Ann. Bot. Fennici 46: 507–515 ISSN 0003-3847 (print) ISSN 1797-2442 (online)
Helsinki 18 December 2009 © Finnish Zoological and Botanical Publishing Board 2009

Preliminary studies on the phylogeny of *Orthotrichum* (Bryophyta) inferred from nuclear ITS sequences

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Received 8 July 2008, revised version received 1 Dec. 2008, accepted 3 Dec. 2008

Sawicki, J., Plášek, V. & Szczecińska, M. 2009: Preliminary studies on the phylogeny of *Orthotrichum* (Bryophyta) inferred from nuclear ITS sequences. — *Ann. Bot. Fennici* 46: 507–515.

The study presents a phylogenetic analysis of species of the moss genus *Orthotrichum*. ITS1 and ITS2 for 30 species were sequenced. The results do not fully reflect the current division of *Orthotrichum* into subgenera and sections. Molecular data divide the genus into two groups of species with superficial and immersed stomata, and indicate a clear distinctness of dioecious species. This suggests that a previous concept postulating that the subgenus *Orthophyllum* should be excluded from the genus *Orthotrichum* might be justified.

Key words: *Orthotrichum*, ITS, molecular systematics, mosses, phylogeny

Introduction

The genus *Orthotrichum* is a widespread moss group, which includes approximately 155 species (Goffinet *et al*. 2007) distributed throughout the world from the Arctic to the Antarctic, except in deserts and wet tropical forests. Species of *Orthotrichum* grow on trees and rocks to a height of ca. 5000 m a.s.l. (Lewinsky 1993). In the most recent revision, Orthotrichaceae was divided into two subfamilies, each comprising two tribes: the Schlotheimieae and Macromitrieae (Macromitrioideae), and the Zygodonteae and Orthotricheae (Orthotrichoidae), and *Orthotrichum* was placed in the latter group (Goffinet & Vitt 1998, Goffinet *et al*. 2004). The subdivision of *Orthotrichum* has been a moot point since the end of the 19th century. Certain taxa have been alternately included in and excluded from the genus in the attempts to divide it into lower taxonomic units, subgenera and sections. The basis for the classification of *Orthotrichum* in a historical perspective was described in detail by Lewinsky (1993) and Lewinsky-Haapasaari and Hedenäs (1998).

According to the latest revision, the genus *Orthotrichum* is divided into seven subgenera (Lewinsky 1993): *Callistoma*, *Exiguifolium*, *Gymnoporus*, *Orthotrichum*, *Orthophyllum*, *Phaneroporum* and *Pulchella*. Based on morphological and molecular data, Goffinet *et al*. (2004) excluded the subgenus *Exiguifolium* from *Orthotrichum* and transferred it to the genus *Letaria*. The subgenera are distinguished based on the following criteria: stoma type (superficial vs. immersed), details of peristome teeth, presence or absence of connecting membrane, cell division of the inner

peristome layer, and ecology. Two of the subgenera, *Gymnoporus* and *Pulchella*, are further divided into sections, *Affinia* and *Leiocarpa*, and *Pulchella*, *Diaphana* and *Rivularia*, respectively. The features determining species affiliation to particular sections are usually the details of the structure of the endostome and leaves.

Despite numerous controversies and ambiguities regarding its division (Vitt 1971, Lewinsky 1993, Lewinsky-Haapasaari & Hedenäs 1998), *Orthotrichum* has never been subject to a phylogenetic analysis. The only available information was provided by an analysis of the family Orthotrichaceae, which comprised only several species of the genus *Orthotrichum* (Goffinet *et al*. 1998, 2004).

The internal transcribed spacer (ITS) region is commonly used in phylogenetic and population genetic studies on bryophytes (Fiedorow *et al*. 1998, Shaw 2000, Shaw & Allen 2000, Shaw *et al*. 2005, Juslén 2006, Goryunov *et al*. 2007, Sawicki & Zieliński 2008, Plášek *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). A review of the applications of the ITS region in bryophyte systematics is given in Vanderpoorten *et al*. (2006). Although ITS sequences have proved effective in phylogenetic studies on bryophytes, it should be noted that single gene phylogeny often disagrees with species phylogeny (Miyamoto & Fitch 1995, Maddison 1997). Those differences may stem not only from differing rates of evolution of particular genome regions (Graur & Li 2000), but also from such phenomena as hybridization, common in bryophytes (Natcheva & Cronberg 2004), or horizontal gene transfer (Gustavsson *et al*. 2005). This concerns primarily chloroplast genes (Shaw *et al*. 2005, Sawicki *et al*. 2008), but cases of such incompatibility among nuclear genes have been also reported (Gustavsson *et al*. 2005, Ghatnekar *et al*. 2006).

Current morphological revisions of various moss taxa are often supported by molecular data (Virtanen 2003, Hyvönen *et al*. 2004, Pedersen & Hedenäs 2005, Cano *et al*. 2005). As compared with morphological data, DNA sequences are not influenced by the changing environmental conditions in which the plants have grown. Hence, molecular data can be used as a powerful tool in resolving taxonomic and systematic problems. Using nuclear ITS sequences from 28 *Orthotrichum* species, we investigated the phylogeny of *Orthotrichum*. The aim of the study was to find out if the genus *Orthotrichum* and its subgenera and sections are monophyletic based on this data.

Material and methods

Material

Our analyses included 30 species representing three genera of the family Orthotrichaceae. The genus *Orthotrichum* was represented by 28 species representing five subgenera. *Zygodon rupestris* was used as outgroup, based on a previous higher-level analysis (Goffinet *et al*. 1998, Goffinet *et al*. 2004).

The list of species used in a molecular analysis, the details concerning voucher data and the GenBank accession numbers are given in Table 1. Since *O. diaphanum* has two substantially different ITS sequences (J. Sawicki unpubl. data), only the sequence showing greater similarity to other species of the genus *Orthotrichum* was used for this analysis. The recently described species *O. moravicum* (Plášek *et al*. 2009) and the doubtful species *O. fastigiatum*, often treated as *O. affine*, were also analyzed.

DNA extraction

Total genomic DNA was extracted from herbarium material. Single stem was ground with silica beads in a FastPrep tissue disruptor for 20 seconds 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

Table 1. Locality, GenBank accession numbers and ITS sequence length of *Orthotrichum* specimens used in analysis. Herbaria: NYBG = New York Botanical Garden, USA; OP = Opava, Czech Republic; OLS = Olsztyn, Poland.

Species	Provenance, herbarium	Accession number ITS1/ITS2	Sequence length ITS1/ITS2
Subgenus *Gymnoporus* section *Leiocarpa*			
O. lyellii	Slovakia, Poloniny Mts., Ruský potok village, herb .OP	EU863206/EU072689	447/480
O. striatum	Czech Rep.,. Moravia, Jihlavské vrchy hills, herb. OP	EU443993/EU072697	428/452
O. vladikavkanum	Tadzhikistan, Dushanbe, in Valle Ramit, herb. NYBG	EU863214/EU871640	429/453
Subgenus *Gymnoporus* section *Affinia*			
O. affine	Czech Rep. , Prov. Bohemia, Pivoň village, herb. OP	EU860400/EU072690	429/452
O. fastigiatum	Czech Rep. , Prov. Moravia, Lázně Jeseník town, herb. OP	EU860401/EU072692	428/452
O. pylaisii	Grenlandia, Godthab distr. Kilearsarfik, herb. NYBG	EU863210/EU871637	420/452
O. sordidum	Armenia, Yerevan, Pambakskij Khrebet, herb. NYBG	EU863212/EU871639	427/452
O. speciosum	Czech Rep. , Prov. Bohemia, Ostrůvek, herb. OP	EU863213/EU072695	416/452
Subgenus *Orthophyllum*			
O. gymnostomum	Canada, Newfoundland, Notre Dame Bay, herb NYBG	EU863204/EU072687	462/484
O. obtusifolium	Slovakia, Nízké Tatry Mts., Liptovský dvôr, herb. OP	EU863208/EU072693	643/484
Subgenus *Pulchella* section *Pulchella*			
O. consimile	USA, WA, Grays Harbor Co., Lake Quinault, herb NYBG	EU443997/EU484066	398/435
O. pulchellum	USA, Clallam Co., Little River Road, herb. NYBG	EU443996/EU484065	405/441
O. scanicum	Czech Rep. , Prov. Bohemia, Český les Mts., herb. OP	EU863211/EU871638	404/434
Subgenus *Pulchella* section *Rivularia*			
O. rivulare	Great Britain, S. Devon, R. Bovey, herb. NYBG	EU484063/EU484070	396/435
Subgenus *Pulchella* section *Diaphana*			
O. alpestre	USA, CA, Mono Co. Inyo National Forest, herb. NYBG	EU443998/EU484067	396/435
O. diaphanum	Poland, Młock near Ciechanów, on Salix sp., herb OLS	EU484077/EU484073	408/431
O. moravicum	Czech Rep. , Prov. Moravia, 3 km NE of Bílá, herb. OP	EU863207/EU072688	403/432
O. pallens	Poland, Góry Bialskie, Bielice village, herb. OP	EU490618/EU072694	396/435
O. patens	Czech Rep. , Prov. Moravia, Zábřeh na Moravě, herb. OP	EU863209/EU871636	397/ 436
O. pumilum	Poland, Prov. Mazowieckie, Ciechanów, herb. OLS	EU443994/EU035537	402/435
O. stellatum	USA, NY, Putnam Co, Fields Farmstead herb. NYBG	EU484081/EU484068	396/437
O. stramineum	Czech Rep. , Prov. Bohemia, Rybník, herb. OP	EU443999/EU072696	397/436
O. tenellum	Canary Islands, Cumpre Neuva, Cumpre, herb. NYBG	EU443995/EU484064	398/436
Subgenus *Phaneroporum*			
O. laevigatum	Canada, Alberta, 3 km E of Rock Lake, herb. NYBG	EU863205/EU871635	429/452
O. rupestre	Czech Rep. , Prov. Bohemia, Branov, Malá Pleš,, herb. OP	EU443991/EU072686	431/454
Subgenus *Orthotrichum*			
O. anomalum	Poland, Góry Bialskie Mts., Stary Gieraltów, herb. OP	EU443992/EU072691	396/435
O. cupulatum	Poland, Pieniny Mts., Kornajowska skała, herb NYBG	EU484072/EU484071	396/435
O. pellucidum	USA, Gunnison Co., Cement Creek, herb NYBG	EU484062/EU484069	396/435
Genus *Ulota*			
Ulota crispa	Czech Rep. , Prov. Moravia, Moravský kras karst, herb. OP	EU863215/EU871641	469/441
Genus *Zygodon*			
Zygodon rupestris	Czech Rep. , Prov. Silesia, Salajka reserve, herb. OP	EU863216/EU871642	304/466

sequences of the applied primers were as follow: ITS1-forward 5´ CAAGGTTTCCGTAGGTGAAC 3´; ITS1-reverse 5´ CAAGAGCCAAGATATCCG 3´; ITS2-forward 5´ CGGATATCTTGGCTCTTG 3´; ITS2-reverse 5´ CCGCTTAGTGATATGCTTA 3´. The ITS region was 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 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).

Data analyses

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. Gaps were excluded from all phylogenetic analyses. MEGA 4 (Tamura *et al*. 2007) was used for the Minimum Evolution (ME) analysis and Maximum Parsimony (MP) analysis. The pairwise distances were estimated with the Maximum Composite Likehood method (Tamura *et al*. 2004) and initial trees generated using a neighbour-joining (NJ) method. The ME tree was searched using the Close Neighbor Interchange (CNI) algorithm (Nei & Kumar 2000) at the search level of 2, and the maximum number of trees retained at each step was set to 100. For parsimony analyses, we an applied branch and bound search as implemented in MEGA 4. Statistical significance of clades within inferred trees was evaluated using the bootstrap method (Felsenstein 1985) with 1000 replicates.

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 (nst = 6) with the proportion of invariable sites in accordance with the best fitted nucleotide evolution model selected on the basis of the 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 four incrementally heated chains, starting from random trees and sampling one out of every 100 generations. Trees were checked for stability, which appeared at around 40 000–50 000 chains, therefore, the first 1000 trees were discarded as burn-ins. Remaining trees were used to construct the Bayesian consensus tree. We consider good bootstrap support > 70% and weak support < 70%. In case of the Bayesian clade, credibility values, significant support was estimated as ≥ 95%.

Results

The length of the ITS1 spacer ranged from 304 bp in *Zygodon rupestris* to 643 bp in *O. obtusifolium*. The shortest ITS1 sequence among *Orthotrichum* species, 396 bp in length, was found in several taxa with immersed stomata: *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*. ITS2 was ca. 20 bp longer in species with superficial stomata than in taxa with immersed stomata.

The alignment had a total length of 1266 bases. The ITS dataset contained 414 variable sites, of which 169 were parsimony informative. A maximum parsimony (MP) analysis resulted in 19 most parsimonious trees of 396 steps, with a consistency index (CI) of 0.7761 and a retention index (RI) of 0.8368. The Minimum Evolution (ME) method (figure not shown) and Bayesian interference (Fig. 1) resulted in very similar trees, differing mostly in the position of *O. lyellii* and *Ulota crispa*. Three main clades were formed. Species of the subgenus *Orthophyllum* formed

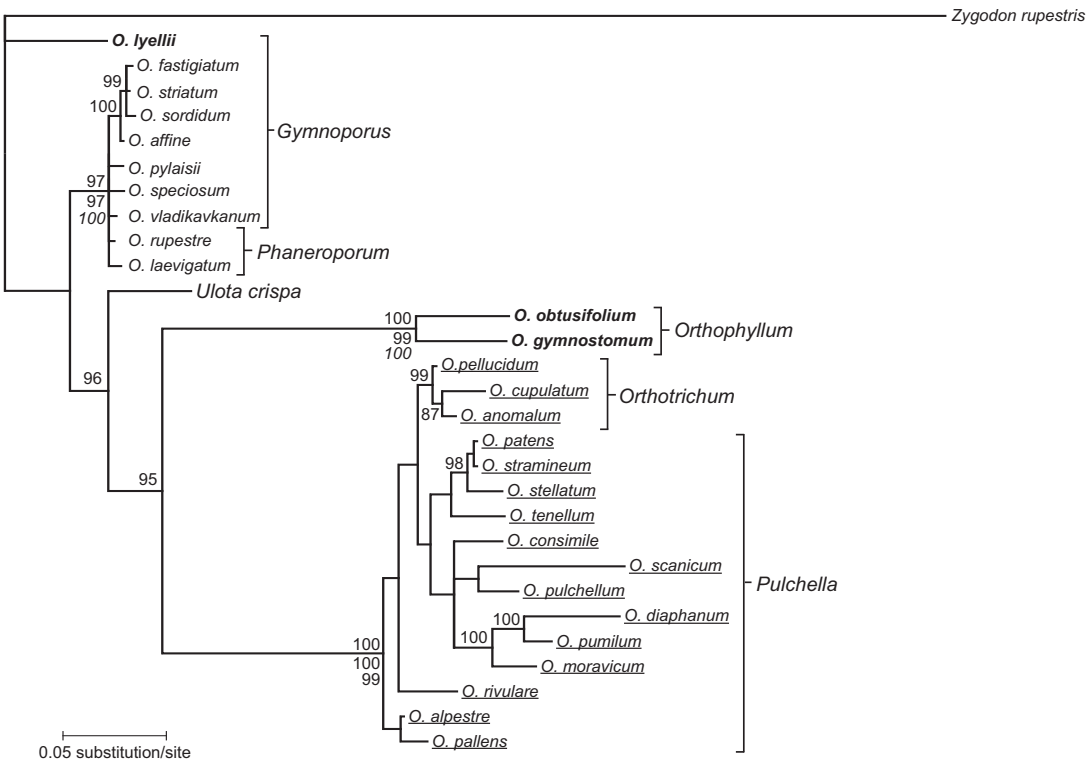

Fig. 1. Phylogram based on the Bayesian approach for 28 *Orthotrichum* species with ITS sequence data. Clade credibility values above 95% are given above the branches. Bootstrap values of clades supported under parsimony and Minimum Evolution (set in italics) are given under the branches. Species with immersed stomata are underlined and names of dioecious species are set in bold face.

a distinct, well-supported clade (MP 99% and ME 100% bootstrap support and Bayesian inference 100% clade credibility). The second clade was formed by species with superficial stomata, including *Ulota crispa* (in the case of MP and ME analysis) which seems to be more closely related to the monoecious species than *O. lyellii*. Within this clade, monoecious species formed a distinct, well-supported group (MP 97% and ME 99% bootstrap support and Bayesian inference 97% clade credibility), however showing no distinct division into subgenera. Only in the case of the minimum evolution analysis, members of the subgenus *Phaneroporum*, *O. laevigatum* and *O. rupestre*, formed their own, but poorly supported clade (ME 44% bootstrap support). Among species of the subgenus *Gymnoporus*, a well-supported clade (MP 92% and ME 93% bootstrap support and Bayesian inference 100% clade credibility) was formed by O. *affine*, *O. fastigiatum*, *O. sordidum* and *O. striatum*, which does not reflect the current division of this subgenus into sections. The third main clade was formed by species with immersed stomata. Three species of the subgenus *Orthotrichum*, *O. anomalum*, *O. cupulatum* and *O. pellucidum*, formed a distinct, well-supported (ME 84% bootstrap support, Bayesian inference 99% clade credibility) to poorly-supported (MP 62% bootstrap support) clade. Among sections of the subgenus *Pulchella*, only the section *Diaphana* seems to be polyphyletic. Three representatives of the section *Pulchella*, *O. consimile*, *O. pulchellum* and *O. scanicum*, formed their own but poorly supported clade (ME 68% and MP 30% bootstrap support).

Discussion

Molecular data do not support the current division of the genus *Orthotrichum* into subgenera. It seems that the only distinguishable subgenus is

Orthophyllum, whose taxonomic distinctness has been frequently postulated before (Hagen 1908, Damsholt *et al*. 1969, Goffinet *et al*. 2004). Its two species *O. gymnostomum* and *O. obtusifolium* were indeed placed in a separate genus, *Stroemia*, by Hagen (1908). They were separated by obtuse leaves with incurved or plane leaf margins and incrassate leaf cells with a stout, central papilla on each side. Since *Stroemia* was an illegitimate name, it was later replaced by *Nyholmiella* (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), as the above features were also observed in other representatives of this genus. The affiliation of these species to the genus *Orthotrichum* was tested by Lewinsky-Haapasaari and Hedenäs (1998) with the use of 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. On the other hand, an analysis of a nuclear ITS sequence conducted in this study suggested that the members of the subgenus *Orthophyllum* are genetically distinct from the other species of *Orthotrichum*. In addition, the investigated sequence revealed a closer relationship between *Ulota crispa* and other *Orthotrichum* species than between these species and *O. gymnostomum* and *O. obtusifolium*, which strongly supports separation of the latter two species from *Orthotrichum*. The distinctness *O. obtusifolium* from the other species of *Orthotrichum* was also revealed by an analysis of sequences from four loci (26S, *nad5*, *rps4* and *trn*L–*trn*F region), which provided a basis for excluding species of the subgenus *Orthophyllum* from *Orthotrichum* (Goffinet *et al*. 2004). Certainly, such a decision should be based on an analysis of a greater number of genes and populations of these species.

Our results also support considerable distinctness of other species with superficial stomata from those with immersed stomata. Stoma type is important in the taxonomy of *Orthotrichum*. They are either level, with surrounding exothecial cells (superficial, phaneropore), or immersed and almost covered by the surrounding cells (immersed, cryptopore). The occurrence of both stomata types on all continents suggests that both types have a long history. However, their historical relationships are still unclear (Lewinsky 1977). Vitt (1971) tried to explain the fact as a genetic change, because the species with superficial stomata have a haploid chromosome number, n = 6, whereas cryptopore species have n = 11. However, autopolyploidy plus the loss of one chromosome seem to be unlikely to create the immersed type of stomata, because it would mean that this feature is controlled by only one chromosome. The separation of the two types of stomata is probably due to a much more complex mechanism, dependent on the interaction between genes in different chromosomes (Lewinsky 1977). According to Paton and Pearce (1957), the development of cryptopore stomata as a result of adaptation to xeric conditions seems unlikely because many species with immersed stomata are found in moist habitats (*O. pulchellum*, *O. consimile*). The position of stomata provided a basis for the division of this genus by Lindberg (1879), who divided it into two subgroups, *Gymnoporus* and *Calyptoporus*, comprising species with superficial and immersed stomata respectively. Within the first of those subgroups, taxa belonging to two subgenera, *Gymnoporus* and *Phaneroporum*, did not form clades in our study. A clear division of the genus *Orthotrichum* into groups of species with immersed and superficial stomata was also confirmed by chloroplast and mitochondrial sequences (Goffinet *et al*. 2004). An analysis of the nuclear locus 26S, mitochondrial locus *nad5* and chloroplast loci *rps4* and *trn*L–*trn*F revealed the distinctness of *O. affine*, *O. laevigatum* and *O. lyellii* from *O. alpestre*, *O. anomalum*, *O. assimile* and *O. macrocephalum*, placing these species in two separate clades.

Molecular data do not support the division of the subgenus *Gymnoporus* into the sections *Leiocarpa* and *Affinia*, as proposed by Lewinsky (1993) and Lewisky-Haapasaari and Hedenäs (1998). The type taxon of the section *Leiocarpa*, *O. striatum*, was included in a moderately supported clade together with *O. affine*, *O. fastigiatum* and *O. sordidum* of the section *Affinia*. Another member of the section *Leiocarpa*, the

dioecious *O. lyellii*, was clearly distinct from the other species of the subgenus *Gymnoporus*. A large number of fixed differences in relation to the other species in the subgenus suggests that this taxon should be placed into a separate infrageneric taxon. Its exclusion from the section *Leiocarpa* and the formation of the section *Lyelliana* by Schimper (1876, followed Lewinsky 1993), seems justified. The distinctness of *O. lyellii* from members of the section *Leiocarpa* was also noted by Vitt (1971), who however did not classify this taxon into a separate section or subgenus. Based on the ITS sequences *Ulota crispa* is closely related to *Orthotrichum* species of the subgenera *Gymnoporus* and *Phaneroporum*. *Gymnoporus* species were found to be more genetically similar to *U. crispa* than to the dioecious *O. lyellii* belonging to the same subgenus. Similar results were obtained while analyzing the chloroplast *rbc*L sequence which showed a close relationship between *Ulota* and *Orthotrichum* species of the subgenus *Gymnoporus* (Goffinet *et al*. 1998). An analysis of four loci from the nuclear (26S), mitochondrial (*nad5*) and chloroplast (*rps4*, *trn*L–*trn*F) genome also showed that *Orthotrichum* species with superficial stomata are closer to species of *Ulota* than to *Orthotrichum* species with immersed stomata of the subgenera *Orthotrichum* and *Pulchella* (Goffinet *et al*. 2004).

A further group was formed by species of the subgenera *Orthotrichum* and *Pulchella*. Unlike species with superficial stomata, representatives of the subgenus *Orthotrichum* formed a clade. As regards morphological characteristics, subgenus *Orthotrichum* differs from subgenus *Pulchella* mostly in having straighter peristome teeth and no connecting membrane (Lewinsky 1993). The placing of species of this subgenus within the '*Pulchella*' group is consistent with the results of Vitt (1971) and with one of the three relationship ideas proposed by Lewinsky-Haapasaari and Hedenäs (1998). In both cases, the authors placed the subgenus *Orthotrichum* within the *Pulchella* clade, based on morphological characters. However, our results did not confirm a close relationship between members of the subgenera *Orthotrichum* and *Phaneroporum*, suggested by Lewinsky (1993). Taxa belonging to these groups are genetically different, despite certain similarities such as the preferred habitats and the characteristics of peristome and calyptra.

For the subgenus *Pulchella*, in contrast to the subgenus *Gymnoporus*, our study resulted in a moderately supported division into sections. The only clearly distinct section was *Pulchella*, whose species were grouped within a single clade in all analyses. The section *Pulchella* could be considered monophyletic, while the largest section, *Diaphana*, is rather paraphyletic. The position of *O. rivulare* may also indicate a genetic distinctness of the section *Rivularia*.

The number and ornamentation of exostome teeth and endostome segments have a central role in the taxonomy of *Orthotrichum*. The ancestors of the genus probably had a very well developed double peristome (with 16 exostome teeth and endostome segments) covered with papillae (Lewinsky 1977). Vitt (1973) suggests that a reduction in peristome characters (mainly in the number of endostome segments and exostome teeth, from 16 to 8 or 0) may result from adaptation to different ecological conditions. According to that author, specialization to xeric habitats, such as the trunks of trees and dry rock surfaces, is the driving force. This view, however, cannot be regarded as generally valid, as a reduction of endostome segments has not been observed in many xeric species growing in dry places (*O. diaphanum*, *O. striatum*), whereas a species (*O. affine*) of shaded and moist places has a reduced peristome of eight teeth and segments. The presence of a preperistome, considered by Vitt (1973) as an advanced character, was subsequently regarded as a primitive state, because it is present in many species with a well-developed, not reduced peristome (Lewinsky 1989). We found no correlation between particular peristome characters and adaptive evolution in *Orthotrichum*.

Although our results do not fully reflect the current taxonomic division, it points to the fact that the former taxonomic concepts were often correct. This refers in particular to the division proposed by Lindberg (1879) and to the separation of the genus *Stroemia* (Hagen 1908), which seems justified. However, this remains a hypothesis with some support and it should be confirmed by further studies.

Acknowledgements

DNA analysis was made possible through financial support from The Lewis B. and Dorothy Cullman Program for Molecular Systematic Studies. We also thank W. R. Buck for lending us herbarium specimens of several *Orthotrichum* species, and two anonymous referees for helpful comments on the manuscript.

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