

## Testing reticulation and adaptive convergence in the Grimmiaceae (Bryophyta)

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Phylogenetic relationships based on plastid DNA sequences have recently been explored for the genus *Grimmia*, revealing a complex evolutionary history and many incongruities with respect to traditional views. Based on empirical observations it was postulated that episodes of allopolyploidy and various hybridization events have triggered speciation in the genus *Grimmia*. Comparisons of genes from different genomes could therefore help to detect putative reticulations that cannot be detected using a single genome. For this purpose phylogenetic inferences, based on the complete ITS region of nuclear ribosomal DNA, were contrasted with plastid (*trnS-trnF*, *trnK/matK*) derived ones. The ITS region proved to be highly variable in *Grimmia*, with various lineage-specific indels interspersed among a considerable number of conserved regions that contained important phylogenetic information. The sectional placement of most species is congruent with previous results based on plastid DNA. However, some species seemingly combine nuclear sequences of one section with chloroplast sequences of another. The species of *Grimmia* subg. *Grimmia*, with the exception of *G. pulvinata*, are nested within *Grimmia* in plastid phylogenies, but are sister to the remaining *Grimmia* groups and closer to *Dryptodon* based on nuclear DNA sequences. According to the Shimodaira-Hasegawa (SH) test an alternative hypothesis in which *Grimmia* subg. *Grimmia* is nested within *Grimmia* could be rejected. Similarly, an alternative topology with *G. tergestina* close to *G. laevigata* as revealed by plastid data was clearly rejected by the SH test, supporting the observation that *G. tergestina* appears to have the nuclear sequence of section *Orthogrimmia* and the chloroplast sequence of section *Guembelia*. We hypothesize that both cases can be best explained by past reticulation events.

**KEYWORDS:** adaptive convergence, Bryophyta, *Dryptodon*, *Grimmia*, Grimmiaceae, ITS, nuclear DNA sequences, reticulation, *Schistidium*

### INTRODUCTION

Current discussions regarding the systematics of *Grimmia* face the same problems as in many other groups: splitting versus lumping. Whereas Muñoz & Pando (2000) as well as Greven (2003) view *Grimmia* as a single large genus, others recognize the subgenera at the generic level (Ochyra & al., 2003; Goffinet & Buck, 2004). Recent molecular studies of the Grimmiaceae (Streiff, 2006; Hernández-Maqueda, 2007; Hernández-Maqueda & al., 2008) have partially resolved questions about generic relationships within the family. For example, *Dryptodon* and *Schistidium* are now considered to be independent genera, whereas the polyphyletic genera *Coscinodon* and *Hydrogrimmia* should be considered as part of *Grimmia* (Hernández-Maqueda & al., 2008), and *Grimmia pitardii* has been transferred to the Campylosteliaceae (Hernández-Maqueda & al., 2007, 2008). Despite the segregation of *Dryptodon* and *Schistidium*, two main issues remain: (1) ancestral character state reconstructions did not identify synapomorphies for the emended *Grimmia*;

(2) the species compositions of the subgenera accepted within *Grimmia* are clearly in conflict with traditional views. The most striking examples are *G. funalis* and *G. elatior*. Both are gametophytically and sporophytically similar to *Dryptodon*, but plastid DNA phylogenies place them nested within *Grimmia*. While adaptive convergence could be an explanation for such a placement, morphological similarities suggest that *Grimmia* could have experienced some episodes of hybridization (Muñoz, pers. obs.). Interspecific hybridization has long been recognized as an important phenomenon in plant evolution (Rieseberg, 1995; Burke & Arnold, 2001). Natcheva & Cronberg (2004) presented an updated overview of bryophyte hybridization, which is rarely considered as an important evolutionary phenomenon in mosses; most often, bryologists consider that the effects of interspecific hybridization in bryophytes are confined to the sporophytic phase with the result of no or sterile spores (e.g., Philibert, 1873). Detection of incongruence between chloroplast and nuclear DNA sequences could be employed to detect putative reticulation events and

thus potential hybrids. Unfortunately, very little bryological literature deals with this topic, an exception being the work on *Sphagnum* by Shaw & Goffinet (2000).

The present study therefore aims to explore the congruence between a nuclear region and the plastid phylogenies already published (Hernández-Maqueda, 2007; Hernández-Maqueda & al., 2008). For this purpose the internal transcribed spacer (ITS) region (18S-5.8S-26S) of the nuclear ribosomal DNA (nrDNA) was sequenced and the obtained phylogenetic results compared with previous studies on plastid DNA (Hernández-Maqueda, 2007; Hernández-Maqueda & al., 2008). The ITS region has been widely used at generic and infrageneric levels in other plant groups (Vanderpoorten & al., 2006; Baldwin & al., 1995), and Quandt & Stech (2003) reviewed its use in bryophytes. In particular, the ITS region has been employed to resolve phylogenetic relationships in *Sphagnum* (Shaw, 2000b), *Amblystegium* (Vanderpoorten & al., 2001), Meteoriaceae (Quandt & al., 2004), *Campylopus* (Stech, 2004), *Trichostomum* and related genera (Werner & al., 2005b), and *Didymodon* (Werner & al., 2005a). From these studies it is evident that sequence variation is largely lineage dependent; for example, the ITS region exhibits similar variation across certain Hypnalean families as is observed among populations of single species of the genus *Mielichhoferia* (Shaw, 2000a). On another matter, the lack of complete concerted evolution, the putative presence of pseudogenes, and paralogy are phenomena that can increase homoplasy in phylogenetic relationships performed with ITS (Álvarez & Wendel, 2003).

The present work attempts to answer the following questions: (1) how useful is the ITS region for studying the phylogeny of *Grimmia*?, (2) how congruent are phylogenetic inferences based on the nuclear ITS region with those derived from plastid DNA, and how do we explain any observed incongruities?, (3) can we propose a solid classification of *Grimmia* considering all available data (morphology, plastid DNA, nuclear DNA)?

## MATERIAL AND METHODS

**Taxon and DNA sampling.** — Forty-nine taxa including species of *Grimmia*, *Schistidium* and *Dryptodon* were included in the analysis. Four *Racomitrium* species were selected as outgroup. Vouchers are deposited in CAS, CHR, MA, MO, and S. GenBank accession and herbarium voucher numbers as well as the origin of specimens are listed in the Appendix. A second reduced dataset (44 terminals) with sequences from both plastid and nuclear DNA was used in a single matrix to evaluate the degree of congruence between both genomes. Plastid sequences derive from previous studies by the same research group (Hernández-Maqueda, 2007; Hernández-Maqueda & al.,

2008). For *Grimmia tergestina*, a critical species in this study, we used sequences from the same two specimens for both cpDNA and nrDNA (MA 14653, MA 25013), and also cpDNA data published by Streiff (2006; GenBank accession numbers: *rps4*, AJ845238; *trnL-F*, AJ847888) from a further specimen. All the sequences were identical across these specimens, therefore only MA 14653 was used in the phylogenetic analyses.

### DNA isolation amplifications and sequencing. —

Protocols for DNA isolation, amplification, purification and sequencing are described in detail in Hernández-Maqueda & al. (2007). Amplification products for the ITS region were generated using the following program: 2 min at 96°C, followed by 30 cycles each with 2 min at 96°C, 1 min at 49°C, and 1 min at 72°C with the annealing temperature increased by 1°C per cycle and stabilized once it reached 60°C. The final extension step at 72°C was set to 7 min. Primers for amplification and sequencing were 18S (forward: 5'-GGA GAA GTC GTA ACA AGG TTT CCG-3') designed by Spagnuolo & al. (1999) and ITS4 (reverse: 5'-TCC TCC GCT TAT TGA TAT GC-3') (White & al., 1990).

### Data analysis. —

Sequences were edited and manually aligned using PhyDE<sup>®</sup> (Müller & al., 2005). Direct sequences were thoroughly screened to detect putative superimposed nucleotide additivity patterns (SNAP) following Whittall & al. (2000). In addition, each sequence was analyzed in order to detect putative pseudogenes. For this purpose obtained sequences were screened for peculiarities in nucleotide composition (GC content) as well as substitution rates as recommended by Álvarez & Wendel (2003) and Bailey & al. (2003).

For phylogenetic inference, all characters were given equal weight and gaps were treated as missing data. Parsimony analyses were conducted using winPAUP\*4b10 (Swofford, 2002) and PRAP (Müller, 2004). The latter program generates command files for PAUP\* that allow parsimony ratchet searches as designed by Nixon (1999) for analysis of large datasets. 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 dataset (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 dataset was analyzed employing a simple indel coding approach as advocated by Simmons & Ochoterena (2000) using the PAUP command file generated by Seqstate (Müller, 2005) and modified later by Müller (2006), with the same options in effect.

Internal branch support was estimated by heuristic bootstrap searches with 1,000 replicates and 10 addition

sequence replicates per bootstrap replicate. Decay values as a further measurement of support for the individual clades were obtained using PRAP in combination with PAUP with the same options in effect as for the ratchet.

Maximum likelihood analyses were executed assuming a general time reversible model (GTR+ $\Gamma$ +I), and rate variation among sites following a gamma distribution. GTR+ $\Gamma$ +I was chosen as the model that best fitted the data according to the Akaike Information Criterion by Modeltest v3.6 (Posada & Crandall, 1998) employing the Windows® interface MTgui (Nuin, 2005). The settings proposed by Modeltest v3.6 were executed in PAUP 4.0b10. For the ITS region the following settings were used: BaseFreq = (0.2252 0.2887 0.2654), Nst = 6, Rmatrix = (1.0 2.8378 0.5930 0.5930 2.8378), Shape = 2.5518, and Pinvar = 0.4323. Internal branch support was estimated by bootstrap searches with 1,000 replicates.

For further measurement of support, posterior probabilities were calculated using MrBayes v3.1 (Huelsenbeck & Ronquist, 2001) employing the GTR model of nucleotide substitution, assuming a rate variation among sites following a gamma distribution and a proportion of invariable sites. 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 (Huelsenbeck & al., 2001, 2002): ten 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 100 generations and the respective trees written to a tree file. Convergence of the chains was inspected graphically by plotting the likelihoods against the number of generations. Calculation of the consensus tree and the posterior probabilities for the clades was based on the trees sampled after the burn-in (stationarity was reached within the first 250,000 generations). Consensus topologies and support values from the different methodological approaches were compiled and drawn using TreeGraph (Müller & Müller, 2004).

**Statistical test for accuracy of the phylogenetic observations.** — *Incongruence length difference (ILD) test.* The degree of congruence between the ITS versus the plastid partition was evaluated based on the obtained ITS data in combination with the *rps4-trnT-trnL-trnF* and *trnK/matK* sequence matrices of Hernández-Maqueda (2007) and Hernández-Maqueda & al. (2007, 2008). We used the test proposed by Farris & al. (1995) based on the incongruence length difference ( $I_{MF}$ ) of Mckeivich & Farris (1981) as implemented in PAUP\* (“partition homogeneity test”). The metric is computed for a number of

random partitions of the combined dataset. When 95% or more of those random partitions show an  $I_{MF}$  smaller than the original, we reject the null hypothesis and conclude that the datasets are significantly heterogeneous. Despite the limitations of this test that have been recently highlighted (Struck & al., 2006), it is still being used and it is especially useful when numerous characters are present and the substitution rate is homogeneous from site to site (Darlu & Lecointre, 2002).

*Shimodaira-Hasegawa (SH) test.* The Shimodaira & Hasegawa (1999) nonparametric test was used to statistically compare alternative phylogenetic hypotheses using the GTR+ $\Gamma$ +I model with the settings proposed by Modeltest. Only taxa that showed conflicting positions with previous studies were explored: *Grimmia* subg. *Grimmia*, *G. funalis*, *G. elatior*, and *G. tergestina*. The analyses were run in winPAUP\*b4.0 using 1,000 bootstrap replicates and full parameter optimization of the model.

## RESULTS

**ITS sequences.** — The ITS sequences of the species studied are highly variable. Length variations range from 515 nt in the species *Racomitrium elongatum* to 670 nt in *Grimmia elongata*. The greatest distance was found between *Schistidium crassipilum* and *Racomitrium elongatum*, with pairwise distances values of up to 11.7%. Variations within taxa range from 2.3% between species of sect. *Montanae* to 6.1% between species of the genus *Dryptodon*. For six species (*Grimmia caespiticia*, *G. funalis*, *G. involuocrata*, *G. montana*, *G. orbicularis*, *G. pulvinata*), multiple populations were sequenced. The highest intraspecific distance was observed in *G. orbicularis* (2.7%), whereas no variation was observed in *G. involuocrata* or *G. pulvinata*. Many structural repeat units were detected in the matrix, ranging in size from 1–2 nt to 86 nt in *Grimmia elongata* (positions 457–543 in the final alignment). Long structural mutations of this type were also observed in other species complicating the alignment process. However, lineage-specific indels often associated with highly variable regions generally alternated with conserved regions. Ambiguously aligned regions were excluded from the analyses. The final matrix included 1,511 characters, positions 1–840 correspond to the ITS1 spacer, 841–1,019 to the 5.8S gene and 1,020–1,511 to the ITS2 spacer.

**Polymorphisms in ITS.** — Although the pherograms were checked carefully by eye no polymorphisms due to SNAP processes could be identified. Questionable double peaks detected for a particular position in some pherograms were unambiguously resolved after analyzing the reverse primer (i.e., *G. involuocrata*, *G. reflexidens*). Moreover, taxa with double peaks were re-sequenced in an attempt to corroborate the obser-



included, whereas values below are based on nucleotide information only. Figure 2 shows the Bayesian tree with posterior probabilities below (with and without indel coding) and bootstrap support values obtained via likelihood above the branches.

Coding of indels as characters according to Simmons & Ochoterena (2000) generally increased the statistical

support for the clades, especially near the tips of the tree, both in the MP and the bayesian analyses. Whereas some clades are largely unresolved in the MP analysis without indel coding, the support for some parts of the tree increases with the sic-matrix appended.

Using *Racomitrium* as outgroup, *Dryptodon*, *Schistidium* and *Grimmia* are grouped with strong support

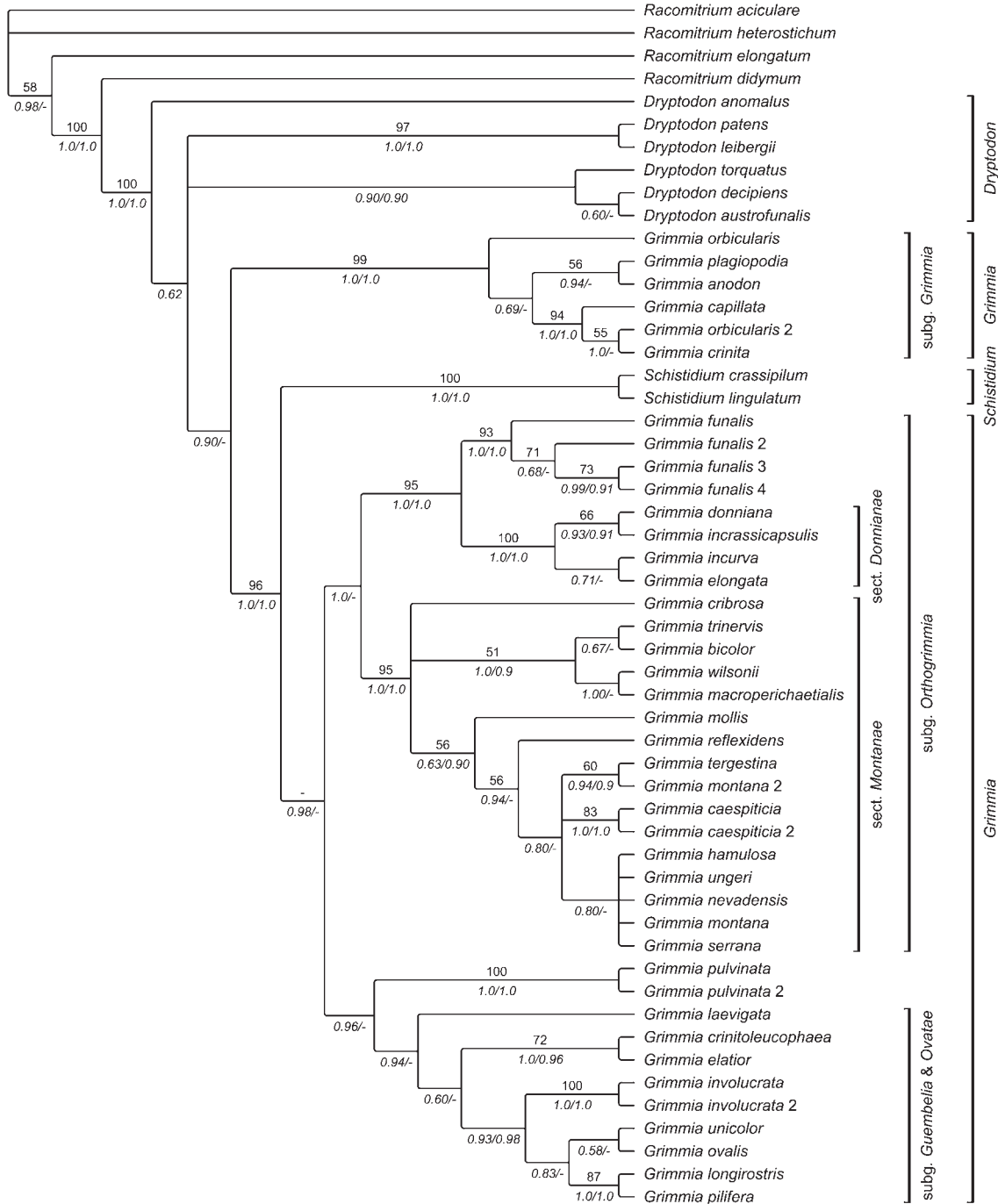


Fig. 2. Bayesian inference of phylogeny based on the ITS dataset with posterior probabilities indicated below (left, with indel coding; right, without indel coding) and ML bootstrap support values above the branches.

(Maximum Parsimony, MP: 100/100 bootstrap support, bs; 13/8 decay value, dv; Maximum Likelihood, ML: 100 bs; Bayesian Inference, BI: 1.0/1.0 posterior probability, pp). In cases where two values are given, the first one refers to the analysis including the sic-matrix. Under parsimony *Dryptodon* is resolved monophyletic, whereas the genus is paraphyletic in model based approaches, but support is lacking in all analyses. *Grimmia* subg. *Grimmia* (including *G. orbicularis*, *G. crinita*, *G. capillata*, *G. anodon*, and *G. plagiopodia*) forms a clade (MP: 100/100 bs, 13/7 dv; ML: 99 bs; BI: 1.0/1.0 pp) sister to the remaining taxa. *Schistidium* and the remaining species of

*Grimmia* form a strongly supported clade (MP: 96/93 bs, 8/5 dv; ML: 96 bs; BI: 1.0/1.0 pp) in which *Schistidium* is maximally supported (MP: 100/100 bs, 31/19 dv; ML: 100 bs; BI: 1.0/1.0 pp), whereas the monophyly of the remaining *Grimmia* is only supported in analyses including the indel matrix (MP: 70/– bs, 5/– dv; ML: – bs; BI: 0.98/– pp). Within the genus *Grimmia*, a clade containing species of the subgenera *Guembelia* and *Ovatae* receives moderate support (MP: 74/– bs, 4/– dv; ML: – bs; BI: 0.94/– pp), but again only in analyses including the indel matrix. Relationships within the clade, however, are largely unresolved and unsupported. *Grimmia pulvinata*

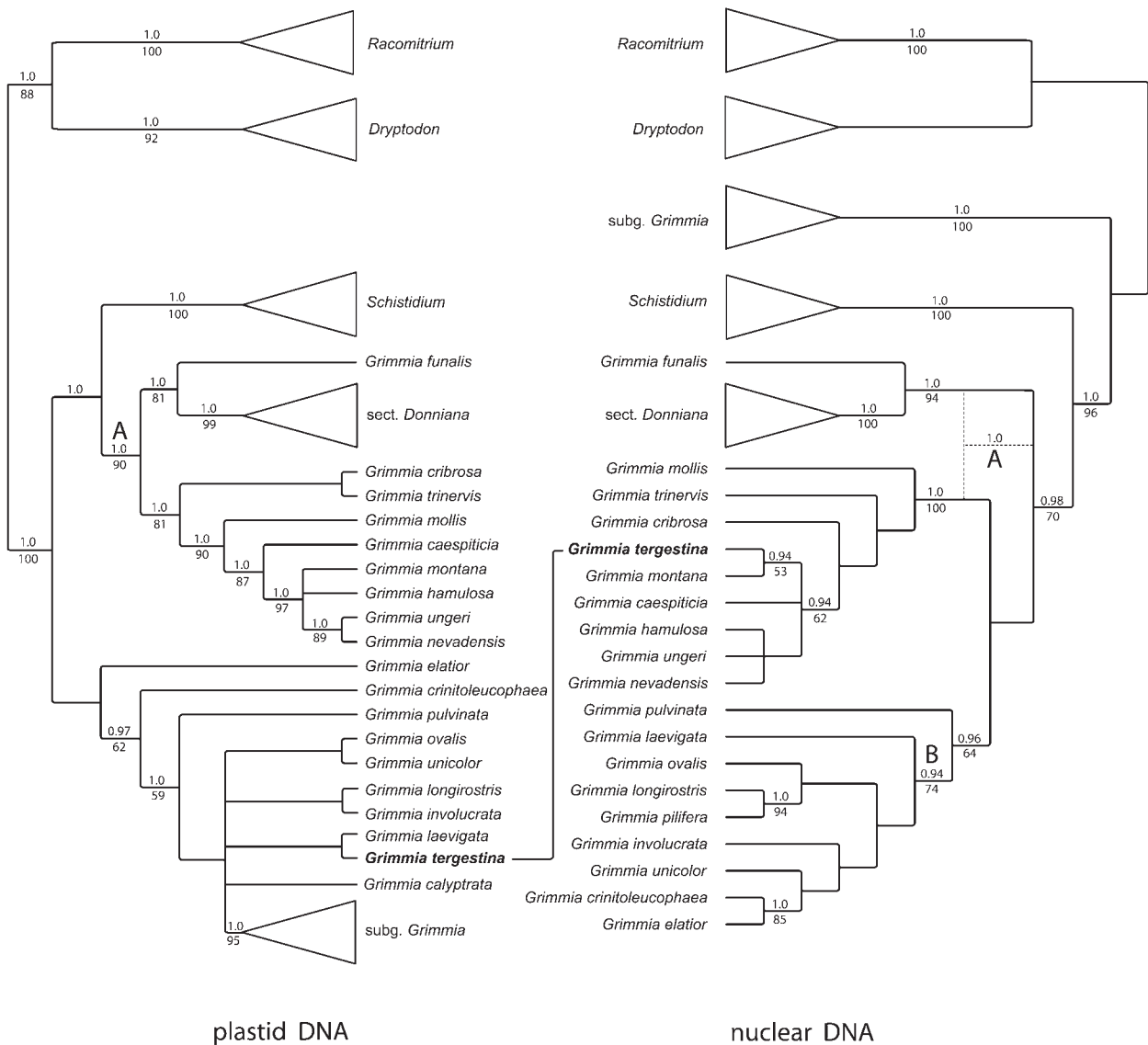


Fig. 3. Strict consensus topologies obtained from separate MP analyses of the ITS region (including indels) and the combined chloroplast regions (*rps4-trnT-trnL-trnF* and *trnK/matK* plus indels), with posterior probabilities above and bootstrap support values below the branches. A indicates subg. *Orthogrimmia*, and B includes species of the subgenera *Guembelia* and *Ovatae*. An alternative ITS topology resolving *Orthogrimmia* monophyletic (indicated by a dashed line) was retrieved in a Bayesian approach, and receives significant support if indels are included in the analysis.

retains a weakly supported sistergroup relation to this clade (MP: 64/– bs, 2/– dv; ML: – bs; BI: 0.96/– pp). Finally, the two sections recognized within subg. *Orthogrimmia* are clearly resolved with high support (sect. *Montanae*: MP: 100/98 bs, 19/7 dv; ML: 95 bs; BI: 1.0/1.0 pp; sect. *Donniana*: MP: 100/100 bs, 8/8 dv; ML: 100 bs; BI: 1.0/1.0 pp). Sister to sect. *Donniana*, *Grimmia funalis* is resolved with high support values (MP: 94/90 bs, 6/4 dv; ML: 95 bs; BI: 1.0/1.0 pp). *Orthogrimmia* itself, however, is only resolved monophyletic in a bayesian approach, and receives significant support if indels are included in the analysis.

**Accuracy of the phylogenetic observations.** — *ILD test.* The trees obtained from the ITS region revealed slightly different topologies from those obtained using plastid DNA (Hernández-Maqueda, 2007; Hernández-Maqueda & al., 2007, 2008). The most significant conflict was found in the position of the clade including *Grimmia orbicularis*, *G. crinita*, *G. capillata*, *G. anodon* and *G. plagiopodia* (*Grimmia* subg. *Grimmia* as considered here). Although *Grimmia* subg. *Grimmia* is resolved monophyletic by plastid data and placed within the genus *Grimmia*, the nuclear sequences place *Grimmia* subg. *Grimmia* sister to the clade including *Schistidium* and the remainder of *Grimmia*. The position of *Grimmia tergestina* is also conflicting. It combines ITS sequences of the *Orthogrimmia*-type and plastid sequences of the *Guembelia*-type (Fig. 3). The *ILD* test showed a significant heterogeneity between the plastid and nuclear datasets ( $P < 0.001$ ). If the problematic species (i.e., *Grimmia* subg. *Grimmia* and *Grimmia tergestina*) were excluded, the  $P$  value increased ( $P = 0.05$ ), indicating homogeneity between the two datasets.

*SH test.* The results of the *SH* test evaluating alternative hypotheses for the placement of *Grimmia* subg. *Grimmia*, *G. funalis*, *G. elatior*, and *G. tergestina* are summarized in Table 1. Of the 5 alternative hypotheses tested, three of them, namely (1) *Grimmia* subg. *Grimmia* within *Grimmia*, (2) the systematic position of *Grimmia funalis* and *G. elatior* nested within *Dryptodon*, and (3) the placement of *G. tergestina* as a close relative of *G. laevigata*, were rejected.

## DISCUSSION

**Molecular variability of the ITS region.** — The variability of the ITS region proved to be high and lineage-dependent in the *Grimmia-Dryptodon-Schistidium* complex. When *Dryptodon* and *Racomitrium* were included in the dataset, a high number of gaps must be introduced into the matrix during alignment. However, a proportionately large number of rather conserved regions guided the alignment and helped to identify larger indels as well as repetitive elements. The data reveals high inter- but very low intragroup variation. The most extreme example of the latter is the sequence variation within sect. *Montanae*, which ranges from 0% to 2.3%. For several taxa having morphological and molecular conflicting signals, namely *G. caespiticia*, *G. funalis*, *G. involucrata*, *G. montana*, *G. orbicularis*, and *G. pulvinata*, more than one sequence was obtained, but the variability detected at population level does not have effects on the results. Two species, *G. pulvinata* and *G. orbicularis*, have polyploid populations ( $n = 13, 14, 26, 26+m$ , cf. Fritsch, 1991), and are therefore prime candidates for intraspecific ITS variability, however pairwise distances between different exemplars are not noteworthy and no evidence for multiple copies per individual were detected. All sequenced populations of *G. pulvinata* cluster in the same clade, which is not the case for *G. orbicularis*. Therefore, the presence of potential pseudogenes in *G. orbicularis* was studied as a source of variability. We rejected this hypotheses based on the higher substitution rates found in ITS1 and ITS2 compared to 5.8S and the lack of significant differences in GC-contents. According to Bailey & al. (2003), functional copies of the ITS region maintain highly conserved functional parts (i.e., the 5.8S gene) relative to the spacers ITS1 and ITS2. In contrast, non-functional copies will show similar substitution rates across the entire region.

**Phylogeny of *Grimmia*.** — Phylogenetic relationships within Grimmiaceae and especially within *Grimmia* and related genera have recently been analysed using plastid DNA and morphology (Streiff, 2006; Hernández-Maqueda, 2007; Hernández-Maqueda & al., 2008). Ac-

**Table 1. Results of Shimodaira-Hasegawa test for comparison of alternative phylogenetic hypotheses.**

Hypotheses	Shimodaira-Hasegawa test		
	–lnL	diff–lnL	<i>P</i> values
ML tree	6348.08584	–	–
<i>Grimmia funalis</i> and <i>Grimmia elatior</i> included in a monophyletic <i>Dryptodon</i>	6418.19311	70.10727	<0.001*
<i>Grimmia</i> subgen. <i>Grimmia</i> nested within <i>Grimmia</i>	6382.23310	34.14725	0.006*
<i>Grimmia</i> subgen. <i>Grimmia</i> nested within <i>Dryptodon</i>	6351.62338	3.53754	0.402
<i>Schistidium</i> as sister to <i>Orthogrimmia</i>	6350.35143	2.26559	0.389
<i>Grimmia tergestina</i> as sister to <i>G. laevigata</i>	6430.97799	82.89210	<0.001*

\*Values of  $P < 0.05$  reject the null hypothesis.

According to these results, *Dryptodon* and *Schistidium* are independent genera, whereas *Coscinodon* and *Hydrogrimmia* are within *Grimmia*, which would include four subgenera: *Grimmia*, *Guembelia*, *Orthogrimmia*, and *Ovatae*. Results using plastid data exhibited conflicts with traditional views, pointing to complex evolutionary processes that cannot be understood in the light of plastid DNA alone.

The current ITS data supports the same basic groups as the plastid DNA, and although the placement of most of the species is also similar, there are some significant differences. Firstly, the monophyly of *Dryptodon* based on the ITS matrix receives no support and is only achieved under parsimony. This might be a consequence of the observed high sequence divergence in *Dryptodon* that in addition resulted in various synapomorphic indel blocks. Besides, the region is quite short and more characters might be required to render high support values to a monophyletic *Dryptodon* as occurs with the plastid data. Secondly, moderate support (Figs. 1, 2) was obtained for a clade containing species of subgenera *Guembelia* and *Ovatae*, which was not supported with plastid DNA. Finally, the most dramatic incongruities with the plastid-derived topology were found with respect to the position of *Grimmia* subg. *Grimmia* and the placement of *G. tergestina*, treated in more detail below.

As the general aspects of the phylogenetic relationships of *Grimmia* have been discussed in detail elsewhere (Hernández-Maqueda & al., 2008), we will focus on taxa for which relationships inferred from ITS data are in conflict with plastid based phylogenies and traditional views.

**Alternative hypotheses for the placement of *Grimmia* subg. *Grimmia*.** — Species in *Grimmia* subg. *Grimmia* are characterized by leaves that are boat-shaped when muticous. According to recent molecular data (Hernández-Maqueda, 2007; Hernández-Maqueda & al., 2007, 2008), *Grimmia* subg. *Grimmia* would include *Grimmia orbicularis*, *G. crinita*, *G. capillata*, *G. plagiopodia*, and *G. anodon*. The position of the morphologically very similar species *G. pulvinata* is still under debate, as published molecular phylogenies fail to resolve its placement. However, a placement of *G. pulvinata* within the subg. *Grimmia* based on molecular data has not been reported, so far. Disregarding *G. pulvinata*, *Grimmia* subg. *Grimmia* was resolved in different positions depending on the dataset employed: nested within an inclusive genus *Grimmia* based on cpDNA, but sister to remaining *Grimmia* subgenera and *Schistidium* employing ITS data (Fig. 3). An additional topology test based on the ITS data clearly rejected the placement of *Grimmia* subg. *Grimmia* nested within an ample *Grimmia*. The observed incongruence could reflect past reticulation events, as polyploids are common in these species (Fritsch, 1991) and thus hybrid origins might be suspected. As mentioned above, the affinities of *G. pulvinata*,

however, that morphologically belongs to *Grimmia* subg. *Grimmia*, are not clear yet, as (1) the cpDNA analyses do not allow a definite decision, and (2) the nuclear phylogeny points towards reticulation of the other species traditionally treated within *Grimmia* subg. *Grimmia*. However, as the accessions of *G. pulvinata* were resolved sister to a clade containing a mixture of *Guembelia* and *Ovatae* (based on ITS data) or within *Guembelia/Ovatae* (cpDNA), we hypothesize that *G. pulvinata* might have been involved in the reticulation events as parental species.

**Alternative hypotheses for the placement of *G. funalis* and *G. elatior*.** — *Grimmia funalis* and *G. elatior* are more closely related to *Dryptodon* spp. on sporophytic grounds, but are resolved within the genus *Grimmia* based on molecular data, next to species that are morphologically very distant. Interestingly, though, *G. funalis* has been occasionally treated by Nordic authors (e.g., Brotherus, 1923) as forming an independent section (sect. *Funales* I. Hagen) related to subg. *Orthogrimmia*, and not to *Dryptodon*. The four sequenced accessions of *G. funalis* show some point mutational differences, making the species a potential candidate for population studies. As an alternative placement of *G. elatior* and *G. funalis*, we tested their inclusion in *Dryptodon*, but this hypothesis was rejected by the SH test ( $P < 0.001$ , Table 1). With the data on hand, it seems that we again partly return to a concept of Brotherus (1923).

**Alternative hypotheses for the placement of *G. tergestina*.** — *Grimmia tergestina* shares nuclear sequences of subgenus *Orthogrimmia* and chloroplast sequences of subgenus *Guembelia*. Several molecular synapomorphies clearly support the observed relationships found with the different datasets, which points to past reticulate events as a possible explanation for such relationships. In addition the SH test clearly rejects the alternative placement of *G. tergestina* as sister of *G. laevigata*.

Additionally, *G. tergestina* is not only of hybrid origin, but we hypothesize that it also acts as a parental species. It naturally hybridizes with other *Grimmia* species and produces normal-looking sporophytes, although it is unknown if they produce viable spores. One such hybrid was described as *G. orbicularis* × *G. tergestina*, an invalid name later validated as *G. philibertii* Giacom. (Philibert, 1873; Giacomini, 1950). Putative hybridization phenomena in *Grimmia* usually seem to involve *G. tergestina* (Culmann, 1926; Loeske, 1930; Greven, 1995). *Grimmia tergestina* strongly resembles *G. americana*, *G. involucreta*, *G. crinitoleucophaea* and *G. ovalis* (subg. *Guembelia*), and cannot be separated from the former three when sterile. *Grimmia americana* differs only in its gonioautoicous sexual condition, whereas *G. involucreta* (gonioautoicous) and *G. crinitoleucophaea* (dioicous) have curved setae and ventricose capsules. We hypothesize that these species have originated by hybridization

with *Grimmia* subg. *Grimmia* members (*G. orbicularis*?) based on the similarity of their sporophytes to some hybrid sporophytes (e.g., *G. philibertii*). The lack of intermediate characters is not a definitive reason to discard a hybrid hypothesis, as hybrids are not necessarily intermediate (Rieseberg, 1995). In bryophytes, gametophytic progeny results from recombination during meiosis and recombinants may inherit different parts of the parental genomes, express various combinations of parental traits, or may be more similar to one or the other parent. We believe that incongruence detected between plastid and nuclear DNA points to past reticulation events affecting the evolution of this species.

## CONCLUSIONS

The ITS region has proved to be a useful molecular marker for inferring phylogenetic relationships within *Grimmia*. Although large structural mutations as well as lineage-specific indels make homology assessment problematic in some regions, especially between different genera (*Dryptodon*, *Racomitrium*, *Grimmia*), conserved regions show relevant phylogenetic signal. Detailed analyses and comparisons of nuclear and plastid DNA reveal that the evolutionary history of the genus *Grimmia* is much more complex than previously thought. The incongruence detected between phylogenetic relationships derived from plastid and nuclear DNA is consistent with reticulate evolution. The placement of *Grimmia* subg. *Grimmia* sister to *Schistidium* and the remainder of *Grimmia* represents an extreme case of reticulate evolution and indicates a complex gene flow in *Grimmia*. Similarly, in the case of *G. tergestina*, reticulate evolution seems to be the best explanation for the detected incongruence. The sectional relationships of both the plastid and the nuclear sequences are supported by numerous base substitutions, and placements in other sections seem implausible. Whether *G. tergestina* is an isolated example within the evolution of the genus should be tested in further investigations. We feel that reticulation events should be taken more into consideration in future research on the systematics of *Grimmia*, and bryophytes in general.

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**Appendix.ö List of investigated specimens, with GenBank accession numbers for the ITS region, including voucher numbers and the herbaria where the specimens are kept. All sequences are new for this study.**

**Species; Voucher or reference; Origin; ITS Genbank Accession n°**

*Dryptodon anomalus* (Schimp.) Loeske; MA-24709; Russia. Altay Republic; EU343751; *Dryptodon austrofunalis* (Müll. Hal.) Ochyra & Zarnowiec; MO-5211690; Bolivia. La Paz; EU343752; *Dryptodon decipiens* (Schultz.) Loeske; MA 32764; Spain. Toledo; EU343753; *Dryptodon leibergii* (Paris) Ochyra & Zarnowiec; MA-25022; U.S.A. California; EU343755; *Dryptodon patens* (Hedw.) Brid.; MO-5142675; U.S.A. Alaska; EU343756; *Dryptodon torquatus* (Drumm.) Brid.; MA-25588; U.S.A. California; EU343757; *Grimmia anodon* Bruch & Schimp.; MA-25617; U.S.A. Nevada; EU343758; *Grimmia bicolor* Herzog; MO-4461458; Bolivia. La Paz; EU343759; *Grimmia caespiticia* (Brid.) Jur.; MA-24716; Spain. Avila; EU343761; *Grimmia caespiticia*2; MA-19713; Spain. Avila; EU343760; *Grimmia capillata* De Not.; MA-24719; Kazajistan. Mangyshlak; EU343762; *Grimmia cribrosa* (Hedw.) Spruce; MO-4441357; USA, Maine; EU343763; *Grimmia crinita* Brid.; MA-22641; Spain. Huesca; EU343764; *Grimmia crinitoleucophaea* Cardot; MA-24655; Siberia. Yakutskaya; EU343788; *Grimmia donniana* Sm.; MA-15356; Italy. Val Venosta; EU343765; *Grimmia elatior* Bruch ex Bals.-Criv. & De Not.; S-B51986; Norway. Trams; EU343754; *Grimmia elongata* Kaulf.; S-B53421; Sweden. Torne Lappmark; EU343766; *Grimmia funalis* (Schwäger.) Bruch & Schimp.; MA-21988; Spain. Huesca; EU343769; *Grimmia funalis*2; MA-22007; Spain. Cantabria; EU343770; *Grimmia funalis*3; S-B64173; Norway, Finmark; EU343767; *Grimmia funalis*4; MA-21468; Russian. Chukotka; EU343768; *Grimmia hamulosa* Lesq.; MA-25701; U.S.A. California; EU343771; *Grimmia incraspicapsulis* B.G. Bell; CHR-503516; New Zealand. Otago; EU343772; *Grimmia incurva* Schwäger.; S-B70022; Sweden. Jamtlands Lam; EU343773; *Grimmia involucrata* Cardot; MA-27659; Mexico. Hidalgo; EU343775; *Grimmia involucrata*2; MA-27658; Mexico. Hidalgo; EU343774; *Grimmia laevigata* (Brid.) Brid.; MA-25401; Spain. Zamora; EU343776; *Grimmia longirostris* Hook.; MA-21394; Siberia. Yakutskaya; EU343777; *Grimmia macroperichaetialis* Greven; MO-513774; Australia; EU343778; *Grimmia mollis* Bruch & Schimp.; S-B6791; Austria. Tirol; EU343779; *Grimmia montana* Bruch & Schimp.; MA-13305; Spain. Asturias; EU343780; *Grimmia montana* 2; MA-14721; U.S.A. California; EU343781; *Grimmia nevadensis* Greven; CAS-C50Grev; U.S.A. California; EU343782; *Grimmia orbicularis* Bruch; MA-25043; U.S.A. California; EU343783; *Grimmia orbicularis*2; MO-5217118; U.S.A. Nevada; EU343784; *Grimmia ovalis* (Hedw.) Lindb.; MO-5217105; U.S.A. Nevada; EU343785; *Grimmia pilifera* P. Beauv.; MA-24934; Russia. Khabarovsk Kray; EU343786; *Grimmia plagiopodia* Hedw.; S-B70024; Sweden. Torne Lappmark; EU343787; *Grimmia pulvinata* (Hedw.) Sm.; MA-25045; U.S.A. California; EU343790; *Grimmia pulvinata*2; MA-25026; U.S.A. California; EU343789; *Grimmia reflexidens* Müll. Hal.; MO-5233641; U.S.A. Colorado; EU343791; *Grimmia serrana* J. Muñoz, Shevock & D.R. Toren; ; EU343792; *Grimmia tergestina* Bruch & Schimp.; MA 14653; Turkey. Antalya; EU343793; *Grimmia trinervis* R.S. Williams; MO Price 1547; Bolivia. Cochabamba; EU343794; *Grimmia ungeri* Jur.; MA-25618; U.S.A. Nevada; EU343795; *Grimmia unicolor* Hook.; S-B51960; Sweden. Västra Götaland; EU343796; *Grimmia wilsonii* Greven; MO-5125736; New Zealand; EU343797; *Racomitrium aciculare* (Hedw.) Brid.; MA-22069; Spain. Cantabria; EU343798; *Racomitrium didymum* (Mont.) Jaeger; MA-25251; Chile. Región de los Lagos; EU343799; *Racomitrium elongatum* Frisvoll.; MA-13319; Spain. Palencia; EU343800; *Racomitrium heterostichum* (Hedw.) Brid.; MO-5125302; U.S.A. California; EU343801; *Schistidium crassipilum* H.H. Blom; MA-14862; Spain. Granada; EU343802; *Schistidium* sp. 'lingulatum' Blom; MA-26281; USA: Washington; EU343750;