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Preliminary results of the taxonomic value of *Tortula densa* (Velen.) J.-P. Frahm inferred from the Internal Transcribed Spacer (ITS) of the nrDNA

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Abstract – The taxonomic concept of *T. ruralis* Hedw., *T. calcicolens* Kramer and *T. densa* (Velen.) J.-P. Frahm of various specialists varies much and was controversely discussed. The species were regarded as synonymous or distinct taxa, or *T. densa* was either placed into synonymy of *T. ruralis* or *T. calcicolens*. A molecular analysis using Internal Transcribed Spacer 1 and 2 (ITS) of selected *Tortula* specimens including specimens referred to *T. ruralis*, *T. calcicolens* and *T. densa* from the same locality, revealed that all three differed in their nuclear DNA sequences. This shows that these morphologically and anatomically distinct specimens are not modifications of *T. ruralis* and *T. densa* is not an intermediate between *T. calcicolens* and *T. ruralis* but a distinct genotype, as indicated by mixed tufts.

Zusammenfassung – Die taxonomischen Konzepte von *T. ruralis* Hedw., *T. calcicolens* Kramer and *T. densa* (Velen.) J.-P. Frahm gehen weit auseinander. Alle drei werden entweder zu einer Art zusammengefasst oder als drei Arten anerkannt, oder *T. densa* wird für ein Synonym von entweder *T. ruralis* oder *T. calcicolens* gehalten. Daher wurde eine molekulare Untersuchung der Internal Transcribed Spacer (ITS) 1 und 2 mehrerer Belege dieser drei Arten vorgenommen. Darunter waren Proben aller drei Arten von demselben Standort. Die Sequenzen zeigen Unterschiede auf dem Artniveau. Das heißt, dass diese Taxa keine Modifikationen von *T. ruralis* sondern distinkte Genotypen sind, was sich auch durch das Auftreten in Mischrasen zeigt.

***Tortula densa* / *Tortula ruralis* Complex / molecular systematics / ITS**

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INTRODUCTION

Taxonomic concepts of species can vary much depending on the author of a flora. This concerns especially taxa of species complexes. For instance, within the *Hypnum cupressiforme* complex, only one species (*H. cupressiforme* agg. – *s.lat*.) is distinguished in the Netherlands (Touw & Rubers, 1989), but 5 (*H. cupressiforme* Hedw. – *s.str*., *H. andoi* A.J.E. Sm., *H. jutlandicum* Holmen & Warnecke, *H. lacunosum* (Brid.) Hoffm. ex Brid., *H. resupinatum* Tayl.) in the neighbouring Germany (Frahm & Frey, 2004). The differentiation of so-called small species (although we would not even call the *Hypnum* species mentioned above as small) makes especially sense, if the name of the species includes ecological information. Because of the wide ecological amplitude of *H. cupressiforme s.lat*., the mentioning of this name in a species list gives no ecological information. However, if *H. lacunosum* is mentioned instead, this name implies the occurrence on a chalk grassland, if *H. andoi* is mentioned, it implies the occurrence on the bark of trees (mainly beech), if *H. jutlandicum* is used, it implies the habitat heathland or acid forest floor, and if *H. resupinatum* is named, it implies an epiphytic taxon in the atlantic part of Europe.

The argument, that these small species are merely modifications of a single species in different habitats can easily be refuted by cultivation experiments or even more easily by the observation of mixed tufts, which indicate at least distinct genotypes (on which taxonomic level ever they will be distinguished, but at least as variety or subspecies). Today, genetic distances between taxa can be estimated by comparing base sequences of suitable molecular markers. Phylogenetic inferences based on the variation in the sequence data then provide the basis for the taxonomic interpretation of the patterns observed.

One of the species complexes in question is the *Tortula ruralis* complex. The taxonomic concepts of this complex vary in many bryological publications between one species (*T. ruralis* (Hedw.) P. Gaertn., E. Mey. & Schreb., Vanderpoorten, 2001), one species with one variety (*T. ruralis* and its var. *submamillosa* Kramer, Gallego *et al*., 2002) two species (*T. ruralis* and *T. calcicolens* Kramer, Touw & Rubers, 1989), two but different species (*T. ruralis* and *T. princeps* De Not., Smith, 2004), and four species (*T. ruralis, T. densa* (Velen.) J.-P. Frahm, *T. calcicolens* and *T. ruraliformis* (Besch.) W. Ingham, Sollmann, 1997).

These varying concepts concern the acceptance of *T. ruraliformis*, the circumscription of *T. calcicolens* and the differentiation of *T. densa*. After publication of the monograph of *Tortula* sect. *Rurales* by Kramer (1980), the attention was drawn on a taxon described earlier by Grebe (1909) as *Tortula calcicola* Grebe, which was renamed by Kramer as *T. calcicolens* because of an earlier homonym. It was said to have shorter, broader leaves, that are broadest in the middle, have a short hyaline leaf base and a leaf margin revolute only 3/4-4/5 of the length.

Years later, the first author studied an isotype of *T. calcicolens* located in BONN, collected by Grebe in Hessia near Hofgeismar and edited in the exsiccate series Bauer, Musci europaei exsiccati no. 897. The study of the type revealed, that these plants were much different from that what was commonly called *T. calcicolens*. The type material consisted of low, dense tufts and differed from the description given by Kramer (1980) by carinate leaves and especially by very few and low papillae upon the upper laminal cells, giving a transparent view

of the areolation. The name *calcicolens* had, based on Kramer's concept, been applied to low dense plants with erect and short, and not carinate leaves but with opaque laminal cells like in *T. ruralis*. This stimulated to search for a name for the plants referred so far erroneously to *T. calcicolens*. In the course of herbarium studies, the first author found that Velenovsky (1897) had described such plants as *T. ruralis* var. *densa*, which is a very illustrative word for these plants that grow in dense low cushions with erect, hardly recurved leaves. Therefore this name was re-introduced (Frahm 1992) for plants formerly determined as *T. calcicolens* and raised to the rank of a species. This was done because *T. ruralis* has been lectotypified by Geissler & Frahm (1995) and the lectotype of *T. ruralis* in the herbarium of Hedwig does not match *T. densa*-like specimens. The lectotype of *T. ruralis* consists of large, loose plants with large remote leaves that are conspicuously recurved. *Tortula densa*, in contrast, consists of low, dense plants with dense foliation, shorter and erect patent leaves. Observations of mixed tufts of *T. ruralis* and *T. densa* as well as of *T. densa* and *T. calcicolens* would support the presence of distinct genotypes. *Tortula densa* revealed to be very common in Central Europe and even more common than *T. ruralis* s.str. The latter is usually confined to natural habitats (chalk grasslands, limestone rocks), whereas *T. densa* is characteristic for man made habitats such as walls, roofs, and very characteristic asphalt of roads. *Tortula calcicolens* in the sense of the type is, however, quite rare and is found on both, natural and artificial habitats.

The acceptance of *T. densa* by bryologists was very different. The Pottiaceae specialist Sollman (1997) accepted this species and included it in a key to the Dutch species of this complex. Werner (1999) also included the species in the check-list of Luxemburg. In contrast, Vanderpoorten (2001) regarded *T. densa* as synonymous with *T. ruralis*. Heinrichs & Geissler (2001) argued that the type of *T. ruralis* var. *densa* fits into the range of variation of *T. ruralis* and that this taxon is not worth of recognition, without having studied the type of *T. calcicolens*. However, it is quite difficult to make such a statement on the range of variation without any further proof, e.g. biometrical studies. Nebel & Philippi (2000) listed *T. densa* as synonymous with *T. ruralis* without giving arguments, however, the description of *T. calcicolens* included *T. densa*. This was especially obvious in the description of the transverse section of the stem ("Zentralstrang in gut 1/4 der Belege vorhanden, in der Regel klein und undeutlich"), of which the presence of a central stand in 1/4 of the specimens refers to *T. calcicolens*, the lack to *T. densa*. The reference list of German bryophytes (Koperski *et al*., 2000) ignored *T. densa*. Gallego *et al*. (2002) published biometrical studies on the species of the *Tortula ruralis* complex and regarded *Tortula densa* as synonymous with *T. calcicolens* (*Syntrichia calcicola*). The biometrical measurements were made, however, on predetermined specimens, not on random specimens. The conclusions were thus based on a circular rational. Thus there was no agreement on the taxonomic status of *T. densa* and the opinion of specialists varied between synonymous with *T. ruralis*, synonymous with *T. calcicolens*, or a distinct taxon, *T. densa*, which was not helpful.

The fact that mixed tufts (Figs 1-3) of *T. ruralis* and *T. densa* and *T. densa* and *T. calcicolens* do occur suggested that *T. densa* may indeed represent a distinct genotype. To test the hypothesis that *T. densa, T. ruralis* and *T. calcicolens* compose three distinct genetic and even phylogenetic entities we sequenced the nuclear ITS region of the rDNA repeat for populations representative of all three morphotypes.


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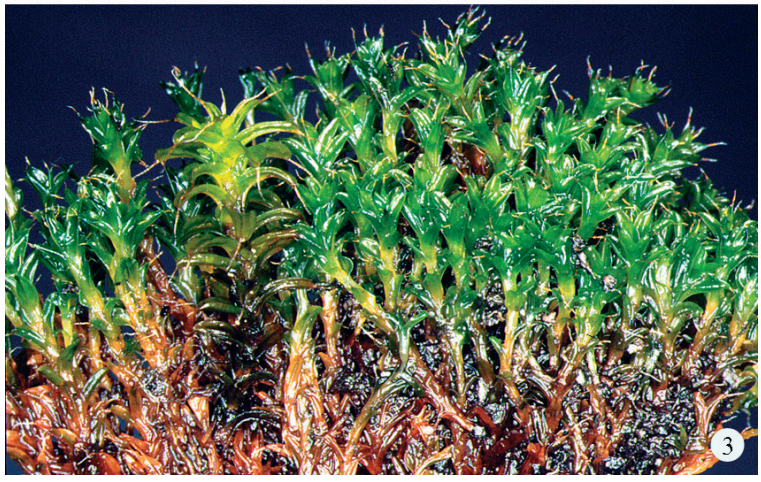
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Table 1. GenBank accession numbers and the collection details of selected specimens.

Species	*Origin*	*GenBank Accession No.*
Tortula densa 1	Germany, North Rhine Westphalia, Eifel Mountains, Nature preserve Eusbach near Mirbach S of Blankenheim, Juniperus heath, 500 m alt., January 21, 2003, *leg. J-P. Frahm s.n.*	AM075191
Tortula densa 2	France, Dépt. Puy de Dôme, between Issoire and St. Nectaire, Montaigut. April 4, 2002, *leg. J-P. Frahm s.n.*	AJ867440
Tortula densa 3	Germany, North Rhine Westfalia, Rhein-Sieg-Kreis, Rommersdorf, auf Mauer, January 25 2003, *leg. J.-P. Frahm s.n.*	AJ867441
Tortula ruralis 1	Germany, North Rhine Westphalia, Eifel Mountains, Nature preserve Eusbach near Mirbach S of Blankenheim, Juniperus heath, 500 m alt., January 21, 2003, *leg. J-P. Frahm s.n.*	AM075192
Tortula ruralis 2	Germany, North Rhine Westfalia, Rhein-Sieg-Kreis, Rommersdorf, auf Mauer, January 25 2003, *leg. J.-P. Frahm s.n.*	AJ867442
Tortula calcicolens	Germany, North Rhine Westphalia, Eifel Mountains, Nature preserve Eusbach near Mirbach S of Blankenheim, *Juniperus* heath, 500 m alt., on the same day, January 21, 2003, *leg. J-P. Frahm s.n.*	AJ867443

MATERIALS AND METHODS

Six specimens were used for the molecular study, three of *T. densa*, two of *T. ruralis* s.str. and one of *T. calcicolens*. Each specimen of all taxa was collected in the same site on the same day and each specimen of *T. densa* and *T. ruralis* s.str. were taken from a mixed tuft. GenBank accession numbers and the details of collection are given in Table 1.

Voucher specimens are deposited in the herbarium BONN.

As molecular marker, the ITS region of nrDNA was chosen because this region has revealed useful in the differentiation of taxa on a species or infraspecific level (Wendel *et al*., 1995; Baldwin & Sanderson, 1998; Baldwin & Markos, 1998; Ainouche & Bayer, 1999; Andreasen & Baldwin, 2001).

Prior to DNA extraction plant material was thoroughly cleaned with distilled water. Remaining algal, fungal or other contaminants were removed manually. DNA was isolated from silica dried fresh material mainly following the method Doyle & Doyle (1990). PCR amplification (Biometra TriBlock thermocycler, PTC-100 or PTC-200 MJ Research) were performed in 50 µl reactions containing 1.5U *Taq* DNA polymerase (Qiagen), 1mM dNTPs- Mix each 0.25 mM (Roth), 1 × buffer (Qiagen), 1.5-2.6Mm $MgCl_2$ (Qiagen) and 12.5 pmol of each amplifivation primer. PCR products were purified using the QIA quick purification kit (Qiagen). For cycle sequencing, half reactions were prepared using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit with Amplitaq-DNA polymerase FS (Perkin-Elmer), applying a standard protocol for

Figs 1-3. Mixed tufts of the *Tortula* taxa. **1.** *Tortula ruralis* Hedw. (single larger yellowish plants) and *T. densa* (Velen.) J.-P. Frahm. **2.** *Tortula ruraliformis* (below) and *T. densa* (Velen.) J.-P. Frahm (above). **3**. *Tortula calcicolens* Kramer and *T. ruralis* Hedw. (single larger plant).

all rection. Exstension products were precipitated with 40 µl 75% (v/v) isopropanol for 15 min at room temperature, centrifuged at 15.000 rpm and washed two times with 250 µl of 75% (v/v) isopropanol. All products were sequenced using two primers in the DNA Sequencing Facility, University of Maine, USA.

For amplifying and sequencing the ITS region the primers ITS4bryo and ITS5bryo (forward ITS 5bryo (5'-GGAAGGAGAAGTCGTAACAAGG-3' and reverse ITS 4bryo (5'-GCAATTCACACTACGTATCGC-3') originally designed by White *et al.* (1990) were used. The entire ITS region was amplified using a protocol consisting of 5 min 94°C, 35 cycles (1 min 94°C, 1 min 48°C and 1 min 72°C) and 4 min 72°C extension time following completion. Cycle sequencing was performed using the following program: 25 cycles (30s 96°C, 15s 50°C and 4 min 60°C).

For the phylogenetic inferences sequences of other *Tortula* species available from Genbank (*T. subulata* Hedw. – AY934570, *T. inermis* (Brid.) Mont. – AY934553, *T. muralis* Hedw. – AY437132, *T. schimperi* M. J. Cano, O. Werner & J. Guerra – AY934579, *T. mucronifolia* Schwaegr. – AY934585, *T. canescens* Mont. – AY934542 and *T. cuneifolia* (Dickson) Turner AY934543) were included.

Sequences were aligned manually. Uncorrected-"p" distance were estimated and used to reconstruct a tree using the Neighbor-joining method as implemented in PAUP 4.0b10 (Swofford, 2002). Support for branches was estimated using the bootstrap approach (Felsenstein 1985) as implemented in Paup, based on 1000 replicates.

RESULTS

The ITS region (ITS1, 5.8S nrDNA, ITS2) varies in length among the accessions. All three specimens of *Tortula densa* have a gap of 42 bp within the ITS2 compared with other selected *Tortula* specimens. The average of %GC content within the same genetic region ranged from 29.01% (*T. densa*) till 31.13% (*T. ruralis*).

Neighbour joining tree (Fig. 4.) based on uncorrected ("p") distance showed that *T. densa, T. calcicolens* and *T. ruralis* compose a well supported clade (i.e., bootstrap of 100%) within which *T. densa* is sister to *T. calcicolens* and *T. ruralis*, which form a lineage supported by a 78% bootstrap-value. Based on uncorrected "p" distance the three samples of *Tortula densa* exhibit a level of genetic identity ranging from 96.9 to 99.2%, which is much higher then the identity (84.4-84.9%) between *T. densa* and *T. ruralis* specimens. The identity percentage between *T. densa a*nd *T. calcicolens* is 87.9-88.3%.

DISCUSSION

The co-occurrence of three morphotypes composing the *T. ruralis* complex in the same locality and even in mixed populations (Figs 1-3) raises the hypothesis that these morphological entities are genetically distinct, rather than mere phenotypes of *T. ruralis*. Inference from ITS sequence is congruent with this hypothesis, and in particular with the distinction of *T. densa*: the three samples compose a single lineage that is defined by a 42bp deletion, and that is sister to the *T. calcicolens/T. ruralis* clade. The comparison of the ITS region of the three taxa studied, especially between the specimens examined from the same locality, shows that there is a significant divergence, and supports that the *T. densa* species

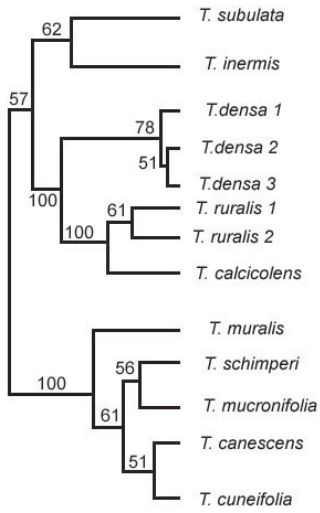

Fig. 4. Relationships among *Tortula* specimens of the *Tortula ruralis complex* estimated by a neighbor joining analysis of the ITS region based on uncorrected ("p") differences. Bootstrap values > 50% from a bootstrap analysis with 1000 replicates are given above branches.

are genetically separated and distinguished on a species level.

Syntrichia glabra J.-P. Frahm & M.T. Gallego was not included in this study, because this species turned out to be a juvenile stage of *T. densa*. First it was obvious that the species could no more be found in the following years at the type locality. Next, decayed specimens of *T. densa* were found, which produced new shoots from the leaf axils of the almost dead plants. These young shoots perfectly matched the description of *T. glabra* with much smaller stems and leaves, shorter apiculus and almost transparent lamina with very low papillae.

Tortula densa (Velen.) J.-P. Frahm, *Fragm. Flor. Geobot*. 39: 393, 1994 (*Syntrichia densa* [Velen.] J.-P. Frahm, *J. Bryol.* 23: 120, 2001)

Syntrichia glabra J.-P. Frahm & M.T. Gallego, *J. Bryol.* 23: 119, 2001, ***syn. nov.***

PROPOSED KEY FOR THE TAXA TREATED

The three taxa can be distinguished by morphological and anatomical characters as follows:

1. Leaves recurved when moist, 5-7 mm long. Stems remote foliate. Tall plants to 8 cm high. Usually on limestone in natural habitats…….. *Tortula ruralis* (Hedw.) P.Gaertn., E.Mey. & Scherb. (*Syntrichia ruralis* [Hedw.] F.Weber & D.Mohr)

1*. Leaves erect-spreading when moist, 2.5-4 mm long. Stems densely foliate. Small plants, 1.5-3 cm high.

2. Upper laminal cells opaque because of dense papillae. Leaves about 4 mm long, plane when moist. Plants medium-sized, robust, densely foliate. Hair-point reddish at base. Stem without central stand. On limestone in natural habitats but more frequently in secondary habitats such as margins of asphalt paved roads, walls and roofs….. *Tortula densa* (Velen.) J.-P.Frahm (*T. ruralis* var. *densa* Velen.)

2*. Upper laminal cells translucent because of low, horseshoe shaped papillae. Leaves 2.5-3.0mm long, keeled. Plants small, slender, loosely foliate. Hair-points without reddish brown base. Stems with central stand. On rocks, walls, concrete and roofs. Much less common than *T. densa*…. *Tortula calcicolens* Kramer (*T. ruralis* var. *calcicola* [J.J.Amann] Barkman, *T. calcicola* Grebe, *Syntrichia calcicola* J. J.Amann)

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