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Taxonomic study of the Eurasian taxa of *Tortula muralis* (*Pottiaceae*, *Musci*) complex

Ph.D. Thesis

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■ Annotation

The thesis aims at clarifying the taxonomic difficulties in the *Tortula muralis* complex. For the first time, the group was studied by means of morphometric analysis, ploidy level assessment by flow cytometry, and molecular analysis based on sequencing of ITS region of nuclear ribosomal DNA. Morphometric and cytometric studies found only two well distinguished groups within the complex. These groups corresponded to the markedly variable species *T. muralis* and to the rather uniform species *T. lingulata*. Variability in ITS sequences suggested extensive gene flow among some of traditional morphologically defined taxa of the complex. Multiple polytopic autopolyploid origin of polyploids was revealed in some taxa. Changes in taxonomic conception of *T. muralis* complex were proposed in order to reflect structure of morphological, karyological, and DNA variability in the group. Natural hybridization is probably an important evolutionary mechanism that generated morphological diversity and taxonomic complexity in the mosses of *T. muralis* complex.

■ Declaration [in Czech]

Prohlašuji, že svoji disertační práci jsem vypracoval samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

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Jiří Košnar

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■ List of papers and author's contribution

The thesis is based on the following papers (listed chronologically):

- I. Košnar J., Kolář F., 2009.** A taxonomic study of selected European taxa of the *Tortula muralis* (Pottiaceae, Musci) complex: variation in morphology and ploidy level. *Preslia* 81, 399–421 (IF=2.792).
Jiří Košnar collected samples in the field, carried out morphometric analyses, participated in cytometric analyses, analysed the data, wrote the manuscript, and edited comments of the co-author and reviewers.

- II. Košnar J., Kučera J., 2010.** The taxonomic identity and typification of *Barbula montenegrina* Breidl. & Szyszyl. (*Bryopsida, Pottiaceae*). *Journal of Bryology* 32, 275–278 (IF=1.222).
Jiří Košnar carried out morphometric analysis, analysed the data, wrote the manuscript, and edited comments of the co-author and reviewers.

- III. Košnar J., Herbstová M., Kolář F., Koutecký P., Kučera J., 2012.** 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. *Taxon* 61, 709–720 (IF=2.782).
Jiří Košnar collected samples in the field, carried out molecular analyses, participated in cytometric analyses, analysed the data, wrote the manuscript, and edited comments of the co-authors and reviewers.

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General introduction

General introduction

The cosmopolitan genus *Tortula* Hedw. is one of the most morphologically diverse groups of the family *Pottiaceae* Schimp. The estimated number of *Tortula* species ranges between approximately 141–163 species worldwide (Crosby et al. 1999, Zander 1993). However, the delimitation of the genus is considerably problematic due to the wide range of morphological diversity and tendency towards frequent reduction of morphological characters in *Tortula* and other related genera within the family. In most of the cases it is impossible to define morphological characters uniquely delimiting particular genera of *Pottiaceae*. As a result, classification of *Pottiaceae* varies among authors and no widely accepted generic concept exists.

With approximately 85 genera and 1400 species worldwide (Zander 1993, The Plant List 2010), the moss family *Pottiaceae* is the major group of bryophytes. The family is considerably diverse and includes various growth forms and life strategies, ranging from short-lived shuttle species and colonists to typical long-lived dominant species with high competitive abilities. Many taxa of *Pottiaceae* are typically found in “harsh environments” such as arid regions, alpine or arctic areas (Zander 1993). The ability to colonize extreme habitats is maintained by various ecological adaptations. Well-known is drought resistance of some of the desert taxa (Li et al. 2010), and morphological adaptations to arid conditions such as ventral leaf photosynthetic organs, incurved leaf margins, and leaves with hyaline hairpoint (Kürschner 2004).

MORPHOLOGICAL DELIMITATION OF THE GENUS *TORTULA*

According to Hedwig’s description (Hedwig 1801), the genus *Tortula* was broadly defined and included stegocarpous mosses with peristome consisting of a single circle of filiform and spirally twisted teeth. The difference from the most similar genus *Barbula* Hedw. was ascribed to gametangial arrangement (monoicous with bud-like male inflorescences

in *Tortula*; dioicous with capituliform male inflorescences in *Barbula*). The concept of genera used by other authors of 19th century was usually even broader, merging in a single genus all taxa of *Pottiaceae* with filiform, spirally twisted teeth and alternatively more or less broader leaves. For instance, Bridel (1818), Bruch et al. (1843), Müller (1849), and Schimper (1860) included such taxa in *Barbula*, whereas Hooker and Greville (1824) or De Notaris (1838, 1862) in *Tortula*. However, these authors often mentioned existence of taxa with characters markedly standing out from the overall *Tortula*-like morphology, and treated such taxa into sections or infrageneric units with no formal taxonomic level.

This approach was a sign of later much narrower definition of the genus *Tortula* proposed by some of the authors at the turn of the 19th and 20th centuries (Juratzka 1882, Limpricht 1890, Roth 1904), which was widely accepted by most authors of 20th century (Brotherus 1923, Podpěra 1954, Saito 1975, Smith 1978b, Nyholm 1989, Frahm and Frey 1992). Several new or formerly not consistently distinguished genera were accepted for the taxa with distinct morphological characters, such as *Aloina* Kindb., *Crossidium* Jur. and *Pterygoneurum* Jur. for the taxa with ventral leaf photosynthetic organs, *Barbula* for dioicous taxa with non-excurrent leaf costa and different shape of leaf transverse section, *Pseudocrossidium* R.S. Williams with strongly recurved leaf margins, *Pottia* (Ehrh. ex Rehb.) Fűrnr. for taxa with different characters of peristome and papilosity of leaf cells, etc. Most of the authors of the 20th century followed concept of Bridel (1801) and distinguished a separate genus *Syntrichia* Brid. involving taxa with high basal membrane of the peristome (e.g. Amann and Meylan 1918, Chen 1941b, Pilous 1948, Podpěra 1954, Pilous and Duda 1960). The segregation of *Syntrichia* was not accepted by the authors in 1970s and 1980s (Saito 1975, Smith 1978b, Nyholm 1989). The narrow concept of the genus *Tortula* was usually defined by combination of following characters: capsule stegocarpous on distinct seta, peristome present and consisting of 32 filiform and often spirally twisted teeth, leaf cells rather small and usually densely papillose, leaf costa often excurrent and having circular shape of transverse section. However, none of above mentioned characters was unique for the genus

Tortula, and the characters were not consistently present in all taxa included in the genus. This led to the hypothesis about non-monophyletic and basal position within *Pottiaceae*, with other genera radiating from *Tortula/Desmatodon*-like ancestor, which was firstly proposed by Hilpert (1933) and followed by phylogenetic reconstructions made by Chen (1941a) and Delgadillo (1975).

An important event in taxonomy of *Pottiaceae* was an overview of the family published by Zander (1993). It was Zander's concept of discovering "morphological transformation series" and emphasizing the importance of gametophytic characters which led to somewhat different generic treatments. Zander included in *Tortula* taxa with gametophyte characters more or less congruent with narrow definition of the genus, but different sporophyte characters, such as *Desmatodon* Brid., and partly also *Phascum* L. ex Hedw. and *Pottia*. The genus *Tortula* was defined by Zander as follows: stem with central strand and lacking sclerodermis and hyalodermis, leaves obovate to spatulate, usually with recurved leaf margins, costal stereid band usually semicircular to rounded in section, hydroid strand present, dorsal costal epidermis usually present, leaf cells rather larger and unobscured by papillae, upper leaf cells with KOH reaction usually yellow, gemmae absent, and sporophyte with tendency to reduction. On the other hand, Zander segregated several former *Tortula* taxa with more or less odd gametophytic morphology into several new genera (*Dolotortula* R.H. Zander, *Chenia* R.H. Zander, *Hilpertia* R.H. Zander, *Sagenotortula* R.H. Zander and *Stonea* R.H. Zander), and insisted on distinguishing *Syntrichia*. Contrary to the previous authors emphasizing the height of basal membrane of peristome, Zander's definition of *Syntrichia* was similar to the concept of Kramer (1980, 1988) and involved only gametophytic characters: red KOH reaction of upper leaf cells, crescent-like shape of costal stereid band, absence of dorsal costal epidermis, and lack of elongate upper laminal marginal cells.

The Zander's concept of *Tortula* was at best only partly accepted by later authors. The only publication fully reflecting Zander's treatment was Ignatov and Ignatova (2003). Other authors agreed with distinguishing *Syntrichia* and Zander's newly recognized genera but rejected merging

some of the traditional former genera with *Tortula* or partly accepted somewhat different definitions of previously recognized genera giving equal weight to gametophyte and sporophyte characters proposed by Guerra and Cano (2000).

Guerra and Cano (2000) distinguished the genera *Pottia* and *Phascum*, although the latter under different definition than most of previous authors of 20th century. Guerra and Cano (2000) also accepted separation of monotypic genus *Mildeella* Limpr. sensu Limpricht (1890), for which new legitimate name *Protobryum* J. Guerra & M.J. Cano was proposed. The genus included *P. bryoides* (Dicks.) J. Guerra & M.J. Cano, considered by Zander (1993) as a member of *Tortula*. Among other recent authors, Cortini Pedrotti (2001) accepted traditional concepts of the genera *Pottia*, *Phascum* and *Desmatodon*, Ochyra et al. (2003) distinguished *Protobryum*, Guerra et al. (2006) distinguished *Pottia*, *Protobryum*, and *Phascum* (sensu Guerra and Cano 2000), and Hill et al. (2006) distinguished *Protobryum* and *Phascum* (sensu Guerra and Cano 2000). Smith (2004) distinguished *Pottia* and *Phascum* (sensu Guerra and Cano 2000), but his definition of *Pottia* was unusual and included only two stegocarpous taxa traditionally included in the genus *Pottia* and considered by Zander (1993) as members of the genus *Microbryum* Schimp.

INFRAGENERIC CLASSIFICATION OF THE GENUS *TORTULA*

Among the treatments closer to recent narrower concepts of *Tortula* (i.e. since the end of 19th century), infrageneric classification of the genus was mostly neglected. Limpricht (1890) and Roth (1904) distinguished two infrageneric units with no formal taxonomic level: *Tortula* and *Syntrichia*, the latter differing from the former in bigger plants and higher basal membrane of the peristome. Brotherus (1923) distinguished three sections: *Tortula*, *Syntrichia* Brid. and *Zygotrichia* Brid. The definition of sect. *Tortula* and sect. *Syntrichia* was somewhat similar to infrageneric units of Limpricht (1890) of the same name but the sect. *Zygotrichia*

involved two taxa segregated from *Syntrichia* sensu Limpricht (namely *T. subulata* Hedw. and *T. mucronifolia* Schwägr.) and differing by shorter stem and only shortly excurrent leaf costa. Nyholm (1989) recognized basically the same three sections as Brotherus (1923) but accepted different sectional names. The name *Tortula* sect. *Cuneifoliae* (Bruch & Schimp.) Spruce was accepted for the former sect. *Tortula*, the name *Tortula* sect. *Rurales* De Not. was accepted for the former sect. *Syntrichia*, and the section with taxa of *Zygotrichia* was labeled as *Tortula* sect. *Tortula*. The different name of the latter *Zygotrichia*-like section referred to the fact that the section contained the newly selected lectotype of *Tortula* (*T. subulata* – Steere 1939).

Among authors who recognized *Tortula* and *Syntrichia* as a separate genera based on differences in height of the basal membrane of peristome, Mönkemeyer (1927) and Podpěra (1954) distinguished two sections within *Tortula*: sect. *Eutortulae* Moenk. and sect. *Crassicostatae* (Schimp.) Podp., the latter differing from the former in costa wider in upper part of the leaf, strongly revolute leaf margins, and shorter and less spiral peristome teeth.

Zander (1993) distinguished four sections which could be attributed to his relatively broader concept of *Tortula*. The Zander's sect. *Tortula* included most of the taxa treated by former authors as sect. *Tortula* sensu Brotherus (1923) or sect. *Cuneifoliae* sensu Nyholm (1989), together with a part of species earlier included in *Desmatodon*, such as its type. This section was characterized by densely papillose and rather small (10–13 µm) upper leaf laminal cells, costa in transverse section with guide cells and 3–6 papillose cells usually forming a convex pad, leaves usually with rounded apices and commonly with long hyaline hairpoint, and usually developed peristome. The sect. *Pottia* (Ehrh. ex Reichenb.) Kindb. included taxa previously ascribed to *Phascum*, *Pottia*, *Desmatodon* p. pte. and partly to *Tortula* sect. *Zygotrichia* sensu Brotherus (1923), and was defined by rather smooth and large (15–20 µm) upper leaf laminal cells, costa in transverse section with guide cells and 2–3 rather smooth and commonly bulging ventral cells, leaves usually with acute apices and usually short yellow-brown awns, and often rudimentary or absent

peristome. The sect. *Schizophascum* (Müll. Hal.) R.H. Zander included taxa with gametophyte similar to sect. *Pottia*, but differing by cleistocarpous capsules and absence of guide cells. The monotypic sect. *Hyophilopsis* (Cardot & Dixon) R.H. Zander was similar to sect. *Pottia*, but differed by strongly developed annulus and elongate upper leaf laminal cells.

As Zander's concept of *Tortula* was not fully accepted by later authors, the same was true for his treatment of infrageneric taxa. Somewhat modified version based on Zander's sections was adopted in regional treatment of Ochya et al. (2003), who accepted his infrageneric subdivision of *Tortula* and found the name *Cuneifoliae* (Schimp.) Ochya to be the correct designation for Zander's sect. *Pottia*. However, these authors treated *T. protobryoides* R.H. Zander as a member of the genus *Protobryum* sensu Guerra and Cano (2000).

MOLECULAR INVESTIGATIONS IN PHYLOGENY OF THE GENUS *TORTULA*

The use of molecular methods in last two decades have revolutionized taxonomy of bryophytes. However, the available molecular phylogenetic studies have brought rather sparse and preliminary information about phylogenetic relations in the genus *Tortula*. This was caused by low resolution power of a single chloroplast DNA marker (*rps4* region) used for phylogenetic analysis of *Pottiaceae*, and incomplete taxa sampling. Accordingly, no changes based on molecular data have been accepted in generic treatments in this group of *Pottiaceae*.

The only more comprehensive work dealing with *Tortula* and several related genera was the study Werner et al. (2002). Among the traditionally recognized genera, results of this study confirmed separate position of *Syntrichia* sensu Zander (1993). On the other hand, virtually all other larger genera and infrageneric taxa recognized in the past were found to be non-monophyletic. The taxa of *Tortula* sect. *Pottia* sensu Zander (1993) formed the most homogeneous group ("*Pottia* clade"), with the exception of misplaced and poorly resolved position of two taxa

(*T. acaulon* (With.) R.H. Zander, *T. mucronifolia*) and nested position of one taxa belonging to *Tortula* sect. *Tortula* (*T. canescens* Mont.). The taxa of polyphyletic *Tortula* sect. *Tortula* were intermingled with taxa of the genera *Pterygoneurum*, *Stegonia* Venturi and *Crossidium*, which is in agreement with hypothesis considering *Tortula* as an ancestral type (Hilpert 1933, Chen 1941a, Delgadillo 1975). Interestingly, polyphyletic nature of *Crossidium* suggests that even relatively complex synapomorphies such as ventral leaf photosynthetic organs characterizing some of the smaller and seemingly well-defined genera have evolved independently several times. It seems likely that other less complex morphological characters such as costa exurrency or leaf cell size and papillosity will exhibit at least similar level of homoplasy, because some of these characters serve as ecological adaptations. Although overall morphology could be still useful for delimitation of closely related taxa, it may be of limited use in reconstruction of phylogenetic relations of higher taxonomic units.

THE TAXONOMY OF *TORTULA MURALIS* COMPLEX

Although there is no widely accepted infrageneric treatment of *Tortula* and also the phylogeny of the genus is far from being well resolved, it is known that some taxa are morphologically more similar to each other and form taxonomically difficult groups. One such group is *T. subulata* complex, which was studied by Cano et al. (2005). Another group of morphologically similar taxa are those putatively closely related to *T. muralis* Hedw., hereafter called *T. muralis* complex.

In Eurasia, the *T. muralis* complex is tentatively defined here to include following taxa: *T. edentula* Ignatova & Ignatov, *T. israelis* Bizot & F. Bilewsky, *T. lingulata* Lindb., *T. montenegrina* (Breidl. & Szyszyl.) Broth., *T. muralis* Hedw. var. *muralis*, *T. muralis* var. *aestiva* Brid. ex Hedw. and *T. obtusifolia* (Schwägr.) Mathieu. The taxa of the *T. muralis* complex share combination of following morphological characters: leaf costa often excurrent and often forming hyaline hairpoint, leaf margins

recurved, upper leaf laminal cells densely papillose and rather small (ca. 10 µm), low basal membrane of the peristome, peristome teeth well developed to reduced, and rather small spores (ca. 9–15 µm). As the most important characters delimiting the taxa of the complex costa excurrency, length of the peristome, presence of leaf border formed by smooth and more thickened cells, and gametangial arrangement were reported. The taxa of the *T. muralis* complex also share epilithic growth, usually on basic substrates. All taxa of the *T. muralis* complex belong to *Tortula* sect. *Tortula* sensu Zander (1993) or Ochyra et al. (2003). According to infrageneric classification of Mönkemeyer (1927) and Podpěra (1954), *T. brevissima*, *T. edentula*, *T. israelis* and *T. muralis* belong to sect. *Eutortulae*, whereas *T. obtusifolia*, *T. lingulata* and *T. montenegrina* to sect. *Crassicostatae*.

As the most morphologically similar, but differing in some of the above mentioned characters delimiting *T. muralis* complex, the following taxa could be considered: *T. brevissima* Schiffn., *T. marginata* Bruch & Schimp., *T. revolvens* (Schimp.) G. Roth and *T. atrovirens* (Turner ex Sm.) Lindb. *T. brevissima* is predominantly terricolous species, differing from most similar *T. muralis* var. *muralis* in slightly shorter basal leaf cells, larger upper leaf laminal cells, larger spores, and bulging ventral cells of the leaf costa. *T. marginata* resembles *T. muralis* var. *aestiva*, but differs in flat leaf margins with multistratose border formed by elongate cells. *T. revolvens* is similar to *T. obtusifolia*, and differs in strongly revolute leaf margins and smaller upper leaf laminal cells. *T. atrovirens* is terricolous or epilithic species most similar to *T. obtusifolia*, differing in markedly bulging ventral cells of the leaf costa, which somewhat resemble photosynthetic organs found in the genus *Crossidium*. Based on results of molecular study of Werner and Guerra (2004), *T. muralis* is paraphyletic, with nested position of *T. vahliana* (Schultz) Mont. However, *T. vahliana* differs from *T. muralis* and other taxa of the complex in nearly all the above mentioned characters, and grows usually on soil instead of rock surface.

Although most of treatments of *Tortula* considered *T. lingulata*, *T. obtusifolia* and *T. muralis* as distinct species, some authors noted clinal

variation and unclear delimitation of some of the taxa of *T. muralis* complex (Culmann 1921, Loeske 1934, Boros and Vajda 1957). The groups *T. muralis* var. *muralis* – *T. muralis* var. *aestiva* – *T. obtusifolia* and *T. obtusifolia* – *T. lingulata* were usually reported as the most problematic. Numerous different chromosome counts were reported within *T. muralis* (Fritsch 1982), indicating putative polyploidy. Moreover, putative cryptic lineages were revealed in *T. muralis* by sequencing of chloroplast DNA (Werner and Guerra 2004). The poorly known taxon *T. montenegrina* was largely neglected in most treatments of *Tortula* or was considered as a subspecies of *T. lingulata* by some authors (Podpěra 1954, van der Wijk et al. 1969) or as a synonyme of *T. lingulata* (Ignatov and Afonina 1992). *T. israelis* was reported to be very similar to *T. muralis*, differing only in extraordinarily developed papilosity of leaf cells (Guerra et al. 1992, Cano et al. 1996). *T. edentula*, the most recently described species of the complex, was considered as being most similar to *T. muralis* var. *aestiva* but partly also to *T. obtusifolia* (Ignatova and Ignatov 2009).

Cryptospeciation, polyploidization, and infraspecific hybridization can be hypothesized as a possible cause of taxonomic difficulties in *T. muralis* complex. These topics are discussed below.

Cryptospeciation

The term “cryptic” or “sibling” species was introduced by Mayr (1942) for taxa which are morphologically indistinguishable but reciprocally reproductively isolated and thus fitting biological species concept firstly proposed by Stresemann (1919). Increasing number of cryptic species has been revealed by means of chromosomal, cytological, and molecular studies in recent decades. Despite its occurrence in all major groups of organisms (Beheregaray and Caccone 2007), it is plausible that the relative contribution of cryptospeciation to diversity varies among geographic regions and different evolutionary lineages (Bickford et al. 2007), as indicated by the recent meta-analysis of Metazoa (Trontelj and

Fišer 2009). For some groups of organisms, it is estimated that cryptic diversity might even twice exceed the known diversity (Oliver et al. 2009). Interestingly, cryptospeciation is not restricted only to organisms with simple morphological structure and limited number of characters, as evidenced by the detection of cryptic lineages even in large mammals (Brown et al. 2007). Although cryptospeciation has been traditionally considered as a result of rather recent radiation or speciation events, some studies have revealed very old cryptic lineages (e.g. Amazonian frog species dated back to late Miocene – Elmer et al. 2007).

The first known case of cryptospeciation in bryophytes was the liverwort *Conocephalum conicum* (L.) Dum. (Szweykowski and Krzakowa 1979). Since then, numerous cases of cryptospeciation have been detected in bryophytes using molecular methods such as isozymes, RAPD, DNA sequencing, or microsatellites (Shaw 2001, Ramaiya et al. 2010). Although the available data still do not allow to assess the real frequency of cryptospeciation in bryophytes, the point of view held by Wyatt et al. (1997) who considered known examples as “the tip of the iceberg” is plausible. In most of the cases, bryophyte cryptospecies showed overlapping distribution, although examples of allopatric or ecologically differentiated lineages are not rare (Szweykowski and Odrzykoski 1990, Boisselier-Dubayle et al. 1995, Shaw and Allen 2000). However, both patterns involving cryptospeciation could potentially explain markedly broad geographic distribution of some bryophyte taxa (Shaw 2001).

Evidence of cryptic speciation has been found in two genera of *Pottiaceae*: *Pleurochaete* Lindb. (Grundmann et al. 2006) and *Tortula* (Werner and Guerra 2004, Cano et al. 2005). The first putative cryptic taxa in *Tortula* were found by sequencing of the *rps4* region of cpDNA in *T. muralis* (Werner and Guerra 2004). The study revealed a total of 18 haplotypes worldwide, exhibiting extraordinarily high level of nucleotide divergence. The distribution pattern ranged from haplotypes found on several continents to those restricted to a certain area. Results of the study further suggested that *T. muralis* might be non-monophyletic and include a nested clade of *T. vahliana*, although the topology received no bootstrap

support. The second case of putative cryptic speciation was detected in *Tortula subulata* complex (Cano et al. 2005), based on phylogenetic analysis using ITS region of ribosomal DNA. Two partly allopatric lineages were found within the morphologically uniform plants of *T. mucronifera*, differing in a considerably high number of indels.

Both above mentioned studies of putative cryptospeciation in *Tortula* suffer from two main drawbacks. First, the evidence for cryptospeciation was inferred from a single locus analysis. It is well known that genealogy of a single genomic region (gene tree) might differ from the real species tree (Rosenberg and Nordborg 2002). Random processes acting at population level such as ancestral polymorphism and incomplete lineage sorting are considered as a main source of incongruence between the gene and species trees (Maddison 1996). For the same reasons, non-monophyly is considered to be the most probable scenario following divergence of two species, and the probability of receiving reciprocal monophyly depends on population size and number of generations (Rosenberg 2003). Second, both studies lack direct evidence of reproductive isolation among the putative cryptic lineages. Both cpDNA and ITS region of nuclear DNA represent multi-copy regions, which are present in thousands copies in a single genome. The cpDNA is supposed to be maternally inherited in bryophytes (Natcheva and Cromberg 2007), providing thus no information about gene flow. On the contrary, the ITS region could be in some cases biparentally inherited and reflect real gene flow, although distinguishing hybridization from incomplete lineage sorting of ancestral polymorphism or pseudogenization might prove difficult (Álvarez and Wendel 2003, Nieto Feliner and Roselló 2007). The biparental inheritance of ITS was routinely used in detection of hybrids in angiosperms (Baldwin et al. 1995, Álvarez and Wendel 2003). However, the absence of individuals heterozygous in their ITS does not necessarily need to be caused by existing reproductive barriers. The biparental inheritance of ITS is not always the rule and heterozygous individuals are usually influenced by the so-called concerted evolution, which tends to homogenize the intraindividual ITS variation (Arnheim 1983, Elder and Turner 1995). The rate of concerted evolution varies among taxa and

evolutionary lineages, which further complicates the reliability of gene flow estimates.

A typical example of a single locus showing misleading information about phylogeny of *Pottiaceae* was detected in multilocus analysis of *Barbula* (Kučera et al. 2013). The ITS dataset pointed out towards deep polyphyly and seeming cryptospeciation in taxa of *Barbula convoluta* Hedw. s.l. On the contrary, dataset of two concatenated chloroplast regions confirmed monophyly of *Barbula convoluta* s.l., which was congruent with morphological observations. Therefore, the existence of cryptospeciation in above mentioned taxa of *Tortula* inferred from single locus studies should be considered with caution and deserves further investigation.

Polyploidy

Polyploidy is considered as an important evolutionary mechanism in vascular plants (Ramsey and Schemske 1998). Multiplication of chromosome sets can act as a speciation event, rapidly forming reproductive barriers between different ploidy levels (Rieseberg and Willis 2007). Moreover, polyploidization is often connected with the rise of new adaptive traits, and polyploid individuals often exhibit altered growth characteristics (Levin 1983) or capability to colonize new habitats and geographic areas (Parisod et al., 2010, Ramsey 2010). The multiplication of chromosomes alone can be the cause of substantial portion of fitness increase in polyploids, as revealed by studies of recently formed neopolyploids sharing nearly completely genetic background with their progenitors (Ramsey 2010).

The overall frequency of polyploidy varies among evolutionary lineages of bryophytes and the estimates for mosses (more than 80%; Smith 1978a) are even higher than those for angiosperms (ca. 40–70%; Masterson 1994, Ramsey and Schemske 1998). The estimated frequency of polyploidy leading to speciation in bryophytes (ca. 5–10% and 6–19% in liverworts and mosses, respectively; Săstăd 2005) is approximately

similar to that in angiosperms (ca. 15%; Wood et al. 2009) and nearly two to tree times lower than in ferns (ca. 31%; Wood et al. 2009). The studies of polyploidy were generally enhanced by development of methods such as flow cytometry (Doležel et al. 1989) that overcome limitations of traditional chromosome counting and enable screening of large numbers of individuals.

Contrary to the view of prevailing autopolyploidy in angiosperms (Ramsey and Schemske 1998), allopolyploidy seems to be much more frequent in bryophytes. Among bryophyte polyploids studied by molecular methods, the vast majority proved to be of allopolyploid origin, i.e. having sets of non-homologous chromosomes due to hybridization (Såstad 2005). Allopolyploidy was detected even in some cryptic species complexes of liverworts, with allopolyploids originating by hybridization between some of the cryptic lineages (Odrzykoski et al. 1996, Boisselier et al. 1998). Although a few cases of putative autopolyploidy were detected in liverworts (Bischler and Boisselier-Dubayle 1993, Bischler and Boisselier-Dubayle 1999, Boisselier-Dubayle and Bischler 1999), no example of autopolyploidy in mosses has been proven by molecular markers so far. This could be partly influenced by limited sampling in mosses when compared to liverworts. Origin of polyploids was examined in four moss families (*Mniaceae* Schwägr., *Polytrichaceae* Schwägr., *Racomitriaceae* Kindb. and *Sphagnaceae* Dumort.), whereas seven families were studied in liverworts (Såstad 2005). Another possible explanation could be simply the overlooking of polyploids due to the frequent morphological indistinguishability from their progenitors (Soltis et al. 2007) or generally low number of bryophyte polyploids studied by molecular markers so far.

Based on variation in chromosome counts, polyploidy seems to be relatively frequent in the genus *Tortula*. The most frequent chromosome counts reported in *Tortula* usually ranges from $n = 20$ to $n = 26$ (Smith 2004), although higher counts are not exceptional. Among the taxa of the *T. muralis* complex, only *T. muralis* has been reported to have several chromosome counts, ranging from $n = 13$ to $n = 66$. This extraordinary variation could be to some extent caused by frequent sampling (Fritsch

1982). Based on the study of Newton (1968), two cytotypes with $n = 26/27$ and $n = 50/52$ could be regarded as the most common ploidy levels. However, the variation in chromosome counts reported in *T. muralis* implies questions concerning the origin of plants with higher chromosome counts. The absence of known cases of autopolyploidy in mosses would favour allopolyploid origin, which would further indicate hybridization causing incongruences in morphological delimitation of the taxa of the complex. On the other hand, even autopolyploidy causing reproductive barriers between different ploidy levels would be possible scenario leading to differentiation of distinct cryptic lineages suggested by cpDNA studies of *T. muralis*. Although interploidal gene flow is known to occur in vascular plants (Ramsey and Schemske 1998), this phenomenon is poorly known in bryophytes and the only detected example was found in the genus *Sphagnum* L. (Flatberg et al. 2006). And finally, the existence of variation in chromosome counts in *T. muralis* raises question about ploidy level and its putative variation in other taxa of the *T. muralis* complex. Among those, chromosome counts are available only for *T. obtusifolia* ($n = 26$; Lazarenko et al. 1968, Lazarenko et al. 1971) and for *T. lingulata* ($n = 24$; Mamatkulov 1967, Lazarenko et al. 1968, Vysotskaja 1975).

Hybridization

Hybridization is undoubtedly another important evolutionary mechanism in sexually reproducing organisms. The frequency of hybrids in vascular plants is considered to be nonrandomly distributed and generally determined by reproductive mode and life strategy (Ellstrand et al. 1996). It was estimated that hybridization was involved in the origin of more than 50% of taxa in vascular plants (Stebbins 1950). Allopolyploidy is the major mechanism of speciation via hybridization in vascular plants, whereas homoploid hybrid speciation is considered to be rather rare (Abbott et al. 2010). Similarly, allopolyploidy seems to be predominant in bryophytes (Såstad 2005), as no evidence of homoploid hybrid speciation

has been found so far. Unfortunately, hybridization in bryophytes is little known and probably underestimated (Natcheva and Cronberg 2004). The low number of known hybrids served for some authors as one of reasons for considering bryophytes as group with low evolutionary potential (Anderson 1963, Crum 1972).

Hybrids in bryophytes were hypothesized since the second half of 19th century, and the first direct evidence was obtained in first half of 20th century by crossing experiments yielding artificial interspecific and intergeneric hybrids (the moss family *Funariaceae* Schwägr. – Wettstein 1923, 1924; the liverwort genus *Sphaerocarpos* Boehm. – Allen 1937). Natcheva and Cronberg (2004) found approximately 60 published reports of hybridization in mosses and noted higher frequency of hybrids in some moss families, including *Pottiaceae*. The authors concluded that these results might be biased by factors such as depth of taxonomic sampling, gametangial arrangement, frequency of sexual reproduction, and extent of sporophyte divergence which enables easier detection of hybrids in sporophytic phase. However, the number of bryophyte hybrids hypothesized in literature is still negligible when compared with vascular plants. Even the advent of molecular methods has not changed dramatically the number of revealed cases of hybridization. This might be due to the prevailing use of non-recombining and uniparentally inherited markers. Most of bryophyte studies rely on DNA sequencing of multi-copy regions (Stech and Quandt 2010), whereas markers with higher potential for detection of hybrids such as SSR, ISSR, or AFLP are much less employed.

Several hybrids were reported in the genus *Tortula*. Based on observations of intermediate sporophytes, Ulychna (1977) suggested hybrids among taxa of *Tortula* sect. *Pottia* and Ros et al. (1994) reported intergeneric hybrids between *Tortula* sect. *Pottia* and *Microbryum* Schimp. Another putative hybrid based on morphological observations of gametophytes involved one member of the *T. muralis* complex. Lobachevskaya and Ulychna (1994) regarded morphologically odd gametophytes as a hybrids between *T. canescens* and *T. muralis*. Considering the putative hybridization of these two morphologically

rather dissimilar species and the fact that all the taxa of the *T. muralis* complex show frequent sexual reproduction, hybridization seems to be a possible explanation of clinal variation among some taxa of the complex.

AIMS OF THE STUDY

The thesis aims at clarifying the taxonomic difficulties in the *Tortula muralis* complex by means of statistical evaluation of morphological characters, ploidy level assessment of gametophyte by flow cytometry (FCM), and molecular analysis based on DNA sequencing. The attention was paid on synthesis of traditional taxonomic concepts based on morphology with new insights from the data obtained by FCM and molecular methods.

The Paper 1 deals with morphological and ploidy level variation of four taxa of the complex (*T. muralis* var. *muralis*, *T. muralis* var. *aestiva*, *T. lingulata* and *T. obtusifolia*). The characters traditionally used for taxa delimitation as well as other potentially valuable characters were analysed by means of multivariate analysis. Several morphotypes of gametophyte were tested using cultivation experiment in order to reveal whether they represent genetically based forms or rather habitat modifications. The taxa were screened for differences in ploidy level, which was for some of the taxa accompanied by traditional chromosome counting.

The Paper 2 is focused on taxonomy of the most poorly known taxon of the *T. muralis* complex, *T. montenegrina*. The type specimen was selected and thorough morphological observation was used to critically evaluate the taxonomic status and synonymy of *T. montenegrina*.

The Paper 3 deals with phylogenetic relationships among taxa of the complex (*T. muralis* var. *muralis*, *T. muralis* var. *aestiva*, *T. lingulata*, *T. obtusifolia*, *T. edentula* and *T. israelis*) and other putatively related taxa of *Tortula* and other genera. The phylogeny was inferred using sequences of ITS region of the nuclear ribosomal DNA. The obtained results elucidated to some extent the evolutionary relations between plants of

different ploidy level. The detected intraindividual variation of ITS sequences was further discussed as a consequence of gene flow among some taxa of the complex.

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I.

A taxonomic study of selected European taxa of the *Tortula muralis* (*Pottiaceae*, *Musci*) complex: variation in morphology and ploidy level

Preslia 81, 399–421

A taxonomic study of selected European taxa of the *Tortula muralis* (Pottiaceae, Musci) complex: variation in morphology and ploidy level

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ABSTRACT

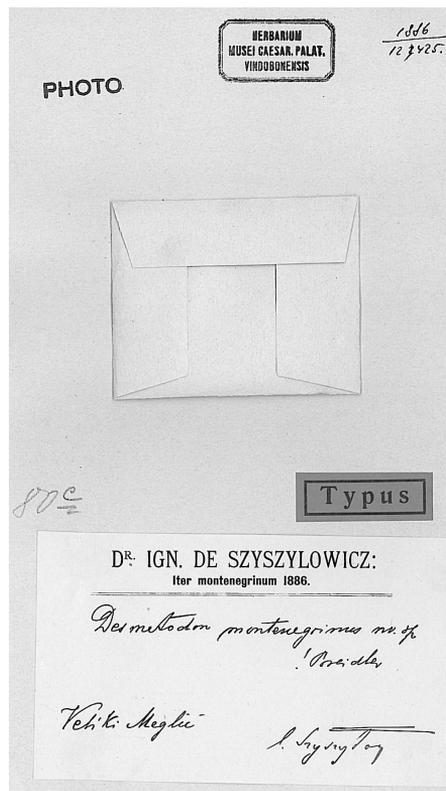
Four European taxa of the *Tortula muralis* complex (*T. lingulata*, *T. muralis* var. *aestiva*, *T. muralis* var. *muralis*, *T. obtusifolia*) were evaluated using multivariate analysis of morphometrical characters, a cultivation experiment and cytological screening (flow cytometry, chromosome counts). This study revealed that only *T. lingulata* is morphologically well defined within the complex and several new sporophytic characters that can be used to distinguish this taxon from the superficially most similar *T. obtusifolia*. The traditionally recognized taxa *T. muralis* var. *muralis*, *T. muralis* var. *aestiva* and *T. obtusifolia* showed continuous variation, with frequent intermediate plants. However, the main character of the gametophyte used for determination (costa excurrency) proved to be stable in cultivation, indicating that this character is under genetic control. Additionally, rather complex and only partly species-specific patterns of ploidy variation were found within the complex. *Tortula lingulata* and *T. obtusifolia* appear to be cytologically homogeneous; plants of *T. lingulata* were found to be diploid, whereas plants tentatively named as *T. obtusifolia* were haploid. In contrast, both haploid and diploid cytotypes were found in both varieties of *T. muralis*, with a marked predominance of diploids in var. *aestiva* and less marked predominance of diploids in var. *muralis*. Current varietal level of the evaluated infraspecific taxa of *T. muralis* was thus found to be warranted.

It is suggested that plants previously recognized as *T. obtusifolia* should be treated as a subspecies of *T. muralis*.

Keywords: chromosome, cultivation experiment, determination key, flow cytometry, hybridization, morphometric analysis, polyploidy, taxonomy.

Následující pasáž o rozsahu 41 stran obsahuje skutečnosti chráněné autorskými právy a je obsažena pouze v archivovaném originálu dizertační práce uloženém na Přírodovědecké fakultě Jihočeské univerzity v Českých Budějovicích.

Publikace vyšla tiskem v časopise Preslia.



II.

The taxonomic identity and typification of *Barbula montenegrina* Breidl. & Szyszyl. (*Bryopsida, Pottiaceae*)

Journal of Bryology 32, 275–278

The taxonomic identity and typification of *Barbula montenegrina* Breidl. & Szyszyl. (Bryopsida, Pottiaceae)

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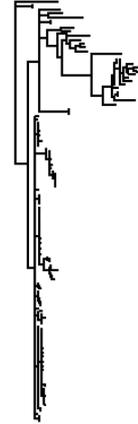
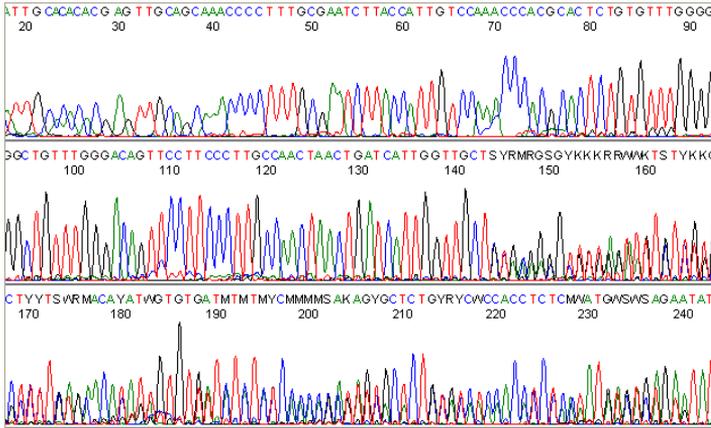
SUMMARY

The taxonomic status of *Barbula montenegrina* Breidl. & Szyszyl., a putative synonym of *Tortula lingulata* Lindb., was re-evaluated, considering its morphological characters and the habitat conditions of the known localities. The taxon is typified here and synonymized with *Tortula muralis* subsp. *obtusifolia* (Schwägr.) Culm.

Keywords: morphology, *Pottiaceae*, synonymization, taxonomy, *Tortula*.

Následující pasáž o rozsahu 12 stran obsahuje skutečnosti chráněné autorskými právy a je obsažena pouze v archivovaném originálu dizertační práce uloženém na Přírodovědecké fakultě Jihočeské univerzity v Českých Budějovicích.

Publikace vyšla tiskem v časopise Journal of Bryology.



III.

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

Taxon 61, 709–720

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.

Key words: bryophytes; gene flow; intragenomic variation; ITS; *Tortula*

Následující pasáž o rozsahu 33 stran obsahuje skutečnosti chráněné autorskými právy a je obsažena pouze v archivovaném originálu dizertační práce uloženém na Přírodovědecké fakultě Jihočeské univerzity v Českých Budějovicích.

Publikace vyšla tiskem v časopise Taxon.

General conclusions

General conclusions

The morphological analyses led to new observations and changes in the traditional concept of *Tortula muralis* complex. Morphometric analysis revealed clinal variation in the group *T. muralis* var. *muralis* – *T. muralis* var. *aestiva* – *T. obtusifolia*. None of the characters, including those traditionally used for determination (costa excurrency and length of the peristome), allowed clear determination of the three taxa. A large portion of specimens showed intermediate characters and/or incongruences among gametophytic and sporophytic character states. Such pattern of morphological variability indicated that all the three taxa were conspecific. However, morphological differentiation of *T. obtusifolia* from both varieties of *T. muralis* was more pronounced than the differentiation between *T. muralis* var. *muralis* and *T. muralis* var. *aestiva*. *T. obtusifolia* was therefore treated as a subspecies of *T. muralis*, whereas the status of variety was maintained in *T. muralis* var. *muralis* and *T. muralis* var. *aestiva*. Interestingly, one of the characters traditionally considered for taxa delimitation (costa excurrency) was found to be stable in cultivation. This led to rejection of previous hypotheses considering some morphotypes as habitat modifications. *T. lingulata* was found to be well morphologically defined species, and new sporophytic characters useful to distinguish this taxon were found. Based on morphological observation of the type specimen and comparison of known ecology and distribution of the taxa of the *T. muralis* complex, the poorly known *T. montenegrina* was synonymized with *T. muralis* subsp. *obtusifolia*.

Flow cytometry analysis was focused on four taxa of the complex (*T. muralis* subsp. *muralis* var. *muralis*, *T. muralis* subsp. *muralis* var. *aestiva*, *T. muralis* subsp. *obtusifolia* and *T. lingulata*) and revealed differences in ploidy level variation. Contrary to various chromosome counts reported in literature, only two main cytotypes were detected: haploid with $n = \text{ca. } 23\text{--}31$, and diploid with $n = \text{ca. } 45\text{--}54$. Unfortunately, technical difficulties of chromosome counting did not allow to assess the counts with higher accuracy. On the other hand, the

estimates of ploidy level revealed distinct pattern of ploidy level variation. Only diploid cytotype was found in *T. lingulata*, whereas *T. muralis* subsp. *obtusifolia* was found to be nearly exclusively haploid. Both haploids and diploids were found in *T. muralis* subsp. *muralis*, with diploids being generally more frequent. Interestingly, the distribution of cytotypes among varieties of *T. muralis* subsp. *muralis* was not equal, with more marked predominance of diploids in var. *aestiva*. Although no geographical pattern was detected and mixed populations of both ploidy levels were not exceptional, no triploid cytotype was detected, indicating the absence of direct interploidal gene flow.

The phylogenetic relations among *T. muralis* complex and related taxa inferred from sequences of ITS region of nuclear ribosomal DNA showed somewhat different topology than previously published phylogeny based on chloroplast *rps4* region. Anyway, analysis of both genomic regions resolved the genus *Tortula* sensu of Zander (1993) as polyphyletic, with nested position of genera *Crossidium*, *Hilpertia*, *Pterygoneurum* and *Stegonia*. Whereas our preliminary sampling of *rps4* region revealed almost no differences in Eurasian samples of the taxa of the complex, ITS sequences were much more variable. The ITS phylogeny resolved a poorly supported clade that included *T. muralis* subsp. *muralis*, *T. muralis* subsp. *obtusifolia*, *T. lingulata* and *T. israelis*. This result is in agreement with morphological definition of the complex and overall similarity in morphological characters. The ITS analysis found no affinity of *T. marginata*, *T. revolvens*, *T. atrovirens*, *T. vahliana* and *T. brevissima* to the *T. muralis* complex.

The resolution within the clade of *T. muralis* complex was rather poor and included several minor lineages. *T. lingulata* was found to be monophyletic, whereas other morphologically defined taxa appeared to be polyphyletic. Unfortunately, poor sampling in *T. israelis* did not allow to make conclusions about its taxonomic status. Its biphyletic nature would suggest that *T. israelis* might be a polytopically arising morphotype sharing only the character of unique papillosity of leaf cells. Anyway, further sampling would be necessary to clarify this question. The polyphyletic nature of *T. muralis* subsp. *muralis* var. *muralis*, *T. muralis*

subsp. *muralis* var. *aestiva* and *T. muralis* subsp. *obtusifolia*, is largely congruent with unclear morphological delimitation of the taxa. The recently described *T. edentula* was synonymized with *T. muralis* subsp. *obtusifolia*, as no morphological and molecular differences between the taxa were found.

Direct sequencing of ITS region further revealed frequent intragenomic variation in all the accessions analysed. Nearly 50% of samples including also other taxa of *Tortula* and related genera showed mixed signal indicating presence of divergent ITS sequences in a single individual. For the taxa standing outside the *T. muralis* complex, the divergent sequences originating from the single individual were usually monophyletic. The only exception was one accession of *T. brevissima*, with intraindividual ITS variation spanning different clades of the phylogeny. However, the intraindividual variation in all other taxa outside the *T. muralis* complex did not exceed infraspecific variation, indicating that the ITS dataset was not biased by obvious incomplete lineage sorting of ancestral polymorphism or pseudogenization. The monophyletic clade of the *T. muralis* complex contained no intraindividual ITS sequences from the taxa outside the complex, which can serve as another support for the current definition of the complex.

The accessions of *T. muralis* complex showed intraindividual ITS variation ranging from closely related monophyletic sequences differing by a few mutations to deeply divergent and polyphyletic intraindividual sequences. The markedly polyphyletic intraindividual ITS sequences were generally less frequent (ca. 16% of samples), and caused complex pattern of reticulations involving nearly all lineages of the *T. muralis* clade. The occurrence of intraindividual ITS variation can be caused by several different mechanisms such as hybridization, incomplete lineage sorting of ancestral polymorphism, or pseudogenization. It seems probable that intraindividual ITS variation in *T. muralis* complex might be caused by gene flow among the lineages leading to heterozygous constitution of ITS genotypes, similarly as in case of infraspecific hybridization. Frequent sexual reproduction, clinal variation in morphology, and complex pattern of ITS reticulation among the lineages of the complex

can be considered as the most powerful indices of the gene flow. The scenario involving incomplete lineage sorting would need numerous independent events of deep divergence in several lineages to generate the complex pattern of reticulations, which seems to be less parsimonious and rather improbable. The influence of pseudogenization could be nearly completely ruled out, as all the ITS sequences had signs of functional molecules.

The existence of putative cryptic species hypothesized based on worldwide diversity of *rps4* sequences still can not be reliably solved, although the phylogeny based on ITS sequences did not indicate presence of any well-defined cryptic lineages. The pattern of intraindividual ITS variation suggested relatively frequent gene flow among the detected lineages of the *T. muralis* complex, which would rather contradict cryptospeciation. However, the sampling for ITS analysis was more limited, covering only a part of the diversity of *rps4* haplotypes.

Both cytotypes in *T. muralis* subsp. *muralis* were found to be polyphyletic. Interestingly, several ITS haplotypes were shared by plants of both ploidy levels. This implies recurrent and polytopic origin of polyploids. Although the frequency of intraindividual ITS variation was higher in diploids than in haploids, the level of intraindividual sequence divergency showed no sign of allopolyploidy. Another evidence of autopolyploidy was found in predominantly haploid *T. muralis* subsp. *obtusifolia*. The only mixed population of both cytotypes shared the same haplotype and was free of intraindividual ITS variation. Therefore, the *T. muralis* complex could be considered as the first case of autopolyploidy in mosses confirmed by molecular markers.

Curriculum vitae

Curriculum vitae

PERSONAL DATA

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EDUCATION

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EMPLOYMENT

2007–2014: Faculty of Science, University of South Bohemia in České Budějovice, part-time job; *position:* skilled employee in research projects in systematic botany and plant ecology; *responsibilities:* DNA analyses.

TEACHING

2011, 2013: Methods of molecular biology in plant ecology and systematics (Faculty of Science, University of South Bohemia in České Budějovice)

2011: Development of microsatellite markers (SSRs) (Faculty of Science, University of South Bohemia in České Budějovice).

2012: Course of 454 pyrosequencing (Faculty of Science, University of South Bohemia in České Budějovice).

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