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The Systematic Position of the Moss *Kingiobryum paramicola* (Pottiaceae) Based on Molecular and Morphological Data

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Abstract. *ITS1 and ITS2 sequences and a morphological analysis confirm the position of the Andean species* Kingiobryum paramicola *H. Rob. within the Pottiaceae and the genus* Didymodon. *It is closely related to* Didymodon australasiae *and* D. umbrosus. *A new combination is proposed:* Didymodon paramicola *(H. Rob.),* comb. nov.

Keywords. *Didymodon paramicola*, ITS, *Kingiobryum*, phylogenetic analysis, Pottiaceae.

Robinson (1967) in his studies on the Bryophytes of Colombia, described from the Colombia Eastern Cordillera, Dept. Cundinamarca, at 3,490 m elevation, the genus *Kingiobryum* that included the new species *K. paramicola* H. Rob. He placed it in the Dicranaceae. He considered his new genus to be most closely related to the group of genera including *Oncophorus* (Brid.) Brid., *Symblepharis* Mont., and *Holomitrium* Brid., and distinguishable from these genera and all other Dicranaceae by the presence of what may be called cancelline areas of enlarged, hyaline, porose cells in the leaf base that had been previously noted only in the Calymperaceae and Leucobryaceae. Florschütz-de-Waard and Florschütz (1979) compiled a list of the moss species known at that time in Colombia and included a new locality for *Kingiobryum paramicola* (Dept. of Meta). New collections bearing capsules from the State of Mérida in Venezuela allowed a more complete study of the taxonomy and ecology of the species (Zander & Cleef 1982). These authors made a detailed morphological description of the gametophyte and the sporophyte, because the original description of Robinson (1967) was brief and limited to the gametophyte. Placement in the Dicranaceae was questioned because of some morphological characteristics typical of the Pottiaceae, such as the papillose upper cells and the acid-base color reaction, which is similar to that of the genus *Erythrophyllopsis* Broth. The aperistomate nature of the capsules of the species prevented more accurate taxonomic placement at the time. They also pointed out that the porose basal cells observed by Robinson, besides appearing in the Calymperaceae and the Leucobryaceae, may also be present in the Pottiaceae and the Encalyptaceae, and mentioned that the intermediate position between Dicranaceae and Pottiaceae is also evident in other genera showing lanceolate leaves with sheathing leaf bases e.g., *Erythrophyllopsis*, *Leptodontium* (Müll. Hal.) Lindb., and *Rhexophyllum* Herzog in the Pottiaceae, and *Holomitrium*, *Oncophorus*, and *Symblepharis* in the Dicranaceae. The authors concluded that, in spite of the above mentioned similarities, the genus *Kingiobryum* should be retained in the Dicranaceae because of the following: ''stem hyalodermis of enlarged rectangular cells; perichaetial leaves smaller than the cauline leaves, oval, prosenchymatous throughout; cauline leaves with upper leaf cells moderately and evenly thickened, heterogeneous in shape; upper laminal papillae bifid and centered over the transverse walls; and costa in cross section with eight guide cells in one layer''. Churchill and Linares (1995) summarized the information concerning the species and pointed out that this Andean species grows on soil or thin layers of soil on rocks of the ''páramos'' from the Eastern Cordillera of Colombia and the Cordillera of Mérida of Venezuela, at 3,490–4,200 m elevation.

Zander (1993), in a re-investigation of the family placement, stated that slits formed by transverse resorption across the longitudinal walls of the medial basal cells, such as those of *Kingiobryum paramicola*, are also present in *Didymodon herzogii* R. H. Zander and *Gertrudiella validinervis* (Herzog) Broth. He emphasized that the latter two species are not closely related, and that further studies were needed to clarify whether *Kingiobryum* belongs to the Pottiaceae or the Dicranaceae. Another species that presents porose basal cells is *Didymodon challaense* R. H. Zander that was previously considered a member of the genus *Erythrophyllopsis* Broth. (Zander 1993).

It was La Farge et al. (2002) who first supported the hypothesis of Zander and Cleef (1982) and Zander (1993), and concluded that *Kingiobryum* be-

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longs to the Pottiaceae in a study of the circumscription of the Dicranaceae based on the chloroplast regions *trn*L-*trn*F and *rps*4. These authors sequenced a total of 84 species, among them, 50 species belonging to 29 genera selected as exemplars from the Dicranaceae sensu Vitt (1984). Exemplar taxa from the Bartramiaceae, Bryaceae, Encalyptaceae, Funariaceae, and Timmiaceae were selected as remote outgroup taxa in this analysis. The study clearly showed that *Kingiobryum* is not closely related to *Holomitrium*, *Oncophorus*, *Symblepharis*, or any other Dicranaceae. *Kingiobryum* was placed in a clade with *Tortula* and *Stegonia* with 100% bootstrap support.

Another study using chloroplast *rps*4 sequences of more than 50 species belonging to the family Pottiaceae confirmed that *Kingiobryum* is a member of this family (Werner et al. 2004). These authors recognized only three of the seven subfamilies proposed by Zander (1993): Merceyoideae, Trichostomoideae, and Pottioideae. The subfamily Timmielloideae was excluded from the Pottiaceae, while the subfamily Gertrudielloideae could not be studied because no plant material was available. The Merceyoideae (in the sense of Zander) are clearly polyphyletic. The genera *Barbula* Hedw. (partly), *Bryoerythrophyllum* P. C. Chen, *Dialytrichia* (Schimp.) Limpr., *Didymodon* Hedw., *Kingiobryum*, *Leptodontium*, *Pseudocrossidium* R. S. Williams, and *Triquetrella* Müll. Hal., all included by Zander in the Merceyoideae, were transferred to the Pottioideae. *Anoectangium*, Schwägr., *Barbula* (partly), *Gymnostomiella* M. Fleisch., *Gymnostomum* Nees & Hornsch., and *Hymenostylium* were included in the Trichostomoideae. Of these genera, only *Scopelophila* (Mitt.) Lindb. remained in the Merceyoideae. The *rps*4 data also suggested that the genus most closely related with *Kingiobryum* is *Didymodon* (Werner et al. 2004). But the insufficient resolution of the *rps*4 sequences and the low number of *Didymodon* species included did not allow the authors to draw final conclusions. Therefore, we decided to use the more variable nuclear ITS region to address this problem. Additionally, *Kingiobryum paramicola* is morphologically compared with other members of the genus *Didymodon*.

Material and Methods

Plant material.—A total of 22 accessions were used in the molecular part of this study, 12 belonging to the genus *Didymodon*, one to *Kingiobryum*, and nine to possible outgroup species. The outgroup species were selected according to previous results obtained during the study of a broader selection of *Didymodon* species. *Trichostomum unguiculatum* (Mitt.) R. H. Zander (subfamily Trichostomoideae) was used to root the trees. Details on geographic origin, voucher data, and GenBank accession numbers are given in Table 1.

For the morphological study, additional specimens were examined:

Kingiobryum paramicola.—COLOMBIA. Cundinamarca. Eastern Cordillera, Municipality of Calera, Hacienda La Siberia, Páramo de Palacio, 3,490 m, *King C-1015, Jaramillo-Mejia & Guevara* (holotype, US; isotype, NY). Meta. Páramo de Sumapaz, Cerro Nevado del Sumapaz, superpáramo del lado W. Lajas, 4,200 m, *Cleef 8073* (NY); lado SW, subida al Alto del Buque, 3,700 m, *Cleef 7691* (NY).

Didymodon herzogii.—BOLIVIA. Cochabamba. Chocayatal, 3,300 m, *Herzog 3606* (holotype of *Trichostomum ferrugineum* Herzog, JE).

Gertrudiella validinervis.—BOLIVIA. La Paz. In den Dornbuschsteppe des Palo, 1,600 m, *Herzog 4344* (holotype of *Gertrudia validinervis* Herzog, JE). Cordillera de Santa Cruz, in schattigen Felsen bei Tres Cruces, 1,400 m, *Herzog 3473* (JE).

DNA extraction.—Total DNA was extracted by the NaOH extraction method described by Werner et al. (2002). Five μl of the crude NaOH extract were diluted by the addition of 45 μl of 100 mM Tris–1 mM EDTA (pH 8.3) and stored frozen at −18°C until the PCR reaction was carried out.

DNA sequencing.—PCR reactions were performed in an Eppendorf Mastercycler using 4 μl of the DNA solution in 50 μl final volume. The reaction mix contained the primers 18S (5′-GGAGAAGTCGTAACAAGGTTTCCG-3′), designed by Spagnuolo et al. (1999) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′; White et al. 1990), at a final concentration of 400 μM, in the presence of 200 μM each of dNTP, 2 mM $MgCl_2$, 2 units Taq polymerase (Oncor Appligene), one μl BLOTTO (10% skimmed milk powder, 0.2% NaN_3 in water), and the buffer provided by the supplier of the enzyme. BLOTTO attenuates the inhibition of PCR by plant compounds (De Boer et al. 1995). Amplification started with 3 min denaturation at 94°C, followed by 35 cycles of 15 s at 94°C, 30 s at 50°C, and one min at 72°C. A final extension step of 7 min at 72°C completed the PCR. The primers were designed to amplify the last bases of the 18s ribosomal RNA gene, ITS-1, 5.8s rRNA gene, ITS-2, and the first few bases of the 26 rRNA gene. Five μl of the amplification products were visualized on a 6% polyacrylamide gel and successful amplifications were cleaned with the QIAquick purification kit (Qiagen). The amplification primers were used in the sequencing reactions with the Big Dye sequencing kit and separated on a ABI-Prism 3700 sequencing machine using standard protocols.

The sequences were aligned using CLUSTALX (Thompson et al. 1997) with the gap open penalty set to 10 and the gap extension penalty set to 0.1. BioEdit (Hall 1999) was used for minor manual adjustment of the alignment. An aligned matrix is available on request from the first author.

MEGA 2.1 (Kumar et al. 2001) was used for the Minimum Evolution (ME) analysis and PAUP* (Swofford 1998) with maximum parsimony (MP) as the optimality criterion. Gaps were excluded from the phylogenetic analysis. In the case of ME, the Kimura 2-parameter distance was used. The maximum number of trees to be retained at each step was set to 100 and the CNI search level to two.

In the case of MP, all characters were given equal weight and the heuristic search used the following settings: steepest descent off, TBR branch swapping, MULTREES on, and 100 random-sequence additions saving an unlimited number of trees per replicate.

Bootstrap analysis (Felsenstein 1985) was carried out

TABLE 1. Collection identification and GenBank accession numbers for the taxa included in the molecular analysis.

Species	Geographic Origin	Voucher Specimen	Genbank Accession No.
Barbula unguiculata Hedw.	Germany, Baden-Württemberg	MUB 10325	AY437129
Bryoerythrophyllum recurvirostrum (Hedw.) P. C. Chen	Italy, Alto Adige	MUB 15351	AY437130
Didymodon acutus (Brid.) K. Saito	Greece, Pelopónnisos	MUB 12954	AY437111
Didymodon australasiae (Hook. & Grev.) R. H. Zander	Morocco, Souss Massa-Draâ	MUB 13217	AY437121
Didymodon bistratosus Hébr. & R. B. Pierrot	Spain, Málaga	MUB 15415	AY437124
Didymodon cordatus Jur.	Spain, Lérida	MUB 8443	AY437115
Didymodon fallax (Hedw.) R. H. Zander	Spain, Balear Islands	MUB 13428	AY437099
Didymodon luridus Hornsch.	Greece, Central Greece and Euboea	MUB 12783	AY437098
Didymodon rigidulus Hedw.	United Kingdom, England	Herb. Blockeel (*Blockeel* 13.06.02)	AY437106
Didymodon sinuosus (Mitt.) Delogne	Italy, Sicily	MUB 13654	AY437090
Didymodon tophaceus (Brid.) Lisa	Greece, Central Greece and Euboea	Herb. Blockeel 29/097	AY437093
Didymodon umbrosus (Müll. Hal.) R. H. Zander	France, Alps Maritimes	Herb. R. Skrzypczak 95600	AY437117
Didymodon vinealis (Brid.) R. H. Zander	Spain, Balear Islands	MUB 13438	AY437103
Kingiobryum paramicola H. Rob.	Venezuela, Mérida	NY 635286	AY437122
Leptophascum leptophyllum (Müll. Hal.) J. Guerra & M. J. Cano	Spain, Murcia	MUB 10427	AY437134
Pseudocrossidium hornschuchianum (Schultz) R. H. Zander	Spain, Almería	MUB 9053	AY437128
Tortula inermis (Brid.) Mont.	Greece, Steréa Ellas	MUB 14049	AY437133
Tortula muralis Hedw.	Yugoslavia, Serbia	MUB 13827	AY437132
Trichostomum unguiculatum (Mitt.) R. H. Zander	South Africa, Western Cape Province	MUB 12254	AY437127
Triquetrella arapilensis Luisier	Spain, Ciudad Real	MUB 6465	AY437126
Triquetrella tristicha (Müll. Hal.) Müll. Hall.	South Africa, Western Cape province	MUB 12218	AY437125

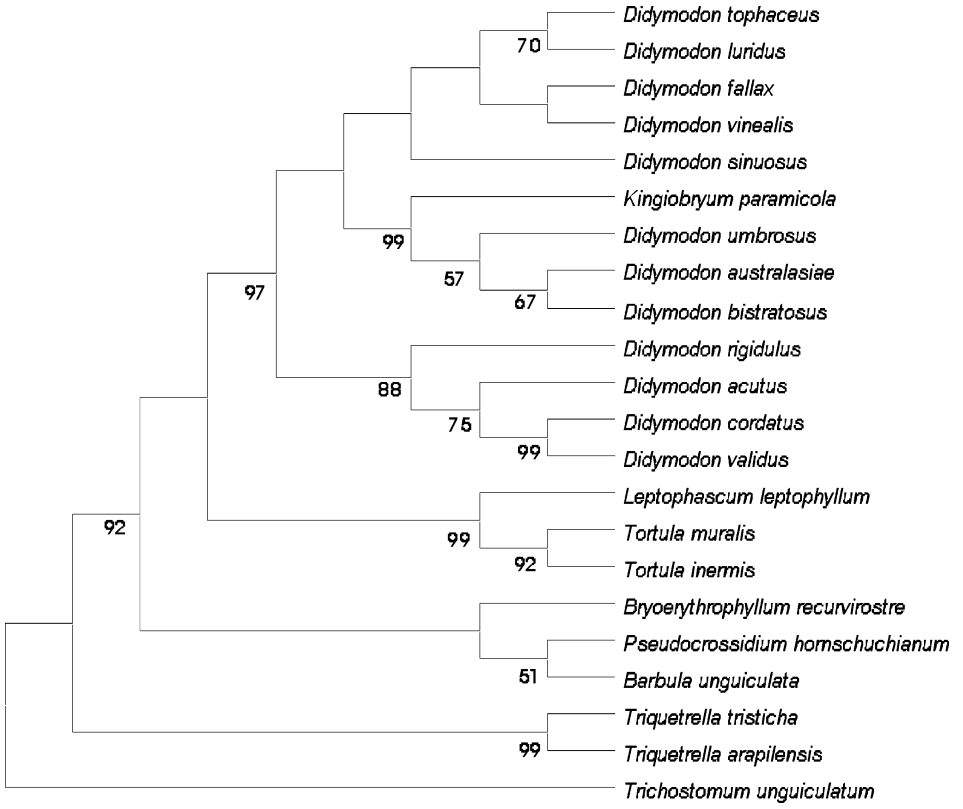

FIGURE 1. One of two most parsimonious MP cladograms (221 steps; CI = 0.507; RI = 0.693). The genus *Didymodon* forms a monophyletic clade and *Kingiobryum paramicola* occupies a position near *Didymodon australasiae* and *Didymodon umbrosus*. Bootstrap support values (1,000 replicates) are given below the clades.

with 1,000 replicates and identical settings for both ME and MP.

RESULTS

DNA sequencing.—The complete region of the 18S rRNA gene (partial sequence)-ITS1 - 5.8S rRNA - ITS2 26S rRNA gene (partial sequence) could be sequenced for all accessions. The length of the region ranged from 735 to 1044 base pairs (bp). The shortest sequence corresponded to *Didymodon sinuosus* (Mitt.) Delogne and the longest sequence to *D. bistratosus* Hébr. & R. B. Pierrot. The partial sequence of the 18S rRNA gene had a uniform length of 26 bp. The ITS1 region was highly variable and contained multiple indels and ranged from 233 bp in *Trichostomum unguiculatum* to 551 bp in *Didymodon bistratosus*. Within the genus *Didymodon*, *D. sinuosus* showed the shortest ITS1 region with 244 bp. The 5.8S rRNA gene had the same length in all accessions (159 bp). The ITS2 region was less variable in length than the ITS1 region, measuring between 264 bp (*Trichostomum unguiculatum*) and 305 bp (*Pseudocrossidium hornschuchianum* (Schultz) R. H. Zander). *Didymodon australasiae* (Hook. & Grev.) R. H. Zander had the longest ITS2 region within this genus with 292 bp and *Didymodon acutus* (Brid.) K. Saito the shortest (273 bp). The alignment had a total length of 1,412 bases.

In all, 893 positions of the aligned sequences were gapped sites and were excluded from further analysis. Of the remaining sites, 397 were constant, 35 variable but parsimony-uninformative and 87 parsimony-informative.

The MP search revealed two most parsimonious trees of 221 steps, CI = 0.507, RI = 0.693 (Fig. 1). The ME criterion resulted in a similar tree with almost identical topology (Fig. 2). The *Didymodon* clade, in both cases, is well supported with bootstrap values of 97% (MP) and 98% (ME). Within *Didymodon*, there are two clades that are probably monophyletic, one of them formed by *D. acutus*, *D. cordatus* Jur., *D. rigidulus* Hedw., and *D. validus* Limpr. (88% bootstrap support with MP and 97% with ME), and the other by *D. australasiae*, *D. bistratosus*, *D. umbrosus* (Müll. Hal.) R. H. Zander, and *Kingiobryum paramicola* (99% bootstrap support with MP and 100% with ME). The position of the remaining species of *Didymodon* is not well resolved and at present it is not clear whether this

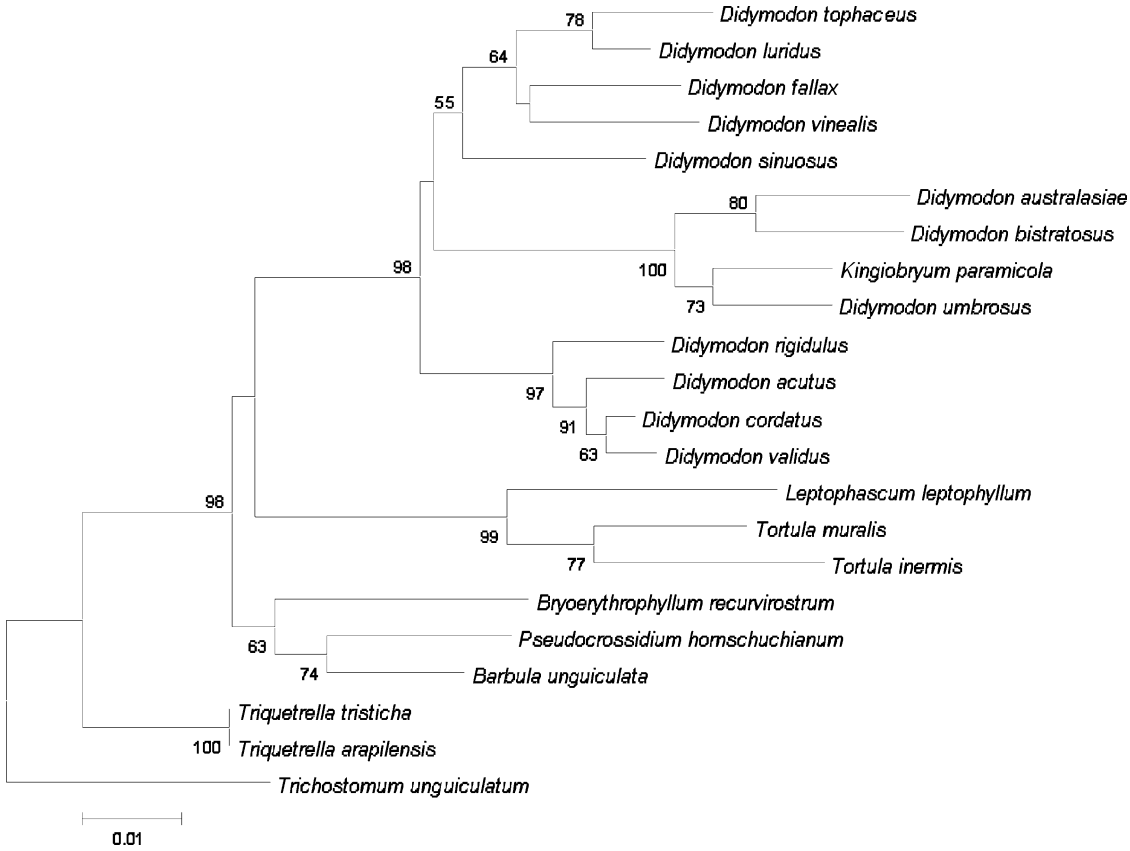

FIGURE 2. ME phylogram. This analysis confirms the monophyly of the genus *Didymodon*. Here, *Kingiobryum paramicola* seems to be more closely related to *Didymodon umbrosus*. Bootstrap support values (1,000 replicates) are given below the clades.

group of species is monophyletic, as suggested by ME, or not.

Also, many morphological similarities were observed between *Kingiobryum paramicola* and some species of the genus *Didymodon*. According to our own observations and the precise description of Zander and Cleef (1982), this species possesses the uniseriate axillary hairs of the leaves, composed of 1–2 brownish basal cells with the remaining 5–7 cells hyaline; this is the most important morphological feature to characterize *Didymodon*. Other morphological characteristics of this genus include laminal cells of leaves well defined in surface view, mostly quadrate to short-oblong cells of the abaxial surface of the costa (Saito 1975), mostly lanceolate to longly lanceolate leaves, and the usually green, shortly rectangular, poorly differentiated basal laminal cells (Zander 1993). The genus *Erythrophyllopsis* can be distinguished from *Kingiobryum paramicola* by bistratose leaf laminae with plane margins; absence of pores in the basal cells; elongate cells on the abaxial face of the costa and on the adaxial face only in the upper ¼ of the leaf; and rudimentary or short peristome.

The most closely related species to *Kingiobryum paramicola* from a morphological point of view are *Didymodon australasiae* and *D. umbrosus*. These three species share most of the characteristics described by Zander (1993) for *Didymodon* sect. Asteriscium—occasional stem hyalodermis; leaves spreading to squarrose when moist from a sheathing base, broadly channeled on the adaxial surface; leaf margins not decurrent, plane to broadly recurved above the sheathing base, bistratose above midleaf; costa ending below the apex to shortly excurrent, adaxial surface convex, with quadrate to elongate cells; upper laminal cells unistratose to bistratose, papillae absent to large, simple to bifid, 1–4 per lumen; transverse section of costa often with hydroids; and KOH color reaction yellow to yellowish orange. The only discrepancy with the set of characteristics proposed by Zander (1993) is the development of the adaxial stereid band in the transverse section of the costa, which, according to Zander (1993), is absent or very small in *Didymodon* sect. Asteriscium, but strongly developed in *Kingiobryum paramicola*.

Discussion

The genus *Didymodon*, as defined by Zander (1978, 1981, 1993), Kučera (2000, 2002) and Jiménez (2003) using morphological characters, has been confirmed as monophyletic within the Pottioideae based on molecular markers (Werner, pers. comm.). According to the nuclear rDNA sequence data presented here, *Kingiobryum paramicola* is placed, with good support, within the genus *Didymodon* by both methods used in this paper for phylogenetic interference. Its closest relatives are a group of species formed by *D. australasiae*, *D. bistratosus*, and *D. umbrosus*. Chloroplast *rps4* data (Werner et al. 2004) also suggest these relationships, but the slower evolution of this region, together with the reduced number of *Didymodon* species included in this study, did not permit a definitive conclusion to be reached about the taxonomic status of *Kingiobryum*. However, of the four species of *Didymodon* included (*D. giganteus* (Funck) Jur., *D. luridus*, *D. sinuosus*, and *D. australasiae*), it was *D. australasiae* that was placed with bootstrap values of 99% (Neighbor-Joining) and 100% (MP) in a monophyletic clade together with *Kingiobryum paramicola*. Based on the *rps4* data, *Kingiobryum paramicola* is not very closely related to *Erythrophyllopsis*. Instead, *E. fuscula* (Müll. Hal.) Hilp. is placed in a clade with *Erythrophyllastrum andinum* (Sull.) R. H. Zander and *Barbula unguiculata* Hedw.

The morphological characteristics that led Robinson (1967) to consider *Kingiobryum paramicola* as a new species were the basal laminal cells differentiated medially, with porose walls. Although uncommon, at least within the family Pottiaceae, these features are present, as mentioned previously, in another two *Didymodon* species. *Didymodon herzogii* was included by Hilpert (1933) in the genus *Gertrudiella* Herzog as *G. ferruginea* Hilp. (basionym: *Trichostomum ferrugineum*, *nom. illeg.*), as well as *G. validinervis*, both of them only known from the Andes. In this work, Hilpert considered that the genus *Gertrudiella* is closely related to the genus *Asteriscium* (Müll. Hal.) Hilp. *nom. illegitimate* [= *Didymodon* sect. Asteriscium (Müll. Hal.) R. H. Zander] because of strongly papillose upper laminal cells, anatomy of the leaf costa, leaf shape, upper laminal cells, thin-walled, long rectangular basal laminal cells, and pluristratose leaf margins (this last characteristic absent from *G. validinervis*). These affinities between the genera *Gertrudiella* and *Asteriscium* described by Hilpert (1933), were later partly corroborated by Zander (1993), who placed *G. ferruginea* in the genus *Didymodon* section Vinealis (Steere) R. H. Zander with a new name: *D. herzogii*, but keeping *G. validinervis* as the only species belonging to this genus. After a study of the type specimens of both species, we have observed that they are morphologically similar to *Kingiobryum paramicola*, although some differences are observed: the adaxial stereid band of the costa section is present in *K. paramicola* but absent from the other species; the number of guide cell layers in the costa section is one in *K. paramicola*, two in *D. herzogii* and four to five in *G. validinervis*; the papillosity in the upper laminal cells is absent or slight in *K. paramicola* and *Gertrudiella validinervis*, but abundant, simple or bifid in *D. herzogii*; and the margins of the leaf are narrowly recurved and bistratose in *K. paramicola*, plane and bistratose in *D. herzogii* and revolute and unistratose in *G. validinervis*. We conclude that the three species share an important set of features and that the differences are within the usual range of variation for related species; furthermore, the segregation of *G. validinervis* into a different genus considered to form an independent subfamily, Gertrudielloideae R. H. Zander, should be more carefully studied. Unfortunately, we have not been able to find appropiate material to be included in this molecular study.

Didymodon challaense, also known from the Andes cordillera, is closely related to *Kingiobryum paramicola*, not only in the porose basal cells, but also the hyaline sheathing leaf base and the bistratose leaf margins—this probably led Zander (1993) to include it within the section Asteriscium of *Didymodon*.

The following nomenclatural change is proposed:

Didymodon paramicola (H. Rob.), *comb. nov.*

Kingiobryum paramicola H. Rob., The Bryologist 70: 9. 1967. Type: ''Colombia. Eastern Cordillera, Department of Cundinamarca, Municipality of Calera, Hacienda La Siberia, Páramo de Palacio, el. 3490 m'' *King C-1015, Jaramillo-Mejia & Guevara* (holotype, US!; isotypes, COL, NY!, U).

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