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The variability of the papillae on the laminal cells of *Barbula indica* (Hook.) Spreng. (Pottiaceae, Musci): a morphological and molecular approach

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Abstract – Morphological investigations including Scanning Electron Microscopy show that *Barbula indica* varies greatly as regards the number, shape and size of its papillae. The validity of these characters for the correct identification of the species, especially in comparison with *B. bolleana*, is discussed based on morphological data and chloroplast *rps*4 gene and *rps*4-*trn*S interspacer sequences.

Musci / *Barbula indica* / *Barbula bolleana* / morphology / Northern Africa / Mauritania

Resumen – Datos morfológicos obtenidos con Microscopía Electrónica de Barrido, sugieren que *Barbula indica* es muy variable en el número, forma y tamaño de las papilas de la lámina del filidio. Basándose en caracteres morfológicos y en secuencias del gen *rps*4 y del espaciador intergénico *rps*4-*trn*S, se discute la validez de los caracteres de las papilas foliares para la correcta identificación de la especie, especialmente en comparación con *B. bolleana*.

Musgos / *Barbula indica* / *Barbula bolleana* / morfología / África del Norte / Mauritania

INTRODUCTION

Barbula indica (Hook.) Spreng. is a Pottiaceous moss species with a wide distribution, especially in the tropical and warm temperate areas of both hemispheres (Zander, 1994). It is very common in Asia (Gangulee, 1974; Saito, 1975; Eddy, 1988; Noguchi, 1988; Frey & Kürschner, 1991; Townsend, 1991; Li & Crosby, 2001), sub-Saharan Africa (Magill, 1981; O'Shea, 1999) and America (Zander, 1979, 1981, 1994). In Europe it is only known from Hungary (Düll, 1992) and in North Africa from Egypt (Ros *et al*., 1999), until its more recent finding in Mauritania

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(González-Mancebo & Ros, 2002). This last record was made in a desert area, the oasis of Tergit, where it grows along the margins of small streams and in soaked sandy soils, where both warm and cold water are found at different springs.

The papillosity of the laminal cells in the family Pottiaceae has long been a matter of discussion. According to some authors, this character is very important in the taxonomy of the family, and not only the number of papillae but also their length and shape should be considered when attempting to separate taxa at species level (Saito, 1975). Examples are *Tortula muralis* Hedw. and *T. baetica* (Casas & R. Oliva) J. Guerra & Ros (= *T. israelis* Bizot & F. Bilewski) (Guerra *et al*., 1992), *Syntrichia princeps* (De Not.) Mitt. and *S. echinata* (Schiffn.) Herrnst. & Ben-Sasson (Herrnstadt *et al*., 1982; Gallego, 2000), *Syntrichia ruralis* (Hedw.) F. Weber & D. Mohr and *S. papillosissima* (Copp.) Loeske (Gallego *et al*., 2002), *Syntrichia ruralis* var. *ruraliformis* (Besch.) Delogne and *S. subpapillosissima* (Bizot & R. B. Pierrot) M. T. Gallego & J. Guerra (Gallego *et al*., 2002), or *Syntrichia virescens* (De Not.) Ochyra and *S. minor* (Bizot) M. T. Gallego *et al.* (Gallego *et al*., 2000). In other cases, it is accepted that the papillosity may vary within a species, e.g. *Phascum cuspidatum* Hedw. (Guerra *et al*., 1991), *Tortula freibergii* Dixon & Loeske (Blockeel & Rumsey, 1990), *Pottia intermedia* (Turner) Fürnr., *P. lanceolata* (Hedw.) Müll. Hal. and *P. truncata* (Hedw.) Bruch & Schimp. (Casares-Gil, 1932).

The Mauritanian material was very difficult to identify because of its variable morphology, especially concerning the leaf cells, which vary from coarsely papillose, as has been described in *B. indica*, to smooth or nearly smooth. Consequently, the authors and some other specialists consulted initially identified the plants as *B. bolleana* (Müll. Hal.) Broth. This last species usually grows on moist or wet substrates, while the ecology of *Barbula indica* includes soil, clay, limestone, coral walls, roadbanks, riverbanks, walls, limepits and tree trunks (Zander, 1979).

Different authors (Saito, 1975; Zander, 1979) have described *Barbula indica* as a polymorphic species, which had already become obvious from its long list of synonyms from both the Old and New World, including combinations in other generic names such as *Hydrogonium* (Müll. Hal.) A. Jaeger, *Semibarbula* Herzog ex Hilp. and *Streblotrichum* P. Beauv. According to Saito (1975), *Barbula indica* is very variable when growing on moist or wet habitats. Also Zander (unpublished), in a provisional version of the Bryophyte Flora of North America, mentions that hygrophytic variants of *Barbula indica* may be confused with *B. bolleana* when incrusted with lime.

The question is whether the specimens from Tergit can be morphologically and molecularly distinguished from *Barbula bolleana*. To solve these questions we decided to carry out a molecular study by sequencing the *rps*4 chloroplastic gene and the *rps*4-*trn*S intergenic spacer of Mauritanian specimens, another typical specimen of *B. indica* from India and of *B. bolleana* from SE Spain. In addition, a morphological study of the papillosity of the upper laminal cells was made using Scanning Electron Microscopy (SEM).

MATERIAL AND METHODS

For the SEM study, the leaves of four specimens collected in the oasis of Tergit (Adrar massif, Mauritania) were used, as mentioned in González-Mancebo & Ros (2002): MUB 11605, 11606, 13135, 13137. These specimens were collected

on soaked soils along the margins of the streams and drier vertical walls. Also a typical, very papillose Indian sample of the species was used: MUB 12234.

For SEM observations of the leaves, the material was fixed in 3% glutaraldehyde with 0.1 M cacodylate buffer at 4º, washed in cacodylate and saccharose buffer, dehydrated in an increasing acetone gradient (30%, 50%, 70%, 90% and 100%), critical-point dried, gold-sputtered with a 200-300Å thick layer and analysed in a Jeol JSM-6100 scanning electron microscope under 10-15 KV.

For the molecular study, two specimens (MUB 11605 and MUB 11609) of *Barbula indica* from the Tergit oasis representing the most extreme cases of papillosity were selected for sequencing, while the specimen from India used for the SEM studies (MUB 12234) and one of *Barbula bolleana* from SE Spain (MUB 11931) were sequenced.

The following procedures were applied:

DNA isolation

Total DNA was extracted from dry material using the DNeasy Plant Mini Kit of Qiagen (Hilden, Germany). The DNA was eluted in 100 µl of 10 mM Tris-buffer (pH 8.5) and stored in the freezer until amplification.

Amplification

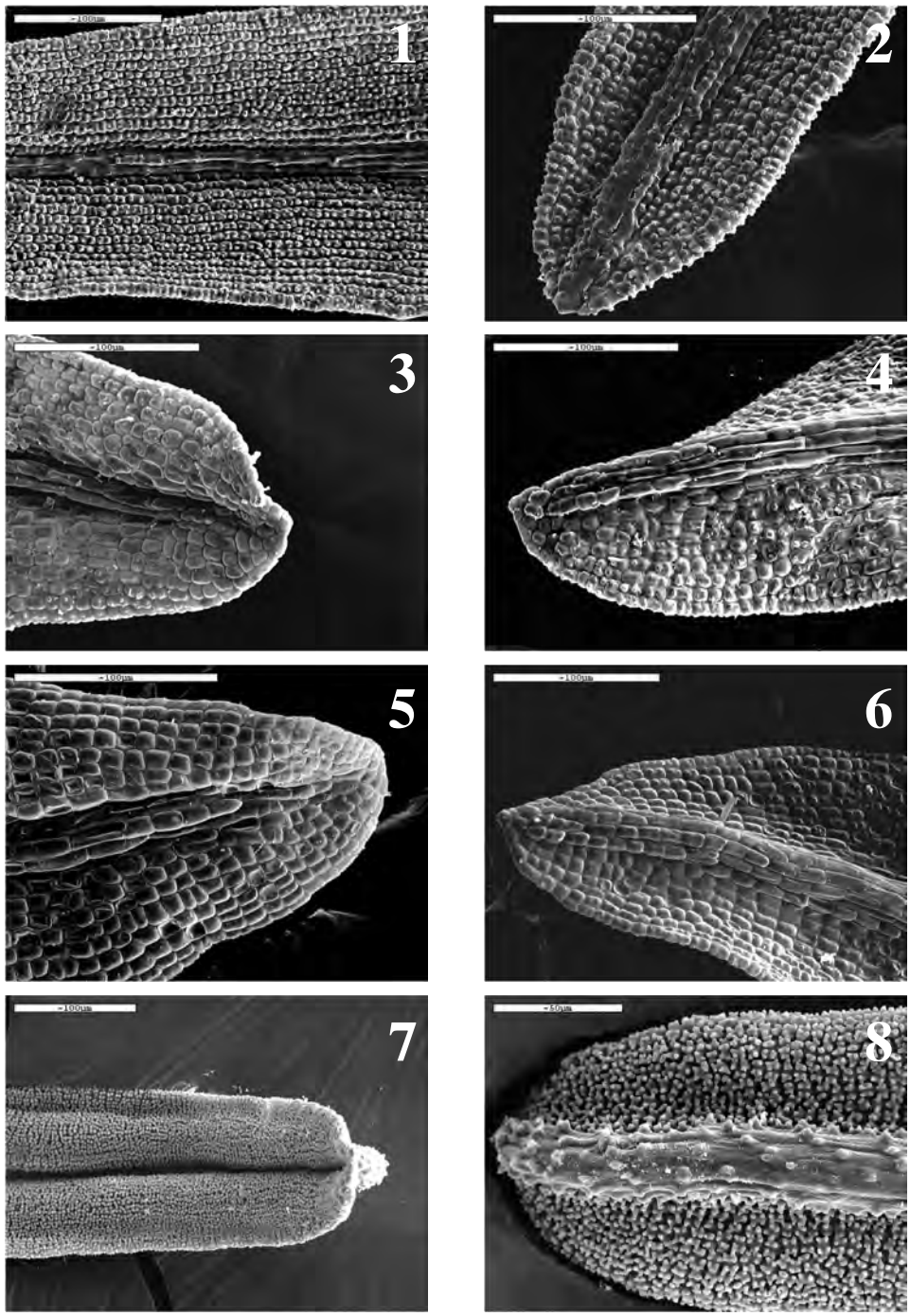
The chloroplast *rps*4 gene and the *rps*4-*trn*S intergenic spacer were amplified in a final volume of 50 µl with the primers rps5 (Nadot et al., 1994) and trnas (Buck *et al.*, 2000), adding. 1 µl of stock DNA as template. The amplification conditions were as follows: 3 min at 94ºC, 35 cycles of 15 sec at 94ºC, 30 sec at 50ºC and 1 min at 72ºC, and a final 7-min extension step at 72ºC. Amplification products were visualized on 8% PAA gels and the successful reactions were cleaned with the help of the High Pure PCR Product Purification Kit of Roche Molecular Biochemicals (Mannheim, Germany).

Sequencing and data analysis

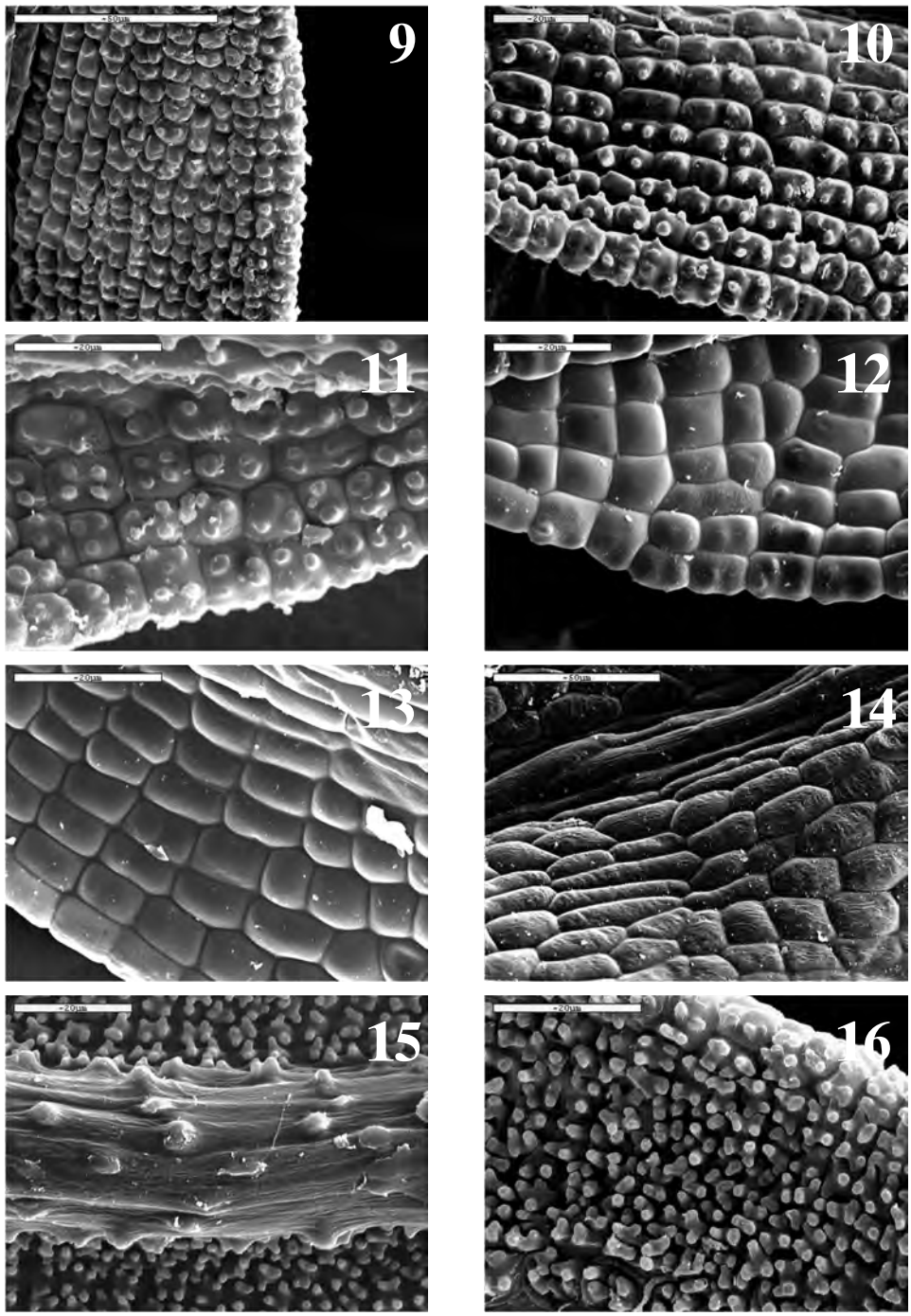
Cycle sequencing was performed with the Big Dyes Sequencing Kit (Perkin Elmer) using a standard protocol and the amplification primers. The annealing temperatures were set to 60ºC in the case of rps5 and 55ºC in the case of trnas. The reaction products were separated on an ABI Prism 3700 automatic sequencer (Perkin Elmer). The sequences were aligned manually.

RESULTS

The SEM study showed the upper laminal cells of the Mauritanian specimens of *Barbula indica* to range from almost smooth on soaked soils (Figs 3-6, 12-14) to very papillose with 1-4 simple or slightly bifurcate papillae per cell in the material from drier vertical walls (Figs 1-2, 9-11). The Indian specimen of *Barbula indica* was much more papillose, with 4-6 papillae per cell and clearly bifurcate (Figs 7, 8, 15, 16).



Figs 1-8. Lamina of the leaf of *Barbula indica* (SEM). **1-6.** Specimens from Mauritania. **7, 8.** Specimen from India. (Fig. 1: MUB 11606; Fig. 2: MUB 13135; Figs 3-6: MUB 11605; Figs 7, 8: MUB 12234).



Figs 9-16. Details of the papillosity in the upper laminal cells and the dorsal side of the nerve of *Barbula indica* (SEM). **9-14.** Specimens from Mauritania. **15, 16.** Specimen from India. (Figs 9, 11: MUB 13137; Fig. 10: MUB 13135; Figs 12, 13: MUB 11605; Fig. 14: MUB 11606; Figs 15, 16: MUB 12234).

The molecular study showed that *Barbula indica* can be easily distinguished from *Barbula bolleana* by its *rps*4 and *rps*4-*trn*S intergenic spacer sequences.

The aligned sequences had a length of 649 bp, 593 bp corresponding to the partial cds of the *rps*4 gene and 56 bp to the *rps*4-*trn*S spacer (Tabl. 1). The sequences of all three sequenced plants of *Barbula indica* were identical. *Barbula indica* and *B. bolleana* differed in two C <->T transitions and two G <->C transversions in the coding region. Three of these changes were nonsynonymous at the aminoacid-level. Furthermore, there were deletions in the noncoding spacer, affecting positions 603 and 612-638 in the case of *Barbula indica.*

DISCUSSION

The sequences of the two populations from Mauritania are identical with the sequence of the morphologically typical form of *Barbula indica* collected in India, and differ in six mutational steps from a morphologically typical sample of *Barbula bolleana* from Spain. The morphological characters of the Mauritanian specimens are similar to those found in the typical form of *B. indica* with the exception of papillosity and are slightly different from *B. bolleana.* Our conclusion is that the Mauritanian plants should be considered as *Barbula indica* and not as *B. bolleana*.

The degree of differentiation between different populations of *Barbula indica* in our sense cannot be estimated with the presented data. It is likely that different populations of *B. indica* could be separated by more variable genetic markers, for example the internal transcribed spacers of the nuclear ribosomal DNA, perhaps in combination with RAPD, ISSR or AFLP. Analyses of this type need a high number of samples, especially in the case of species with a very wide distribution such as *B. indica*.

We found a certain degree of sequence variation in the same chloroplast DNA regions of other Pottiaceae (*Crossidium seriatum* H.A. Crum & Steere, *Tortula muralis* Hedw. and *Tortula vahliana* (Schultz) Mont.) (Werner *et al*., unpublished data and Werner *et al*., 2002). On the other hand, no identical sequences were found in closely related species, for example in the aggregate of *Pottia lanceolata* (Hedw.) Müll. Hal. Therefore the variability of the *rps*4 gene and the contiguous spacer is, at least in these cases, sufficient for separating species.

Papillae on the leaf lamina varied widely in the populations analysed. They were more developed in the plants growing on vertical walls than on soaked soils, a finding that led us to the conclusion that the papillosity of this Pottiaceous species may be much more variable than expected. A relationship between papillosity and habitat conditions was observed by Chen (1941), who, while considering papillae and mamillae as important characters for taxon distinction at genus level, pointed out that morphological variations depend to a certain extent on the external conditions of the habitat, especially when the plants are immersed.

Tabl. 1. Comparison of chloroplast *rps*4 gene sequences in *Barbula bolleana* (GenBank accession number AF481033) and *Barbula indica* (GenBank accession numbers AF481034, AF481035, AF 481036). Sequence divergences are underlined and marked in bold type. ▶

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                . . . . | . . . . |. . . . | . . . . |. . . . | . . . . |. . . . | . . . . |. . . . | . . . . |
                        10        20        30        40        50
Barbula_bo      GACCTCGTCT AAGAATAATA CGCCGTTTAG GAACTTTACC AGGACTAAGC
Barbula_in      GACCTCGTGT AAGAATAATA CGCCGTTTAG GAACTTTACC AGGACTAAGC
                . . . . | . . . . |. . . . | . . . . |. . . . | . . . . |. . . . | . . . . |. . . . | . . . . |
                        60        70        80        90        100
Barbula_bo      AATAAAACAC CACATTTAAA ATCTAGTTCT ACTAATCAAT CAAGTTCTAA
Barbula_in      AATAAAACAC CACATTTAAA ATCTAGTTCT ATTAATCAAT CAAGTTCTAA
                . . . . | . . . . |. . . . | . . . . |. . . . | . . . . |. . . . | . . . . |. . . . | . . . . |
                        110       120       130       140       150
Barbula_bo      TAAAAAAATT TCTCAGTATC GCATTCGTTT AGAAGAAAAACAAAAATTGC
Barbula_in      TAAAAAAATT TCTCAGTATC GCATTCGTTT AGAAGAAAAACAAAAATTGC
                . . . . | . . . . |. . . . | . . . . |. . . . | . . . . |. . . . | . . . . |. . . . | . . . . |
                        160       170       180       190       200
Barbula_bo      GTTTTCATTA CGGAATAACA GAAAGACAATTACTAAATTA TGTACGTATT
Barbula_in      GTTTTCATTA CGGAATAACA GAAAGACAATTACTAAATTA TGTACGTATT
                . . . . | . . . . |. . . . | . . . . |. . . . | . . . . |. . . . | . . . . |. . . . | . . . . |
                        210       220       230       240       250
Barbula_bo      GCTAGAAAAGCAAAAGGATC AACAGGGTTA ATTTTATTAC AATTACTAGA
Barbula_in      GCTAGAAAAGCAAAAGGATC AACAGGGTTA ATTTTATTAC AATTACTAGA
                . . . . | . . . . |. . . . | . . . . |. . . . | . . . . |. . . . | . . . . |. . . . | . . . . |
                        260       270       280       290       300
Barbula_bo      AATGCGCTTA GATAACGTTA TTTTTCGATT AGGTATGGCT CCTACAATTC
Barbula_in      AATGCGTTTA GATAACGTTA TTTTTCGATT AGGTATGGCT CCTACAATTC
                . . . . | . . . . |. . . . | . . . . |. . . . | . . . . |. . . . | . . . . |. . . . | . . . . |
                        310       320       330       340       350
Barbula_bo      CTGGAGCAAG ACAATTAGTA AATCATAGAC ATATTTTAGT AAATAATCGC
Barbula_in      CTGGAGCAAG ACAATTAGTA AATCATAGAC ATATTTTAGT AAATAATCGC
                . . . . | . . . . |. . . . | . . . . |. . . . | . . . . |. . . . | . . . . |. . . . | . . . . |
                        360       370       380       390       400
Barbula_bo      ATAGTAAATA TTCCAAGTTA CCGTTGTAAA CCTCAGGATT TTATTACTAT
Barbula_in      ATAGTAAATA TTCCAAGTTA CCGTTGTAAA CCTGAGGATT TTATTACTAT
                . . . . | . . . . |. . . . | . . . . |. . . . | . . . . |. . . . | . . . . |. . . . | . . . . |
                        410       420       430       440       450
Barbula_bo      CAAAGATCGA AAAAAATCTC AAGTTATGGT TACTAAAAAT TTAAATTTTT
Barbula_in      CAAAGATCGA AAAAAATCTC AAGTTATGGT TACTAAAAAT TTAAATTTTT
                . . . . | . . . . |. . . . | . . . . |. . . . | . . . . |. . . . | . . . . |. . . . | . . . . |
                        460       470       480       490       500
Barbula_bo      CTCAAAAATC TAAAATACCA AATCATTTAA CTTTTAATTC TTTAGAAAAA
Barbula_in      CTCAAAAATC TAAAATACCA AATCATTTAA CTTTTAATTC TTTAGAAAAA
                . . . . | . . . . |. . . . | . . . . |. . . . | . . . . |. . . . | . . . . |. . . . | . . . . |
                        510       520       530       540       550
Barbula_bo      AAAGGACTAATTAATCAGAT ATTAGATCAA GAATCAATTG GTTTAAAAAT
Barbula_in      AAAGGACTAATTAATCAGAT ATTAGATCAA GAATCAATTG GTTTAAAAAT
                . . . . | . . . . |. . . . | . . . . |. . . . | . . . . |. . . . | . . . . |. . . . | . . . . |
                        560       570       580       590       600
Barbula_bo      AAACGAATTG TTAGTTGTAG AATATTATTC TCGTCAAGCT TAACTAAAAA
Barbula_in      AAACGAATTG TTAGTTGTAG AATATTATTC TCGTCAAGCT TAACTAAAAA
                . . . . | . . . . |. . . . | . . . . |. . . . | . . . . |. . . . | . . . .
                        610       620       630       640
Barbula_bo      ATTAAAAAAAATTGAAAATTT TTTTATGTAA AAAAAAAAT TTATAAAGAA
Barbula_in      AT-AAAAAAA T−−−−−−−− −−−−−−−−−−−−−−−−T TTATAAAGAA
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According to Zander (1979, 1981, unpublished data), the most relevant morphological features for distinguishing *B. indica* and *B. bolleana* are the upper laminal cells and the leaves. In *B. indica* the upper laminal cells are 7-12 µm wide, firm, quadrate, usually distinctly and strongly papillose, while the leaves are usually firm when wet. In *B. bolleana*, the cells are 11-15 µm wide, often lax, quadrate to rectangular, rarely or weakly papillose; its leaves are rather flaccid when wet. In spite of these differences, in the analysed material, only the width of the upper laminal cells suggested their attribution to *Barbula indica*. On this basis, *Barbula indica* shows a remarkable variability of leaf papillosity and the most important character for differentiating this species from *B. bolleana* is the width of the upper laminal cells. It might be necessary to re-evaluate the importance of this character, although its relative importance could well differ from one to another species.

On the basis of our data, it is not possible to conclude whether the leaf papillosity is determined by environmental or genetical factors in the case of *Barbula indica*. It might be that different allelic forms of the responsible genes are present in various populations (e.g. Mauritania). Alternatively, the genes controlling this character may allow strong modificatory adaptions in response to environmental conditions, although it will not be possible to answer this question without sequencing genes directly related to this character. For example, if leaf papillosity is genetically determined, and this gene is in linkage equilibrium with the genes used for sequencing, no correlation of the molecular markers and leaf papillosity, will be observed despite the fact that leaf papillosity is strictly genetically determined. This type of question is best answered by cultivating genetically identical plants under different environmental conditions.

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