Ptychomitriaceae, two conflicting topologies were resolved by the combined analysis: one of them groups *Ptychomitrium* and *Campylostelium* and has been termed "Ptychomitriaceae monophyletic", PM, Fig. 1, left tree), whilst the other branches *Campylostelium* first, grouping *Ptychomitrium* with Grimmiaceae ("*Campylostelium* first", CF, Fig. 1, right tree). In nine MPTs Ptychomitriaceae were monophyletic (PM; –ln 3548.23205) whereas in the other nine MPTs the clade comprising *Campylostelium* and *Grimmia pitardii* branched first, followed by *Ptychomitrium* and *Grimmiaceae* (–ln 3549.36277). Figure 2 shows one of the 18 MPTs with decay values and bootstrap support (with and without indel coding) along the branches. As the hypothesis with Ptychomitriaceae being monophyletic had the better likelihood score and was in addition independently retrieved by the strict consensus of the simple indel coding approach as well as the maximum likelihood and

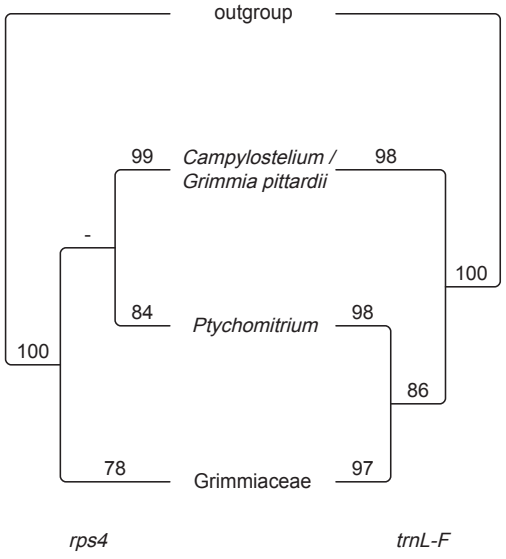

Fig. 1. Strict consensus topologies obtained from separate MP analyses of the *trnL-F* and *trnS-rps4* regions, with bootstrap support values along the branches

Bayesian analyses of the combined data, one of the nine MPTs showing the Ptychomitriaceae monophyletic hypothesis was choosen for illustration (Fig. 2). The maximum likelihood tree (–ln 3549.55239) with bootstrap support as well as posterior probabilities (with and without indel coding) is depicted in Fig. 3.

Separate analyses of the *trnS-rps4* and *trnL-F* matrices revealed a conflicting signal regarding Ptychomitriaceae between both data sets (Fig. 1). Whereas the *trnS-rps4* matrix favoured the PM-hypothesis (although without support), the *trnL-F* matrix resolved the *Campylostelium* first (CF) hypothesis with moderate support (BS 86). With almost equal amount of parsimony informative sites in each data partition, the observed conflicting signal might explain why neither the PM nor the CF hypothesis receives significant support in the combined analyses.

All analyses reveal *Grimmia pitardii* sister to *Campylostelium strictum* with maximal statistical support. Apart from *Campylostelium* (including *Grimmia pitardii*), both

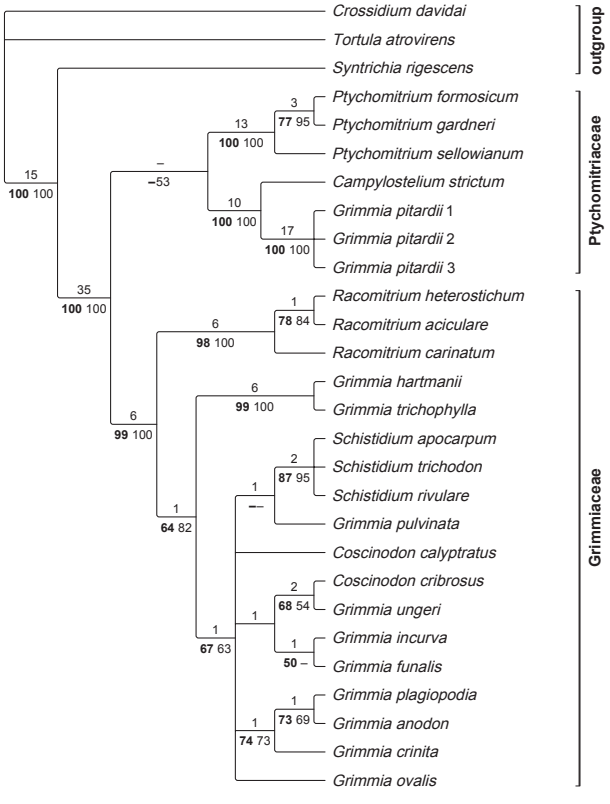

Fig. 2. One of 18 most parsimonious trees (length = 385, CI = 0.735, RI = 0.839, RC = 0.617) of the combined data. Decay indices are depicted above the branches; bootstrap support values are shown below the branches. The second value refers to bootstrap support obtained with the sic-indel matrix appended (sic = simple indel coding; Simmons & Ochoterena, 2000) as implemented in SeqState (Müller, 2004b).

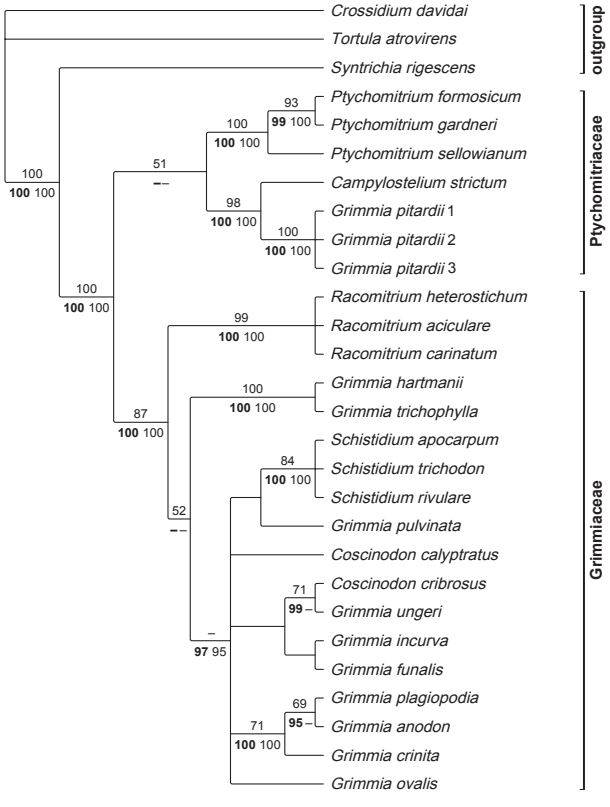

Fig. 3. Maximum likelihood (ML) tree of the combined data (–ln 3549.55239) with ML bootstrap support shown above the branches and posterior probabilities (with and without indel coding) below. The second value refers to bootstrap support obtained with the sic-indel matrix appended (sic = simple indel coding; Simmons & Ochoterena, 2000) as implemented in SeqState (Müller, 2004b). Only significant posterior probabilities ≥95 are depicted.

Ptychomitrium and Grimmiaceae (including *Racomitrium, Coscinodon*, *Grimmia,* and *Schistidium*) form maximally supported groups in all analyses. Generally, *Campylostelium* (including *Grimmia pitardii*) is grouped with *Ptychomitrium*, although without significant support. Within Grimmiaceae, *Racomitrium* is resolved monophyletic with maximal support and branching first, followed by a maximally supported clade consisting of *Grimmia hartmanii* Schimp. and *G. trichophylla* Grev. *Grimmia* is thus revealed paraphyletic with species of *Coscinodon* and the monophyletic *Schistidium* (MP: DC 2, BS 87/95; ML/Bayes: BS 93, PP 100/100) nested within.

DISCUSSION

Based on our results, the *Grimmiaceae*, as defined by Buck & Goffinet (2000), form a monophyletic group with high to maximal statistical support. However, the genus *Grimmia* is resolved paraphyletic based on (1) the position of *G. hartmanii* and *G. trichophylla*, and (2) the position of *Schistidium* and *Coscinodon* nested within *Grimmia*. *Grimmia hartmanii* and *G. trichophylla* are considered by various authors (e.g., Ochyra & al., 2003) as representatives of the genus *Dryptodon* Brid. (Type: *D. patens* (Hedw.) Brid.), which could indeed represent an independent genus. To this end, an in-depth study using more DNA regions (ITS, *trnK/matK*, as well as the *rps4-trnT-trnL* spacers) from a large number of members of all the taxa traditionally recognized within Grimmiaceae is currently under way to resolve the phylogenetic relationships within the family (Hernández-Maqueda & al., in prep.).

Regarding *Grimmia pitardii,* phylogenetic inferences unambiguously resolve this species sister to *Campylostelium strictum,* and hence as a member of Ptychomitriaceae rather than Grimmiaceae. Other representatives of *Grimmia* subg. *Grimmia* (i.e., *G. anodon, G. plagiopodia,* and *G. crinita*), a subgenus which currently comprises *Grimmia pitardii* (Loeske, 1930) belongs to the Grimmiaceae clade, and are thus not closely related to the *Campylostelium strictum*-*Grimmia pitardii* clade. The phylogenetic inferences based on molecular characters support the interpretation by Maier (1998) of morphological characters (i.e., plurilobed mitrate calyptra, leaf costa with median guide cells larger than the ventral cells, peristome with basal membrane, and outer peristome layer as thick as the inner peristome layer). Consequently, we agree that *G. pitardii* should be transferred to *Campylostelium* and that the correct treatment of this taxon is *Campylostelium pitardii* (Corb.) E. Maier, as Maier (1998) proposed.

Regarding the relationships of *Ptychomitrium* and *Campylostelium*, *trnL-F* alone favors with moderate support the CF-hypothesis in agreement with the classification of Limpricht (1890). However, the two genera are resolved sister to *Grimmiaceae* when *trnS-rps4* and *trnL-F* sequences are used in combination, in agreement with recent molecular studies (Tsubota & al., 2003; Hedderson & al., 2004). The low support for grouping *Ptychomitrium* and *Campylostelium* does not allow conclusions concerning the phylogenetic relationships between these genera, especially as the phylogenetic signal provided by *trnL-F* matrix clearly supports the CF-hypothesis (Fig. 1). More sequence data of different regions from a denser sampling within Ptychomitriaceae might resolve this issue (Hernández-Maqueda & al., in prep.).

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