Differentiation in DNA fingerprinting and morphology among species of the pleurocarpous moss genus, *Rhytidiadelphus* (Hylocomiaceae)

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DNA fingerprinting techniques including inter simple sequence repeats (ISSR) and restriction digest patterns from 18S-26S nuclear ribosomal DNA internal transcribed spacers (ITS) were combined with a detailed morphological analysis to seek characters that discriminate closely related species in the pleurocarpous moss genus, *Rhytidiadelphus*. The two sibling species, *R. subpinnatus* and *R. squarrosus*, were indistinguishable based on ITS markers but displayed a clear genetic discontinuity based on ISSR markers. Overall, genetic divergence was similar among *R. squarrosus* and *R. subpinnatus* on the one hand and *R. loreus* and *R. triquetrus* on the other. The results clearly support the specific status of *R. subpinnatus* and *R. japonicus* within the controversial *R. squarrosus* complex. New divergent morphological characters were found between *R. subpinnatus* and *R. squarrosus*. The results suggest that ISSR markers may be an alternative for distinguishing sibling moss species when sequences of the most variable genomic regions traditionally used at low taxonomic level, such as ITS, do not provide the appropriate degree of polymorphism.

KEYWORDS: ISSR, ITS, mosses, PCR-RFLP, *Rhytidiadelphus*, species-level systematics.

INTRODUCTION

In many cases where minor morphological divergence among moss taxa was traditionally interpreted as a result of environmental variation [e.g., *Climacium americanum* Brid. and *C. kindbergii* (Ren. & Card.) Grout (Shaw & al., 1994); *Polytrichum commune* Hedw. and *P. uliginosum* Wallr. (Bijlsma & al., 2000)], molecular markers, including isozymes, PCR-based DNA fingerprinting techniques, microsatellites, and DNA sequences have increasingly revealed subtle patterns of sibling species (see Shaw, 2001, and Shaw & al., 2002, for review). Such investigations are, however, currently hampered by the low level of variability of many molecular markers that have been investigated at low taxonomic levels in bryophytes, or by technical difficulties in the use of highly variable markers such as microsatellites. The latter were recently successfully used to address issues at the population level in *Polytrichum*-related taxa when allozymes did not provide the appropriate level of polymorphim (van der Velde & al., 2001), but the difficulties in designing suitable specific primers indeed make this marker still largely unemployed in mosses.

In this context, PCR-based DNA fingerprinting methods, although limited in their use for phylogenetic inference due to difficulties in homology assessment, are well suited for examining patterns of genetic diversity and differentiation of similar species (see Harris, 1999, for review). RAPD, by far the most widely used DNA fingerprinting technique, has been used in bryophytes to survey population genetic structure and dispersal (see Skotnicki & al., 2000, 2001, for review), phylogeographic patterns (Freitas & Brehm, 2001), and species relationships (Boisselier-Dubayle & Bischler, 1994; Boisselier-Dubayle & al., 1995).

As a less widely used PCR-based marker, inter simple sequence repeats (ISSR) exhibit a few advantages over other markers. ISSR primers anneal to simple sequence repeats that are abundant throughout the eukaryotic genome and evolve rapidly, and hence may reveal a high level of polymorphism (Zietkiewicz & al., 1994; Li & Ge, 2001). In addition, ISSRs may produce more reliable and reproducible bands than RAPDs because of the higher annealing temperature and longer primer sequences (Qian & al., 2001). ISSR markers have therefore proved their usefulness for population genetic studies, especially in detecting clonal diversity (e.g., Esselman & al., 1999) and in resolving taxonomic relationships at or below the species level (e.g., Blair & al., 1999).

In this study, DNA fingerprinting techniques including ISSR and digest patterns of the internal transcribed spacers of 18S-26S rDNA (ITS) were combined with a

detailed morphological analysis to seek characters that discriminate closely related species in *Rhytidiadelphus* (Limpr.) Warnst., one of the 12 genera of the family, Hylocomiaceae (Rohrer, 1985). Initially, the genus comprised three species, namely *R. squarrosus* (Hedw.) Warnst., *R. triquetrus* (Hedw.) Warnst, and *R. loreus* (Hedw.) Warnst., until Koponen (1971) split the *R. squarrosus* complex into three distinct species including *R. squarrosus* s. str., *R. subpinnatus* (Lindb.) T. Kop., and *R. japonicus* (Reimers) T. Kop., which mostly differ in habitat preference and macroscopic features.

Examination of the pattern of morphological variation in the context of a tree of genetic similarity derived from ITS and ISSR markers allowed us to test the hypothesis that morphological variation in the genus corresponds to genetic differences rather than to habitat conditions in order to provide a clearer picture of species delineation.

MATERIALS AND METHODS

Taxon sampling and morphological survey. — Taxon sampling included representatives of the five *Rhytidiadelphus* species, namely *R. japonicus*, *R. loreus*, *R. squarrosus*, *R. subpinnatus*, and *R. triquetrus* (Table 1). Morphological and anatomical characters of the sporophyte and the gametophyte were studied in detail in all five species and scored as binary or trinary characters (Tables 2, 3). Wherever possible, morphological characters were scored from the same specimens used in the molecular analyses. *Rhytidiadelphus* species are, however, rarely fruiting so that all the sporophytic characters were scored on other specimens kept at S.

Molecular protocols. — PCR-RFLP of the ITS were performed according to the protocol described in Patterson & al. (1998). Two restriction enzymes, Hae3 and Hin6 I, were selected after examination of restriction site polymorphism on the whole sequences of *R. japonicus*, *R. loreus*, *R. squarrosus*, and *R. triquetrus* (GenBank accession numbers for ITS1: AJ288330, AJ288329, AJ288326, AJ288429; ITS2: AJ288544, AJ288543, AJ288541, AJ277241, respectively).

Five primers described in Blair & al. (1999) were tested for ISSR amplification in *Rhytidiadelphus*. Two of them (R2: ACA CAC ACA CAC ACA CTG; and R3: GAC AGA CAG ACA GAC A) were subsequently selected for exhibiting scorable and polymorphic band patterns. PCR reactions were carried out in a total volume of 25 µl containing 13.8 µl H_2O, 0.2 µl Taq, 1 µl $MgCl_2$, 2.5 µl 10× buffer, 2 µl primer, 2.5 µl of 25 mM dNTPs, 1 µl 25 mM bovine serum albumin, and 1 µl including 15–30 ng DNA (quantified by fluorimetry). PCR amplifications included 35 cycles of 1 min at 94°C, 1 min at 40°C and 2 min at 72°C, preceded by a period of 5 min at 94°C and completed by a final extension of 4 min at 72°C. The ISSR amplification products were stained by ethidium bromide, run for 2h at 135V on 2% agarose gel, and visualized by UV.

Data analysis. — Polymorphic bands from the ITS and ISSR analyses were scored for presence/absence (Table 4). For each of the molecular and morphological datasets, a phenetic tree of genetic similarity was obtained by neighbor-joining (NJ), and the resulting trees

Table 1. Voucher information of the 16 collections included in a combined molecular and morphological assessment of the five species of *Rhytidiadelphus*.

Species	Voucher	Locality	Habitat
R. japonicus	*Schofield & al. 103679* (S)	U.S.A., Alaska	Crowberry heath hummock
R. loreus	*Vanderpoorten 2229* (S)	France, Ile de France	On humus in atlantic beech-oak forest with *Ruscus aculeatus*
	Vanderpoorten 4951 (S)	U.S.A., Alaska	Peaty fen, with *Sphagnum* spp., *Straminergon stramineum*
	Vanderpoorten R2 (S)	Belgium, Luxembourg	Wet, peaty spruce forest with *Sphagnum* spp.
	Vanderpoorten R3 (S)	Belgium, Luxembourg	Wet, peaty forest brook margin with *Sphagnum* spp.
R. squarrosus	*Vanderpoorten R1* (S)	Belgium, Luxembourg	Wet, peaty spruce forest with *Sphagnum* spp.
	Vanderpoorten R4 (S)	Belgium, Luxembourg	Wet, peaty spruce forest with *Sphagnum* spp.
	Vanderpoorten R6 (S)	Belgium, Brabant wallon	Grassland
R. subpinnatus	*Bisang B70172* (S)	Sweden, Södermanland	Spring area in forest
	Hedenäs B62882 (S)	Sweden, Västergötland	Swampy forest
	Hedenäs B13894 (S)	Sweden, Hälsingland	Along small forest brook
	Hedenäs B38393 (S)	Sweden, Hälsingland	Brook shore
	Schumacker 960906/1 (LGHF)	Belgium, Liège	Along forest brook
	Vanderpoorten R5 (S)	Belgium, Luxembourg	Wet, peaty forest brook margin with *Sphagnum* spp.
R. triquetrus	*Vanderpoorten R3* (S)	Belgium, Luxembourg	On humus in beech forest
	Vanderpoorten R8 (S)	Belgium, Luxembourg	On humus in beech forest

Table 2. Polymorphic morphological and anatomical characters scored on the five species of *Rhytidiadelphus*.

1. Plants mainly prostrate (0), or erect (1).
2. Plants more or less regularly pinnately (0), or irregularly (1) branched.
3. Stem leaves in basal part varying around ovate to broadly ovate (0), or around triangular to cordate (1).
4. Stem leaves slightly (0), or strongly (1) constricted at insertion.
5. Stem leaves gradually narrowed towards leaf apex (0), or with clearly differentiated acumen (1).
6. Stem leaves smooth (0), or at least sometimes slightly to strongly plicate (1) in basal portion.
7. Stem leaves slightly to strongly falcate (0), or slightly to strongly squarrose (1).
8. Margin slightly denticulate to denticulate (0), or denticulate to coarsely so (1).
9. Median leaf lamina cells narrow, width varying between 4.0–8.5 µm (0), or wide, varying between 6.0–9.5 µm (1).
10. Median leaf lamina cells short, length varying between 24.0–77.5 µm (0), intermediate, 27.5–98.5 µm (1), or long, length between (42.0–)52.0–128.0 µm (2).
11. Alar groups well differentiated (0), or indistinct (1).
12. Stem cortex (including epidermis) of up to 4 or 5 layers (0), or (1–)2–3-stratose (1).
13. Calyptra 3–4-stratose (0), or 4–6-stratose (1).
14. Distance between transverse walls on outside of basal endostome membrane up to 10.0 µm (0), or more than 10.0 µm (1).
15. Costa smooth (0), or ending in a spine (1).
16. Margin near insertion recurved (0), or plane (1).
17. Median lamina cells smooth (0), or strongly prorate (1).
18. Seta frequently more than 20 mm long (0), or up to 20 mm (1).
19. Lower outside of exostome cross-striolate (0), or reticulate (1).
20. Exostome border in lower portion of teeth normal (0), or very broad (20–25% of exostome width) (1).
21. Endostome basal membrane mostly higher than 38% of total endostome height (0), or shorter (1).

Table 3. Matrix of the 21 morphological characters scored on the five species of *Rhytidiadelphus*. See Table 2 for character numbers.

	Characters																				
Species	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
R. japonicus	0	1	1	1	?	1	?	1	0	0	0	0	-	0	0	0	0	0	0	0	0
R. loreus	0	1	0	0	0	1	0	0	1	2	1	0	1	1	0	0	0	0	0	0	0
R. squarrosus	1	1	0	0	1	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	1
R. subpinnatus	0	0	1	0	1	0	1	0	0	1	0	1	0	0	0	0	0	1	0	0	0
R. triquetrus	1	0	1	1	0	1	0	1	0	1	1	0	1	1	1	1	1	0	1	1	0

were compared to assess whether the different datasets yielded congruent information.

RESULTS

In *Rhytidiadelphus*, analysis of the five polymorphic restriction fragments of ITS produced by Hae3 allowed the recognition of three haplotypes corresponding to (1) *R. loreus*; (2) *R. triquetrus*; and (3) the three other species, *R. japonicus, R. squarrosus* and *R. subpinnatus* (Table 4). The six polymorphic restriction fragments of ITS produced by Hin6 I allowed the recognition of three other haplotypes, corresponding to (1) *R. squarrosus* and *R. subpinnatus*; (2) *R. loreus*; and (3) *R. japonicus* and *R. triquetrus*. Hence, the combination of both restriction fragment patterns discriminated all species except *R. squarrosus* and *R. subpinnatus*. NJ analysis of the ITS dataset suggested that the former two species formed a clade with *R. japonicus*, which was separated by a fairly long branch from a clade including *R. loreus* and *R. triquetrus* (Fig. 1a).

ISSR patterns using the R2 and R3 primers produced 13 and 8 scorable polymorphic fragments, respectively (Table 4). These patterns were consistently obtained in all the investigated populations of the different species and provided markers allowing the distinction of each individual species. NJ analysis of the ISSR dataset resulted in an almost star-like tree (Fig. 1b). Hence, combining the ITS and ISSR datasets resulted in a tree reflecting the structure of the ITS dataset, but where *R. subpinnatus* and *R. squarrosus* were distinct due to different ISSR patterns. The degree of genetic differentiation within the clades including *R. loreus* and *R. triquetrus* on the one

Table 4. Scoring of the band patterns consistently obtained in all the populations of each of the five species of *Rhytidiadelphus*. Fragments (a–m) that are polymorphic among species for each of the PCR-RFLP markers employing the Hae3 and Hin6 I restriction enzymes and ISSR primers R2 and R3 are scored as present (1) or absent (0).

	japonicus	*loreus*	*squarrosus*	*subpinnatus*	*triquetrus*
Hae3					
a	1	1	1	1	0
b	0	0	0	0	1
c	1	0	1	1	0
d	1	1	1	1	0
e	0	1	0	0	1
Hin6 I					
a	0	0	1	1	0
b	1	0	0	0	1
c	0	1	0	0	0
d	0	1	0	0	0
e	1	0	1	1	1
f	0	1	0	0	0
R2					
a	1	0	0	0	0
b	0	1	1	0	1
c	0	1	1	0	1
d	0	0	0	0	1
e	1	1	1	0	0
f	1	1	1	1	0
g	1	1	0	1	0
h	0	0	1	0	0
i	0	1	0	1	0
j	0	0	0	1	0
k	0	0	1	1	0
l	0	0	1	0	0
m	0	0	1	1	0
R3					
a	0	1	0	1	1
b	0	0	1	1	1
c	0	0	1	0	0
d	0	0	1	0	0
e	1	1	0	1	0
f	1	0	1	1	0
g	0	0	1	0	0
h	0	0	0	1	1

hand, and *R. subpinnatus* and *R. squarrosus* on the other, was similar.

Twenty-one morphological characters proved to be informative for species delineation (Tables 2, 3). These characters mostly concerned gametophyte habit and shape (Fig. 2), but characters of stem anatomy, leaf areolation pattern, and a few sporophytic characters also tended to differ among species. The NJ analysis of these characters resulted in a phenetic tree where *R. squarrosus* and *R. subpinnatus* formed a group distinct from *R. loreus* and *R. triquetrus*, with *R. japonicus* occupying an intermediate position between these two units (Fig. 1c).

The morphological character states were mapped on the phenetic tree of genetic similarity resulting from the NJ analysis of the combined ITS and ISSR markers (Fig. 1d). *Rhytidiadelphus squarrosus* and *R. subpinnatus* differ from the three other species by smooth, squarrose stem leaves with a clearly differentiated acumen and a 3–4 stratose calyptra. *Rhytidiadelphus subpinnatus* can be morphologically distinguished from *R. squarrosus* by a mostly prostrate and regularly branched habit, a triangular to cordate basal part of stem leaves, a 2–3 stratose stem cortex, narrow median leaf laminal cells ranging between 4.0 and 8.5 µm, a basal membrane of the endostome that is mostly less than 38% of total endostome height, and a seta that is frequently shorter than 20 mm. *Rhytidiadelphus loreus* and *R. triquetrus* morphologically differ from the other species of the genus by slightly to strongly falcate leaves that are gradually narrowed towards the apex, indistinctly differentiated alar groups, a 4–6 stratose calyptra, and a distance between transverse walls on outside of basal endostome membrane of more than 10 µm. *Rhytidiadelphus loreus* differs from *R. triquetrus* by a mostly prostrate, irregularly pinnately branched habitus, slightly denticulate to denticulate stem leaves that have an ovate to broadly ovate basis and are slightly constricted at insertion, as well as wide (6.0–9.5 µm) and long (52.0–128.0 µm) median leaf lamina cells, a recurved leaf margin near leaf insertion, a smooth costa, smooth median laminal cells, a cross-striolate lower outside part of the exostome, and the lower exostome border less than 20–25% of exostome width. *Rhytidiadelphus japonicus* differs from all the other species by short (24.0–77.5 µm) leaf lamina cells and shares a series of features with both the *squarrosus-subpinnatus* cluster (well differentiated alar groups and a distance between transverse walls on outside of basal endostome membrane up to 10 µm) and the *loreus-triquetrus* cluster (slightly to strongly plicate stem leaves in their basal portion).

DISCUSSION

In *Rhytidiadelphus*, ITS and ISSR markers were diagnostic for each of the five species and strongly support Koponen's (1971) species concept. The employed markers led to the recognition of two pairs of closely related species including *R. loreus* and *R. triquetrus* on the one hand and *R. squarrosus* and *R. subpinnatus* on the other. These species pairs are also supported by cytological data. *Rhytidiadelphus loreus* and *R. triquetrus* indeed possess 5–6 chromosomes whereas *R. squarrosus* and *R. subpinnatus* possess 6, 8 or 10 chromosomes (Fritsch, 1991). These species clusters also differ in several gametophytic characters, namely calyptra thickness and leaf stance, plication, and areolation.

Both ITS and ISSR markers distinguished *R. loreus* and *R. triquetrus*. The species were clearly morphologically delimited and differed by 12 of the 21 variable morphological characters. *Rhytidiadelphus subpinnatus* and *R. squarrosus* were indistinguishable based on the ITS

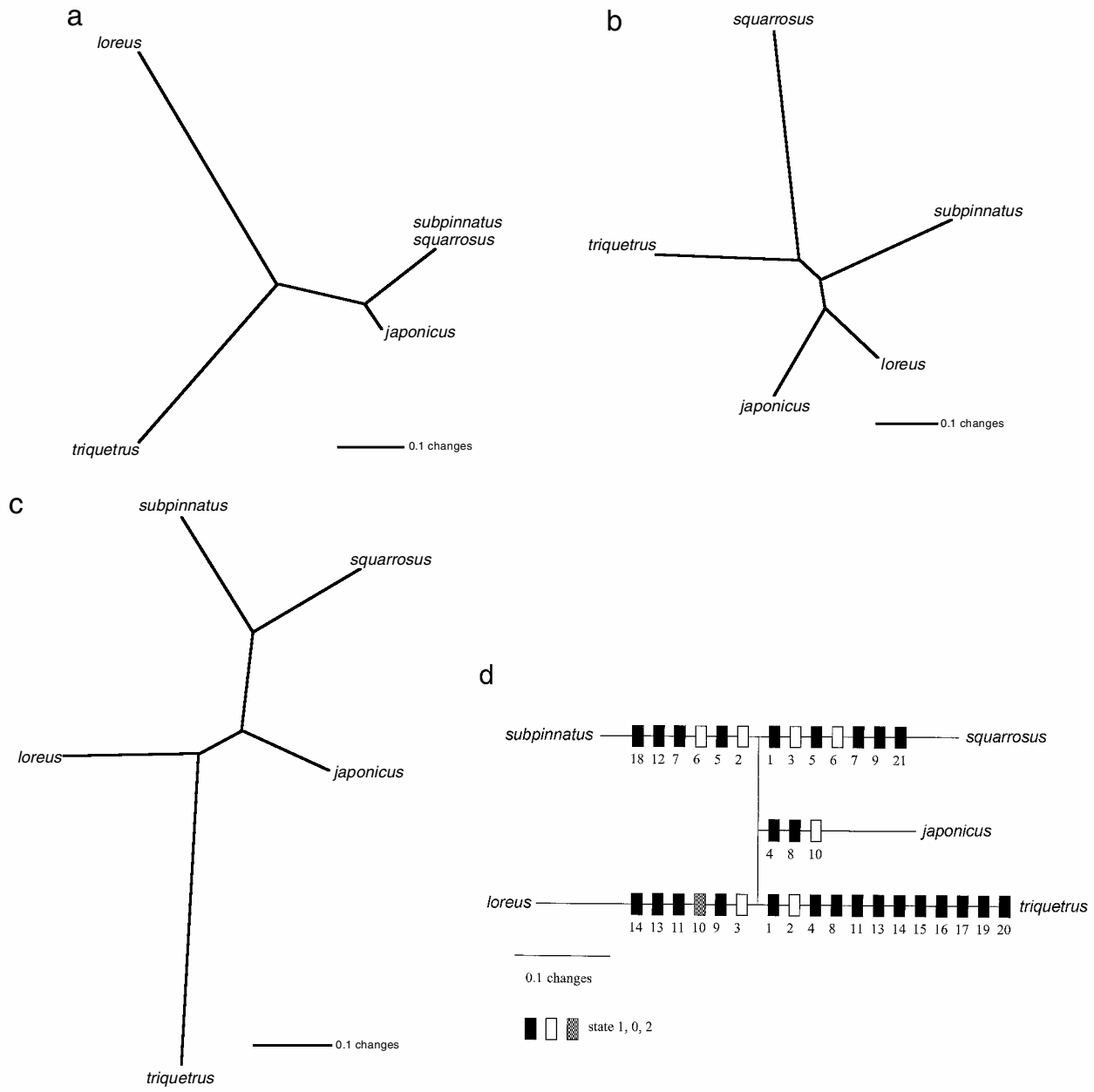

Fig. 1. Unrooted neighbor-joining trees resulting from the analysis of a, ITS, b, ISSR, and c, morphological datasets. d, morphological character states mapped on the phenetic tree of genetic similarity based on the neighbor-joining analysis of the combined polymorphic ITS and ISSR markers. See Table 2 for character numbers. Character states that are only present in one or two species are represented by bars on the species branches, the alternative states being present in the other species.

markers but displayed a clear genetic discontinuity based on the ISSR markers. Overall, genetic divergence was similar among *R. squarrosus* and *R. subpinnatus* on the one hand and *R. loreus* and *R. triquetrus* on the other. The results thus clearly suggest that *Rhytidiadelphus subpinnatus* deserves specific status.

Despite a clear genetic differentiation, morphological distinction among *R. subpinnatus* and *R. squarrosus* is rather subtle (Fig. 2). The species differed by seven of the 21 variable morphological characters. Two of these characters, however, belong to the sporophytic phase and are thus of limited taxonomic assistance in these dioecious, rarely fertile species. The diagnostic characters are mostly macroscopic features related to habit that may be difficult to define and are often prone to habitat variation. In particular, van der Hoeven & al. (1998) showed that

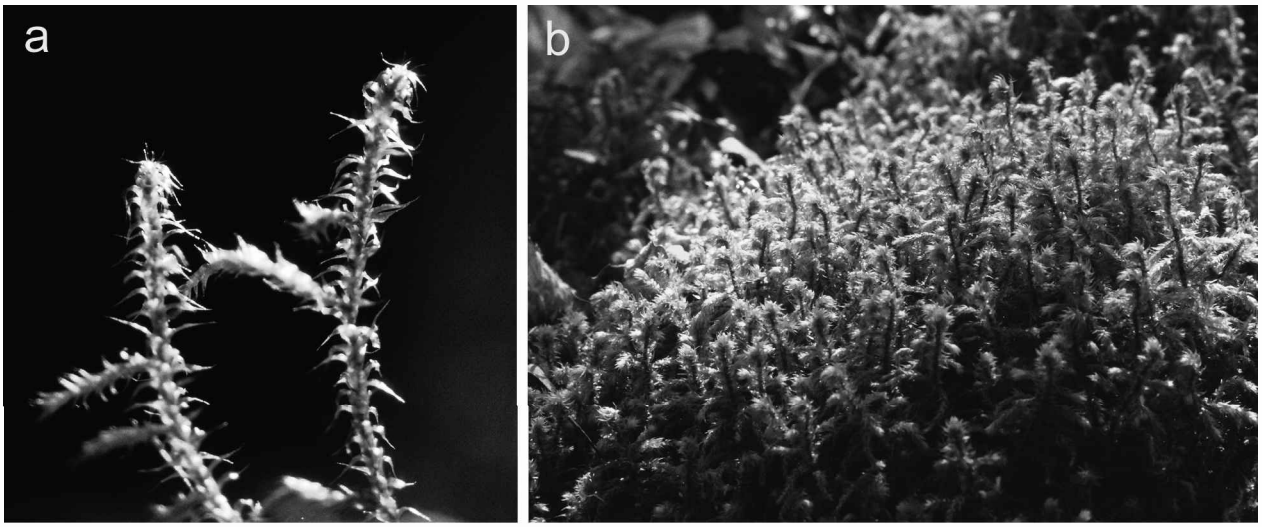

Fig. 2. *Rhytidiadelphus squarrosus* (a) and *R. triquetrus* (b) from Sweden, Södermanland. Both exhibit an erect habit but differ, in addition to microscopical features, by many macroscopical characters including branching pattern [irregularly branched (a) vs. more or less regularly pinnately branched (b)], stem leaf shape [with a clearly differentiated acumen (a) vs. gradually narrowed towards apex (b)], and stance [slightly to strongly squarrose (a) vs. slightly to strongly falcate (b)].

the habit of *R. squarrosus* is highly plastic depending on irradiance. Hence, *R. subpinnatus* has sometimes been interpreted as a morphological expression of *R. squarrosus* under wet and shaded conditions (Crum & Anderson, 1981; Anderson & al., 1990). Even the authors who maintain both taxa at the specific (Koponen, 1971; Smith, 1978; Schofield & Talbot, 1991, Noguchi, 1994; Blockeel & Long, 1998; Dirkse & al., 1999; Koperski & al., 2000) or subspecific level (Jensen, 1939) acknowledge that taxon identification can be difficult or even impossible due to the existence of a series of intermediates (Augier, 1966; Smith, 1978). Indeed, although typical expressions of *R. subpinnatus* are clearly different from *R. squarrosus* and remotely resemble *Hylocomium brevirostre* (Brid.) B. & S. (Touw & Rubers, 1989; Nebel & Philippi, 2000), some intergrading specimens with sub-ascendent shoots and ill-definable branching pattern are more difficult to identify. The analysis of species circumscriptions presented here, however, suggests that, in addition to the features documented in Koponen (1971), two features, including the shape of the stem leaves at base and the number of layers of the stem cortex, may assist with species identification when habit characters are insufficient. Thus, although habitat conditions may influence the appearance of the plants, the hypothesis that morphological variation among these two species only results from plasticity can be rejected.

The results suggest that ISSR markers may be an alternative for distinguishing among sibling moss species when traditional markers used at low taxonomic level do not provide the needed level of polymorphism. For instance, variation in ITS, the most variable region that has been commonly sequenced in mosses, exceedingly varies from one taxon to another, elucidating relationships from the population to the familial level depending on the investigated taxon (Shaw & al., 2002). In certain taxa, such as *Mielichhoferia* Hornsch. and *Fontinalis* Hedw., the ITS displayed enough variability to provide strong evidence for the existence of cryptic species in the absence of any morphological divergence (Shaw, 2000; Shaw & Allen, 2000). Similarly, ITS variation was sufficient to sink species whose morphological circumscription did not fit with the patterns of genetic variability in the genus *Leucobryum* Brid. (Vanderpoorten & al., in press). In certain other taxa, conversely, ITS variation was almost absent and the lack of sequence divergence has been sometimes used as evidence for the need of taxonomic rearrangements [e.g., the reduction of *Thamnobryum maderense* (Kindb.) Hedenäs as a variety of *T. alopecurum* (Hedw.) Nieuwl. (Stech & al., 2001)] or even reduction to synonymy [e.g., *Platyhypnidium mutatum* Ochyra & Vanderpoorten and *P. riparioides* (Hedw.) Dix. (Nebel & Philippi, 2000, based on Stech & Frahm, 1999); *Weymouthia billardieri* (Hamp.) Broth and *W. cochlearifolia* (Schwaegr.) Dix. (Quandt & al., 2001)]. The lack of sequence variation in ITS has also been interpreted as evidence for strain identity among disjunctly distributed populations from different environments of the moss, *Eurhynchium crassinervium* (Tayl.) B. & S. (Frahm & al., 2000). We believe that the lack of genetic variation within a small portion of the genome is not sufficient to support any reduction to synonymy, as markers

that display the relevant level of polymorphism are obviously needed to draw taxonomic conclusions from resolved and supported phylogenetic patterns. When morphological characters suggest that different taxa may be involved, the lack of differentiation in a limited set of molecular markers is thus not necessarily a reason to lump taxa together. Different moss species with a distinct morphological identity have been recognized despite the lack of variation in molecular markers [e.g., *Hypnum heseleri* Ando & Higuchi (van Zanten & Hofman, 1994); *Palustriella pluristratosa* Stech & Frahm (Stech & Frahm, 2001)]. In this context, highly polymorphic markers screening the whole genome such as ISSRs may provide additional information that can help in resolving such taxonomic issues.

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