Plant Syst Evol (2009) 279:125–137
DOI 10.1007/s00606-009-0153-0

ORIGINAL ARTICLE

Molecular evidence does not support the current division of *Orthotrichum* subgenus *Gymnoporus*

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Received: 11 September 2008 / Accepted: 25 January 2009 / Published online: 25 March 2009

Abstract Eight *Orthotrichum* species of subgenus *Gymnoporus* were compared using the internally transcribed spacer regions-1 and -2 and the chloroplast *trn*H-*psb*A region. A phylogenetic analysis did not reflect the current division of this subgenus into sections *Affinia* and *Leiocarpa*. The investigated sequences revealed a close relationship between *O. striatum*, a typical species of section *Leiocarpa* and *O. affine*, a typical species of section *Affinia*. An easily distinguishable group was formed by samples of the dioecious *O. lyellii*, placed into section *Leiocarpa*. A large number of fixed differences between *O. lyellii* and other species of subgenus *Gymnoporus* raises doubts concerning its position within this subgenus. No marker mutations enabling to differentiate *O. fastigiatum* from *O. affine* have been found. In absence of such mutations for *O. affine* and *O. striatum*, the status of *O. fastigiatum* cannot be determined unambiguously.

Keywords *Orthotrichum* · ITS · *trn*H-*psb*A · Genetic diversity · Molecular taxonomy

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Introduction

The genus *Orthotrichum* is a widespread moss group which includes approximately 155 species (Goffinet et al. 2007). Taxa belonging to this genus are found throughout the world from the Arctic to the Antarctic, avoiding only deserts and wet tropical forests. Species of the genus *Orthotichum* grow on trees and rocks to a height of ca. 5,000 m.a.s.l (Lewinsky 1993). The most recent revision divided Orthotrichaceae into Macromitrioideae, Zygodontoideae and Orthotrichoidae, placing *Orthotrichum* into the last group (Goffinet et al. 1998). The subdivision within this genus has been a matter of a continuing debate since the end of the nineteenth century. Certain taxa have been alternately included in and excluded from the genus *Orthotrichum* in the attempt to divide it into lower taxonomic units, subgenera and sections. The basis for the classification of the genus *Orthotrichum* in a historical perspective has been described in detail by Lewinsky (1993) and Lewinsky-Haapasaari and Hedenäs (1998).

According to the latest revision, the genus *Orthotrichum* Hedw. is divided into seven subgenera (Lewinsky 1993): *Calistoma* (Iwats. and Sharp) Lewinsky, *Exguifolium*, *Orthotrichum*, *Orthophyllum* Delonge, *Gymnoporus* (Braihw.) Limpr., *Phaneroporum* Delonge and *Pulchella* (Schimp.) Vitt. The subgenera are distinguished based on the following criteria: stomata location, location of peristome teeth, presence or absence of connecting membrane, cell division of the inner peristome layer and ecology.

One of the most interesting subgenera is *Gymnoporus*, which includes 55 species (Lewinsky 1993). Species within the subgenus *Gymnoporus* are usually described as autoicous (except for *O. lyellii*), with superficial stomata, leaves acute to acuminate, leaf margins reflexed to revolute

(rarely erect or incurved), leaf papillae simple or branched (never C-shaped), peristome teeth reflexed to recurved when dry, capsules not widest at mouth. A lower number of chromosomes ($n = 6$) and the absence of m-chromosomes suggest that this subgenus belongs to a phylogenetically primitive group.

The internal division of the subgenus *Gymnoporus* and the taxonomic status of some of its species have been the subject of considerable discussion over the past years (Nyholm 1969; Vitt 1971, 1973; Lewinsky 1993, Lewinsky-Haapasaari and Hedenäs 1998). Vitt (1971) did not distinguish the subgenus *Gymnoporus* and placed all of its species into subgenus *Phaneroporum*, divided into two sections. The first section, *Leiocarpa* Mol., included, e.g., *O. striatum*, *O. speciosum* or *O. lyellii*. The other section, *Rupestria*, contained, e.g., *O. affine*, *O. sordidum* or *O. pylaisii*. However, the division line between these two sections is not so stark, because the morphological features often fade into one another. According to Lewinsky (1993), subgenus *Gymnoporus* includes two sections: *Leiocarpa* Mol. and *Affinia* Schimp. They can be differentiated mainly based on the differences in endostome segments and leaf features. Section *Leiocarpa* has endostome segments that are usually well-developed and ornamented on both sides, whereas the other section has endostome segments that are usually less developed and smooth or only slightly ornamented on the outside. Venturi (1887) additionally divided section *Affinia* into two subsections, *Affinia* and *Arctica*, placing *O. pylaisii* and *O. sordidum* (which occur mostly in the North) into the latter.

The affiliation of particular taxa to sections has been also widely debated, the most controversial issue being the classification of *O. speciosum* and *O. lyellii*. The former was included into the section *Affinia* (Nyholm 1969; Lewinsky 1993; Lewinsky-Hapaasaari 1995) or into the section Leiocarpa (Vitt 1971; Lewinsky-Haapasaari and Hedenäs 1998). Kindberg (1897) proposed to transfer *O. speciosum* into a separate section, *Speciosa*. As regards the dioecious *O. lyellii*, most bryologists associate it with section *Leiocarpa* (Nyholm 1969; Vitt 1973; Lewinsky 1993), but alternative concepts stress the distinctness of this species resulting primarily from its dioeciousness, the presence of propagules and leaf morphology (Vitt 1971).

The separate species status of some taxa of subgenus *Gymnoporus*, e.g., *O. affine* and *O. fastigiatum*, is often questioned by botanists since the ranges of variability of their key characters overlap (Lewinsky 1993). Based on intermediary morphological characters (hair of calyptra, seta length), Nyholm (1969) suggested the possible hybrid origin of *O. affine*, pointing to *O. fastigiatum* and *O. speciosum* as potential parents. Despite numerous controversies and ambiguities regarding its division, subgenus *Gymnoporus* has never been analyzed with the use of molecular techniques. The main objective of this study was to clarify the taxonomic relationships among *Orthotrichum* species of the subgenus *Gymnoporus* using nuclear and chloroplast sequences.

The internal transcribed spacer (ITS) region is commonly used in phylogenetic and population genetics studies on bryophytes (Shaw 2000; Shaw and Allen 2000; Shaw et al. 2005; Hartmann et al. 2006; Goryunov et al. 2007; Hedenäs 2008; Plášek et al. 2009). A review of the applications of the ITS region in bryophyte systematics is given in Vanderpoorten et al. (2006). 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 chloroplast *trn*H-*psb*A sequence, which is a candidate region for plant bar-coding, characterized by relatively high variability (Newmaster et al. 2008; Sawicki et al. 2008), was also used in the present study. The main goals of this study were (1) to determine phylogenetic relationships among European taxa within the subgenus *Gymnoporus*, (2) to provide insight, based on molecular data, regarding the division of this subgenus into sections, and (3) to verify the taxonomic status of *O. fastigiatum*.

Materials and methods

Materials

Our analyses included 29 specimens representing ten species of the genus *Orthotrichum*. The subgenus *Gymnoporus* was represented by eight species belonging to two sections. *Orthotrichum pulchellum* and *O. stamineum* were used as outgroup taxa, based on a previous higher-level analysis (Plášek et al. 2009; Sawicki et al. 2009). The number of specimens of each species in the subgenus *Gymnoporus* ranged from 1, in the case of the rare *O. vladikavkanum* to 5, in that of *O. fastigiatum*. The list of species used in a molecular analysis, the details concerning voucher data and the GenBank accession numbers are given in Appendix 1.

DNA extraction

Total genomic DNA was extracted from herbarium material. Single stems were ground with silica beads in a FastPrep tissue disruptor for 20 s and subsequently treated processed using the DNAEasy® Plant Mini Kit (Qiagen) following the manufacturer's protocol. Extraced DNA samples were stored at −20°C.

ITS amplification and sequencing

For amplification and sequencing of ITS we used the primers of Fiedorow et al. 1998. The ITS were amplified in a volume of 25 µl containing 20 mM $(NH_4)SO_4$, 50 mM Tris–HCl (pH 9.0 at 25°C), 1.5 mM $MgCl_2$, 1 µl BSA, 200 µM each dATP, dGTP, dCTP, dTTP, 1.0 µM of each primer, one unit of Taq polymerase (Qiagen) and 1 ml of the DNA solution. The reaction was processed at 94°C for 1 min. 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 step of 72°C for 5 min. Finally, 5 µl of the amplification products were visualized on 1.5% agarose gel with ethidium bromide staining. Purified PCR products were sequenced in both directions using ABI BigDye 3.1 Terminator Cycle Kit (Applied Biosystems) and then visualized using an ABI Prism 3130 Automated DNA Sequencer (Applied Biosystems).

*trn*H-*psb*A amplification and sequencing

For amplification and sequencing of *trn*H-*psb*A we used the primers of Sang et al. 1997. The ITS were amplified in a volume of 25 µl containing 20 mM $(NH_4)SO_4$, 50 mM Tris–HCl (pH 9.0 at 25°C), 1.5 mM $MgCl_2$, 1 µl BSA, 200 µM each dATP, dGTP, dCTP, dTTP, 1.0 µM of each primer, one unit of Taq polymerase (Qiagen) and 1 µl of the 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 step of 72°C for 5 min. Next stages were carried out as with the ITS sequences.

Data analysis

Electropherograms were edited and assembled using Sequencher 4.5 (Genecodes Inc.). The assembled sequences were aligned using Muscle 3.6 (Edgar 2004) and manually adjusted with BioEdit 7 (Hall 1999). Phylogenetic analyses were conducted using maximum parsimony (MP), minimum evolution (ME) and Bayesian inference (Rannala and Yang 2007). Gaps were excluded from all phylogenetic analyses. MEGA 4 (Tamura et al. 2007) was used for the Minimum Evolution (ME) analysis (Rzhetsky and Nei 1992) and MP analysis. The pairwise distances were estimated with the Kimura 2-parameter method (Kimura 1980) and initial trees were generated using the neighbor-joining (NJ) method. The ME tree was searched using the close neighbor interchange (CNI) algorithm (Nei and Kumar 2000) at a search level of 2, and the maximum number of trees retained at each step was set to 100. 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 method (Felsenstein 1985) with 1,000 replicates.

Bayesian inference was performed using MrBayes 3.12 (Huelsenbeck and Ronquist 2001). The parameters of the likelihood model were those of the general time reversible model (nst = 6) with the proportion of invariable sites in accordance with the best fitted nucleotide evolution model selected on the basis of Akaike Information Criterion (Akaike 1974) scores in the Modeltest 3.7 (Posada and Crandall 1998). The MCMC algorithm was run for 1,000,000 generations with 4 incrementally heated chains, starting from random trees and sampling one out of every 100 generations. Stationarity was determined to have occurred after 40,000–50,000 generations in each analysis by plotting likelihood scores, and the first 1,000 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 >70%, moderate <70% and >50%, and poor <50%. In the case of Bayesian clade credibility values, significant support was estimated at ≥95%.

Incongruence between the *trn*H-*psb*A and ITS datasets was assessed by comparing clade support on the consensus tree. For example, if species A was included in clade A with significant bootstrap support (or posterior probability) 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. The agreement between the trees obtained by different phylogenetic methods was analyzed in the same way.

Sequence polymorphism and molecular diversity indices were estimated using Arlequin 3.11 (Excoffier et al. 2005). The number of fixed nucleotide differences among species and of nucleotide sites with shared polymorphic nucleotides was estimated for all pairwise combinations of species using the Sites program (Hey and Wakeley 1997).

Results

Molecular variation of nuclear ITS sequences

The length of ITS sequences was found to be moderately variable (Table 1). The longest ITS1 and ITS2, 445–447 and 478–480 bp in length, respectively, were present in *O. lyellii*. Among species of the subgenus *Gymnoporus*, the shortest ITS1 sequence was found in *O. speciosum* (416 bp); however *O. stramineum* and *O. pulchellum* of subgenus *Pulchella,* used as outgroup taxa, had considerably shorter ITS1, 396–398 and 404 bp, respectively. Except for *O. lyellii*, all species of subgenus *Gymnoporus* have ITS2 of 451 or 453 bp in length. No length variation

Table 1 Summary of polymorphic sites in the ITS1, ITS2 and *trn*H- *psb*A sequences of studied *Orthotrichum* species

Species	*N*	Sequences length			Mutations			Molecular diversity			
		ITS1	ITS2	*trnH-psbA*	ITS1	ITS2	*trnH-psbA*	ITS1	ITS2	ITS1 + ITS2	*trnH-psbA*
Orthotrichum affine	4	427–428	452	234	1ts + 3tv + 1ind	1ts + 1tv	2ind	0.00624	0.00295	0.00455	0.00000
Orthotrichum fastigiatum	5	427–428	451–452	234	2ts + 1ind	1ts + 3tv + 1ind	1ind	0.00187	0.00443	0.00318	0.00001
Orthotrichum lyellii	4	445	478–480	234	1ts + 2tv + 2ind	2ts + 1tv + 5ind	2ts + 1tv + 1ind	0.00374	0.00314	0.00344	0.00630
Orthotrichum pulchellum	1	404	441	235	–	–	–	–	–	–	–
Orthotrichum pylaisii	2	438	422	234	No variation	No variation	No variation	–	–	–	–
Orthotrichum sordidum	2	441	423	233–234	5ts + 4ind	2ts + 2tv + 2ind	1ts + 2ind	0.01174	0.00887		0.00429
Orthotrichum speciosum	4	416	452	235	No variation	No variation	No variation	–	–	–	–
Orthotrichum stramineum	3	396–398	434–436	233–234	8ts + 1tv + 14ind	3ts + 11ind	1ts + 3ind	0.01528	0.00463	0.01289	0.00282
Orthotrichum striatum	4	427–429	451–452	233–234	5ts + 1tv + 6ind	4ts + 1tv + 3ind	1tv	0.00940	0.00740	0.00911	0.00284
Orthotrichum vladikavkanum	1	439	422	236	–	–	–	–	–	–	–

was observed in the ITS sequences of *O. pylaisii* and *O. speciosum*.

The studied species had different levels of intraspecific nucleotide variation. Two species, *O. speciosum* and *O. pylaisii,* showed no variation in ITS, although they were represented by widely separated populations from different countries (Appendix 1). Among species of subgenus *Gymnoporus,* the highest variation was observed in *O. striatum* (Table 1). Many more mutations occurred in the populations of *O. stramineum,* including 12 substitutions and 8 indels in ITS1 and 4 substitutions and 8 indels in ITS2. Most of the analyzed species had more variable ITS1 than ITS2, except for *O. fastigiatum* in which two more substitutions were found in ITS2.

The level of nucleotide diversity within species of subgenus *Gymnoporus* ranged from 0.0000 (*O. speciosum* and *O. lyellii*) to 0.0091 (*O. striatum*). The highest nucleotide diversity was found in *O. stramineum* of subgenus *Pulchella* (0.0129). Two doubtful species, *O. affine* and *O. fastigiatum*, differed in molecular diversity, particularly with respect to the ITS1 sequence; the value of 'π' was over threefold higher in *O. affine* than in *O. fastigiatum*. The reverse situation was observed in ITS2; *O. fastigiatum* was marked by greater variation than *O. affine*.

Molecular variation of the chloroplast *trn*H-*psb*A spacer

The length of the *trnH-psbA* spacer ranged from 233 bp in *O. affine* to 239 bp in *O. lyellii*. Infraspecific length variation was noted in *O. affine* (233–235 bp), *O. fastigiatum* (234–235 bp) *O. lyellii* (238–239 bp) and *O. stramineum* (235–238 bp). Nucleotide polymorphism at the intraspecific level was low. No mutations were observed among samples of *O. speciosum* and *O. pylaisii*, and only one or two indels were found among samples of *O. fastigiatum* and *O. affine* (Table 1). A higher number of mutations was reported in *O. lyellii* (4), *O. sordidum* (3) and *O. stramineum* (4). The levels of nucleotide diversity within species of the subgenus *Gymnoporus* ranged from 0.0000 (*O. affine*, *O. fastigiatum*, *O. speciosum O. pylaisii*) to 0.0063 (*O. lyellii*).

Phylogenetic relationships

The aligned, combined ITS1 and ITS2 dataset contained 984 base pairs including indels. The total dataset contained 100 parsimony informative characters and 30 variable, but uninformative characters. The mean number of fixed nucleotide differences between species was 29.9 across the ITS dataset and 19.6 across species of the subgenus *Gymnoporus*. Three species, *O. pulchellum*, *O. stramineum* and *O. lyellii,* had a significantly higher number of fixed

differences in relation to other species (Table 2). The number of fixed differences for *O. pulchellum* ranged from 62 to 18, respectively, for *O. affine* and *O. stramineum*. The number of fixed differences for *O. stramineum* ranged from 53 (*O. affine* and *O. speciosum*) to 18 (*O. pulchellum*). The dioecious *O. lyellii* had a higher than average number of fixed differences in relation to monoecious species of this subgenus, ranging from 44 (*O. striatum*) to 48 (*O. affine*, *O. fastigiatum*, *O. pylaisii*, *O. speciosum*). No fixed differences were found between *O. affine* and *O. fastigiatum,* and—surprisingly—between them and the well morphologically defined *O. striatum*. Six fixed differences were observed for *O. speciosum* and other species of the section Affinia and only three between *O. speciosum* and *O. striatum*. Only one site with shared polymorphism was found. Three species, *O. affine*, *O. fastigiatum* and *O. striatum,* had A-T transversion at site 394 of the ITS2 sequence.

A maximum parsimony (MP) analysis resulted in 190 most parsimonious trees [length 96, consistency index (CI) 0.9883, retention index (RI) 0.9791]. The strict consensus tree with bootstrap values for supported nodes is presented in Fig. 1. The minimum evolution (ME) method (tree not shown) and Bayesian interference (Fig. 2) resulted in very similar trees. No incongruence was found between Bayesian, ME and MP trees. The subgenus *Gymnoporus* clade was well supported with bootstrap values of 99–100% (MP and ME, respectively) and a clade credibility value of 100% under Bayesian inference. Within the *Gymnoporus* clade, two well-supported clades were formed: one by populations of the dioecious *O. lyellii* (MP 99% and ME 100% bootstrap support and Bayesian inference 100% clade credibility), the other including monoecious species (MP 99% and ME 100% bootstrap support and Bayesian inference 100% clade credibility). Among monoecious taxa, only samples of *O. speciosum* and *O. pylaisii* formed separate clades with moderate to good support (*O. speciosum* clade, MP 85% and ME 92% bootstrap support and Bayesian inference 100% clade credibility; *O. pylaisii* clade, MP 66% and ME 97% bootstrap support and Bayesian inference 97% clade credibility). Within a well-supported clade (MP 84% and ME 93% bootstrap support and Bayesian inference 99% clade credibility) formed by most samples of *O. affine*, *O. fastigiatum* and *O. striatum,* two clades containing two samples of *O. affine* (MP 65% and ME 99% bootstrap support and Bayesian inference 100% clade credibility) and *O. fastigiatum* (ME 65% bootstrap support and Bayesian inference 97% clade credibility) were recognized. This well-supported clade of *O. affine*, *O. fastigiatum* and *O. striatum* does not, however, include one population of *O. striatum* from Poland, which differs from the other two populations with respect to several mutations. Another sample of *O. striatum* included

Table 2 Fixed differences among studied *Orthotrichum* species

	Orthotrichum affine	*Orthotrichum fastigiatum*	*Orthotrichum lyellii*	*Orthotrichum pulchellum*	*Orthotrichum pylaisii*	*Orthotrichum sordidum*	*Orthotrichum speciosum*	*Orthotrichum stramineum*	*Orthotrichum striatum*	*Orthotrichum vladikavkanum*
O. affine		0	0	4	1	1	4	1	0	2
O. fastigiatum	0		0	4	1	1	4	1	0	2
O. lyellii	48	48		3	0	0	2	0	0	2
O. pulchellum	62	61	58		4	3	5	3	3	6
O. pylaisii	6	6	48	60		1	3	1	0	2
O. sordidum	0	0	47	61	4		1	0	0	2
O. speciosum	6	6	48	61	6	3		2	2	3
O. stramineum	53	52	47	18	50	51	53		0	3
O. striatum	0	0	45	59	4	0	3	50		2
O. vladikavkanum	4	4	46	61	4	1	3	52	1	

*trn*H-*psb*A (above) and ITS (below)

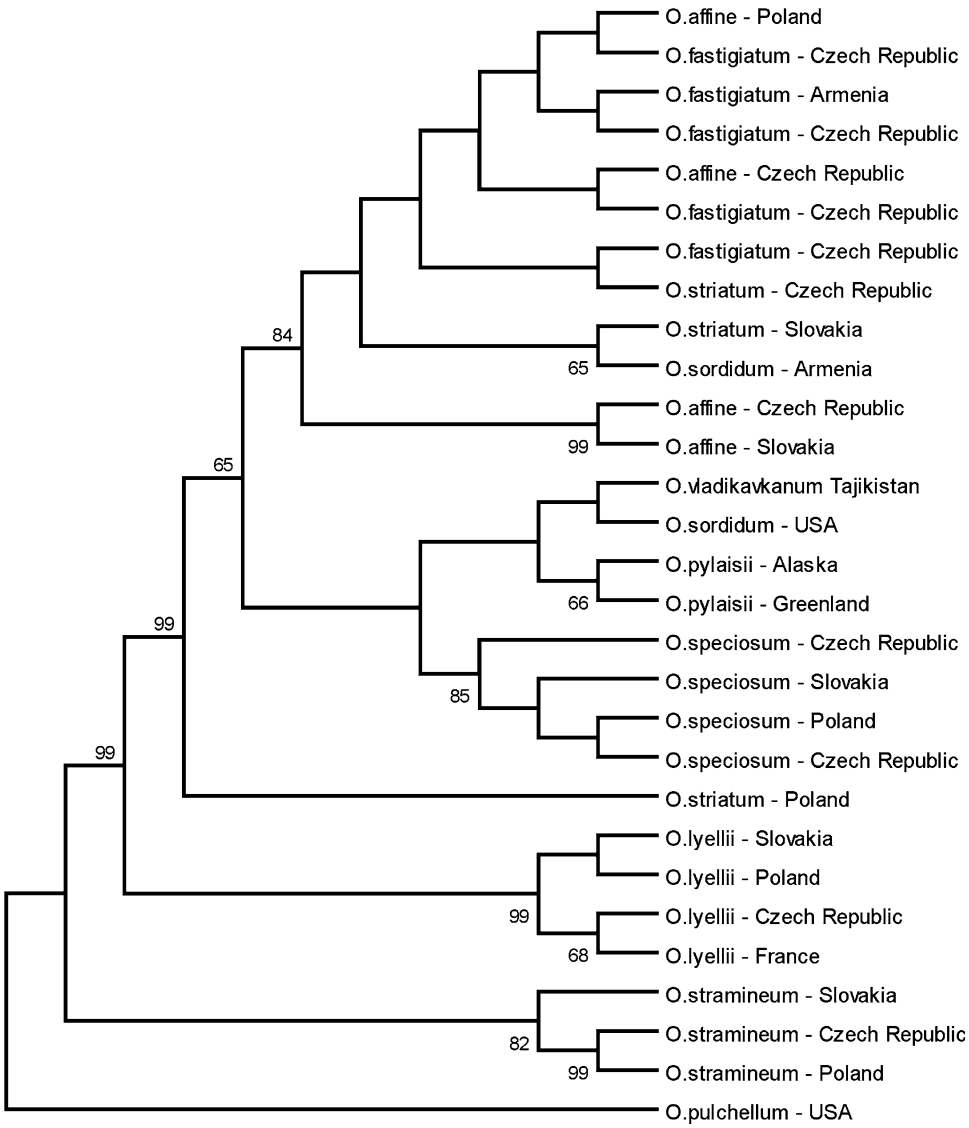

Fig. 1 Strict consensus of 190 most parsimonious trees with tree length of 96 steps (CI = 0.9883, RI = 0.9791) based on ITS sequences. Bootstrap values above 50% are given below branches

in the *O. affine–O. fastigiatum* clade formed a moderately to well-supported group (MP 65% and ME 78% bootstrap support and Bayesian inference 97% clade credibility) together with *O. sordidum* from Armenia.

The total length of the aligned *trn*H-*psb*A spacer was 242 base pairs including indels. Only 10 (4.13%) variable sites were found and 6 (2.48%) of them were parsimony informative. The mean number of fixed nucleotide differences between species was 1.8 across the entire dataset and 1.3 across species of subgenus *Gymnoporus*. Most fixed differences were found for outgroup taxa (*O. pulchellum* and *O. stramineum*) and species of subgenus *Gymnoporus*. Within subgenus *Gymnoporus,* the highest number of fixed differences was reported between *O. speciosum* and other taxa of this subgenus. For many pairs of species no fixed differences were noted (Table 2).

A maximum parsimony (MP) analysis resulted in 335 most parsimonious trees [length 12, consistency index (CI) 0.9167, retention index (RI) 0.9655]. The subgenus *Gymnoporus* clade was not supported on the basis of *trn*H-*psb*A data. Only three clades had moderate bootstrap support (Fig. 3): clades contain samples of *O. speciosum*, three out of four samples of *O. lyellii* and two samples of *O. stramineum*. Similar results were obtained with regard to the minimum evolution tree and to the Bayesian tree (trees not shown); however, in this case more clades were supported. Apart from clades supported by a MP analysis, moderate bootstrap support was reported for the clade formed by samples of *O. pylaisii* (ME 65% bootstrap support) and most samples of *O. affine* and *O. fastigiatum* (ME 66% bootstrap support). The *trn*H-*psb*A spacer analysis showed also a close relationship between *O. speciosum* and a sample of *O. sordidum* from the United States (ME 84% support).

Phylogenetic trees developed based on the chloroplast and nuclear genomes were found to be incongruent. The

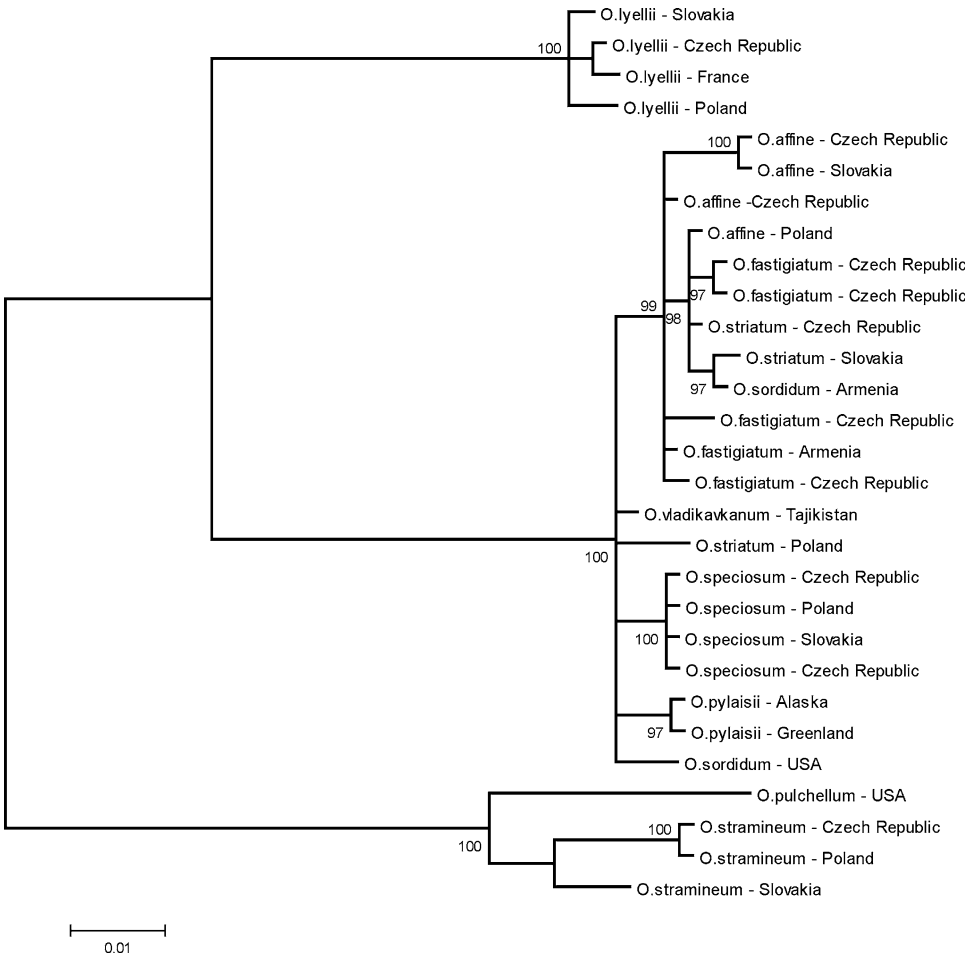

Fig. 2 Phylogram based on the Bayesian approach for the studied *Orthotrichum* species with ITS sequence data. Clade credibility values above 95% are given below the branches

incongruence concerned a single accession of *O. lyellii* from France, and it was a consequence of a four nucleotide inversion, which—combined with a low number of parsimony informative sites—resulted in the exclusion of this sample from the *O. lyellii* clade.

Discussion

Genetic variation

The analyzed *Orthotrichum* species differed with regard to the intraspecific polymorphism of ITS sequences. The values of the molecular diversity coefficient (π) of the most variable species, *O. stramineum* ($\pi = 0.01$) and *O. striatum* ($\pi = 0.009$), remained within the mid-range of variation determined for species of the genus *Sphagnum* section *Acutifolia* (Shaw and Cox 2005). Species of *Orthotrichum* section *Affinia*, *O. affine* and *O. fastigiatum*, were found to be less diverse; their nucleotide diversity was comparable to that of the least diverse peat mosses. Goryunov et al. (2007) analyzed variation of the ITS1 sequence of the *Schistidium apocarpum* complex and found that the number of mutations was smaller than in the examined species of *Orthotrichum*. Similarly as in the case of *Orthotrichum*, also among *Schistidum* there were species for which no intraspecific variation of ITS1 sequences was observed, despite a considerable geographic distance between the tested populations. However, none of the nine *Schistidium* species had the number of mutations equal to that detected in the most diverse *O. striatum* and *O. stramineum*.

A considerably higher degree of variation was observed in *Hylocomium splendens* (Chiang and Schaal 1999). An analysis of the ITS2 sequence across the populations of this species confirmed the existence of as many as 434 transitions and 407 transversions. In the majority of cases, the pairwise distances between the populations of *H. splendens* were higher than between *Orthotrichum* species belonging to different subgenera. Only slightly lower variation of ITS sequences was reported in *Mielichhoferia elongata* and *M. mielichhoferiana,* in which the distances between populations ranged from 0 to 0.34 and from 0 to 0.14, respectively (Shaw 2000). In these species, similarly as in *Orthotrichum* species analyzed in this study, the ITS1 sequence was

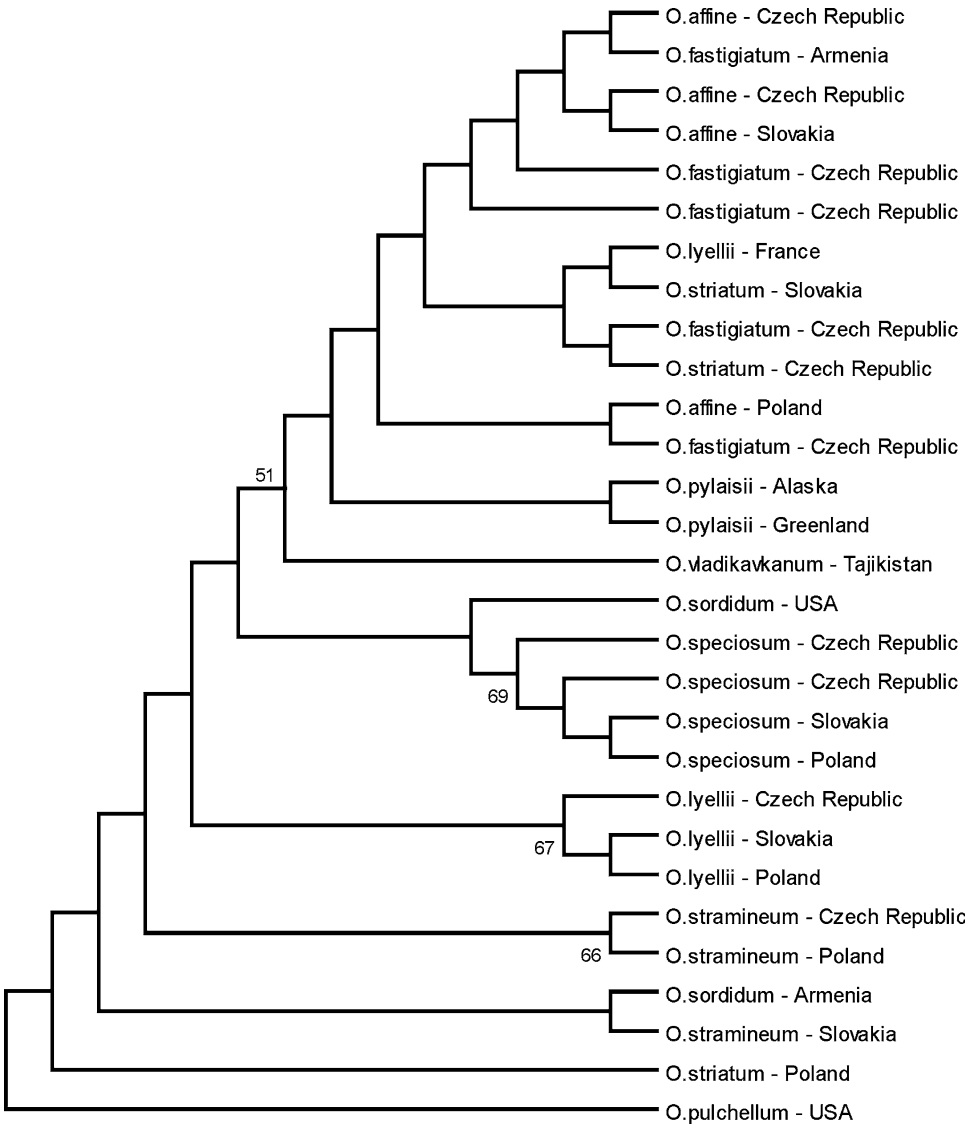

Fig. 3 Strict consensus of 335 most parsimonious trees with tree length of 12 steps (CI = 0.9167, RI = 0.9655) based on *trn*H-*psb*A sequences. Bootstrap values above 50% are given below branches

found to be more variable than the ITS2 sequence. The opposite tendency was noted in species of the family Fontinaliaceae, among which greater variation was observed with respect to the ITS2 sequence (Shaw and Allen 2000). The only species of the subgenus *Gymnoporus* characterized by higher variation in ITS2 than in ITS1 was *O. fastigiatum* (Table 1).

Although the ITS sequence is considered to be the most variable among the loci used for phylogenetic analyses of bryophytes (Shaw 2000; Shaw and Allen 2000; Vanderpoorten et al. 2001; Heinrichs et al. 2004; Shaw et al. 2005; Goryunov et al. 2007), its analysis did not show variation between four distant populations of *O. speciosum*. An analysis of the genetic structure of this species with AFLP markers demonstrated high variation at the intra-population level (Snall et al. 2004). A total of 65.8% polymorphic loci and genetic diversity of 0.196 were observed among 79 analyzed samples, which contradicts the results of the present study. The divergence between the above findings could result from the use of different categories of DNA markers. In bryophytes, where seven chloroplast (*trn*L) and nuclear (*ITS*, *Leafy1*, *Leafy2*, *RapdA*, *RapdB*, *RapdF*) loci were analyzed, the nucleotide diversity of *Sphagnum fimbriatum* and *S. squarrosum* was $\pi = 0.0219$ and $\pi = 0.0031$, respectively. An analysis of three chloroplast loci (*trn*LF, *trn*SG, *rpl*16) of the European populations of two peat moss species showed much lower values of molecular diversity, $\pi = 0.00028$ and $\pi = 0.00104$, respectively (Szovenyi et al. 2006). A similar tendency was observed in this study; in the majority of species much lower variation was noted in the chloroplast *trn*H-*psb*A sequence than in the nuclear DNA sequence. The reverse situation was observed only in *O. lyellii* whose chloroplast sequence showed twofold higher variation than nuclear sequences.

Division and phylogeny of the subgenus *Gymnoporus*

Molecular data do not support the division of the subgenus *Gymnoporus* into the sections *Leiocarpa* and *Affinia* in its current shape. ITS sequences fail to resolve our accessions of *O. affine*, *O. fastigiatum* and *O. striatum* as single monophyletic lineages. The type taxon of section *Leiocarpa*, *O. striatum*, was included into a well-supported clade together with *O. affine*, *O. fastigiatum* and *O. speciosum* of the section *Affinia*. Moreover, a low genetic distance and the lack of fixed differences between this species and *O. affine* of section *Affinia* suggest their close relationship, despite the existence of many morphological characters permitting an easy and reliable classification. The absence of significant differences in the ITS sequence between *O. striatum* and *O. affine* is highly surprising, especially that even species of the *Schistidium apocarpum* complex, morphologically hardly distinguishable and frequently questioned by taxonomists (Blom 1996), differ considerably with regard to their ITS1 sequences (Goryunov et al. 2007). Such a situation was also encountered in the morphologically well defined *Sphagnum fimbriatum* and *S. girgensohnii* whose values of genetic similarity (Cronberg 1996; Sawicki and Zieliński 2008) and the lack of fixed differences in the seven sequenced loci (Shaw and Cox 2005) do not support taxonomic distinctness. Polok et al. (2005) showed that RAPD and ISJ variation among the populations of *S. girgensohnii* was greater than between *S. girgensohnii* and *S. fimbriatum*. However, in contrast to the examined species of *Orthotrichum*, the above peat mosses belong to the group of green species within section *Acutifolia*. There were also no genetic differences between morphologically well defined species within the family Amblystegiaceae. Vanderpoorten and Tignon (2000) demonstrated that AFLP variation among the populations of *Hygroamblystegium tenax* was greater than between *H. tenax* and the morphologically distinguishable *H. fluviatile*. A later analysis of the ITS sequences of species of the genera *Amblystegium* and *Hygroamblystegium* has revealed that they are very closely related (Vanderpoorten et al. 2001). Moreover, ITS sequences fail to resolve accessions of the genera *Amblystegium* and *Hygroamblystegium* as single monophyletic lineages. Incongruence between morphological and molecular data was found also in the genus *Fontinalis*. The monophyly of groups of species within this genus defined by leaf morphology was not supported by ITS and *trn*L sequences (Shaw and Allen 2000). Conflicting molecular and morphological data have been also reported within Bryaceae (Holyoak and Pedersen 2007). An analysis of four chloroplast sequences has revealed a closer relationship of *Bryum bornholmense* and *B. rubens* to species of the genus *Plagiobryum* than to other *Bryum* taxa. The obtained data fail to resolve the accession of *B. radiculosum* as a monophyletic group. Similar patterns of ITS variation at the intra- and inter-specific levels have been described in other plants groups (Samuel et al. 1998; Manos 1999; Cullings 2000; Resetnik et al. 2007).

This result may be interpreted in several ways. One of the reasons may be hybridization, often observed in bryophytes, including in mosses and liverworts (Natcheva and Cronberg 2004). The occurrence of hybrids has been also reported in several species of the genus *Orthotrichum*. The following hybrids were described based on morphological characters: *O. anomalum* and *O. straminеum* (Ruthe 1873), *O. diaphanum* and *O. sprucei* (Philibert 1883), *O. gymnostomum* and *O. obtusifolium* (Hedderson 1986). Nyholm (1969) suggested the hybrid origin of *O. affine*, with *O. fastigiatum* and *O. speciosum* as potential parents.

The results of this study point to the occurrence of cryptic species in *O. affine*, *O. fastigiatum*, *O. sordidum* and *O. striatum*. Research on the intraspecific diversity in bryophytes demonstrated the existence of cryptic species among liverworts, morphologically similar but differing significantly with respect to electrophoretic phenotypes. Cryptic species were found in *Conocephalum conicum* (Szweykowski et al. 1981a, Akiyama and Hiraoka 1994), *Pellia epiphylla* (Szweykowski et al. 1981b), *Pellia endiviifolia* (Zieliński 1987), *Riccia dictyospora* (Dewey 1989), *Aneura pinguis* (Szweykowski and Odrzykoski 1990, Bączkiewicz et al. 2008), *Marchantia* (Boisselier-Dubayle et al. 1995), *Porella* (Therrien et al. 1998) and within the genus *Reboulia* (Boisselier-Dubayle et al. 1998). The presence of cryptic species was also reported in mosses of the genera *Mielichhoferia* (Shaw and Rooks 1994; Shaw 2000), *Mnium* (Wyatt et al. 1997), *Neckera* (Appelgren and Cronberg 1999) and *Plagiomnium* (Wyatt and Odrzykoski 1998) as well as in the species *Fontinalis antipyretica* (Shaw and Allen 2000).

The opposite situation was observed in *O. speciosum*, which was included into a separate clade. Despite its high morphological similarity to *O. affine* and *O. fastigiatum*, this taxon differs from these species at several sites of the ITS sequence. Nyholm (1969) considered the above taxa to be closely related, and even suggested that *O. affine* could be a hybrid of *O. fastigiatum* and *O. speciosum*. The authors of more recent taxonomic revisions differed in their opinions about the position of *O. speciosum*. Vitt (1973) placed this species into the section Leiocarpa, while Lewinsky (1993) postulated its inclusion into section *Affinia* due to peristome ornamentation and cell division of the inner peristome layer. A cladistic analysis of the genus *Orthotrichum*, based on morphological and ecological data, grouped *O. speciosum* together with species of section *Leiocarpa* (Lewinsky-Haapasaari and Hedenäs 1998).

Another member of section *Leiocarpa*, the dioecious *O. lyellii,* formed its own well-supported clade, and it was clearly distinct from other species of the subgenus *Gymnoporus*. A large number of fixed differences and the values of pairwise distances in relation to other species of the studied subgenus suggest that this taxon should be placed into a separate section, *Lyelliana* Schimp. (Schimper 1876). The exclusion of *O. lyellii* from section *Leiocarpa* is also supported by its morphological features. This taxon is easily distinguished from all other species of the analyzed subgenus by its dioecious sexual condition. Other features that distinguish this species from among members of section *Leiocarpa* are plane leaf margins and size reaching from 5–6 cm (Nyholm 1969; Lewinsky 1993) to even 13 cm (Vitt 1971). *O. lyellii* is also the only species within section *Leiocarpa* reported to produce gemmae (Lewinsky 1993). Moreover, the production of gemmae from the cells of rhizoids has been reported (Correns 1899; Piccioli 1932), which is quite unusual in other species of the genus *Orthotrichum*. Vitt (1968) found *O. lyellii* to be anisosporous, with spores of two different size classes, while other *Orthotrichum* species were usually isosporous (Lewinsky 1993). The investigated sequences did not differentiate section *Artica*, including *O. pylaisii* and *O. sordidum*, proposed by Venturi (1887). These species did not form a common clade, and the two analyzed specimens of *O. sordium* differed substantially with respect of the examined sequences, which may suggest the paraphyletic nature of this taxon. It should be noted, however, that the analyzed samples of *O. sordidum* represented different continents, in contrast to the previously discussed *O. affine*, *O. fastigiatum* and *O. striatum*, which was reflected in the results of phylogenetic analyses (Shaw et al. 2005; Shaw and Cox 2005).

Taxonomic status of *O. fastigiatum*

The identification of *O. fastigiatum* and its differentiation from the similar *O. affine* may be difficult considering the variability of morphological features of these species. Only one morphological character is commonly used by bryologists, i.e., seta length (Nyholm 1969). Other features are very similar and in consequence *O. fastigiatum* and *O. affine* are often considered as one species named *O. affine*.

The conducted analyses did not provide an unequivocal answer regarding the distinctness of *O. fastigiatum* and *O. affine*. Phylogenetic analyses grouped the populations of these species within a single clade. The same analyses did not differentiate two populations of *O. striatum* whose taxonomic distinctness has not been challenged to date. The absence of significant genetic differences and the presence of diagnostic morphological features are observed relatively frequently in the plant world, both among bryophytes (Shaw et al. 2005) and higher plants (Polok 2005).

Hybridization, mentioned above, may hinder the determination of the taxonomic status of *O. fastigiatum*. Among the tested samples, two populations of *O. affine* and two populations of *O. fastigiatum* formed two easily distinguishable clades within a common group. These populations may be pure species, while the remaining ones could be formed as a result of hybridization. Hybridization processes hindered also the determination of the taxonomic status of *Sphagnum rubellum* (Nyholm 1969, Daniels and Eddy 1990; Smith 1996), and only population genetics studies provided a basis for distinguishing this taxon (Cronberg 1996, 1998; Sawicki and Zieliński 2008).

Despite the fact that no fixed nucleotide differences were noted between *O. affine* and *O. fastigatum*, these species differed with regard to the degree of ITS sequence variation. ITS1 sequence variation was over threefold higher in *O. affine* than in *O. fastigiatum*. The opposite situation was observed in the ITS2 sequence, which was found to be more variable in *O. fastigiatum*.

The lack of significant differences in the analyzed sequences, accompanied by differences in their variation, are not sufficient to assess the taxonomic status of *O. fastigiatum*, whose determination requires further investigations at the population level, with the use of more variable molecular markers. Both taxa may be at an early stage of divergence, and differences between them could be based mostly on the frequency of particular alleles.

More research at the population genetics level is needed to fully resolve relationships among species of the subgenus *Gymnoporus* and to understand the evolutionary processes occurring within this group. The results of this study clearly show that the current division of the subgenus *Gymnoporus* into two sections, *Leiocarpa* and *Affinia*, should be regarded as unfounded. Both of these sections should be merged into one, known under the name of Leiocarpa, respecting the description of Molendo (1875). Section *Affinia* was reported 1 year later by Schimper (1876). According to the new concept, section *Leiocarpa* includes species showing significant similarity at the molecular level, with the exception of one species, *Orthotrichum lyellii*, which based on recent studies should be transferred into a separate section.

Appendix 1

Accession data for plants included in the molecular analysis of *Orthotrichum* subg. *Gymnoporus*. GenBank accession numbers are given in the following sequence: ITS1, ITS2, *trn*H-*psb*A.

O. affine Schrad. *ex* Brid., Opava-4, Czech Republic: Pivon, FJ159248, FJ168668, FJ036876;

O. affine Schrad. *ex* Brid., Opava-9, Czech Republic: Bohemia, EU860400, EU072690, FJ036878

O. affine Schrad. *ex* Brid., Opava-5, Poland: Bialskie Mts, FJ159249, FJ168669, FJ036877

O. fastigiatum Bruch *ex* Brid., Opava-6, Czech Republic: Cesky Les, FJ159253, FJ168671, FJ036880

O. fastigiatum Bruch *ex* Brid., Opava-7, Czech Republic: Morava, EU860401, EU072692, FJ036881

O. fastigiatum Bruch *ex* Brid., Opava-8, Czech Republic: Silesia, FJ159254, FJ168672, FJ036882

O. fastigiatum Bruch *ex* Brid., Opava-10, Czech Republic: Jasenik, FJ159251, FJ168673, FJ036883

O. fastigiatum Bruch *ex* Brid., NYBG-36, Armenia: Razdan, FJ159252, FJ168674, FJ036884

O. lyellii Hook. *et* Taylor, Opava-12, Slovakia: Poloniny MTS, EU863206, EU072689, FJ036872

O. lyellii Hook. *et* Taylor, Opava-13, Czech Republic: Bohemia, FJ159245, FJ168679, FJ036873

O. lyellii Hook. *et* Taylor, OLS-14, Poland: Tuczki, FJ159246, FJ168680, FJ036874

O. lyellii Hook. *et* Taylor, NYBG-42, France: Herault, FJ159247, FJ168681, FJ036875

O. pulchellum Brunt., NYBG-00462757, USA: Clallam Country, EU443996, EU484065, FJ036888

O. pylaisii Brid., NYBG-00151764, Greenland: Godthab, EU863210, EU871637, FJ036869

O. pylaisii Brid., NYBG-00151773, Alaska: Simeonof Island, FJ159260, FJ168667, FJ036868

O. sordidum Sull. *et* Lesq., NYBG-54, Armenia: Yerevan, EU863212, EU871639, FJ036871

O. sordidum Sull. *et* Lesq., NYBG-00760280, USA: Connecticut, FJ159261, FJ168666, FJ036870

O. speciosum Nees, Opava-1, Czech Republic: Bohemia, EU863213, EU072695, FJ036863

O. speciosum Nees, Opava-2, Poland: Bialskie Mts, FJ159258, FJ168663, FJ036866

O. speciosum Nees, Opava-3, Slovakia: Liptovice, FJ159257, FJ168664, FJ036865

O. speciosum Nees, Opava-11, Czech Republic: Silesia, FJ159259, FJ168665, FJ036864

O. stramineum Hornsch. *ex* Brid., Opava-19, Slovakia: Durkovice, FJ159243, FJ168678, FJ036889

O. stramineum Hornsch. *ex* Brid., Opava-20, Poland: Bialskie Mts, FJ159244, FJ168677, FJ036890

O. stramineum Hornsch. *ex* Brid., Opava-21, Czech Republic: Bohemia, EU443999, EU072696, FJ036891

O. striatum Hedw., Opava-153827, Czech Republic: Moravia, EU443993, EU072697,FJ036885

O. striatum Hedw., Opava-16, Poland: Bialskie Mts, FJ159256, FJ168676, FJ036886

O. striatum Hedw., Opava-17, Slovakia: Liptovice, FJ159255, FJ168675, FJ036887

O. vladikavkanum Venturi, NYBG-50, Tajikistan: Dushanbe, EU863214, EU871640, FJ036867

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