SYSTEMATICS AND PHYLOGENY

A case study of intragenomic ITS variation in bryophytes: Assessment of gene flow and role of polyploidy in the origin of European taxa of the *Tortula muralis* (Musci: Pottiaceae) complex

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Abstract For the first time in bryophyte studies, we performed comprehensive cloning of the ITS region to reveal intraindividual variation of ITS sequences. We assessed relationships among morphologically defined taxa of the polyploid complex of the moss *Tortula muralis*. Our results detected a monophyletic *T. muralis* complex comprising *T. muralis* subsp. *muralis, T. muralis* subsp. *obtusifolia*, *T. lingulata, T. israelis,* and *T. edentula*. The single accession of *T. edentula* was found nested within *T. obtusifolia*, and biphyletic *T. israelis* was found to be nested within *T. muralis*. With the exception of *T. lingulata*, intragenomic ITS sequence variation was high in the *T. muralis* complex. Most intraindividual sequences were nevertheless only weakly divergent, suggesting their origin via mutations exceeding the rates of concerted evolution. Markedly divergent sequences found within a single individual most probably resulted from gene flow among distant lineages of the complex. Such pattern of ITS variation challenges the traditional morphology-based taxonomy. No phylogenetic signal was associated with ploidy-level variation, suggesting a polytopic origin of the diploids. Interestingly, the pattern of ITS variation together with morphological evidence indicate the autopolyploid origin of some lineages, which renders the *T. muralis* complex the first group of mosses in which autopolyploidy is implied by molecular markers.

Keywords bryophytes; gene flow; intragenomic variation; ITS; *Tortula*

Supplementary Material Figures S1–S3 (in the Electronic Supplement) and the alignment are available in the Supplementary Data section of the online version of this article (http://www.ingentaconnect.com/content/iapt/tax).

■ INTRODUCTION

The internal transcribed spacer (ITS) of 18S-26S nuclear ribosomal DNA is one of the most widely used sequence markers in bryophyte studies (Stech & Quandt, 2010). As a non-coding part of the 18S-26S operon, the ITS region is a true multi-copy marker with hundreds to thousands of copies arranged in tandem arrays of the operon (Álvarez & Wendel, 2003). Despite its multi-copy nature, the homogeneity of individual ITS copies is driven by concerted evolution (Arnheim, 1983; Elder & Turner, 1995). However, the rate of concerted evolution varies greatly, and intragenomic variation of ITS copies (ITS paralogs sensu Álvarez & Wendel, 2003) is not exceptional (Buckler & al., 1997; Álvarez & Wendel, 2003; Nieto Feliner & Rosselló, 2007).

There are two main possible explanations for the occurrence of intragenomic ITS variation, both assuming incomplete concerted evolution of nrDNA arrays. First, the occurrence of intragenomic ITS variation might result from the hybridization between parents containing different ITS sequences (Baldwin & al., 1995; Sang & al., 1995). Second, divergent intraindividual sequences might arise by molecular processes unrelated to hybridization, such as the accumulation of mutations that exceeds the rate of concerted evolution, nrDNA array multiplication, or pseudogenization (Álvarez & Wendel, 2003; Nieto Feliner & Rosselló, 2007). These molecular mechanisms might result in polymorphisms which together with incomplete lineage sorting processes may obscure phylogenetic analysis, especially when non-orthologous sequences or apparent pseudogenes are not recognized (Buckler & al., 1997). The intragenomic variation of ITS sequences is challenging, because the assumption of orthology is crucial for the correct reconstruction of phylogeny. Numerous studies addressed intragenomic ITS variation in vascular plants (Álvarez & Wendel, 2003). However, little is known about intragenomic ITS variation in bryophytes. To the best of our knowledge, this phenomenon has been detected only in the genus *Plagiomnium* T.J. Kop. (Harris, 2008).

Recently, we have found intragenomic ITS variation in the European taxa of the *Tortula muralis* complex. According to a morphological study by Košnar & Kolář (2009), the complex was defined to include *T. muralis* Hedw. subsp. *muralis* with var. *muralis* and var. *aestiva* Brid. ex Hedw., *T. muralis* subsp. *obtusifolia* (Schwägr.) Culm., and *T. lingulata* Lindb. The detected clinal variation and poor morphological differentiation

among the taxa of the *T. muralis* complex might result from gene flow among taxa, or might reflect cryptic speciation, i.e., the existence of additional, genetically divergent lineages that are poorly or not at all defined morphologically, as has been revealed frequently in all major groups of bryophytes studied using molecular markers (Shaw, 2001). The latter hypothesis was proposed in a study of molecular variation in *Tortula muralis* using *rps4* sequences (Werner & Guerra, 2004), where several morphologically undefined lineages were detected. These lineages were hypothesized to represent putative cryptic species because one of the nested clades included the morphologically well-defined and generally accepted *Tortula vahliana* (Schultz) Mont. Unfortunately, low variability of chloroplast *rps4* sequences poorly reflects patterns of genetic variability in closely related taxa of Pottiaceae (Köckinger & Kučera, 2011). Therefore, such hypothesis needs to be substantiated using more variable molecular markers.

In addition, a distinct pattern of ploidy variation and habitat preferences has been detected among subspecies and varieties of *T. muralis* (Košnar & Kolář, 2009). Plants evaluated as subsp. *obtusifolia* were exclusively haploid, whereas both haploid and diploid cytotypes were found in both varieties of *T. muralis* subsp. *muralis*. The morphological variability in the broader distribution area in Eurasia comprises several other taxa, including *T. israelis* Bizot & F. Bilewski, known from the Mediterranean region and the Near East, and the recently described *T. edentula* Ignatova & Ignatov from the Kuril Islands. Other putatively closely related taxa, including, e.g., *T. vahliana* and *T. brevissima* Schiffn. (Werner & al., 2002a; Werner & Guerra, 2004), were also included for further consideration, as described below.

The objectives of the current study were to: (i) evaluate intragenomic ITS variation in the *T. muralis* complex and related taxa; (ii) determine the phylogeny of the *T. muralis* complex, including putatively related Eurasian species of *Tortula* and related genera; and (iii) determine the relationship between ploidy level and genetic lineages in the *T. muralis* complex, i.e., determine whether diploids arose recurrently from different haploid ancestors.

■ MATERIALS AND METHODS

Plant material. — A total of 159 herbarium specimens were selected for molecular analysis (Appendix). Most specimens were collected in Europe but a few were from Asia. Definition of the taxa in the *T. muralis* complex followed the morphological concept suggested in our previous study (Košnar & Kolář, 2009). In cases when plants from a single collection were markedly heterogeneous morphologically, plants of each analysed morphotype were considered a separate sample. **S**amples of morphologically uniform plants collected at one locality were treated as a population.

To incorporate our data into a broader phylogenetic context, we included samples of other species of *Tortula* sensu Zander (1993), together with selected taxa of *Crossidium* Jur., *Pterygoneurum* Jur. and *Stegonia latifolia* (Schwägr.) Venturi ex Broth. The nomenclature follows Zander (1993) and Cano (2006).

Flow cytometry. — Ploidy levels of plants tentatively assigned to the *T. muralis* complex were determined using flow cytometry (FCM). Usually 1 to 3 moss shoots were chopped together with the internal standard (*Glycine max* (L.) Merr. 'Polanka', 2C = 2.50 pg) in LB01 buffer (Doležel & al., 1989) containing 4,6-diamidino-2-phenylindol (DAPI). Analyses were performed on a Partec PA II flow cytometer (Partec, Münster, Germany), and data were processed using Partec FloMax v.2.4d software. For details on the FCM protocols, see Košnar & Kolář (2009).

Molecular protocols. — Total genomic DNA was extracted from one moss shoot or occasionally from 2 to 10 shoots (see Appendix) using the NaOH method (Werner & al., 2002b) or the Invisorb Spin Plant Mini Kit (Invitek, Berlin, Germany). In addition to ITS, 17 samples including all morphologically defined taxa of the *T. muralis* complex were selected for preliminary analysis of the *rps4* chloroplast region. The PCRs for ITS were performed according to the protocol by Köckinger & Kučera (2011), and the protocol by Werner & Guerra (2004) for *rps4*. Direct sequencing was performed as described in Köckinger & Kučera (2011).

When data obtained from direct ITS sequencing indicated a mixed template, and more than two polymorphic positions within one sequence were detected, molecular cloning was performed. For approximately half of the cloned samples, both DNA extraction and PCR reactions were repeated on a different day to ensure reproducibility (see below). Repeated PCR reactions were performed as above, except that only 30 cycles and a 2-minute cycle extension step were used in order to reduce formation of chimeric sequences. PCR products were cloned using the pGEM-T Vector System I (Promega, Madison, Wisconsin, U.S.A.). Clone sampling and sequencing were usually performed until all variation detectable on direct sequences was recovered. No differences were found between sequences and clones obtained from repeated DNA extractions and PCR reactions of the same sample, indicating the absence of artificial ITS variation originating from sample cross-contaminations or other sources.

Data analysis. — Sequences were edited using BioEdit v.7.0.9.0 (Hall, 1999) and preliminarily aligned using Clustal W v.1.4 with default options (Thompson & al., 1994). The raw alignments were trimmed according to the shortest sequence in the dataset. This led to exclusion of the first 9 bp of ITS1 and the last 7 bp of ITS2, which could not be aligned with certainty. The first 22 bp of the *rps4* amplicon were excluded because of the shorter length of some of the sequences. The ITS dataset was subsequently aligned by MAFFT v.6 (Katoh & al., 2002; available online at http://mafft.cbrc.jp/alignment/server/) using the Q-INS-i algorithm with the 200PAM / κ = 2 scoring matrix. The gap opening penalty was set to 1, and the offset value was set to 0.0. For accessions in which up to two polymorphic sites within one direct sequence were detectable in both forward and reverse directions, reconstructed sequences with all possible combinations of polymorphic sites were used. For accessions obtained by cloning, autapomorphic changes unique to a single accession at a non-variable position of the alignment were considered *Taq* errors (Hengen, 1995) and were overwritten

according to the direct sequence. The *rps4* dataset was aligned manually, and sequences were assigned to haplotypes following Werner & Guerra (2004).

Using ITS data, phylogenetic relationships were assessed using maximum parsimony (MP) as implemented in TNT v.1.1 (Goloboff & al., 2008) and Bayesian inference as implemented in MrBayes v.3.1.2. (Huelsenbeck & al., 2001). All characters were given equal weight, and gaps were coded as missing data. The MP analysis was run using the heuristic New Technology search with the following settings: Sectional Search = ON (including active RSS, CSS, and XSS), Ratchet = ON, Drift = ON, Tree Fusing = ON, Maxtrees = 10,000, random additions with 10,000 replicates. A bootstrap analysis (Felsenstein, 1985) was performed with 1000 replicates using the heuristic search strategy as described, except for random addition with 20 replicates. For Bayesian inference, the best-fit model of sequence evolution was selected using the Bayesian information criterion (Schwarz, 1978) calculated in jModelTest v.0.1.1 (Posada, 2008). The general time-reversible model (Rodríguez & al., 1990) with a discrete gamma distribution was selected. Two runs with 10,000,000 generations starting with a random tree and employing 12 simultaneous chains each (one hot, eleven cold) were executed. The temperature of a hot chain was set empirically to 0.01, and every 100th tree was saved. The analysis was considered to be completed when the average standard deviation of split frequencies dropped below 0.01. The first 25,000 trees (25%) were discarded as the burn-in phase, and the remaining 75,000 trees were used for construction of a 50% majority consensus tree. Based on recent phylogenetic studies (Werner & al., 2002a, 2004) and our preliminary analysis of ITS data of related taxa, *Chenia leptophylla* was used as outgroup. To test the phylogenetic signal in intragenomic ITS variation, alternative topological hypotheses were evaluated. For Bayesian inference, monophyly of markedly polyphyletic intraindividual ITS sequences (see Appendix) was tested by calculating the posterior probability (PP) of the set of trees containing such monophyly (Huelsenbeck & Imennov, 2002).

TCS v.1.18 (Clement & al., 2000) was used to produce a parsimony network of *rps4* haplotypes with a 95% confidence limit. Based on results by Werner & Guerra (2004), suggesting that *rps4* sequences of *T. muralis* and *T. vahliana* are closely related, the *rps4* dataset included taxa of the *T. muralis* complex together with *T. vahliana*. Gaps were treated as missing data, but potentially informative indels were scored (present/absent) and the data were added to the matrix.

■ RESULTS

All products of the ITS amplification were full length, spanning the ITS1 region, the 5.8S rDNA gene, and the ITS2 region. The aligned sequences had a length of 1036 bp, of which 382 characters were variable and 300 parsimony-informative. The lowest variation was observed in the 5.8S gene, which had only two variable positions. The strict consensus tree obtained from MP was generally more resolved than the 50% consensus Bayesian tree (Figs. S1–S2 in the Electronic Supplement; and Figs. 1–2, respectively). Both trees showed similar general topologies and differed only in poorly supported internal branches, which were better resolved by MP. For simplicity, only the Bayesian tree is presented here (Figs. 1–2), and only those groups resolved by both methods are discussed.

The aligned *rps4* data matrix contained 655 characters, of which 37 were variable and 17 parsimony-informative.

Occurrence of intragenomic ITS variation. — Intragenomic variation was detected in approximately 46% of the samples belonging to the *T. muralis* complex and in 50% of the samples of the taxa related to the complex. For the *T. muralis* complex, the intraindividual ITS sequences of 22 samples (16%) were markedly polyphyletic and caused eight reticulations among the most distinct lineages (Fig. 2; see below). As evaluated using posterior probability, hypotheses assuming monophyly of such markedly polyphyletic sequences were found to be significantly worse than the topology observed in the 50% consensus Bayesian tree. The highest PP of monophyly of intraindividual ITS sequences was found in sample M37 (PP = 0.026), and in other samples the PP was lower than 0.000 (for list of analysed samples, see Appendix).

Delimitation of the *T. muralis* complex based on ITS data. — Taxa of the *T. muralis* complex together with *T. israelis* and *T. edentula* form a poorly supported (PP = 0.92, BS = 51%) monophyletic group, here called the "*T. muralis* clade" (Figs. 1–2). This clade notably does not include *T. vahliana* and *T. brevissima*, and is sister to a clade comprising the remaining taxa of *Tortula* and related genera (PP = 0.81, BS < 50%) with the exception of *T. marginata*. The genera *Tortula, Crossidium,* and *Pterygoneurum* are apparently polyphyletic. The most distinct lineage in the ITS tree is a long and well-supported "*Pottia* clade" (PP = 1.00, BS = 69%), comprising *Crossidium squamiferum, Stegonia latifolia, Pterygoneurum* taxa, and several terricolous *Tortula* taxa, belonging to section *Pottia* (Rchb.) Kindb., together with *Hilpertia velenovskyi, T. brevissima,* and *T. mucronifolia*. Interestingly, ITS sequences of *T. brevissima* appeared to be polyphyletic. Although three of the four cloned sequences obtained from two Spanish samples of *T. brevissima* cluster together in a well-supported clade, the remaining sequence is sister to a clade consisting of *T. acaulon, T. mucronifolia, Crossidium squamiferum, Stegonia latifolia,* and *Pterygoneurum* taxa.

Relationships within the *T. muralis* complex based on ITS data. — The pattern of relationships based on the analysis of ITS sequences (Fig. 2) does not agree with the previously suggested classification based on a morphometric analysis. An exception to this is *T. lingulata*, which forms a monophyletic clade (PP = 0.98, BS = 69%) consisting of two haplotypes that differ by a single nucleotide substitution. No intragenomic ITS variation was detected in *T. lingulata*.

The most distinct ITS clade, hereafter called the "*obtusifolia* 1 clade", is a well-supported branch (PP = 0.98, BS = 95%) that contains a high frequency of *T. muralis* subsp. *obtusifolia* morphotypes (Fig. 2). Sequences from 70% of the populations identified morphologically as subsp. *obtusifolia* belong here, together with sequences from 23% of populations of morphs intermediate between *T. muralis* subsp. *obtusifolia* and *T. muralis*

subsp. *muralis* var. *aestiva*. Nevertheless, the *obtusifolia* 1 clade also contains sequences from 30% of the populations of *T. muralis* subsp. *muralis* morphs (both varieties and irrespective of ploidy level). The single sequence of *T. edentula*, which morphologically resembles *T. muralis* subsp. *obtusifolia,* is also nested in the *obtusifolia* 1 clade. ITS sequences of *T. muralis* subsp. *muralis* and *T. muralis* subsp. *obtusifolia* commonly were part of markedly polyphyletic assemblages of intragenomic ITS variation from individual amplifications. Thus, 36% of *T. muralis* subsp. *muralis* and one sample of *T. muralis* subsp. *obtusifolia* nested in the *obtusifolia* 1 clade are parts of intraindividual ITS variation appearing on distant branches of the *T. muralis* clade. Those polyphyletic sequences were strongly divergent, sharing a rather low number of identical nucleotides with *obtusifolia* 1 sequences (86.2%–92.2%).

Tortula muralis subsp. *obtusifolia* is clearly polyphyletic because accessions not contained in the *obtusifolia* 1 clade appear in other lineages (Fig. 2). Although most accessions from the "*obtusifolia* 2 clade" contain the sequences from morphs of subsp. *obtusifolia*, the frequency of plants with the clear morphology of subsp. *obtusifolia* in this clade (sequences from 30% of its populations) was lower than in the *obtusifolia* 1 clade (sequences from 70% of its populations; Fig. 2), while the frequency of plants intermediate between subsp. *obtusifolia*

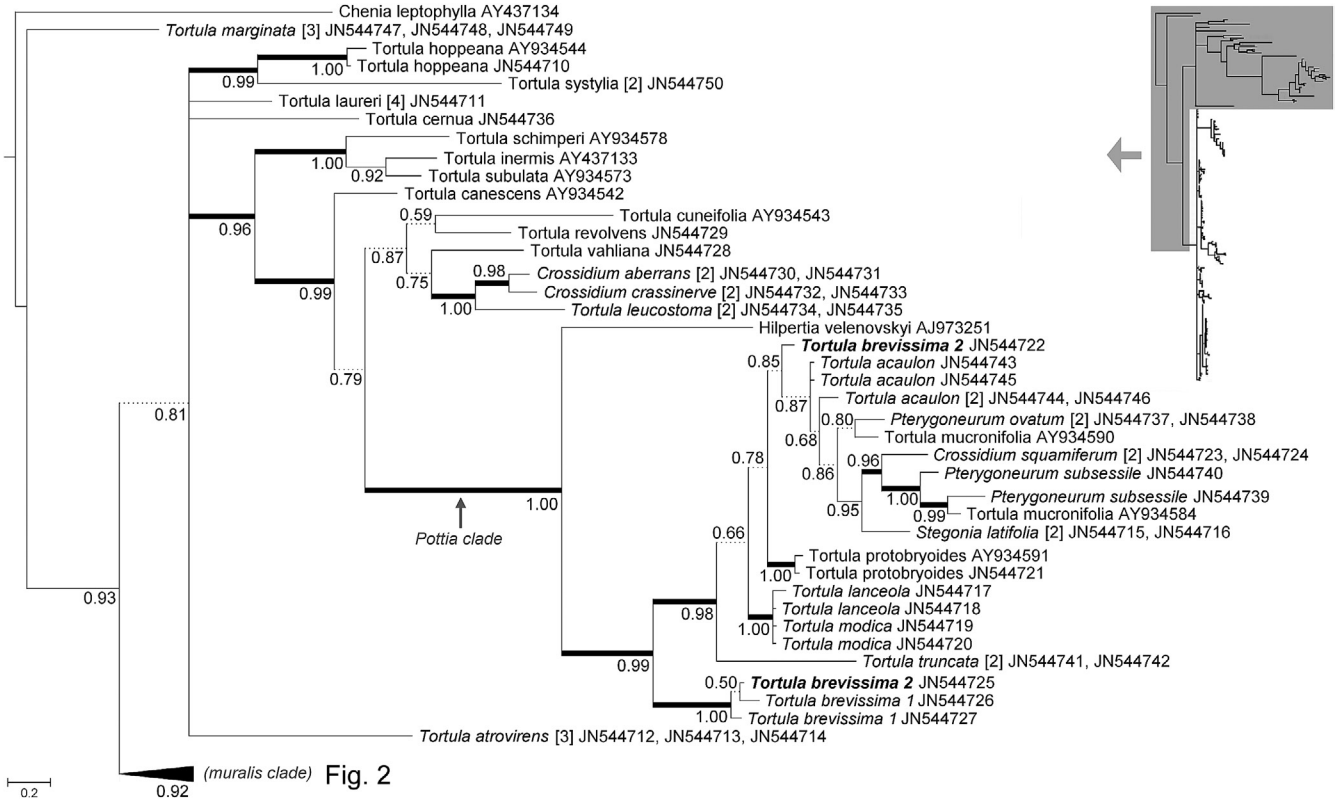

Fig. 1. Phylogenetic tree of the *Tortula muralis* complex and related taxa based on ITS sequence data. The tree was constructed using Bayesian inference and was rooted with *Chenia leptophylla*. Numbers on branches indicate posterior probabilities. Dotted lines indicate branches with posterior probabilities <0.90, and bold lines indicate branches with posterior probabilities >0.95. Sequences obtained by molecular cloning are in italics. Samples containing polyphyletic intragenomic sequences belonging to different major clades are in bold. Monophyletic clades containing sequences that originated from a single specimen with intragenomic ITS variation were compressed and considered a single sequence; numbers in square brackets indicate the number of such monophyletic sequences. Numbers after taxa correspond to GenBank accession numbers. For detailed voucher information, see Appendix.

Fig. 2. Subtree showing the *Tortula muralis* clade of the ITS tree. The tree was constructed using Bayesian inference. Numbers on branches of major lineages indicate posterior probabilities. Dotted lines indicate branches with posterior probabilities <0.90, and bold lines indicate branches with posterior probabilities >0.95. Graphs indicate the percentage of populations of a given morphotype containing the ITS sequence of each particular group (only percentages >10% are shown). Sequences obtained by molecular cloning are in italics. Samples containing polyphyletic intragenomic sequences belonging to different major clades are in bold. Lines in the right part of the figure indicate reticulations among main groups caused by samples containing markedly polyphyletic intragenomic sequences of different clades of the tree (numbers refer to number of such samples). Monophyletic clades containing sequences that originated from a single specimen with intragenomic ITS variation were compressed and considered a single sequence; numbers in square brackets indicate the number of such monophyletic sequences. Known *rps4* haplotypes are underlined and in parentheses. "x" indicates haploid cytotypes, and "2x" diploid cytotypes (for detailed voucher information, see Appendix). ▶

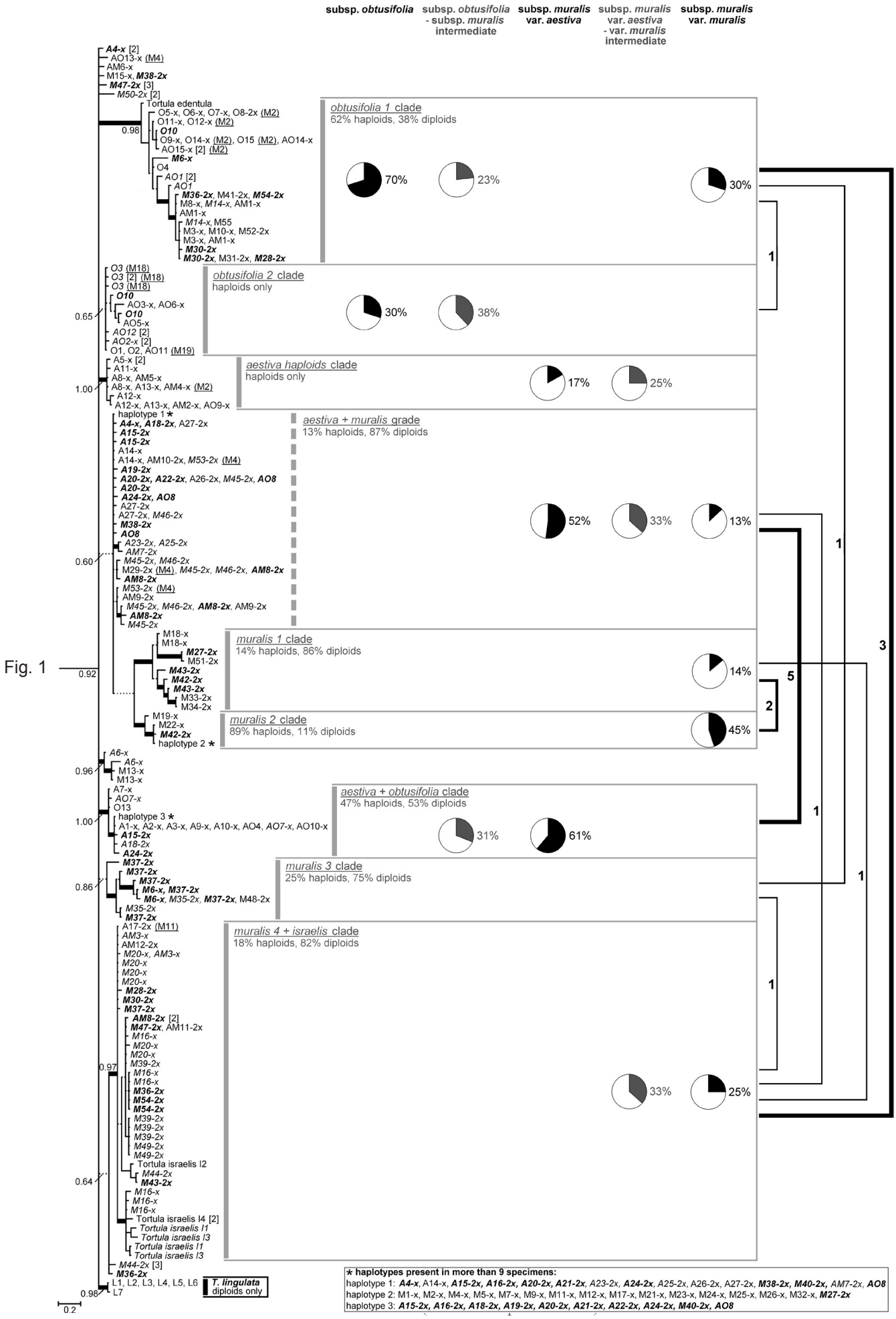
subsp. obtusifolia
subsp. obtusifolia - subsp. muralis intermediate
subsp. muralis var. aestiva
subsp. muralis var. aestiva - var. muralis intermediate
subsp. muralis var. muralis
obtusifolia 1 clade
62% haploids, 38% diploids
70%
23%
30%
obtusifolia 2 clade
haploids only
30%
38%
aestiva haploids clade
haploids only
17%
25%
aestiva + muralis grade
13% haploids, 87% diploids
52%
33%
13%
muralis 1 clade
14% haploids, 86% diploids
14%
muralis 2 clade
89% haploids, 11% diploids
45%
aestiva + obtusifolia clade
47% haploids, 53% diploids
31%
61%
muralis 3 clade
25% haploids, 75% diploids
muralis 4 + israelis clade
18% haploids, 82% diploids
33%
25%
A4-x [2]
AO13-x (M4)
AM6-x
M15-x, M38-2x
M47-2x [3]
M50-2x [2]
Tortula edentula
O5-x, O6-x, O7-x, O8-2x (M2)
O11-x, O12-x (M2)
O10
O9-x, O14-x (M2), O15 (M2), AO14-x
AO15-x [2] (M2)
M6-x
O4
AO1 [2]
AO1
M36-2x, M41-2x, M54-2x
M8-x, M14-x, AM1-x
AM1-x
M14-x, M55
M3-x, M10-x, M52-2x
M3-x, AM1-x
M30-2x
M30-2x, M31-2x, M28-2x
O3 (M18)
O3 [2] (M18)
O3 (M18)
O10
AO3-x, AO6-x
O10
AO5-x
AO12 [2]
AO2-x [2]
O1, O2, AO11 (M19)
A5-x [2]
A11-x
A8-x, AM5-x
A8-x, A13-x, AM4-x (M2)
A12-x
A12-x, A13-x, AM2-x, AO9-x
haplotype 1 *
A4-x, A18-2x, A27-2x
A15-2x
A15-2x
A14-x
A14-x, AM10-2x, M53-2x (M4)
A19-2x
A20-2x, A22-2x, A26-2x, M45-2x, AO8
A20-2x
A24-2x, AO8
A27-2x
A27-2x, M46-2x
M38-2x
AO8
A23-2x, A25-2x
AM7-2x
M45-2x, M46-2x
M29-2x (M4), M45-2x, M46-2x, AM8-2x
AM8-2x
M53-2x (M4)
AM9-2x
M45-2x, M46-2x, AM8-2x, AM9-2x
AM8-2x
M45-2x
M18-x
M18-x
M27-2x
M51-2x
M43-2x
M42-2x
M43-2x
M33-2x
M34-2x
M19-x
M22-x
M42-2x
haplotype 2 *
A6-x
A6-x
M13-x
M13-x
A7-x
AO7-x
O13
haplotype 3 *
A1-x, A2-x, A3-x, A9-x, A10-x, AO4, AO7-x, AO10-x
A15-2x
A18-2x
A24-2x
M37-2x
M37-2x
M37-2x
M6-x, M37-2x
M6-x, M35-2x, M37-2x, M48-2x
M35-2x
M37-2x
A17-2x (M11)
AM3-x
AM12-2x
M20-x, AM3-x
M20-x
M20-x
M20-x
M28-2x
M30-2x
M37-2x
AM8-2x [2]
M47-2x, AM11-2x
M16-x
M20-x
M20-x
M39-2x
M16-x
M16-x
M36-2x
M54-2x
M54-2x
M39-2x
M39-2x
M39-2x
M49-2x
M49-2x
Tortula israelis I2
M44-2x
M43-2x
M16-x
M16-x
M16-x
Tortula israelis I4 [2]
Tortula israelis I1
Tortula israelis I3
Tortula israelis I1
Tortula israelis I3
M44-2x [3]
M36-2x
L1, L2, L3, L4, L5, L6
L7
T. lingulata diploids only
0.92
0.60
0.65
1.00
0.96
1.00
0.86
0.97
0.64
0.98
0.2
1
1
5
2
3
1
1
1
* haplotypes present in more than 9 specimens:
haplotype 1: A4-x, A14-x, A15-2x, A16-2x, A20-2x, A21-2x, A23-2x, A24-2x, A25-2x, A26-2x, A27-2x, M38-2x, M40-2x, AM7-2x, AO8
haplotype 2: M1-x, M2-x, M4-x, M5-x, M7-x, M9-x, M11-x, M12-x, M17-x, M21-x, M23-x, M24-x, M25-x, M26-x, M32-x, M27-2x
haplotype 3: A15-2x, A16-2x, A18-2x, A19-2x, A20-2x, A21-2x, A22-2x, A24-2x, M40-2x, AO8

Fig. 1

and subsp. *muralis* (sequences from approximately 38% of its populations) was somewhat higher than in the *obtusifolia* 1 clade (sequences from 23% of its populations, Fig. 2). In a single collection from France, plants of both the *obtusifolia* 1 and *obtusifolia* 2 clades were detected. This collection was morphologically heterogeneous, containing plants with the morphology of subsp. *obtusifolia* (O4; *obtusifolia* 1 clade) together with plants intermediate between subsp. *obtusifolia* and subsp. *muralis* (AO12; *obtusifolia* 2 clade).

Although some clades contained plants with the morphology of var. *muralis* ("*muralis* 1 clade", "*muralis* 2 clade", "*muralis* 3 clade"), both varieties of *T. muralis* subsp. *muralis* are apparently polyphyletic. Moreover, several ITS sequences were shared by plants which morphologically belonged to one or the other variety.

A biphyletic nature was observed for *T. israelis*, which is nested within one of the moderately supported *T. muralis* subsp. *muralis* clades that contained mostly var. *muralis* morphotypes ("*muralis* 4+*israelis* clade", PP = 0.97, BS = 52%).

Only one major clade (considering those with sequences from more than two samples) was completely free of reticulations caused by intragenomic ITS variation. This clade, here called the "*aestiva* haploids clade" (PP = 1.00, BS = 84%), consists predominantly of var. *aestiva* samples. Interestingly, plants of this clade tend to occur in natural habitats (base-rich rocks).

No geographical pattern was detected in the phylogenetic relationships based on ITS sequences of the *Tortula muralis* complex. The only exception to this was the clade that contained predominantly eastern European samples of *T. lingulata*.

Distribution of ploidy levels on the ITS tree of the *T. muralis* complex. — No phylogenetic pattern was detected in the distribution of haploids and diploids on the phylogenetic tree constructed with ITS data (Fig. 2). Both cytotypes were detected in six of the nine major subclades of the *T. muralis* clade. Moreover, nine haplotypes were shared by haploid and diploid individuals, including four diploid samples without intragenomic variation of ITS.

Intragenomic variation in ITS was more frequent in diploids (71% of the analysed samples) than in haploids (30%). The same was also true for markedly polyphyletic intragenomic ITS sequences (i.e., sequences of the major well-supported lineages).

No intermediate (triploid) ploidy level was detected in the *T. muralis* clade.

Variation in the chloroplast *rps4* region. — Among the 17 samples sequenced, six *rps4* haplotypes were revealed. Interestingly, two of them (M18, M19) were not recorded in the earlier study by Werner & Guerra (2004), while the remaining four had been previously recorded among the 17 haplotypes detected among samples of the world-wide distribution area. The distribution of *rps4* haplotypes is not consistent with the ITS tree (Fig. 2; Fig. S3 in the Electronic Supplement). The most common haplotype M2 was found in 10 samples that included both cytotypes and morphotypes of *T. muralis* subsp. *obtusifolia* and *T. muralis* subsp. *muralis* var. *aestiva*, morphotypes intermediate between *T. muralis* subsp. *obtusifolia* and *T. muralis* subsp. *muralis* var. *aestiva*, morphotypes intermediate between both varieties of *T. muralis* subsp. *muralis*, and *T. lingulata*. Similarly, haplotype M4 (differing by a single mutation from M2) was found in three samples from two independent ITS lineages, including both cytotypes and plants of different morphotypes. Haplotypes M1 and M11 were each found in a single sample.

■ DISCUSSION

Origin of intragenomic ITS variation in *Tortula* and related taxa. — When investigating intragenomic ITS variation, it is necessary to use a single individual for molecular analysis. Even in small bryophytes, one shoot is usually sufficient for DNA extraction. In our study we used a single moss shoot for most DNA extractions, and it is therefore unlikely that variation in sequences was caused by sampling of several individuals with different genotypes. This is especially evident for those samples in which markedly polyphyletic intraindividual ITS sequences were detected; in all these cases, only one shoot was used for DNA extraction (see Appendix for details).

Sampling of pseudogenes is also improbable in our study, because all the obtained sequences have signs of functional nrDNA, including a conserved 5.8S gene (Harpke & Peterson, 2008). In approximately 50% of our samples, the non-identical ITS sequences from a single sample proved to be more or less closely related and often were resolved within a monophyletic clade. This pattern indicates a rather recent differentiation, which resulted from only few mutations within nrDNA arrays. In other cases, however, we observed relatively large differences among intragenomic ITS sequences, which are difficult to explain by stepwise molecular processes or ancestral polymorphism and rather might result from hybridization. According to Nieto Feliner & al. (2004), the existence of concerted evolution affecting multicopy regions reduces the possibility of incomplete lineage sorting of ancestral polymorphisms. The presence of concerted evolution in our case can be inferred from the existence of plants lacking intragenomic ITS variation. The probable existence of gene flow among ITS lineages is in accordance with the usually sexual reproduction within the *T. muralis* complex. In addition, the poorly resolved topologies with low support that were detected in our dataset might also be caused by occasional ITS recombination following hybridization, because recombinant signal in some cases may result in more trees with a larger number of polytomies (Funk, 1985; McDade, 1992).

Remarks on the phylogeny of *Tortula* and related taxa inferred from ITS data. — The phylogeny inferred from the ITS sequences was partly different from that based on *rps4* (Werner & al., 2002a). Both phylogenies contain a well-supported *Pottia* clade, which comprises *Tortula* sect. *Pottia* sensu Zander (1993), i.e., a clade that includes *Protobryum* sensu Guerra & Cano (2000) together with *Stegonia latifolia*. According to the ITS data, this clade moreover contains *Hilpertia, Tortula mucronifolia, Crossidium squamiferum* (type of *Crossidium*), *Pterygoneurum ovatum* (type of *Pterygoneurum*), and *P. subsessile*, which were not analysed by Werner & al. (2000a). However, several taxa had different relationships in

the two phylogenies. Discrepancies between ITS and *rps4* data notably include *Tortula brevissima* and *T. acaulon* (*Phascum cuspidatum* sensu Guerra & Cano, 2000, the type species of *Phascum*), which are nested within *Pottia* according to ITS but appear in a sister clade (*T. acaulon*) or even in different clades of Pottioideae (*T. brevissima*) according to *rps4*.

Evolution of the *T. muralis* complex and taxonomic implications. — ITS data demonstrated that the morphologically defined *T. muralis* complex, as delimited by Košnar & Kolář (2009), is indeed monophyletic. The complex further includes *T. israelis* and *T. edentula* but not *T. vahliana*, as postulated by Werner & Guerra (2004). Taxa of the complex share the usually epilithic growth, small (9–12 µm) and densely papillose leaf cells, markedly revolute leaf margins, isodiametric marginal leaf cells, absence of photosynthetic outgrowths on the ventral side of the costa, and rather small spores (8.5–12.0 µm, but 11–15 µm in *T. lingulata*). These characters allow to distinguish superficially similar but phylogenetically distant taxa, such as *T. brevissima, T. vahliana*, or *T. marginata*. Although the monophyly of the *T. muralis* complex received poor statistical support in the ITS analysis, it is supported by the pattern of intragenomic ITS variation. Even though the intraindividual sequences detected in taxa within the *T. muralis* clade were commonly recorded on distant branches within this clade, they never occurred in other clades of *Tortula*.

As discussed above, phylogenetic analysis of ITS data resulted in a complex pattern suggesting the existence of gene flow among lineages of the *T. muralis* complex, together with some level of ancestral polymorphism. Thus, with the exception of *T. lingulata*, the taxonomic status of the taxa analysed remains critical. The variability of chloroplast *rps4* sequences was too low for reconstructing the species-level phylogeny of the *T. muralis* complex. Our sampling, however, did not include non-European plants (except for *T. edentula*, which was nested within *T. muralis* subsp. *obtusifolia* in the ITS tree). In consequence, we refrain from drawing conclusions about possible cryptic speciation within *T. muralis*, as hypothesized by Werner & Guerra (2004). On the other hand, the virtual absence of reproductive isolation among lineages can be considered important evidence contradicting the cryptic speciation hypothesis in the *T. muralis* complex, at least within the geographical scope of our analysis.

Evolutionary relationships between haploids and diploids in the *T. muralis* complex. — In most cases, both haploids and diploids were found in individual subclades (Fig. 2), which suggests a polytopic and recurrent origin of diploids. Recurrent polyploidization enhances unidirectional inter-ploidy gene flow, which might be followed by homoploid hybridization among the distinct polyploid (in our case gametophytic diploid) lineages, further increasing their variability (Soltis & Soltis, 1999). Such processes might have further obscured the relationships within the *T. muralis* complex.

In some clades, one cytotype prevails. *Tortula lingulata,* as discussed above, seems to be strictly diploid. Interestingly, one German population, previously considered to be probably *T. lingulata* by Meinunger & Schröder (2007), contains both haploids and diploids. These plants were collected far from the distribution centre of *T. lingulata*, which lies in the eastern Baltic region. Their morphology is intermediate between *T. muralis* subsp. *obtusifolia* and *T. lingulata*, but the spores are heterogeneous in size. Spore size was found to be the most important character for distinguishing between the two taxa (Košnar & Kolář, 2009). The spore size of haploid plants was within the range of *T. muralis* subsp. *obtusifolia,* whereas the diploid plants had the larger spores typical of *T. lingulata*. The ITS haplotype of both cytotypes was identical. Therefore, the likely explanation is that the German population consists of haploid plants of *T. muralis* subsp. *obtusifolia* that in situ gave rise to autodiploid progeny. The same explanation might apply to *T. edentula*, which is reported to differ from *T. muralis* subsp. *obtusifolia* by having larger spores (typical for diploids) and by lacking a peristome. Unfortunately, the *T. edentula* material was too old to provide FCM data, but the variation of all important morphological characters, including the absence of a peristome, is identical to that of the above-described German '*T. lingulata*'. An autodiploid origin is thus a plausible hypothesis to explain the larger spores. Moreover, the phylogenetic analysis places *T. edentula* within the *obtusifolia* 1 clade, and we therefore consider *T. edentula* to be identical with *T. muralis* subsp. *obtusifolia* (see Taxonomic Changes below).

The overall frequency of markedly divergent intragenomic ITS sequences was considerably higher in diploids (38% of the samples) than in haploids (3%). Diploids with intragenomic ITS variation are most likely hybrids of different lineages of the ITS tree; although divergent, all are nested within the *T. muralis* clade. On the other hand, approximately 29% of the diploids lacked intragenomic ITS variation, and four of them shared ITS sequences with haploids. This is consistent with the autopolyploid origin of diploids from closely related haploids. Autopolyploidy is clearly evident at least in two cases of mixed populations of both cytotypes sharing the same ITS sequence: the above discussed German population of *T. muralis* subsp. *obtusifolia*, and a Czech population of *T. muralis* var. *muralis,* i.e., samples M9 and M32, respectively. Even when the intragenomic ITS sequences isolated from diploid individuals were not identical, they had not diverged much, which also indicates an autopolyploid origin. Autopolyploidy is further supported by the almost identical morphology of both cytotypes (Košnar & Kolář, 2009) and the frequent existence of populations with mixed ploidy (J. Košnar & al., unpub. data). Based on these facts, we consider the *T. muralis* complex to be the first case of autopolyploidy in mosses that is supported by molecular marker data. The demonstration of autopolyploidy in mosses contrasts with the allopolyploid (i.e., hybrid polyploid) origin proposed for almost all other bryophyte groups that have been studied by molecular markers (Såstad, 2005; Shaw, 2009).

■ TAXONOMIC CHANGES

Tortula muralis subsp. *obtusifolia* (Schwägr.) Culm. in Rev. Bryol. 48: 22. 1921 = *Tortula edentula* Ignatova & Ignatov in Arctoa 18: 135. 2010 ('2009').

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Appendix. List of herbarium specimens used for sequencing and FCM analysis. Samples with ITS paralogs appearing markedly polyphyletic in the ITS phylogeny are in bold. +, more than one moss shoot used for DNA extraction; *, sample tested for monophyly of intraindividual ITS sequences by calculating the posterior probability of monophyly using Bayesian inference; *x*, haploid gametophyte; 2*x*, diploid gametophyte; 3*x*, triploid gametophyte. GenBank accession numbers of ITS are in normal font, *rps4* sequences are in italics, with haplotype designations in brackets; for accession numbers of previously published sequences, see Fig. 1 and Fig. S1. Specimens collected by Košnar and Kučera are deposited in CBFS.

Sample	Ploidy	GenBank accession	Locality	Substrate	Voucher
Tortula edentula					
E1	–	JN544826	Russia: Shikotan Island	Cliffs on sea coast	*Bakalin K-49-2-07* (MHA)
Tortula israelis					
I1	–	JN544880, JN544882	Greece: Athens	Nitrophilous vegetation	*Cano & al. 12104* (MUB)
I2	–	JN544879	Spain: Pontevedra		*Gallego 11866* (MUB)
I3	–	JN544881, JN544883	Spain: Cádiz	Wall	*Cano 1386* (MUB)
I4	–	JN544897	Spain: Murcia		*Rams 10421* (MUB)
Tortula lingulata					
L1+	–	JN544837	Czech Rep.: Peruc	Sandstone boulder	*Košnar 577*
L2+	–	JN544837	Latvia: Krimulda	Sandstone rock	*Košnar 772*
L3+	–	JN544837	Latvia: Sigulda	Sandstone rock	*Košnar 786*
L4+	–	JN544837	Latvia: Ieriķi	Sandstone rock	*Košnar 795*
L5+	2*x*	JN544837	Latvia: Kārļi	Sandstone rock	*Košnar 797*
L6+	–	JN544837	Estonia: Toila	Wall (sandstone)	*Ingerpuu* 24.6.2005 (TU)
L7+	–	JN544838	Russia: Sablino	Sandstone rock	*Abramov & Abramova s.n.* (TAM)
L8+	2*x*	*JN581668 (M2)*	Latvia: Cīrulīši	Sandstone rock	*Košnar 802*
Tortula muralis subsp. *muralis* var. *aestiva*					
A1+	*x*	JN544804, *JN581673 (M2)*	Czech Rep.: Dolní Adršpach	Wall (sandstone)	*Košnar 724*
A2	*x*	JN544804	Czech Rep.: České Žleby	Wall (granite)	*Košnar 1647*
A3	*x*	JN544804	Czech Rep.: Vilémovice	Limestone rock	*Košnar 1713*
A4	*x*	JN544771, JN544789, JN544790, JN544793	Czech Rep.: Trhanov	Bridge (concrete)	*Košnar 1888*
A5	*x*	JN544763	Czech Rep.: Velké Hydčice	Limestone rock	*Košnar 1904*
A6	*x*	JN544773, JN544774	Germany: Neusatz	Wall (granite)	*Košnar 1601*
A7	*x*	JN544808	Hungary: Dömös	Andesite rock	*Košnar 746*
A8	*x*	JN544766, JN544768	Hungary: Hont	Andesite rock	*Košnar 1825*
A9	*x*	JN544804	Hungary: Királyháza	Wall (andesite)	*Košnar 1838*
A10	*x*	JN544804	Latvia: Krimulda	Wall (limestone)	*Košnar 775*
A11	*x*	JN544764	Romania: Băile Olăneşti	Wall	*Košnar 1918*
A12	*x*	JN544765, JN544767	Romania: Cozia	Sandstone rock	*Košnar 1920*
A13	*x*	JN544767, JN544768	Romania: Cozia	Sandstone rock	*Košnar 1921*
A14	*x*	JN544771, JN544781, JN544782	Slovakia: Čabraď	Wall (andesite?)	*Košnar 635*
A15	2*x*	JN544769, JN544770, JN544771, JN544775, JN544776	Czech Rep.: Nebákov	Wall (Sandstone)	*Košnar 560*
A16*	2*x*	JN544771, JN544775	Czech Rep.: Kost	Wall (sandstone)	*Košnar 561*
A17	2*x*	JN544845, *JN581680 (M11)*	Czech Rep.: Kralupy n. Vltavou	Wall (sandstone)	*Košnar 817*
A18	2*x*	JN544775, JN544793, JN544805	Czech Rep.: Bohumilice	Wall (concrete)	*Košnar 1294*
A19	2*x*	JN544775, JN544890	Czech Rep.: Bílek	Wall (mortar)	*Košnar 1508*
A20	2*x*	JN544771, JN544775, JN544785, JN544815	Czech Rep.: Rabštejn n. Střelou	Phyllitic schist rock	*Košnar 1572*
A21	2*x*	JN544771, JN544775	Czech Rep.: Josefov	Wall (mortar)	*Košnar 1723*
A22	2*x*	JN544775, JN544785	Hungary: Mt. Csóványos	Andesite boulder	*Košnar 1842*
A23+	2*x*	JN544771, JN544777	Hungary: Mt. Csóványos	Andesite rock	*Košnar 1847*
A24	2*x*	JN544771, JN544775, JN544786, JN544787	Latvia: Krimulda	Wall (limestone)	*Košnar 778*
A25+	2*x*	JN544771, JN544777, *JN581667 (M2)*	Slovakia: Čabraď	Wall (andesite?)	*Košnar 648*
A26	2*x*	JN544771, JN544785	Slovakia: Kečovo	Wall (concrete)	*Košnar 1007*
A27	2*x*	JN544771, JN544778, JN544793, JN544814	Slovakia: Buková	Wall (limestone)	*Košnar 1017*
Tortula muralis subsp. *muralis* var. *muralis*					
M1	*x*	JN544812	Bosnia and Hercegovina: Vlasenica	Limestone rock	*Košnar 1360*
M2	*x*	JN544813	Bosnia and Hercegovina: Police	Limestone rock	*Košnar 1363*
M3	*x*	JN544829, JN544831	Czech Rep.: Templštejn	Wall (concrete)	*Košnar 418*

Appendix. Continued.

Sample	Ploidy	GenBank accession	Locality	Substrate	Voucher
M4+	*x*	JN544813	Czech Rep.: Žďárky	Concrete	*Košnar 741*
M5	*x*	JN544813	Czech Rep.: Zlatý kůň	Limestone rock	*Košnar 1263*
M6*	*x*	JN544791, JN544792, JN544828	Czech Rep.: Srbsko	Limestone rock	*Košnar 1280*
M7	*x*	JN544813	Czech Rep.: Sudslavice	Limestone rock	*Košnar 1301*
M8	*x*	JN544830	Czech Rep.: České Žleby	Wall (granite)	*Košnar 1648*
M9	*x*	JN544813	Czech Rep.: Bechyně	Granite rock	*Košnar 1897*
M10	*x*	JN544829	Czech Rep.: Nerestce	Limestone rock	*Košnar 1899*
M11	*x*	JN544813	Czech Rep.: Nerestce	Limestone rock	*Košnar 1900*
M12	*x*	JN544812	Switzerland: Meiringen	Bridge (concrete)	*Košnar 990*
M13	*x*	JN544772	Germany: Neusatz	Wall (granite)	*Košnar 1599*
M14	*x*	JN544827, JN544830	Hungary: Drégelyvár	Wall (andesite)	*Košnar 1831*
M15	*x*	JN544817	Italy: Anguillara Sabazia		*Košnar 1907*
M16	*x*	JN544847, JN544848, JN544854, JN544855, JN544856, JN544857	Montenegro: Mratinje	Wall (concrete)	*Košnar 1365*
M17	*x*	JN544812	Montenegro: Plav	Wall (concrete)	*Košnar 1392*
M18	*x*	JN544839	Montenegro: Djurkovići	Wall (mortar)	*Košnar 1405*
M19	*x*	JN544816	Montenegro: Žabljak	Wall (concrete)	*Košnar 1409*
M20	*x*	JN544862, JN544870, JN544871, JN544872, JN544873, JN544874	Norway: Runde	Concrete	*Košnar 1906*
M21	*x*	JN544813	Romania: Măcin	Granite rock	*Košnar 1188*
M22	*x*	JN544811	Romania: Răstoliţa	Bridge (concrete)	*Košnar 1348*
M23	*x*	JN544813	Slovakia: Čenkov	Wall (concrete)	*Košnar 993*
M24	*x*	JN544813	Slovakia: Turňa n. Bodvou	Wall (limestone)	*Košnar 1010*
M25	*x*	JN544813, *JN581666 (M1)*	Slovakia: Buková	Limestone rock	*Košnar 1016*
M26	*x*	JN544813	Switzerland: Luzern	Wall (mortar)	*Košnar 991*
M27	2*x*	JN544813, JN544843	Armenia: Tatev	Wall	*Košnar 1646*
M28	2*x*	JN544836, JN544846	Czech Rep.: Senorady	Wall (concrete)	*Košnar 416*
M29	2*x*	JN544795, *JN581679 (M4)*	Czech Rep.: Tachov	Wall (concrete)	*Košnar 771*
M30*	2*x*	JN544834, JN544835, JN544836	Czech Rep.: Peruc	Sandstone rock	*Košnar 874*
M31	2*x*	JN544836	Czech Rep.: Český Krumlov	Wall (mortar)	*Košnar 885*
M32	2*x*	JN544812	Czech Rep.: Bechyně	Granite rock	*Košnar 1898*
M33	2*x*	JN544842	Czech Rep.: Nerestce	Limestone rock	*Košnar 1901*
M34	2*x*	JN544841	Czech Rep.: Nerestce	Limestone rock	*Košnar 1902*
M35	2*x*	JN544792, JN544889	France: Montpellier	Wall	*Košnar 1033*
M36	2*x*	JN544833, JN544875, JN544876	Hungary: Drégelyvár	Wall (andesite)	*Košnar 1832*
M37*	2*x*	JN544791, JN544792, JN544892, JN544893, JN544894, JN544895, JN544896	Hungary: Poroszló		*Košnar 1912*
M38	2*x*	JN544771, JN544794, JN544817	Italy: Monte Chianti		*Košnar 1908*
M39	2*x*	JN544865, JN544866, JN544867, JN544868	Italy: Sicily, Police		*Košnar 1909*
M40	2*x*	JN544771, JN544775	Latvia: Krimulda	Wall (limestone)	*Košnar 777*
M41	2*x*	JN544833	Montenegro: Mratinje	Wall (concrete)	*Košnar 1367*
M42*	2*x*	JN544810, JN544840	Montenegro: Djurkovići	Wall (limestone)	*Košnar 1404*
M43*	2*x*	JN544878, JN544884, JN544885	Montenegro: Žabljak	Wall (concrete)	*Košnar 1408*
M44	2*x*	JN544858, JN544859, JN544860, JN544861	Montenegro: Riječani	Wall (concrete)	*Košnar 1417*
M45	2*x*	JN544779, JN544780, JN544785, JN544795, JN544891	Poland: Wisełka	Concrete	*Košnar 1905*
M46	2*x*	JN544778, JN544779, JN544780, JN544795	Romania: Răstoliţa	Bridge (concrete)	*Košnar 1347*
M47	2*x*	JN544869, JN544886, JN544887, JN544888	Romania: Capaţini Mts., Stogsoara	Limestone rock	*Košnar 1916*
M48	2*x*	JN544792	Spain: Madrid	Wall (concrete)	*Košnar 1255*
M49	2*x*	JN544863, JN544864	Spain: Bullas, Río Mula	Concrete	*Kučera 13671*
M50	2*x*	JN544761, JN544762	Slovakia: Čenkov	Brick	*Košnar 992*
M51+	2*x*	JN544844	Slovakia: Turňa n. Bodvou	Wall (limestone)	*Košnar 1009*

Appendix. Continued.

Sample	Ploidy	GenBank accession	Locality	Substrate	Voucher
M52	2*x*	JN544829	Slovakia: Stakčín	Wall (concrete)	*Košnar 1018*
M53	2*x*	JN544782, JN544783, *JN581678 (M4)*	Slovakia: Belina	Wall (concrete)	*Košnar 1021*
M54	2*x*	JN544833, JN544852, JN544853	Slovakia: Hajnáčka	Basalt rock	*Košnar 1023*
M55	–	JN544827	Slovakia: Devín	Limestone boulder	*Košnar 1042*
Tortula muralis subsp. *muralis*—plants intermediate between var. *aestiva* and var. *muralis*					
AM1	*x*	JN544830, JN544831, JN544832	Czech Rep.: Luže	Wall (brick)	*Košnar 466*
AM2	*x*	JN544767	Czech Rep.: Karlštejn	Limestone rock	*Košnar 1287*
AM3	*x*	JN544862, JN544877	Germany: Neusatz	Wall (granite)	*Košnar 1600*
AM4	*x*	JN544768, *JN581674 (M2)*	Hungary: Dömös	Andesite rock	*Košnar 747*
AM5	*x*	JN544766	Romania: Capaţini, Stogsoara	Limestone rock	*Košnar 1917*
AM6	*x*	JN544798	Slovakia: Stožok	Andesite rock	*Košnar 630*
AM7	2*x*	JN544771, JN544788	Czech Rep.: Hrubá Vrbka	Concrete	*Košnar 710*
AM8*	2*x*	JN544780, JN544795, JN544796, JN544797, JN544850, JN544851	Czech Rep.: Kralupy n. Vltavou	Sandstone rock	*Košnar 832*
AM9	2*x*	JN544780, JN544799	Germany: Ruhestein	Wall (sandstone)	*Košnar 1598*
AM10	2*x*	JN544782	Hungary: Dobogókö	Andesite rock	*Košnar 744*
AM11	2*x*	JN544869	Romania: Băile Olăneşti	Wall	*Košnar 1919*
AM12	2*x*	JN544849	Romania: Oradea	Wall (concrete)	*Košnar 1353*
Tortula muralis subsp. *obtusifolia*					
O1	*x*	JN544751	Austria: Zalußenalm	Base-rich schist rock	*Košnar 926*
O2	–	JN544751	France: Mt. Cenis		*De Zuttere 22169* (priv. herb.)
O3	–	JN544800, JN544801, JN544802, JN544803, *JN581681 (M18)*	France: Mt. Cenis		*Skrzypczak 03424* (priv. herb.)
O4	–	JN544821	France: Mt. Cenis, Grotte percée		*Skrzypczak 98395* (priv. herb.)
O5	*x*	JN544825	Germany: Schwarzwald	Sandstone rock	*Košnar 1586*
O6	*x*	JN544825	Germany: Schwarzwald	Sandstone rock	*Košnar 1588*
O7	*x*	JN544825	Germany: Schwarzwald	Sandstone rock	*Košnar 1589*
O8+	2*x*	JN544825, *JN581676 (M2)*	Germany: Schwarzwald	Sandstone rock	*Košnar 1587*
O9+	*x*	JN544824	Hungary: Mt. Csóványos	Andesite rock	*Košnar 1845*
O10*	–	JN544758, JN544759, JN544760	Iceland: Rangárvallasýsla	Rock	*Johansson s.n.* (S)
O11	*x*	JN544822	Romania: Călimani Mts.	Andesite rock	*Košnar 1324*
O12+	*x*	JN544822, *JN581675 (M2)*	Romania: Călimani Mts.	Andesite rock	*Košnar 1330*
O13	–	JN544807	Romania: Răstoliţa	Andesite rock	*Košnar 1349*
O14	*x*	JN544824, *JN581671 (M2)*	Slovakia: Stožok	Andesite rock	*Košnar 631*
O15	–	JN544824, *JN581669 (M2)*	Slovakia: Čabraď	Andesite rock	*Košnar 639*
Plants intermediate between *Tortula muralis* subsp. *muralis* var. *aestiva* and *Tortula muralis* subsp. *obtusifolia*					
AO1+	–	JN544818, JN544819, JN544820	Armenia: Garni		*Vašák s.n.* (B)
AO2	*x*	JN544752, JN544753	Armenia: Tatev	Wall	*Košnar 1646*
AO3	*x*	JN544757	Austria: Mt. Leiterkopf	Base-rich schist rock	*Košnar 1543*
AO4	*x*	JN544804	Austria: Leiterbach	Base-rich schist rock	*Košnar 1551*
AO5	*x*	JN544756	Austria: Kleinfleißbach	Base-rich schist rock	*Košnar 1556*
AO6+	*x*	JN544757	Austria: Kleinfleißbach	Base-rich schist rock	*Košnar 1565*
AO7+	*x*	JN544804, JN544806, *JN581670 (M2)*	Czech Rep.: Lažánky	Limestone rock	*Košnar 601*
AO8	–	JN544771, JN544775, JN544784, JN544785, JN544785, JN544786	Czech Rep.: Kralupy	Sandstone rock	*Košnar 824*
AO9	*x*	JN544767	Czech Rep.: Holštejn	Limestone rock	*Košnar 1533*
AO10	*x*	JN544804	Czech Rep.: Příběnice	Erlan rock	*Košnar 1903*
AO11	–	JN544751, *JN581682 (M19)*	France: Mt. Cenis	Rock	*Skrzypczak 03455* (priv. herb.)
AO12	–	JN544754, JN544755	France: Mt. Cenis, Grotte percée		*Skrzypczak 98395* (priv. herb.)
AO13	*x*	JN544809, *JN581677 (M4)*	Hungary: Dömös	Andesite rock	*Košnar 749*
AO14	*x*	JN544824	Hungary: Dömös	Andesite rock	*Košnar 750*
AO15	*x*	JN544823, *JN581672 (M2)*	Hungary: Visegrád	Andesite rock	*Košnar 756*

Appendix. Continued.

Sample	Ploidy	GenBank accession	Locality	Substrate	Voucher
Crossidium aberrans	–	JN544730, JN544731	Spain: Sierra de Cazorla	Rock	*Kučera 5747*
C. crassinerve	–	JN544732, JN544733	Spain: Las Torres de Cotillas	Calcareous soil	*Kučera 13662*
C. squamiferum	–	JN544723, JN544724	Montenegro: Virpazar	Limestone rock	*Košnar 1414*
Pterygoneurum ovatum	–	JN544737, JN544738	Czech Rep.: Němčičky	Loess	*Košnar 319*
P. subsessile	–	JN544739, JN544740	Czech Rep.: Čejkovice	Loess	*Košnar 1913*
Stegonia latifolia	–	JN544715, JN544716	Austria: Mt. Hohe Dock	Bare soil	*Košnar 1448*
Tortula acaulon	–	JN544743, JN544744, JN544745, JN544746	Czech Rep.: Horní Bojanovice	Bare soil	*Košnar 317*
T. atrovirens	–	JN544712, JN544713, JN544714	Spain: Cabo de Gata		*Kučera 5338*
T. brevissima 1	3*x*	JN544726, JN544727	Spain: Las Torres de Cotillas	Calcareous soil	*Kučera 13662*
***T. brevissima* 2**+	–	JN544722, JN544725	Spain: Cabo de Gata	Soil	*Kučera 5332*
T. cernua	–	JN544736	Norway: Svalbard, Petuniabukta	Soil	*Košnar 1914*
T. hoppeana	–	JN544710	Austria: Mt. Waldhorn	Gneiss rock	*Kučera 12892*
T. lanceola	–	JN544717, JN544718	Czech Rep.: Nové Dobrkovice	Soil	*Košnar 245*
T. laureri	–	JN544711	Austria: Mt. Scharnock	Soil	*Kučera 9218*
T. leucostoma	–	JN544734, JN544735	Norway: Svalbard, Petuniabukta	Soil	*Košnar 1915*
T. marginata	–	JN544747, JN544748, JN544749	Italy: Sicily, Scopello	Wall	*Košnar 1910*
T. modica	–	JN544719, JN544720	Czech Rep.: Nové Dobrkovice	Soil	*Košnar 250*
T. protobryoides	–	JN544721	Czech Rep.: Horní Němčí	Soil	*Košnar 1245*
T. revolvens	–	JN544729	Spain: Rambla de Tabernas		*Kučera 5386*
T. systylia	–	JN544750	Italy: Mt. Col del Cuc	Soil	*Kučera 7278*
T. truncata	–	JN544741, JN544742	Germany: Hub	Soil	*Košnar 1605*
T. vahliana	–	JN544728, *JN581683 (V2)*	Netherlands		*Vanderpoorten 4835* (priv. herb.)