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Molecular studies resolve *Nyholmiella* (Orthotrichaceae) as a separate genus

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Abstract Two *Orthotrichum* species of the subgenus *Orthophyllum* were compared with other representatives of this genus using internally transcribed spacer regions 1 and 2, the chloroplast *trn*H–*psb*A region, and inter-simple sequence repeat (ISSR) and intron–exon splice conjunction (ISJ) markers. The ISSR and ISJ markers used revealed many bands and mutations specific only to *O. gymnostomum* and *O. obtusifolium*. Phylogenetic analysis clearly supported previous concepts postulating that species of the subgenus *Orthophyllum* should be recognized as the separate genus *Nyholmiella*.

Key words molecular taxonomy, *Nyholmiella*, *Orthotrichum*, phylogeny.

The genus *Orthotrichum* Hedw. is a widespread moss group that includes approximately 155 species (Goffinet et al., 2007). Taxa belonging to this genus are found throughout the world, from the Arctic to the Antarctic, except in deserts and wet tropical forests. Species of the genus *Orthotrichum* grow on trees and rocks to a height of approximately 5000 m above sea level (Lewinsky, 1993). In the most recent revision, Orthotrichaceae was divided into Macromitrioideae, Zygodontoideae, and Orthotrichoidae, and *Orthotrichum* was placed in the last group (Goffinet et al., 1998, 2004). The subdivision within this genus has been a matter of a continuing debate since the end of the 19th century. Certain taxa have been alternately included in and excluded from the genus *Orthotrichum* in an attempt to divide it into lower taxonomic units, subgenera and sections. The basis for the classification of the genus *Orthotrichum* from a historical perspective has been described in detail elsewhere (Lewinsky, 1993; Lewinsky-Haapasaari & Hedenäs, 1998).

According to the latest revision, the genus *Orthotrichum* is divided into seven subgenera (Lewinsky, 1993): *Callistoma*, *Exiguifolium*, *Gymnoporus*, *Orthotrichum*, *Phaneroporum, Pulchella*, and *Orthophyllum*. The inclusion of *Orthophyllum* into the genus *Orthotrichum* is a matter of contention. The distinctness of species belonging to the subgenus *Orthophyllum* from other taxa in the genus *Orthotrichum* was noted by Delogne (1885), who was the first to place two species, namely *O. gymnostomum* and *O. obtusifolium*, into the subgenus *Orthophyllum*. Hagen (1908) went one step further, forming a separate genus for *O. gymnostomum* and *O. obtusifolium*, namely *Stroemia* Hag. These plants were distinguished by obtuse leaves with incurved or plane leaf margins and incrassate leaf cells with a stout central papilla on each side. Because *Stroemia* was an illegitimate name, it was later replaced by *Nyholmiella* Holmen & Warncke (Damsholt et al., 1969). A later revision of the genus *Orthotrichum* resulted in the inclusion of *O. gymnostomum* and *O. obtusifolium* into *Orthotrichum* (Vitt, 1973), because the features noted above were also observed in other representatives of this genus. The affiliation of these species with the genus *Orthotrichum* was tested by Lewinsky-Haapasaari and Hedenäs (1998) using cladistic methods. However, analysis of selected morphological characters of the above taxa in subgenus *Orthophyllum* did not confirm their distinctness sufficiently to place them into a separate genus.

Molecular studies have shed new light on the assignment of species in the subgenus *Orthophyllum* to the genus *Orthotrichum*. Goffinet et al. (2004) examined phylogenetic relationships within the family Orthotrichaceae and noted that dissimilarity of *O. obtusifolium* from other members of this genus, thus suggesting the need to exclude the subgenus *Orthophyllum* from the genus *Orthotrichum*. Sawicki et al. (2009a) arrived at a similar conclusion while analyzing internal transcribed spacer (ITS) sequences in the genus *Orthotrichum*.

Analysis of ITS sequences has revealed a closer relationship between *Ulota crispa* and other *Orthotrichum* species than between these species of the genera *Ulota*

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and *Orthotrichum* and *O. gymnostomum* and *O. obtusifolium*, which strongly supports the separation of taxa of the subgenus *Orthophyllum* from those of the genus *Orthotrichum*. However, in both cases the analysis was limited to single specimens of one (Goffinet et al., 2004) or two (Sawicki et al., 2009a) representatives of the subgenus *Orthophyllum.*

Current morphological revisions of various moss taxa are often supported by molecular data (Virtanen, 2003; Hyvönen et al., 2004; Cano et al., 2005; Pedersen & Hedenäs, 2005). Compared with morphological data, DNA sequences are not affected by changes in the environmental conditions under which the plants are grown. Hence, molecular data can be used as a powerful tool to resolve taxonomic and systematic problems. The aim of the present study was to determine, using molecular analysis with DNA markers, whether representatives of the subgenus *Orthophyllum* should be excluded from the genus *Orthotrichum* and whether the genus status of *Nyholmiella* should be resurrected.

The ITS region is commonly used in phylogenetic and population genetics studies on bryophytes (Shaw, 2000; Vanderpoorten et al., 2003; Hedenäs, 2008; Heinrichs et al., 2009). In plants, the ITS region is grouped into arrays consisting of hundreds to thousands of tandem repeats. This region includes two spacers, ITS1 and ITS2, that separate the 18S, 5.8S and 26S genes of nuclear ribosomes (Baldwin et al., 1995). The sequences of nuclear ribosomal DNA have been used successfully in previous studies of *Orthotrichum* (Plášek et al., 2009; Sawicki et al., 2009a,b).

The chloroplast *trn*H–*psb*A sequence, which is a candidate region for plant bar coding but is characterized by relatively high variability (Erickson et al., 2008; Newmaster et al., 2008), was also used in the present study.

In addition, genome-scanning intron–exon splice junction (ISJ) and inter-simple sequence repeat (ISSR) markers were used. The ISSR markers have been widely used in both taxonomic and population genetics studies on bryophytes (Vanderpoorten et al., 2003; Gunnarsson et al., 2005). In contrast, the ISJ markers, which have been proven effective in studies of both bryophytes (Sawicki & Szczecińska, 2007; Bączkiewicz et al., 2008; Sawicki et al., 2008; Plášek & Sawicki, 2010) and higher plants (Szczecińska et al., 2006, 2009), have been used less frequently. The ISJ primers are partly complementary to the sequences on the exon-intron boundary and can therefore can the genome fragments containing functional genes, including those responsible for the phenotype.

1 Material and Methods

1.1 Material

Our analyses included 32 species representing three genera of the family Orthotrichaceae. The genus *Orthotrichum* was represented by 28 species belonging to five subgenera. Two *Zygodon* species were used as outgroup, based on previous analyses (Goffinet et al., 1998, 2004; Sawicki et al., 2009a).

The list of species used in the molecular analysis, details regarding voucher data and GenBank accession numbers are given in Appendix 1.

1.2 DNA extraction

Total genomic DNA was extracted from herbarium material. A single stem was ground with silica beads in a FastPrep tissue disruptor (MP Biomedicals, Solon, OH, USA) for 20 s and subsequently treated using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Extracted DNA samples were stored at −20°C.

1.3 Amplification and sequencing of the ITS

For amplification and sequencing of the ITS, we used the primers described by Fiedorow et al. (1998); Table 1). The ITS regions were amplified in a volume of 25 μL containing 20 mmol/L $(NH_4)_2SO_4$, 50 mmol/L Tris-HCl (pH 9.0 at 25°C), 1.5 mmol/L $MgCl_2$, 1 μL bovine serum albumin (BSA), 200 μmol/L dNTPs (dATP, dGTP, dCTP, and dTTP), 1.0 μmol/L of each primer, 1 unit Taq polymerase (Qiagen), and 1 mL DNA solution. The reaction was processed at 94°C for 1 min,

Table 1 Sequences of internal transcribed spacer, *trn*H–*psb*A, intron–exon splice conjunction, and inter-simple sequence repeat primers used in the present study

Primer	Sequence (5′–3′)
ITS1-F	CAAGGTTTCCGTAGGTGAAC
ITS1-R	CAAGAGCCAAGATATCCG
ITS2-F	CGGATATCTTGGCTCTTG
ITS2-R	CCGCTTAGTGATATGCTTA
*psb*A-F	GTTATGCATGAACGTAATGCTC
*trn*H-R	CGCGCATGGTGGATTCACAAATC
IS810	$(GA)_8T$
IS813	$(CT)_8T$
IS822	$(TC)_8A$
IS825	$(AT)_8G$
IS828	$(TG)_8A$
IS831	$(ACC)_6$
IS843	CATGGTGTTGGTCATTGTTCCA
IS846	$GGGT(GGGGT)_2G$
ISJ 2	ACTTACCTGAGGCGCCAC
ISJ 4	GTCGGCGGACAGGTAAGT
ISJ 5	CAGGGTCCCACCTGCA
ISJ 6	ACTTACCTGAGCCAGCGA

ITS, internal transcribed spacer; ISJ, intron–exon splice conjunction.

followed by 30 cycles at 94°C for 1 min, 59°C for 1 min, and 72°C for 1.5 min, with a final extension at 72°C for 5 min. Finally, 5 μL of the amplification products was visualized on a 1.5% agarose gel with ethidium bromide staining. Purified polymerase chain reaction (PCR) products were sequenced in both directions using the ABI BigDye 3.1 Terminator Cycle Kit (Applied Biosystems, Foster City, CA, USA) and then visualized using an ABI Prism 3130 Automated DNA Sequencer (Applied Biosystems).

1.4 Amplification and sequencing of *trn*H–*psb*A

For the amplification and sequencing of *trn*H–*psb*A, we used the primers of Sang et al. (1997), as listed in Table 1. The amplifications were performed in a volume of 25 μL containing 20 mmol/L $(NH_4)_2SO_4$, 50 mmol/L Tris-HCl (pH 9.0 at 25°C), 1.5 mmol/L $MgCl_2$, 1 μL BSA, 200 μmol/L dNTPs, 1.0 μmol/L of each primer, 1 unit Taq polymerase (Qiagen) and 1 μL DNA solution. The reaction was processed at 94°C for 1 min, followed by 35 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 5 min. The next stages were performed as for the amplification of the ITS sequences.

1.5 ISJ and ISSR markers

Two types of markers were used to analyze genetic variation in the present study, namely microsatellite ISSR markers developed by Ziętkiewicz et al. (1994) and ISJ markers. Similar to random amplified polymorphic DNA and amplified fragment length polymorphism markers, the target sequences of ISSR markers do not require prior identification, which makes the ISSR markers suitable for studying species for which species-specific primers amplifying microsatellite loci (i.e. simple sequence repeats (SSR)) have not yet been developed. In constrast with SSR markers, ISSR primers are complementary to repeated sequences rather than to fragments flanking those sequences. Semispecific ISJ markers are based on sequences that are commonly found in plants and are crucial for post-transcription DNA processing (Weining & Langridge, 1991). The ISJ primers are partly complementary to the sequences on the exon–intron boundary. The sequences of the ISSR and ISJ primers used for DNA amplification in the present study are given in Table 1.

The ISSR-PCR and ISJ-PCR reactions were performed in a reaction volume of 20 μL containing 40 ng genomic DNA, 1.0 μmol/L primer, 1.5 mmol/L $MgCl_2$, 200 μmol/L dNTPs, 1× PCR buffer, 1 μL BSA, and 1 U Genomic Red Taq polymerase (Sigma, St Louis, MO, USA). The ISSR marker reactions were performed as follows: initial denaturation for 5 min at 94°C; 34 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 50°C, and elongation for 1 min and 30 s at 72°C; and a final elongation for 7 min at 72°C. The reaction conditions for ISJ primers were as follows: initial denaturation for 3 min at 94°C, denaturation for 1 min at 94°C, annealing for 1 min at 48°C, elongation for 1 min and 30 s at 72°C, and a final elongation for 5 min at 72°C. The sequences of the primers used in these reactions are given in Table 2. The products of the ISSR-PCR were separated on 2% agarose gels, whereas the products of the ISJ-PCR were separated on 1.5% agarose gels, followed by DNA staining with ethidium bromide. After being rinsed in deionized water, the agarose gels were analyzed in a transilluminator under ultraviolet light at a wavelength of 302 nm using the Felix 1010 system (Biotec-Fischer, Reiskirchen, Germany).

Table 2 Numbers of specific substitution, indels, and bands for each group analyzed

Taxa	ITS1	ITS2	*trn*H–*psb*A	ISSR+ISJ
Zygodon	11s, 23i	22s, 33i	3s, 2i	21
Ulota	3i	1i	–	–
Orthophyllum	13s, 24i	5s, 7i	1s	18
Gymnoporus	–	–	–	–
Phaneroporum	–	–	–	–
Orthotrichum	–	1s	–	–
Pulchella	–	–	–	–

s, substitutions; i, indels; –, no specific mutations or bands; ITS, internal transcribed spacer; ISJ, intron–exon splice conjunction; ISSR, inter-simple sequence repeat.

1.6 Data analysis

Electropherograms were edited and assembled using Sequencher 4.5 (Genecodes Inc., Ann Arbor, MI, USA). The sequences assembled were aligned using Muscle 3.6 (Edgar, 2004) and adjusted manually with BioEdit 7 (Hall, 1999). Regions of ambiguous alignment and incomplete data (i.e. at the beginning and end of the sequences) were excluded from analyses. Gaps were excluded from all phylogenetic analyses. Phylogenetic analyses were conducted using maximum parsimony (MP) as implemented in MEGA 4 (Tamura et al., 2007) and Bayesian inference of phylogeny as implemented in MrBayes 3.12 (Huelsenbeck & Ronquist, 2001). For parsimony analyses, we applied branch and bound search as implemented in MEGA 4. The statistical significance of clades within inferred trees was evaluated using the bootstrap (BS) method (Felsenstein, 1985) with 2000 replicates.

Incongruence from ITS and *trn*H–*psb*A data was assessed by comparing clade support on the consensus MP tree. For example, if Species A was included in Clade A with significant BS support based on interference in the ITS region but resolved as a member of Clade B with significant support based on the

*trn*H–*psb*A region, the phylogenetic trees based on these loci were considered incongruent. To identify incongruence in phylogenetic signals we used the 70% BS criterion. Because incongruence was not observed, the ITS and *trn*H–*psb*A datasets were combined for subsequent phylogenetic analyses. Congruence between the trees obtained by different phylogenetic methods was analyzed in the same way.

Bayesian inference was performed using MrBayes 3.12 (Huelsenbeck & Ronquist, 2001). The parameters of the likelihood model were those of the general time reversible model (no. states = 6) with the proportion of invariable sites in accordance with the best fitted nucleotide evolution model selected on the basis of Akaike Information Criterion scores in Modeltest 3.7 (Posada & Crandall, 1998). The Markov chain Monte Carlo (MCMC) algorithm was run for 6 000 000 generations with four incrementally heated chains, starting from random trees and sampling one of every 200 generations. Stationarity was determined to have occurred after 100 000–150 000 generations in each analysis by plotting likelihood scores, and the first 6000 trees were excluded as the burn-in. The remaining trees were used to construct the Bayesian consensus tree. Bootstrap support was considered to be good at >70%, moderate at <70% and >50%, and poor at <50%. In the case of Bayesian clade credibility values, significant support was estimated at ≥95%.

The ISJ and ISSR bands were scored for their presence (1) or absence (0) and transformed into a 0/1 binary character matrix. Fragments that could not be scored unambiguously were excluded from analysis. Phylogenetic analysis of a binary data was accomplished by using maximum parsimony in PAUP* 4.0b10 (Swofford, 2003). Multiple MP trees were summarized with a strict consensus tree and bootstrapped with 2000 replicates.

As another measure of distinctness, the number of fixed nucleotide differences among genera and subgenera was estimated for all pairwise combinations of species using the Sites program (Hey & Wakeley, 1997). In case of binary data, GenAlex 6.1 (Peakall & Smouse, 2006) was used to find species- and subgenus-specific bands.

2 Results

2.1 ITS region

The length of the ITS1 spacer ranged from 304 bp in *Zygodon rupestris* to 643 bp in *Orthotrichum obtusifolium*. The shortest ITS1 sequence among *Orthotrichum* species (396 bp) was found in several taxa with immersed stomata, namely *O. alpestre*, *O. anomalum*, *O. cupulatum*, *O. pallens*, *O. pellucidum*, and *O. rivulare*. The length of the ITS2 spacer ranged from 432 bp in *O. moravicum* to 484 bp in *O. gymnostomum* and *O. obtusifolium*.

The alignment of the ITS region had a total length of 1291 bases. The ITS dataset contained 523 variable sites, of which 348 were parsimony informative. An MP analysis resulted in 20 MP trees of 868 steps, with a consistency index (CI) of 0.7134 and a retention index (RI) of 0.8969.

The MP method and Bayesian interference (data not shown) resulted in trees with similar topology. The methods used enabled the formation of three main clades. Species of the subgenus *Orthophyllum* formed a distinct, well-supported clade (MP 99% BS support and Bayesian inference 100% clade credibility). The second clade was formed by monoecious species with superficial stomata (MP 90% BS support); however, there was no distinct division evident into subgenera *Gymnoporus* and *Phaneroporum*. The third main clade was formed by species with immersed stomata. Three species of the subgenus *Orthotrichum*, namely *O. anomalum*, *O. cupulatum*, and *O. pellucidum*, formed a distinct clade (MP 99% BS support but Bayesian inference only 58% clade credibility). Three representatives of the section *Pulchella* of subgenus *Pulchella*, namely *O. consimile*, *O. pulchellum*, and *O. scanicum*, formed their own, but unsupported, clade.

Apart from the three main clades, separate groups were also formed by *Ulota bruchii* and *U. crispa* (MP 99% BS support and Bayesian inference 99% clade credibility), and the dioecious *O. lyellii*, which seems to be distinct from other representatives of the subgenus *Gymnoporus* (MP 99% BS support and Bayesian inference 100% clade credibility).The third species of the genus *Ulota*, the dioecious *U. phyllantha*, shows a close relationship with *O. lyellii*; however, their common clade was unsupported.

2.2. Chloroplast *trn*H–*psb*A region

The length of the *trn*H–*psb*A spacer ranged from 236 bp in *O. affine*, *U. crispa*, and *U. phyllantha* to 247 bp in *Zygodon rupestris*. The total length of the aligned *trn*H–*psb*A spacer was 256 bp including indels. Only 48 variable sites were found and, of these, 16 were parsimony informative. The MP method and Bayesian interference resulted in very similar trees (data not shown). Only three clades had a significant BS support. The first well-supported clade (MP 97% BS support and Bayesian inference 100% clade credibility) included the genera *Orthotrichum* and *Ulota*. Within this group, only two clades were supported: the clade of species of the subgenus *Orthopyllum* (MP 51% BS support, 91% Bayesian inference) and the clade of *O.*

lyellii (MP 59% BS support, 90% Bayesian inference). Because chloroplast and nuclear data were congruent, a joint *trn*H–*psb*A and ITS analysis was performed.

2.3 Combined ITS and *trn*H–*psb*A data

The combined ITS and *trn*H–*psb*A alignment had a total length of 1547 bp. The ITS + *trn*H–*psb*A dataset contained 587 variable sites, of which 364 were parsimony informative. The MP analysis resulted in 17 MP trees of 936 steps, with a CI of 0.7075 and an RI of 0.8897. A strict consensus tree with BS values for supported nodes is shown in Fig. 1. The MP method and Bayesian interference (Fig. 2) resulted in similar trees, differing mostly in the position of *O. lyellii* and *U. phyllanta*. The methods used enabled three main clades to be recognized. Species of the subgenus *Orthophyllum* formed a distinct, well-supported clade (MP 100% BS support and Bayesian inference 100% clade credibility). The second clade (MP 100% BS support and Bayesian inference 100% clade credibility) was formed by species with immersed stomata, although no distinct division into subgenera *Orthotrichum* and *Pulchella* was evident. The third main clade (MP 89% support, not supported under Bayesian inference) was formed by species with superficial stomata, representing the subgenera *Gymnoporus* and *Phaneroporum*. Similarly, as in the case of species with immersed stomata, groups corresponding to particular subgenera were not established.

The MP and Bayesian analyses resulted in a different position of *O. lyellii* and *U. phyllantha*. Based on MP analysis, *O. lyellii* formed a common, although poorly supported (MP 56% BS support), clade with species of the subgenera *Gymnoporus* and *Phaneroporum*, whereas Bayesian analysis placed specimens of *O. lyellii* in a separate, first-class clade. According to the results of MP analysis, *U. phyllanta*, a dioecious species in the genus *Ulota*, formed a common, unsupported clade with *O. lyellii*. Based on Bayesian analysis, *U. phyllantha* formed a common clade (unsupported) with *Orthotrichum* species with superficial stomata. The remaining monoecious species of this genus, namely *U. bruchii* and *U. crispa*, formed a separate, well-supported clade (MP 100% BS support and Bayesian inference 100% clade credibility).

2.4 ISJ and ISSR markers

Analysis of 39 specimens, performed using 20 primers representing two DNA marker categories, enabled 270 bands to be distinguished, of which 95.5% were polymorphic. Six ISJ primers amplified a total of 66 bands (11 bands per primer), whereas 14 ISSR primers revealed 204 bands (14.6 bands per primer). All primers amplified fragments across the 39 samples studied, with the number of fragments amplified ranging from eight (ISJ-2) to 19 (ISSR-807). The greatest number of bands was identified in *U. crispa* (71), followed by *O. pylaisii* (68) and *U. bruchii* (65). The lowest number of bands was amplified in specimens of *O. gymnostomum* (35, 33, and 32).

The MP tree had three main clusters (Fig. 3). The first moderately supported clade (BS 65%) was formed by monoecious *Ulota* and *Orthotrichum* of the subgenera *Gymnoporus* and *Phaneroporum*. Within this clade, species of the genus *Ulota* formed a common, well-supported subclade (BS 95%), similar to *Orthotrichum* species (BS 94%). Two species of the subgenus *Phaneroporum*, namely *O. rupestre* and *O. laevigatum*, formed a common, although poorly supported, clade (BS 58%). The second well-supported clade (BS 90%) was formed by monoecious *Orthotrichum* species with immersed stomata, members of *Orthotrichum* and *Pulchella*. Within this clade, only the easily distinguishable taxa of the section *Pulchella*, subgenus *Pulchella*, formed their own well-supported subclade (BS 96%). Representatives of the subgenus *Orthotrichum* formed a common, moderately supported clade (BS 66%). The third main clade with 97% BS support was formed by species of the subgenus *Orthophyllum*. The remaining dioecious species, namely *O. lyellii* and *U. phyllantha*, were not included in any of these clades.

2.5 Molecular identification of genera and subgenera

Specific substitutions, indels and bands were not found for any of the recognized *Orthotrichum* subgenera (Table 2). The greatest number of specific changes was observed for the genus *Zygodon*. The molecular identification of this species was possible based on 36 substitutions (11 in ITS1, 22 in ITS2, and three in *trn*H–*psb*A) and 58 indels. The ISSR and ISJ markers revealed 22 specific bands in this genus. A slightly lower number of specific mutations was determined for members of the subgenus *Orthophyllum*. Their molecular identification was possible based on 19 substitutions (13 in ITS1, five in ITS2, and one in *trn*H–*psb*A) and 31 indels (24 in ITS1 and seven in ITS2). With regard to the subgenus *Orthophyllum*, analysis with ISJ and ISSR markers revealed 18 specific bands. The molecular identification of the genus *Ulota* was possible based on only four indels (three in ITS1 and one in ITS2). Among the other subgenera investigated, only *Orthotrichum* had one specific substitution in ITS2.

2.6 Pairwise differences

The number of pairwise differences between the groups identified ranged from 0 in the subgenera

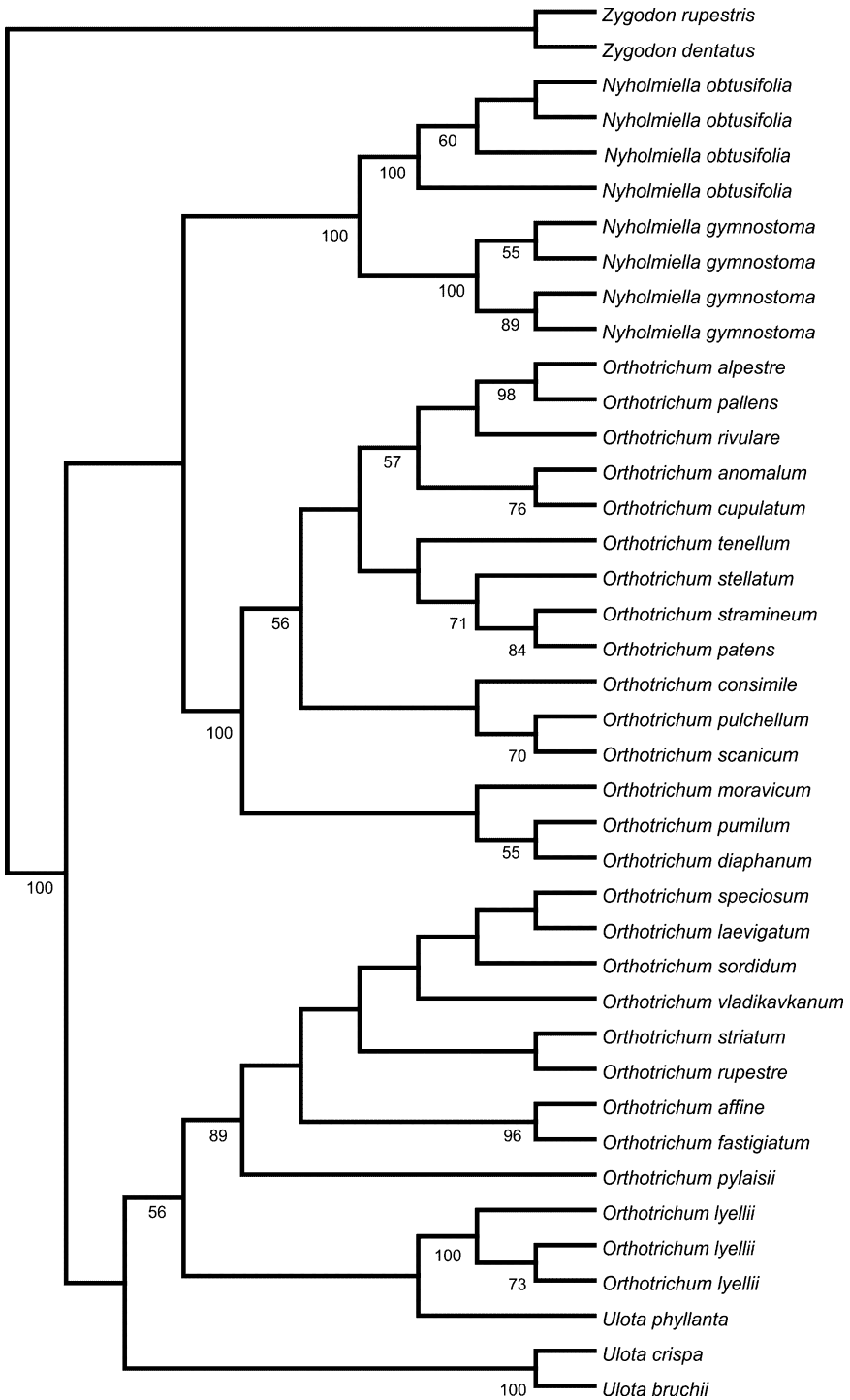

Fig. 1. Strict consensus of 17 most parsimonious trees with tree length of 936 steps (consistency index = 0.7075; retention index = 0.8897) based on internal transcribed spacer (ITS) and *trn*H–*psb*A sequences. Bootstrap values above 50% are given below the branches.

Gymnoporus and *Phaneroporum* to 224 in the subgenus *Orthophyllum* and representatives of the genus *Zygodon*, which served as an outgroup in the present study (Table 3).

Among the markers used, the largest number of fixed differences was revealed by the ITS1 sequence (a total of 1051), whereas the chloroplast spacer *trn*H–*psb*A was found to be least effective, with only 48

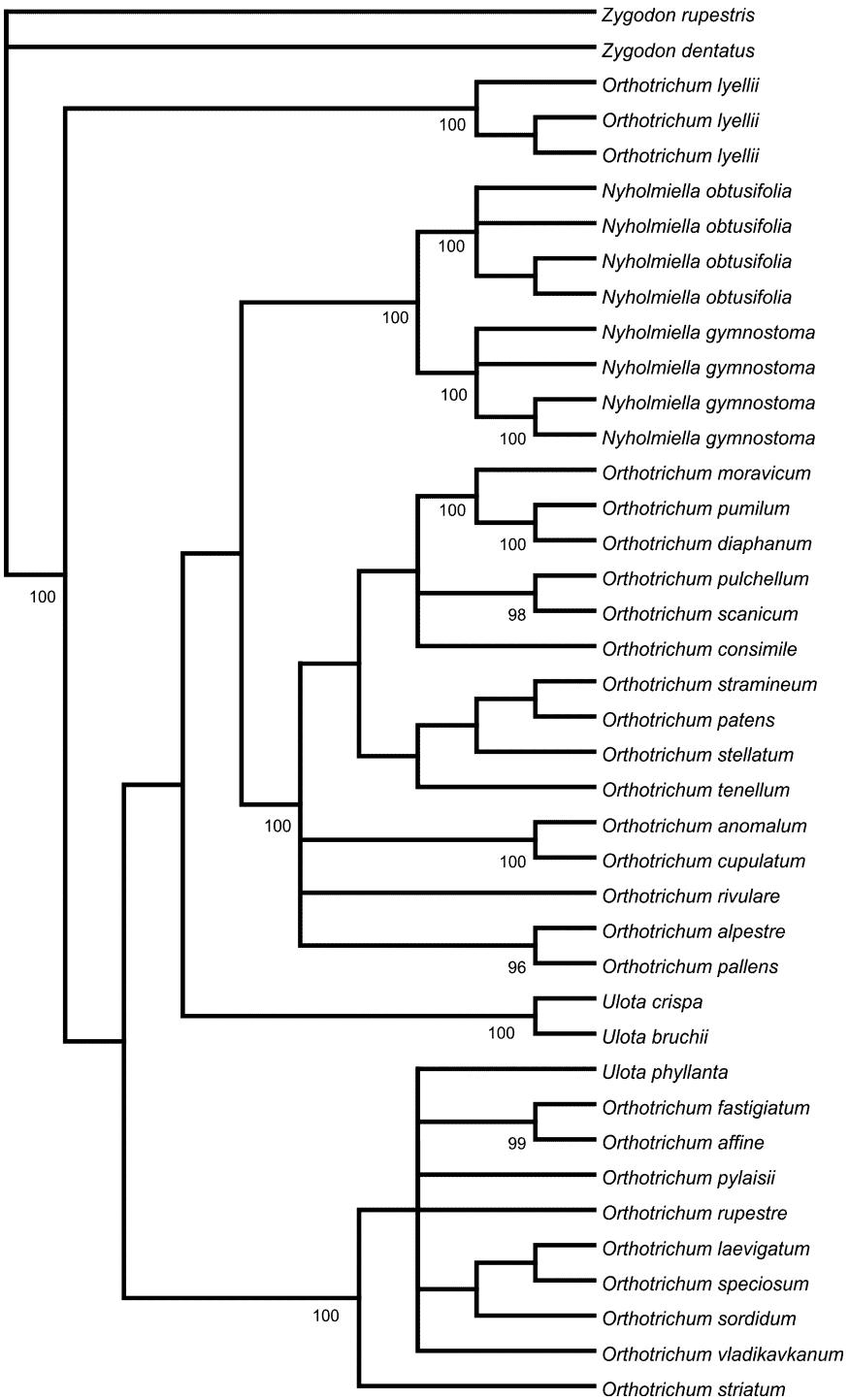

Fig. 2. Phylogram based on the Bayesian approach for the *Orthotrichum* species studied with internal transcribed spacer (ITS) and *trn*H–*psb*A sequence data. Clade credibility values above 95% are given below the branches.

fixed differences. A low number of fixed differences (i.e. three) was noted for the subgenera *Orthotrichum* and *Pulchella*, as well as the genus *Ulota* and the subgenus *Gymnoporus* (12). Among the groups analyzed, the greatest number of fixed nucleotide and allelic differences was observed for the genus *Zygodon* (which served as an outgroup) and the subgenus *Orthopyllum*. With regard to the latter, the smallest number of fixed

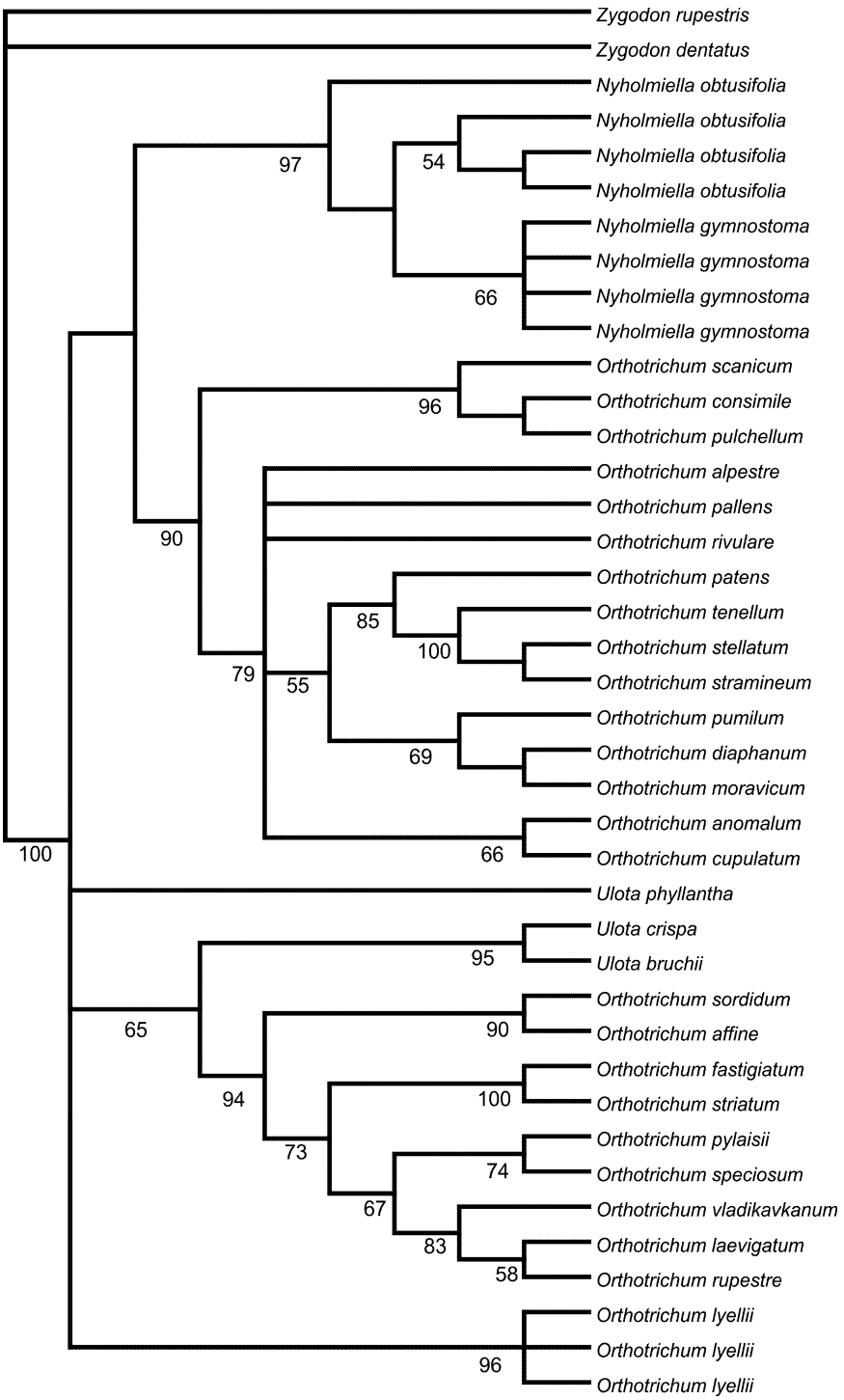

Fig. 3. Strict consensus cladogram showing maximum parsimony relationships among the *Orthotrichum* samples studied based on inter-simple sequence repeat and intron–exon splice conjunction markers. Numbers below the branches are bootstrap values.

differences was determined for the subgenus *Gymnoporus* (73), the subgenus *Pulchella* (87), and the genus *Ulota* (87). In total, 107 and 117 fixed differences were found for the subgenus *Orthophyllum* and the subgenera *Orthotrichum* and *Phaneroporum*, respectively. *Ulota* species, considered a separate genus, had a substantially lower number of fixed differences, varying from 12 (subgenus *Gymnoporus*) to 87 (*Orthophyllum*). No

Table 3 Fixed pairwise nucleotide and allelic differences among genera and subgenera analyzed

Taxa pair	ITS1	ITS2	*trn*H–*psb*A	ISSR+ISJ	Total
Orthophyllum vs. *Gymnoporus*	42	18	1	12	73
Orthophyllum vs. *Phaneroporum*	56	21	1	39	117
Orthophyllum vs. *Orthotrichum*	60	26	1	20	107
Orthophyllum vs. *Pulchella*	56	19	1	11	87
Orthophyllum vs. *Ulota*	46	21	1	19	87
Orthophyllum vs. *Zygodon*	97	91	8	28	224
Gymnoporus vs. *Phaneroporum*	0	0	0	0	0
Gymnoporus vs. *Orthotrichum*	44	11	0	19	74
Gymnoporus vs. *Pulchella*	37	8	0	10	55
Gymnoporus vs. *Ulota*	5	4	0	3	12
Gymnoporus vs. *Zygodon*	68	66	7	14	155
Phaneroporum vs. *Orthotrichum*	50	11	1	52	114
Phaneroporum vs. *Pulchella*	41	0	0	34	75
Phaneroporum vs. *Ulota*	9	8	0	14	31
Phaneroporum vs. *Zygodon*	75	66	6	36	183
Orthotrichum vs. *Pulchella*	0	3	0	0	3
Orthotrichum vs. *Ulota*	45	13	0	26	84
Orthotrichum vs. *Zygodon*	91	53	7	36	187
Pulchella vs. *Ulota*	42	8	0	18	68
Pulchella vs. *Zygodon*	84	56	7	26	173
Ulota vs. *Zygodon*	69	76	7	22	174

ITS, internal transcribed spacer; ISJ, intron–exon splice conjunction; ISSR, inter-simple sequence repeat.

fixed differences were noted for the subgenera *Gymnoporus* and *Phaneroporum*, and only three fixed differences were confirmed for the subgenera *Orthotrichum* and *Pulchella*.

3 Discussion

Analysis of molecular data often contributes to the resolution of taxonomic problems pertaining to a given taxonomic group. The implementation of advanced molecular methods has shed new light on the taxonomy of numerous groups of both vascular plants (Dillon et al., 2007; Mols et al., 2008) and bryophytes (Goffinet et al., 2004; Hedenäs, 2008; Heinrichs et al., 2009), for which the solutions offered by classical morphological analysis had proven insufficient.

The results of the present study suggest that *O. gymnostomum* and *O. obtusifolium* are clearly distinct from other species of the genus. This hypothesis has been confirmed by both phylogenetic analyses of the nuclear ITS region and the chloroplast *trn*H–*psb*A region, and a relationship analysis performed using ISJ and ISSR markers. A high number of specific mutations and ISJ/ISSR bands also supports the exclusion of members of the subgenus *Orthophyllum* from the genus *Orthotrichum*.

The affiliation of these species with the genus *Orthotrichum* was tested by Lewinsky–Haapasaari & Hedenäs (1998) using cladistic methods. However, an analysis of the selected morphological characters of the above taxa did not confirm their distinctness sufficiently to place them into a separate genus. It should be noted that in that study, the genus *Ulota* served as an outgroup. As shown by later molecular analyses, this genus is very closely related to *Orthotrichum* species of the subgenera *Gymnoporus* and *Phaneroporum* (Goffinet et al., 1998, 2004; Sawicki et al., 2009a), which could significantly affect the final conclusions reached by Lewinsky–Haapasaari & Hedenäs (1998). The molecular analysis conducted in the present study suggests that members of the subgenus *Orthophyllum* are genetically distinct from other species of the genus *Orthotrichum*. In addition, the sequence investigated revealed a closer relationship between *U. crispa* and other *Orthotrichum* species than between these two groups and *O. gymnostomum* and *O. obtusifolium*, which strongly supports the separation of taxa of the subgenus *Orthophyllum* from those of the genus *Orthotrichum*. The results of the present study are also consistent with earlier findings referring to different genome regions. Goffinet et al. (2004) investigated the family Orthotrichaceae using four loci from three genomes (26S, *nad*5, *rps*4, and *trn*L–*trn*H) and reported that *O. obtusifolium* was clearly distinct from other species of this genus. These authors also suggested that the subgenus *Orthophyllum* should be excluded from the genus *Orthotrichum*. However, that study focused on one sample only (*O. obtusifolium*). A clear distinctness between *O. obtusifolium* and *O. gymnostomum* was also validated by analysis of ITS sequences (Sawicki et al., 2009a), thus resolving the subgenus *Orthophyllum* as a sister clade of the subgenera *Orthotrichum* and *Pulchella*.

The large number of specific mutations and bands also points to the distinctness of species within the subgenus *Orthophyllum*. In total, 50 specific mutations and

18 subgenus-specific bands were determined for *O. obtusifolium* and *O. gymnostomum*. No such mutations were found for other subgenera, except for a single mutation in the subgenus *Orthotrichum*. The *Ulota* species considered as separate genus had only four specific mutations. The number of fixed nucleotide differences between the subgenera analyzed indicates that *O. gymnostomum* and *O. obtusifolium* should be excluded from the genus *Orthotrichum* and included in *Nyholmiella*.

Species of the new genus *Nyholmiella* differ from other species within the genus *Orthotrichum* mainly in having leaf margins incurved or involute (not plane or recurved like all other *Orthotrichum* taxa). The shape of the leaves (obtuse or rounded at the apex) is typical, but it is not a unique distinguishing feature because some other species within *Orthotrichum*, such as *O. macrocephalum* (cf. Lara et al., 1994), *O. cyathiforme* (cf. Lewinsky, 1984), or *O. crenulatum* (cf. Lewinsky, 1992), have similar leaf characters. *Nyholmiella obtusifolia* and *N. gymnostoma* invest a significant part of their energy in vegetative reproduction. They produce a large number of gemmae and, in this respect, are comparable probably only to *O. lyellii*.

Dioecious species of the genus *Nyholmiella* rely primarily on vegetative propagation probably because they produce sporophytes much less frequently than monoecious species of the genus *Orthotrichum* (Nyholm, 1969; Smith, 1996). The taxonomic status of the only (following the exclusion of *Nyholmiella* species) dioecious species, *Orthotrichum lyellii*, also remains unclear because molecular data suggest that it differs considerably from monoecious *Orthotrichum* species (Goffinet et al., 2004; Plášek et al., 2009; Sawicki et al., 2009a,b). However, this species has a different evolutionary pathway than *Nyholmiella*, as confirmed by both morphological (Vitt, 1971; Lewinsky, 1993; Lewinsky–Haapasaari & Hedenäs, 1998) and molecular (Goffinet et al., 2004; Sawicki et al., 2009b) data. The results of the present study also indicate that dioecious *Orthotrichum* and *Ulota* species do not form a monophyletic group. The dioecious *U. phyllantha* was grouped with *O. lyellii* only in the case of MP, but their common clade was not supported statistically (Fig. 1).

Despite the fact that species of the genus *Nyholmiella* share certain features with *O. lyellii* and *U. phyllantha*, including those considered important for the evolution of Orthotrichaceae (e.g. the position of the stomata and dioecism; Vitt, 1971; Lewinsky, 1993; Goffinet et al., 2004; Sawicki et al., 2009b), their morphological and molecular distinctness seems to fully justify the ranking of *Nyholmiella* as a separate genus. Finally, it should be noted that the genus *Nyholmiella* is currently treated as valid by some taxonomists, such as the authors of the most recent identification keys to the genus *Orthotrichum* for the territory of Europe (Lara et al., 2009), who follow the approach of Goffinet et al. (2004).

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References

Baldwin BG, Sanderson MJ, Porter JM. 1995. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. Annals of the Missouri Botanical Garden 82: 247–277.

Bączkiewicz A, Sawicki J, Buczkowska K, Polok K, Zieliński R. 2008. Application of different DNA markers in studies on cryptic species of *Aneura pinguis* (Hepaticae, Metzgeriales). Cryptogamie Bryologie 29: 3–21.

Cano MJ, Werner O, Guerra J. 2005. A morphometric and molecular study in *Tortula subulata* complex (Pottiaceae, Bryophyta). Botanical Journal of Linnean Society 149: 333–350.

Damsholt K, Holmen K, Warncke E. 1969. A list of the bryophytes of Denmark. Botanisk Tidsskrift 65: 163–183.

Delogne CH. 1885. Flore cryptogamique de Belgique. Annales de la Société Belge de Microscopie 9: 7–220.

Dillon SL, Lawrence PK, Henry RJ, Price J. 2007. *Sorghum* resolved as a distinct genus based on combined ITS1, *ndh*F and *Adh*1 analyses. Plant Systematics and Evolution 268: 29–43.

Edgar RC. 2004. Muscle: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research 32: 1792–1797.

Erickson DL, Spouge J, Resch A, Weigt LA, Kress WJ. 2008. DNA barcoding in land plants: Developing standards to quantify and maximize success. Taxon 57: 1304–1316.

Felsenstein J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39: 783–791.

Fiedorow P, Odrzykoski I, Szweykowska-Kulińska Z. 1998. Phylogenetic studies of liverworts using molecular biology techniques. In: Małuszyńska J ed. Plant cytogenetics. Katowice: Silesian University Press. 244–249.

Goffinet B, Bayer RJ, Vitt DH. 1998. Circumscription and phylogeny of the Orthotrichales (Bryopsida) inferred from *rbc*L sequence analyses. American Journal of Botany 85: 1324–1337.

Goffinet B, Shaw AJ, Cox CJ, Wickett NJ, Boles S. 2004. Phylogenetic inferences in the Orthotrichoideae (Orthotrichaceae: Bryophyta) based on variation in four loci from all genomes.

Monographs in Systematic Botany from the Missouri Botanical Garden 98: 270–289.
Goffinet B, Buck WR, Wall MA. 2007. *Orthotrichum freyanum* (Orthotrichaceae, Bryophyta), a new epiphytic species from Chile. Nova Hedwigia 131: 1–11.
Gunnarsson U, Hassel K, Söderström L. 2005. Genetic structure of the endangered peat moss *Sphagnum angermanicum* in Sweden: A result of historic or contemporary processes? Bryologist 108: 194–203.
Hagen I. 1908. Forarbejder til en norsk lovmosflora. I. Orthotrichacea. Det Kongelige Norske Videnskabers Selskabs Skrifter 1907, 13: 1–100.
Hall TA. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41: 95–98.
Hedenäs L. 2008. Molecular variation in *Drepanocladus aduncus* s.l. does not support recognition of more than one species in Europe. Journal of Bryology 30: 108–120.
Heinrichs J, Klugmann F, Hentschel J, Schneider H. 2009. DNA taxonomy, cryptic speciation and diversification of the Neotropical-African liverwort, *Marchesinia brachiata* (Lejeuneaceae, Porellales). Molecular Phylogenetics and Evolution 53: 113–121.
Hey J, Wakeley J. 1997. A coalescent estimator of the population recombination rate. Genetics 145: 833–846.
Huelsenbeck JP, Ronquist FR. 2001. MRBAYES: Bayesian inference of phylogeny. Bioinformatics 17: 754–755.
Hyvönen J, Koskinen S, Merrill GLS, Hedderson TA, Stenroos S. 2004. Phylogeny of Polytrichales (Bryophyta) based on simultaneous analysis of molecular and morphological data. Molecular Phylogenetics and Evolution 31: 915–928.
Lara F, Garilleti R, Mazimpaka V. 1994. *Orthotrichum macrocephalum* sp. nov., a new moss of section *Diaphana* from the Iberian Peninsula. Bryologist 97: 402–408.
Lara F, Garilleti R, Medina R, Mazimpaka V. 2009. A new key to the genus *Orthotrichum* Hedw. in Europe and the Mediterranean region. Cryptogamie Bryologie 30: 129–142.
Lewinsky J. 1984. The genus *Orthotrichum* Hedw. (Musci) in Australia. A taxonomic revision. Journal of the Hattori Botanical Laboratory 56: 369–460.
Lewinsky J. 1992. The genus *Orthotrichum* Hedw. (Orthotrichaceae, Musci) in Southeast Asia. A taxonomic revision. Journal of the Hattori Botanical Laboratory 72: 1–88.
Lewinsky J. 1993. A synopsis of the genus *Orthotrichum* Hedw. (Musci, Orthotrichaceae). Bryobrothera 2: 1–59.
Lewinsky-Haapasaari J, Hedenäs L. 1998. A cladistic analysis of the moss genus *Orthotrichum*. Bryologist 101: 519–555.
Mols JB, Keßler PJA, Rogstad SH, Saunders RMK. 2008. Reassignment of six *Polyalthia* species to the new genus *Maasia* (Annonaceae): Molecular and morphological congruence. Systematic Botany 33: 490–494.
Newmaster SG, Fazekas AJ, Steeves RAD, Janovec J. 2008. Testing candidate plant barcode regions in the Myristicaceae. Molecular Ecology Resources 8: 480–490.
Nyholm E. 1969. Illustrated moss flora of Fennoscandia. Lund: Gleerup.
Peakall R, Smouse PE. 2006. GenAlEx 6: Genetic analysis in Excel. Population genetic software for teaching and research. Molecular Ecology Notes 6: 288–295.
Pedersen N, Hedenäs L. 2005. Taxonomic and nomenclatural implications of phylogenetic studies of the Bryaceae based on molecular data and morphology. Bryologist 108: 123–128.
Plášek V, Sawicki J. 2010. Is the hairy vaginula an diagnostic feature in the taxonomy of the genus *Orthotrichum*? Acta Societatis Botanicorum Poloniae 79: 75–80.
Plášek V, Sawicki J, Trávníčková V, Pasečná M. 2009. *Orthotrichum moravicum* (Orthotrichaceae), a new moss species from the Czech Republic. Bryologist 112: 329–336.
Posada D, Crandall KA. 1998. Modeltest: Testing the model of DNA substitution. Bioinformatics 14: 817–818.
Sang T, Crawford DJ, Stuessy TF. 1997. Chloroplast DNA phylogeny, reticulate evolution, and biogeography of *Paeonia* (Paeoniaceae). American Journal of Botany 84: 1120–1136.
Sawicki J, Szczecińska M. 2007. Semi-specific intron–exon splice junction markers in bryophyte studies. Biodiversity Research and Conservation 5–8: 25–30.
Sawicki J, Nadolski Ł, Hościk M, Zieliński R. 2008. Identification of *Conocephalum conicum* and *C. salebrosum* (Hepaticophyta) based on DNA markers. Časopis Slezskeho Zemskeho Muzea Opava (A) 57: 259–264.
Sawicki J, Plášek V, Szczecińska M. 2009a. Preliminary studies on the phylogeny of the genus *Orthotrichum* inferred from nuclear ITS sequences. Annales Botanici Fennici 46: 507–515.
Sawicki J, Plášek V, Szczecińska M. 2009b. Molecular evidence do not support the current division of *Orthotrichum* subgenus *Gymnoporus*. Plant Systematics and Evolution 279: 125–137.
Shaw AJ. 2000. Molecular phylogeography and cryptic speciation in the mosses *Mielichhoferia elongata* and *M. mielichhoferiana* (Bryaceae). Molecular Ecology 9: 595–608.
Smith AJE. 1996. The moss flora of Britain and Ireland. Cambridge: Cambridge University Press.
Swofford DL. 2003. PAUP: Phylogenetic analysis using parsimony (and other methods) 4.0 Beta. Sunderland: Sinauer.
Szczecińska M, Sawicki J, Polok K, Hołdyński C, Zieliński R. 2006. Comparison of the *Polygonatum* species from Poland on the basis of different DNA markers. Annales Botanici Fennici 43: 379–388.
Szczecińska M, Sawicki J, Wąsowicz K, Hołdyński Cz. 2009. Genetic variation of the relict and endangered population of *Chamaedaphne calyculata* (Ericaceae) in Poland. Dendrobiology 62: 23–33.
Tamura K, Dudley J, Nei M, Kumar S. 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596–1599.
Vanderpoorten A, Hedenäs L, Jacquemart A-L. 2003. Differentiation in DNA fingerprinting and morphology among species of the pleurocarpous moss genus, *Rhytidiadelphus* (Hylocomiaceae). Taxon 52: 229–236.
Virtanen V. 2003. Phylogeny of Bartramiaceae based on morphology and on *rbc*L, *rps*4 and *trn*L-F sequence data. Bryologist 106: 280–296.
Vitt DH. 1971. The infrageneric evolution, phylogeny, and taxonomy of the genus *Orthotrichum* (Musci) in North America. Nova Hedwigia 21: 683–711.
Vitt DH. 1973. A revision of genus *Orthotrichum* in North America, north of Mexico. Bryophytorum Bibliotheca 1: 1–208.

Weining S, Langridge P. 1991. Identification and mapping of polymorphisms in cereals based on polymerase chain reaction. Theoretical and Applied Genetics 82: 209–216.
Ziętkiewicz E, Rafalski A, Labuda D. 1994. Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics 20: 176–183.

Appendix 1

Accession data for plants included in the molecular analysis. GeneBank accession numbers are given in the following sequence: ITS1, ITS2, *trn*H–*psb*A.

Nyholmiella gymnostoma (Bruch *ex* Brid.) Holmen et Warncke, USA, Arizona, herb NYBG GU827721, GU807602, GQ370549

N. gymnostoma (Bruch *ex* Brid.) Holmen et Warncke, Canada, Newfoundland, herb NYBG, GU827722, GU807603, GQ370548

N. gymnostoma (Bruch *ex* Brid.) Holmen et Warncke, Sweden, herb OLS, GU827723, GU807604, GQ370356

N. gymnostoma (Bruch *ex* Brid.) Holmen et Warncke, Canada, Newfoundland, herb NYBG, EU863204, EU072687, GQ370355

N. obtusifolia (Brid.) Holmen et Warncke, Slovakia, herb. OP, EU863208, EU072693, GQ370351

N. obtusifolia (Brid.) Holmen et Warncke, Czech Republic, herb. OP, GU827718, GU807599, GQ370350

N. obtusifolia (Brid.) Holmen et Warncke, Poland, herb OLS, GU827719, GU807600, GQ370349

N. obtusifolia (Brid.) Holmen et Warncke, USA, NY, herb OLS, GU827720, GU807601, GQ370348

Orthotrichum affine Schrad. *ex* Brid., Czech Republic, herb OP, FJ159248, FJ168668, FJ036876

O. alpestre Bruch et Schimp., USA, CA, Mono Co., herb. NYBG, EU443998, EU484067, GQ370336

O. anomalum Hedw., Poland, herb. OP, EU443992, EU072691, GQ370333

O. consimile Mitt., USA, WA, herb NYBG, EU443997, EU484066, GQ370337

O. cupulatum Hoffm. *ex* Brid., Poland, Pieniny Mts., herb NYBG, EU484072, EU484071, GQ370334

O. diaphanum Schrad. *ex* Brid., Poland, herb OLS, EU484077, EU484073

O. fastigiatum Bruch *ex* Brid., Czech Republic, herb OP, FJ159253, FJ168671, FJ036880

O. laevigatum J.E. Zetterst., Alberta, Canada, herb. NYBG, EU863205, EU871635 429, GQ370347

O. lyellii Hook. et Taylor, Slovakia: Poloniny Mts., herb OP, EU863206, EU072689, FJ036872

O. lyellii Hook. et Taylor, Czech Republic, Bohemia, herb. OP, FJ159245, FJ168679, FJ036873

O. lyellii Hook. et Taylor, Poland, herb OLS, FJ159246, FJ168680, FJ036874

O. moravicum Plášek & Sawicki, Czech Republic, herb. OP, EU863207, EU072688, GQ370343

O. pallens Bruch *ex* Brid., Poland, herb. OP, EU490618, EU072694, GQ370338

O. patens Bruch *ex* Brid., Czech Republic, herb. OP EU863209, EU871636, GQ370340

O. pulchellum Brunt., USA, Clallam Co., herb NYBG, EU443996, EU484065, FJ036888

O. pumilum Sw. *ex* anon., Poland, Ciechanów, herb. OLS, EU443994, EU035537, GQ370342

O. pylaisii Brid., Greenland, herb NYBG, EU863210, EU871637, FJ036869

O. rivulare Turner, Great Britain, herb. NYBG, EU484063, EU484070, GQ370344

O. rupestre Schleich. *ex* Schwägr., Czech Republic, herb. OP, EU443991, EU072686, GQ370346

O. scanicum Grönvall, Czech Republic, herb. OP EU863211, EU871638, GQ370341

O. sordidum Sull. et Lesq., USA, CON, herb NYBG, FJ159261, FJ168666, FJ036870

O. speciosum Nees, Czech Republic, herb OP, EU863213, EU072695, FJ036863

O. stellatum Brid., USA, NY, herb. NYBG, EU484081, EU484068, GQ370345

O. stramineum Hornsch. *ex* Brid., Slovakia, herb OP, FJ159243, FJ168678, FJ036889

O. stramineum Hornsch. *ex* Brid., Poland, herb OP, FJ159244, FJ168677, FJ036890

O. stramineum Hornsch. *ex* Brid., Czech Republic, herb OP, EU443999, EU072696, FJ036891

O. striatum Hedw., Poland, Bialskie Mts, herb OP, FJ159256, FJ168676, FJ036886

O. tenellum Bruch *ex* Brid., Canary Islands, herb. NYBG, EU443995, EU484064, GQ370339

O. vladikavkanum Venturi, Tajikistan, herb. NYBG, EU863214, EU871640, FJ036867

Ulota bruchii Brid., Czech Republic, herb. OP, GQ389645, GQ389655, GQ370357

U. crispa (Hedw.) Brid., Czech Republic, herb. OP, EU863215, EU871641, GQ370358

U. phyllanta Brid., Norway, herb. OP, GQ389646, GQ389656, GQ370356

Zygodon dentatus (Limpr.) Karttunen, Czech Republic, herb. OP, GQ389644, GQ389664, GQ370359

Z. rupestris Lor., Czech Republic, herb. OP EU863216, EU871642, GQ370360