Universidade de Brasília

INSTITUTO DE CIÊNCIAS BIOLÓGICAS
DEPARTAMENTO DE BOTÂNICA
PROGRAMA DE PÓS-GRADUAÇÃO EM BOTÂNICA

DNA Barcode dos Gêneros *Schlotheimia* Brid. e *Macromitrium* Brid. para o Brasil

DAIANE VALENTE VALENTE

Brasília, fevereiro de 2020.

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AGRADECIMENTOS

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DNA Barcode dos Gêneros *Schlotheimia* Brid. e *Macromitrium* Brid. para o Brasil

Resumo

DNA Barcoding é um método molecular utilizado para facilitar a identificação de espécies, que consiste em obter sequências curtas de DNA a partir de uma região padronizada do genoma, que atenda aos seguintes critérios: variabilidade genética suficiente a nível das espécies, sequência curta para facilitar a extração e amplificação de DNA e presença de regiões conservadas para o desenvolvimento de *primers*. Para elaborar uma estratégia de DNA Barcoding para musgos utilizamos dois gêneros da família Orthotrichaceae: (i) *Schlotheimia*, devido a identificação morfológica das espécies somente ser possível se a planta estiver fértil e (ii) *Macromitrium*, devido a sua complexidade morfológica, e a falta de conhecimento sobre a real diversidade brasileira desse grupo. Este trabalho teve como objetivo principal elaborar uma estratégia de DNA Barcoding ou ferramenta molecular para identificação das espécies dos gêneros *Schlotheimia* e *Macromitrium,* testando 3 marcadores moleculares (*trn*L-F, *trn*G-R e ITS) visando à solução de possíveis problemas taxonômicos, contribuindo com dados moleculares para o desenvolvimento futuro de trabalhos em larga escala de DNA Barcoding. Como objetivo secundário, verificar os 64 nomes de *Macromitrium* citados para o Brasil (20 nomes aceitos e 44 nomes excluídos da flora do Brasil) buscando esclarecer sua validade e identificação correta, contribuindo para o conhecimento da diversidade de espécies de *Macromitrium* ocorrentes no Brasil. Para DNA Barcoding foram utilizadas 87 sequências para *Schlotheimia* e 108 para *Macromitrium.* Para morfologia e filogenia de *Macromitrium* foram analisados o material typus para 64 espécies e utilizadas 111 sequências de 4 marcadores moleculares (*trn*L-F, *rps*4, *nad*5 e 26S), respectivamente. Foram realizadas análises de máxima parcimônia, máxima verossimilhança, inferência bayesiana, neighbour-joining, Automatic Barcode Gap Discovery (ABGD) e variação intra e interespecífica. Nossos dados evidenciaram que o gênero *Schlotheimia* é monofilético. Já o gênero *Macromitrium* não é um grupo monofilético ocorrendo a formação de três grupos diferentes: MG1 (gênero *Macromitrium* verdadeiro), MG2 (novo gênero *Pseudomacromitrium*) e MG3 (novo gênero *Aureomacromitrium*). O melhor candidato a marcador de DNA Barcoding foi *trn*G-R devido à sua fácil amplificação, boa qualidade das sequências e capacidade de discriminação das espécies de ambos os gêneros. O marcador nuclear ITS foi fácil de amplificar e apresentou maior variação em relação aos marcadores plastidiais, porém foi difícil de alinhar e apresentou sequências de baixa qualidade devido a trechos de polinucleotídeos ou contaminação por fungos. *Trn*L-F teve a pior performance entre os marcadores testados, apresentando baixo potencial de discriminação para todos os grupos. Em contrapartida foi um marcador fácil de amplificar, com sequências de boa qualidade. Nossos dados contribuíram para re-circunscrição de *Macromitrium*, descrição de dois novos gêneros e conhecimento da real diversidade brasileira, onde das 64 espécies listadas para o Brasil, 22% são boas espécies, 53% são sinônimos de outras espécies, 16% são excluídos da flora brasileira e 9% não foram possíveis verificar. Com isso, ocorreu uma redução do número de espécies de 20 para 14, sendo que três dessas são conhecidas somente pelo typus, podendo ter sido extintas. Com relação á *Schlotheimia* ocorreu redução de 13 para 11 espécies e os dados serviram de base para a revisão taxonômica do gênero, além de ser uma importante ferramenta para a identificação das espécies.

Palavras-chave: marcadores moleculares, identificação, taxonomia, Orthotrichaceae, musgos.

DNA Barcode of the Genera *Schlotheimia* Brid. and *Macromitrium* Brid. to Brazil

Abstract

DNA Barcoding is a molecular method used to facilitate species identification, which consists of obtaining short DNA sequences from a standardized region of the genome that meets the following criteria: sufficient genetic variability at species level, short sequence to facilitate DNA extraction and amplification and presence of conserved regions for primer development. To elaborate a DNA barcoding strategy for mosses use two genera of Orthotrichaceae family: (i) *Schlotheimia* due to morphological identification of species only be possible if the plant is fertile and (ii) *Macromitrium* due to the complex morphology and lack of knowledge of the Brazilian real diversity. The main objective of this work was to elaborate a Dna Barcoding strategy or molecular tool for the identification of *Schlotheimia* and *Macromitrium* species, testing three molecular markers (*trn*L-F, *trn*G-R and ITS) aiming at solving possible taxonomic problems, contributing to molecular data for the future development work in large-scale DNA Barcoding. As a secondary objective, to verify the 64 names of Brazilian *Macromitrium* (20 accepted names and 44 names excluded from the Brazilian flora) in order to clarify their validity and correct identification, contributing to the knowledge of the diversity of *Macromitrium* species occurring in Brazil. For DNA barcoding, a sampling of 87 sequences for *Schlotheimia* and 108 for *Macromitrium* was used. For morphology and phylogeny of *Macromitrium* the typus material was analyzed for 64 species and 111 sequences of 4 molecular markers (*trn*L-F, *rps*4, *nad*5 and 26S) were used. Maximum parsimony, maximum likelihood, Bayesian inference, neighbor-joining, Automatic Barcode Gap Discovery (ABGD) and intra and interspecific variation analyzes were performed. Our data showed that *Schlotheimia* is monophyletic and *Macromitrium* not monophyletic, occurred with the formation of three different groups: MG1 (true *Macromitrium* genus), MG2 (new genus, *Pseudomacromitrium*) and MG3 (*Aureomacromitrium*, new monospecific genus). The best candidate for DNA Barcoding marker was *trn*G-R due to its easy amplification, good sequence quality and species discrimination ability of both genera. The ITS nuclear marker was easy to amplify and showed greater variation than plastid markers, but was difficult to align and presented poor quality sequences due to polynucleotide stretches or fungal contamination. *Trn*L-F had the worst performance among the markers tested, presenting low discrimination potential for all groups. In contrast, it was an easy-to-amplify marker with good quality sequences. Our data contributed to *Macromitrium* re-circumscription, description of two new genera and knowledge of the real Brazilian diversity, where of the 64 species listed for Brazil, 22% are good species 53% are synonymous with other species; 16% are excluded from the Brazilian flora, and 9% could not be verified. Thus, there was a reduction in the number of species from 20 to 14, and three of these are known only by typus, and may have been extinct. Regarding to *Schlotheimia* there was a reduction from 13 to 11 species and the data served as the basis for the taxonomic revision of the genus, besides being an important tool for species identification.

Key words: molecular markers, identification, taxonomy, Orthotrichaceae, mosses.

1 INTRODUÇÃO

Briófitas são plantas avasculares, cujos estudos moleculares demonstram estarem representadas em três divisões: Marchantiophyta (hepáticas), Anthocerotophyta (Antóceros) e Bryophyta (musgos) (Stech & Frey 2008; Goffinet & Shaw 2009). Representam o segundo maior grupo de plantas terrestres, apresentando entre 15.000-18.000 espécies no mundo (Gradstein *et al.* 2001; Shaw & Goffinet 2000). No Brasil foram catalogadas 1.570 espécies, das quais 331 são endêmicas. Desse total de espécies conhecidas no país, 882 são de musgos (Flora do brasil online, http://floradobrasil.jbrj.gov.br/).

Apesar do levantamento das espécies brasileiras (Costa & Luizi-Ponzo 2010; Costa *et al.* 2011; Costa & Peralta 2015), a identificação correta desses grupos é uma problemática constante e entrave para muitos estudos, pois as briófitas são muitas vezes consideradas difíceis de identificar em nível de espécie, devido ao seu tamanho pequeno, caracteres morfológicos pouco conspícuos e considerável plasticidade morfológica em resposta a fatores ambientais, tornando necessária nova abordagem para o reconhecimento de táxons (Bergamini & Peintinger 2002; Hebert *et al.* 2003; Buryová & Shaw 2005; Hassel *et al.* 2005).

Sistemas de identificação molecular, através da análise de um pequeno segmento do genoma, representam uma abordagem extremamente promissora para o diagnóstico da diversidade biológica (Herbert *et al.* 2003). Esse método molecular para identificação de espécies conhecido como "DNA *Barcoding"* consiste em sequências curtas de DNA a partir de uma região padronizada do genoma que atenda aos seguintes critérios: variabilidade genética suficiente a nível das espécies, sequência curta para facilitar a extração e amplificação de DNA e presença de regiões conservadas para o desenvolvimento de primers (Kress *et al.* 2005). Esta técnica tem sido considerada uma ferramenta viável para recorrentes problemas de identificação com animais, porém para plantas a ainda precisa ser aprimorada (Hebert *et al.* 2003).

No Brasil, foram desenvolvidos ensaios de DNA *barcoding* somente para identificação de plantas com flores (Gonzalez *et al.* 2013; Vivas *et al.* 2014; Palhares *et al.* 2015; Bolson *et al.* 2015; Silva *et al.* 2015; Rivera-Jiménez *et al.* 2017). Estudos com briófitas, no entanto, estão apenas começando (Dantas *et al.* 2018), sendo esse trabalho o primeiro de DNA *Barcoding* de briófitas para o Brasil.

Considerando o desenvolvimento de novas ferramentas de fácil identificação para briófitas, foi elaborado um projeto pioneiro de *Barcoding* para briófitas do Brasil em parceria com o *Naturalis Biodiversity Center* da Holanda, intitulado "DNA Barcoding of Brazilian bryophytes – A case study to improve the identification of tropical bryophyte species",

aprovado através do edital 71/2013 do MEC/MCTI/CAPES/CNPq/FAPs. O presente trabalho faz parte desse projeto maior, onde estão sendo investigados os gêneros *Schlotheimia* Brid. e *Macromitrium* (Brid.), ambos pertencentes a família Orthotrichaceae.

Schlotheimia é um gênero com distribuição pantropical com aproximadamente 120 espécies (Frey & Stech 2009). No Neotrópico são registradas 56 espécies, onde estima-se que provavelmente menos de 30 sejam realmente confirmadas (Gradstein *et al.* 2001). De acordo com a Flora do Brasil (2020) são reconhecidas 12 espécies de *Schlotheimia* das quais cinco são endêmicas (Costa & Peralta 2015). A escolha desse gênero foi devido a identificação morfológica de algumas espécies de *Schlotheimia* requererem o estudo do esporófito (Peralta *et al.* 2020 dados não publicados) condição essa, nem sempre encontrada nas plantas em campo ou herbários.

Macromitrium é um gênero com aproximadamente 350 espécies, amplamente distribuídas em regiões tropicais e subtropicais (Vitt & Ramsay 1985a,b). No Neotrópico, são estimadas cerca de 125 espécies para o gênero (Gradstein *et al.* 2001). Para o Brasil foram citadas 64 espécies, porém destas, 44 foram indicadas como pouco conhecidos por Costa *et al.* (2011) e não aparecem na lista das espécies do Brasil. Atualmente na Flora do Brasil (2020) são estimadas 20 espécies, das quais cinco são endêmicas. A morfologia desse gênero é bastante complexa, e os caracteres utilizados para identificação de espécies não estão bem estabelecidos. Assim, a verdadeira diversidade de espécies que ocorrem no Brasil ainda é desconhecida.

Este trabalho visa auxiliar na delimitação das espécies e circunscrição do gênero *Macromitrium* para o Brasil, e também servir como uma ferramenta promissora para facilitar a identificação das espécies dos gêneros *Schlotheimia* e *Macromitrium.*

Dessa forma serão apresentados 3 capítulos:
1) Molecular tools to identify tropical mosses: a case study of the Brazilian species of *Schlotheimia* Brid. (Bryophyta, Orthotrichaceae)

O primeiro capítulo foi publicado na revista Systematics and biodiversity (Qualis A2), visando testar o potencial de três marcadores moleculares, sendo uma região nuclear ITS, e duas regiões plastidiais *trn*L-F e *trn*G-R, (primeira vez usado em musgos) para resolver as relações filogenéticas e delimitações de espécies dentro de *Schlotheimia* no Brasil. Além disso, buscamos avaliar o conceito de espécies morfológicas com base nos dados moleculares para auxiliar a revisão taxonômica em andamento da *Schlotheimia* no Brasil (trabalho extra tese) realizados em colaboração com pesquisadores do Instituto de Botânica de São Paulo.

2)   Taxonomic Notes on Brazilian *Macromitrium* Brid. (Bryophyta, Orthotrichaceae)

O segundo capítulo, foi submetido para a revista Phytotaxa (Qualis B2). Foi um trabalho de taxonomia clássica, realizado como um projeto paralelo, durante o doutorado sanduíche no *Naturalis Biodiversity Center* na Holanda, em que foi possível ter acesso ao material typus do gênero *Macromitrium* depositados em diversas coleções como BM, E, G, GOET, L, NY, PC. Nosso objetivo neste trabalho foi verificar todos os 64 nomes de *Macromitrium* brasileiro (20 nomes aceitos e 44 nomes excluídos da flora do Brasil) buscando esclarecer sua validade e identificação correta, contribuindo para o conhecimento da diversidade de espécies de *Macromitrium* ocorrentes no Brasil.

3)   *Macromitrium* Brid. (Bryophyta, Orthotrichaceae) in Brazil: A Molecular Approach

O terceiro capítulo foi submetido para a revista *Plant Systematics and Evolution* (Qualis B1), e emprega um conjunto de dados moleculares de quatro marcadores dos três compartimentos genômicos para estudar a circunscrição e delimitação de espécies de *Macromitrium* no Brasil, visando testar se as espécies brasileiras de *Macromitrium* formam um grupo monofilético. Caso não seja um grupo monofilético, delimitar quais as espécies brasileiras pertencem ao verdadeiro *Macromitrium* e testar o potencial de Marcadores *trn*G-R, *trn*L-F e ITS para identificação molecular das espécies brasileiras de *Macromitrium.*

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2 OBJETIVOS

2.1 Objetivo geral

Elaborar uma estratégia de DNA Barcoding ou ferramenta molecular para identificação das espécies dos gêneros *Schlotheimia* e *Macromitrium,* visando à solução de problemas taxonômicos, contribuindo com dados moleculares para o desenvolvimento futuro de trabalhos em larga escala de DNA Barcoding.

2.2 Objetivos específicos

a) Analisar o potencial de discriminação ao nível de espécie dos marcadores de DNA usados, selecionando o melhor marcador ou a combinação de marcadores ideais para os gêneros em estudo;

b) Ajudar na solução de problemas de identificação taxonômica dos gêneros *Schlotheimia* e *Macromitrium* através de uma abordagem integrada morfo-molecular;

c) Testar o monofiletismo gênero *Macromitrium* inserindo as espécies que ocorrem no Brasil na filogenia existente;

d) Testar o monofiletismo do gênero *Schlotheimia* inserindo as espécies que ocorrem no Brasil na filogenia existente;

e) Inferir circunscrições moleculares e testar o estado das espécies endêmicas brasileiras dos gêneros *Schlotheimia* e *Macromitrium;*

f) Comparar a variação molecular interespecífica x intraespecífica e detectar possíveis casos de especiação críptica;

g) Otimizar protocolos de extração e amplificação de DNA, se necessário.

CAPÍTULO I

Molecular tools to identify tropical mosses: a case study of the Brazilian species of *Schlotheimia* Brid. (Bryophyta, Orthotrichaceae).

3 MOLECULAR TOOLS TO IDENTIFY TROPICAL MOSSES: A CASE STUDY OF THE BRAZILIAN SPECIES OF *SCHLOTHEIMIA* BRID. (BRYOPHYTA, ORTHOTRICHACEAE)

3.1 Abstract

Species of the moss genus *Schlotheimia* are often difficult to identify morphologically because it is necessary to study sporophytic characters, but fertile plants are quite rare. In this paper we aim to infer the potential of *trn*G-R, *trn*L-F, and ITS markers to resolve phylogenetic relationships and species delimitations within *Schlotheimia* in Brazil, using different tree-based analysis methods and Automatic Barcode Gap Discovery (ABGD). For the first time in bryophytes the *trn*G-R spacer was sequenced together with the *trn*G intron (*trn*G-R region). Furthermore, we aim to evaluate the morphological species concept based on the molecular data, to aid the ongoing taxonomic revision of *Schlotheimia* in Brazil. The combined analysis of all three markers resolved eleven clades corresponding to *Schlotheimia* species, which was corroborated by ABGD and morphological characters. The best candidate marker for DNA barcoding was *trn*G-R due to its easy amplification and ability to discriminate all but one species. While *trn*G-R is sufficient for routine identification, the combination *trn*G-R + ITS should be used if all Brazilian *Schlotheimia* species should be identified with high statistical support. The nuclear marker ITS was easy to amplify and more variable than the plastid markers, but a higher percentage of low quality sequences due to polynucleotide stretches or fungal contamination is a potential drawback. *Trn*L-F had a low discrimination potential. The analysis of the studied molecular markers provides a baseline for the taxonomic revision of *Schlotheimia* and is an important tool for the identification of sterile specimens.

Key words: barcode, ITS, molecular information, species identification, *trn*G-R, *trn*L-F.

3.2 Introduction

Bryophytes are often difficult to identify at species level, due to their generally small size, relatively few and inconspicuous morphological characters, frequent absence of sporophytic characters, considerable morphological plasticity in response to environmental factors, and still unclear species delimitations and taxonomy in many groups (e.g. Bergamini & Peintinger, 2002; Buryová & Shaw, 2005; Hassel, Pedersen & Söderström, 2005).

Species identification based on DNA sequences (DNA barcoding) is a useful tool to overcome problems with morphological identification (Hebert, Cywinska, Ball & Dewaard, 2003). DNA barcoding of plants is generally performed based on a combination of two or more genetic loci. From a pool of several DNA regions tested mainly for angiosperms, the chloroplast loci *rbc*L and *mat*K were chosen as core plant barcoding markers (CBOL Plant Working Group, 2009), and the chloroplast *psb*A-*trn*H as well as nuclear ribosomal ITS regions are two widely used supplementary barcode loci (e.g. Kress, Wurdack, Zimmer, Weigt & Janzen, 2005; Sass, Little, Stevenson & Specht, 2007). For bryophytes, no consensus has been achieved yet: a part of the DNA barcoding studies of bryophytes used the same four markers recommended for angiosperms, whereas others tested different marker combinations (Table 1). In particular, recent large-scale DNA barcoding studies in Germany and the Netherlands relied on the marker combination *trn*L-F and ITS (and *rpl*16 in Germany), due to partly low discrimination capacity of *rbc*L and *psb*A-*trn*H, and amplification problems with *mat*K in bryophytes.

DNA barcoding studies of bryophytes so far focused mainly on extra-tropical regions (Table 1), although species diversity is highest in the tropics. Brazil, for example, hosts at least 1567 bryophyte species (http://floradobrasil.jbrj.gov.br/), but DNA barcoding of plants in Brazil has so far been restricted entirely to angiosperms (e.g. Palhares et al., 2015; Rivera-Jiménez et al., 2017). The potential of DNA barcoding to catalogue and better understand the tropical bryophyte biodiversity, especially in mega-diverse countries like Brazil, remains to be investigated.

As part of a pilot project to investigate the utility of DNA barcoding for Brazilian bryophytes (see Dantas, Valente, Carvalho-Silva & Câmara, 2018), the present study addresses species identification in *Schlotheimia* Brid. (Orthotrichaceae, subfamily Macromitrioideae), a pantropical moss genus with approximately 120 species (Frey & Stech, 2009). For the Neotropics, about 56 species are recorded, but the genus is in need of taxonomic revision in the Neotropics, and probably less than 30 species are actually justified (Gradstein, Churchill &

Salazar-Allen, 2001). In Brazil, 13 species were considered to occur, six of which are endemic (Costa & Peralta, 2015). In a first step of revising *Schlotheimia* in Brazil, one species was newly described and another reduced to synonymy (Peralta & Ristow, 2017). However, morphological species delimitation in *Schlotheimia* is difficult based on gametophytic characters, and sporophytes are frequently absent in herbarium collections and living plants in the field. Therefore, molecular data are important to test species delimitations and guide the taxonomic revision.

To infer molecular species delimitations in Brazilian *Schlotheimia*, we selected three DNA regions, the chloroplast *trn*L-F and *trn*G as well as the nuclear ribosomal ITS region. We considered markers that were either frequently used in previous studies or tested already in Orthotrichaceae (Table 1), and were supposed to perform well in terms of sequencing success and species discrimination capacity. In contrast to the study of Li, Guo & Yu (2013), and for the first time in bryophytes, we employed the extended *trn*G-R region, so that all three potential DNA barcoding markers comprise two non-coding parts each (*trn*L-F: *trn*L group I intron and *trn*L-*trn*F intergenic spacer, *trn*G-R: *trn*G group II intron and *trn*G-*trn*R intergenic spacer, ITS: internal transcribed spacers ITS1 and ITS2). Besides, the *trn*G-R region presented good results for DNA barcoding in ferns (Pryer et al., 2010).

Hence, the aims of this study are to (i) test the potential of *trn*G-R, *trn*L-F, and ITS markers to resolve the phylogenetic relationships and species delimitations within *Schlotheimia* in Brazil, and (ii) evaluate the morphological species concept based on the molecular data to aid the ongoing taxonomic revision of *Schlotheimia* in Brazil.

To discriminate species from the molecular data we follow a step-wise approach. Firstly, putative species are inferred from tree-based analysis methods employed in earlier DNA barcoding studies (neighbour-joining, maximum parsimony, maximum likelihood, Bayesian inference), based on topological congruence between (combinations of) markers and statistically significant clade support (bootstrap support ≥95% and posterior probability ≥ 0.95). Secondly, an automated species delimitation approach, Automatic Barcode Gap Discovery (ABGD; Puillandre, Lambert, Brouillet & Achaz, 2012), which uses a pairwise genetic distance-based method to find non-overlapping intra- and interspecific genetic distance distributions within the sequence dataset, is employed as an alternative to construct hypothetical candidate species from the molecular data. Finally, the distribution of morphological character states is analysed by ancestral state reconstruction to infer whether the putative species distinguished by DNA barcoding are recognizable morphologically.

3.3 Materials and methods

Sampling

We sequenced 34 specimens of *Schlotheimia* and two specimens of *Macromitrium* (Orthotrichaceae, Macromitrioideae) (Goffinet, Bayer & Vitt, 1998) from herbarium collections from UB and SP as well as fresh samples collected during field work. *Schlotheimia* was resolved in a basal position within Macromitrioideae, indicating that taxa from the subfamily Orthotrichoideae should be used as outgroup representatives. However, we used species of *Macromitrium* as outgroup representatives because sequences from both subfamilies were difficult to align, especially for the variable marker ITS. We sequenced 2−4 individuals for each ingroup species, depending on availability of material as well as DNA extraction and amplification success. Voucher information and Genbank accession numbers are listed in Appendix S1.

DNA extraction, amplification and sequencing

DNA extraction and PCR amplification (Mullis & Faloona, 1987) were performed at the Laboratory of Molecular Biology of Plants, Botany Department, University of Brasília. Total genomic DNA was extracted using the mini-CTAB protocol (Doyle & Doyle, 1987), with modifications (Câmara, 2010).

Three molecular markers (chloroplast *trn*L-F and *trn*G-R, nuclear ribosomal ITS) were amplified with primers Cm / Fm (Frey, Stech & Meißner, 1999), B (Pačak & Szweykowska-Kulińska, 2000) / TRNR22R (Nagalingum, Schneider & Pryer, 2007), and 18F / 25R (Stech & Frahm, 1999), respectively. The PCR amplification mixture had a total volume of 50 μl and contained 5 μl of 5× thermophilic buffer, 5 μl of 50 mM $MgCl_2$, 0.5 μl *Taq* polymerase (Promega, Madison, Wisconsin, U.S.A.), 2 μl of BSA (10 mg/ml), 4 μl of 1 mM dNTP, 2.5 μl of each primer (10 μM), 2.0 μl of DNA and 26.5 μl of water. The PCR profile for all markers was 95°C (30 sec), 48°C–56°C (45 sec), 72°C (1 min) for 35 cycles, always preceded by an initial melting step of 1 min at 95°C and with a final extension of 72°C for 5 min. Primer annealing temperatures were 56°C for *trn*L-F, 53°C for *trn*G-R and 48°C for ITS. If no PCR products were obtained, the PCR was repeated with other annealing temperatures for *trn*L-F (50 or 55 °C), *trn*G-R (50, 51 or 55 °C) and ITS (54 or 58 °C), respectively, and 100% DMSO (Dimethyl sulfoxide) or Betaine (concentration 0.5, 1 and 2M) was added. If still no success was achieved, the DNA of the respective samples was re-extracted.

PCR products were purified and sequenced by Macrogen (Seoul, Korea). Sequences were assembled and edited using Geneious v.6.1.6 (Biomatters, 2010).

Molecular species discrimination

Sequences were initially aligned using Clustal X (Higgins & Sharp, 1988), then manually adjusted using PhyDE v.0.9971 (Müller, Quandt, Müller & Neinhuis, 2006) and exported as Nexus files. Intraspecific and interspecific variation were inferred from pairwise distances, calculated using the Kimura 2-parameter (K2P) model (Kimura, 1980) in MEGA7 (Kumar, Stecher & Tamura, 2015).

Tree-based methods to infer species delimitations included neighbour-joining (NJ) as well as phylogenetic approaches (maximum parsimony [MP], maximum likelihood [ML], Bayesian inference [BI]). Analyses were carried out for each marker separately as well as for the combinations *trn*L-F + *trn*G-R, *trn*L-F + ITS, *trn*G-R + ITS, and all three markers combined.

Neighbour-joining (Saitou & Nei, 1987) and MP analyses were performed in PAUP* v.4.0b10 (Swofford, 2002). Heuristic searches under MP were performed with 1,000 random addition replicates and tree-bisection-reconnection (TBR) branch swapping, saving a maximum of 10,000 trees. All characters were unordered and equally weighted, and gaps were either treated as missing data or coded as informative by simple indel coding (SIC; Simmons & Ochoterena, 2000) as implemented in SeqState (Müller, 2005).

 For ML and BI analyses, the best-fit model of evolution for each locus was obtained based on the Akaike information criteria using jModeltest 3.06 (Posada, 2008). Maximum likelihood analyses were carried out using RAxML v7.2.6 (Stamatakis, 2006; Silvestro & Michalak, 2012). Clade support for NJ, MP and ML was assessed from bootstrap analyses with 1000 replicates (Felsenstein, 1985).

Bayesian inference analyses were carried out in MrBayes v. 3.2.6 (Ronquist et al., 2012). Two runs with four Markov Chain Monte Carlo chains each were run for 5,000,000 generations. Chains were sampled every 1,000 generations and the respective trees were written to a tree file. Convergence of runs was verified by ensuring that the average standard deviation of split frequencies was <0.01. Tracer 1.5 (Rambaut & Drummond, 2013) was used to determine when the tree sampling stabilized. The first 25% of the trees were discarded as 'burn-in'. A majority rule consensus tree and posterior probabilities were calculated from the resulting trees.

Automatic Barcode Gap Discovery (ABGD; Puillandre et al., 2012) analyses were carried out on the online web server (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html). The dataset of three markers combined (*trn*L-F + *trn*G-R + ITS) was used with the input file in fasta format and the Kimura-2-parameter model and a range of different settings employed. Since the latter resulted in the same number of initial partitions, the final parameters were set as follows: Pmin = 0.001, Pmax = 0.01, Steps = 50, X = 1.1, Nb bins = 100.

Ancestral state reconstruction

We scored the character states of 54 gametophytic and sporophytic characters for the sequenced *Schlotheimia* specimens. For sterile specimens, sporophytic character states were added based on the information from the fertile specimens or the literature. Of the 54 characters, 37 were constant across all species and excluded from further analysis. The remaining 17 variable characters (Appendix S2) were used for ancestral state reconstruction under maximum parsimony in Mesquite v.351 (Maddison & Maddison, 2015). A maximum parsimony consensus tree in nexus format, calculated in PAUP based on the combined molecular matrix (*trn*L-F + *trn*G-R + ITS) and using the same parameters as described above, and the character matrix of standard categorical data were used as input data. The Trace Character History option was used to map the distribution of ancestral character states on the phylogeny.

3.4 Results

Sequencing success

PCR amplification and sequencing success using a single primer pair was high (94–100%) for all three markers. The *trn*G-R region was the easiest to analyse, since PCR products could be obtained from 97% of the samples in a single PCR round, and all of them resulted in high-quality sequences. Both *trn*L-F and ITS required greater laboratorial effort. For *trn*L-F, high-quality sequences could be obtained from 100% of the samples as well, 82% of which after the first-round PCR and 18% after a second PCR with modifications or after re-extracting the DNA. For ITS, only 65% of the samples yielded good sequences from the first PCR product, whereas the remainder had to be redone, either by repeating the PCR or by re-extracting the DNA. All samples could be sequenced after a maximum of four attempts, but still 6% of the ITS sequences were of insufficient quality. Finally, 29 samples for which high-quality sequences of all three markers were obtained, were included in the further analyses (Fig. 1). Characteristics of sequence lengths and variability of all three markers are summarized in Table

2. The nuclear ITS marker is 48/72% larger and 60/76% more variable relative to the plastid markers *trn*G-R and *trn*L-F, respectively.

Molecular species discrimination

Phylogenetic trees of each individual marker (*trn*L-F, *trn*G-R and ITS) did not show incongruence in terms of well-supported branches (data not shown), indicating that the markers could be combined. The Bayesian inference tree based on the maximum amount of information (three markers combined with indels coded by SIC), is shown in Fig. 1, together with a summary of the results obtained from all applied tree-based and ABGD species delimitation methods.

Eleven clades corresponding to putative morphological species (A – *Schlotheimia appressifolia*, B – *S. tecta*, C – *S. gracilescens*, D – *S. merkelii*, E – *S. spinomitria*, F – *S. rugifolia*, G – *S. jamesonii*, H – *S. elata*, I – *S. pseudoaffinis*, J – *S. torquata*, and K – *S. trichomitria*) were resolved in the combined analysis (Fig. 1). Of these, nine received high support (bootstrap support [BS] ≥95% and posterior probability [PP] ≥0.95) in at least three of the four analyses. The remaining two clades D and H received high PP values, but moderate bootstrap support (BS ≥85−≤94%). Relationships among these putative species received generally lower (BS ≤84%) or no support, except for the sister group relationships of clades A / B and D / E, respectively.

None of the individual markers was able to resolve all 11 clades with high support in all analyses (Fig. 1). In the analyses with indels included (Appendix S3), the ITS marker separated all 11 clades with high support in the BI analysis. Bootstrap support was high in at least one of the three analyses (NJ, MP, ML) for eight clades and moderate for clade E. The *trn*G-R marker resolved eight clades with high support and six clades with high BS in at least one analysis (most often in ML). The *trn*L-F marker performed worst, with in total only four clades receiving high support. Both chloroplast markers together separated ten clades with high support, while the combinations of either chloroplast marker plus ITS discriminated all 11 clades with high support, at least in the BI analyses. Results of the analyses with indels excluded (Appendix S4) were similar to the analyses including indels in terms of support for the 11 clades. Differences occurred mainly in clades D, E, and K, with generally higher support with indels for the former two clades, but higher support without indels in few analyses for clade K (BI of *trn*G-R and the combination *trn*L-F + *trn*G-R).

Ranges of intraspecific versus interspecific pairwise nucleotide distances according to the K2P model and overlap for the individual markers and combinations are shown in Table 3. All markers and combinations showed overlap between the maximum intraspecific and minimum interspecific distance, with a minimum overlap of 0.003 for the combinations *trn*L-F + *trn*G-R and *trn*L-F + *trn*G-R + ITS, and a maximum overlap of 0.009 in ITS. The overlap was due to the small intraspecific distance between clades D (*S. merkelii*) and E (*S. spinomitria*) in contrast to a large intraspecific distance within clade A (*S. appressifolia*).

The species delimitation method ABGD (Figure 1) revealed a clear "barcode gap" at Pmax = 0.0010, delimiting eleven putative species.

Ancestral state reconstruction

Character state changes ranged between one and nine among the 17 variable characters (Appendix S5), indicating different degrees of homoplasy in most characters. Five characters displayed apomorphic states for a single molecular clade corresponding to a *Schlotheimia* species (7 – vegetative leaf [VL] apex aristate in *S. pseudoaffinis*, 8 – VL margin dentate in *S. appressifolia*, 9 – VL costa long excurrent in *S. pseudoaffinis*, 11 – perichaetial leaf apex acuminate in *S. torquata*, 11 – calyptra surface rugose in *S. merkelii*, spinose with foliose trichomes in *S. spinomitria*, and pilose with filamentose trichomes in *S. trichomitria*; Appendix S2). The combination of four characters (6 – vegetative leaves lamina, 10 – perichaetial leaves lamina, 14 – capsule exposure, and 17 – calyptra surface), of which the ancestral state reconstructions are shown in Figure 2, allowed to distinguish all 11 molecularly separated species except *S. elata* and *S. gracilescens*. The latter two species are distinguishable by a number of other characters (characters 1–5 and 9; cf. Appendix S2).

3.5 Discussion

The evaluation of the potential of genetic loci for DNA barcoding of plants should take into account the general barcoding criteria (sufficient genetic variability at the species level, short sequence to facilitate DNA extraction and amplification, and presence of conserved flanking regions for the development of universal primers (Kress et al., 2005). For bryophytes there is not a single universal marker matching all these characteristics that allows the identification of all species. Most existing studies on bryophytes have compared the highly variable ITS and various markers of the chloroplast genome (Table 1). While some studies concluded that it may take several markers to distinguish all species of a bryophyte genus with statistical support (e.g. Arctic *Dicranum* species; Lang et al., 2014), other studies suggested that

a single marker out of a set of markers tested might suffice. For example, ITS was sufficient to distinguish all studied species of the liverwort genus *Herbertus* (Bell et al., 2012) and the *Racomitrium canescens* complex of the moss family Grimmiaceae (Stech et al., 2013). Given the different results in different bryophyte lineages, however, testing the variability and utility for species delimitation of commonly used markers still appears necessary for newly studied genera.

In *Schlotheimia*, ITS was an easy-to-amplify marker with high variability for identification at the species level, discriminating all 11 putative species with significant support at least under Bayesian inference. However, problems regarding sequence quality were encountered due to poly-C, -T and -A nucleotide stretches of approximately 50 base pairs in ITS1, and sometimes fungal contamination, which demanded greater efforts to obtain sequences of good quality. These problems have already been pointed out by Hollingsworth, Graham & Little (2011) as a limitation to the use of ITS as a core barcode. In the gymnosperm order Cycadales, the presence of polynucleotide stretches was a negative factor for the use of ITS for DNA barcoding as well (Sass et al., 2007).

The *trn*L-F region was considered as an easy to amplify marker, but with low variation in angiosperms (Kress et al., 2005). Subsequent DNA barcoding studies of bryophytes confirmed that *trn*L-F sequences could be obtained easily and were of high quality (e.g. Liu et al., 2010), which was also true for *Schlotheimia*. The species discrimination capacity of *trn*L-F varied considerably in different bryophyte genera, from 53% in *Dicranum* (Lang et al., 2014) to 89% in 49 species of mosses (Liu et al., 2010). In Orthotrichaceae, this marker alone was able to discriminate only 57% of the Chinese species of *Macromitrium* (Li et al., 2013), and in Brazilian *Schlotheimia* the discrimination potential is even lower (Fig. 1, Appendix S3, S4).

The *trn*G-R region was the marker that best met the barcoding criteria in the present study, being easy to amplify, with excellent sequence quality, and with sufficient variation to discriminate the Brazilian *Schlotheimia* species (all but two clades resolved with statistical support in at least one analysis; Fig. 1). Until now, only the *trn*G intron has been used in molecular studies of mosses (e.g. Câmara & Shaw 2013; Carter, 2010, 2012; Medina, Lara, Goffinet, Garilleti & Mazimpaka, 2013). In Chinese *Macromitrium*, the discrimination potential of *trn*G was almost 86% (Li et al., 2013). As in *trn*L-F, the *trn*G intron is more conserved in *Schlotheimia* than in *Macromitrium*, which would preclude its use for DNA barcoding, but the addition of the *trn*G-*trn*R intergenic spacer significantly increases the discrimination potential. The *trn*G-R has been used successfully for phylogenetic inference (Nagalingum et al., 2007;

Leon, Rothfels, Arakaki, Young & Pryer, 2013) and DNA barcoding (Pryer et al., 2010) in ferns, and it is suggested to employ the same region in future studies of bryophytes as well, instead of only sequencing the *trn*G intron.

The combination *trn*L-F + *trn*G-R did not result in greater discrimination potential compared to *trn*G-R alone (Appendix S3, S4). Similar results were found by Liu et al. (2010), where some combinations of chloroplast regions did not increase their discrimination capacity. If all Brazilian *Schlotheimia* species should be distinguished with high statistical support, independent from the type of analysis performed, the combinations *trn*G-R and ITS should be used (Fig. 1). However, *trn*G-R is sufficient for routine identification of *Schlotheimia* specimens from Brazil, only in some cases where additional sequencing of ITS may be required.

The discrimination of 11 *Schlotheimia* species was congruent among all phylogenetic methods used and the ABGD species delimitation method. The intraspecific variation of *S. appressifolia* (clade A) is greater than the interspecific variation between *S. merkelii* (clade D) and *S. spinomitria* (clade E), which leads to overlap of intra- and interspecific distances in the total dataset (all three markers combined; Table 3). However, the clades of all species are well supported by molecular data and (combinations of) morphological characters (see below), indicating that an overall comparison of pairwise distances is less meaningful than the phylogenetic and species delimitation methods. The intraspecific molecular variation in *S. appressifolia* may be a case of cryptic speciation, which is increasingly documented in mosses (Carter, 2012; Fernandez, Shevock, Glazer & Thompson, 2006; Hedenäs & Eldenäs, 2007; McDaniel & Shaw, 2003; Myszczyński et al., 2017; Schwarzera & Joshi, 2017; Shaw, 2000).

The molecular approach shows that the majority of the plants used in this study were incorrectly identified, which probably holds for other collections deposited in Brazilian herbaria as well. Frequent misidentification based on morphology may be due to an oversimplified use of few gametophytic characters in Brazil, where *Schlotheimia* plants with smooth to undulate vegetative leaves were commonly identified as *S. jamesonii* and plants with rugose vegetative leaves as *S. rugifolia.* The analysis of sporophytic characters was not a routine practice for identification of Brazilian *Schlotheimia* species. The present study corroborates that gametophytic characters alone are not sufficient for reliable identification, whereas a combination of gametophytic and sporophytic characters allows to distinguish all 11 molecularly separated clades (Fig. 2, Appendix S2).   The perichaetial leaves lamina and apex, capsule exposure, and calyptra surface are among the most important characters for identification of *Schlotheimia* species occurring in Brazil, together with characters of the

vegetative leaves (Fig. 2, Appendix S2). In the absence of perichaetial leaves and sporophytes, however, routine identification based on DNA sequences would be necessary for some species that are gametophytically identical (*S. rugifolia*, *S. spinomitria* and *S. trichomitria*, Appendix S2).

Schlotheimia appressifolia and *S. tecta*, the two species of the Neotropical subg. *Stegotheca* (Mitt.) Broth. (Atwood, 2009) have immersed capsules and mitrate-campanulate calyptrae, in contrast to exserted capsules and large campanulate calyptrae in subg. *Schlotheimia* (Fig. 2-C). The latter is resolved as paraphyletic due to the nested position of the well-supported clade of subg. *Stegotheca* (Fig. 1), but analyses based on more markers are necessary to resolve subgeneric relationships with confidence. Of the gametophytic characters that Atwood (2009) used to distinguish *S. appressifolia* and *S. tecta*, the margin and costa of the vegetative leaves do not show any difference between the specimens analysed here, whereas the dentate versus entire leaf margin, and, in addition, the distally undulate versus rugose perichaetial leaf surface does allow to distinguish both species (Appendix S2).

The specimens of clade C were originally identified as *S. rugifolia*, but the morphological characteristics (undulate to distally plane vegetative leaves, distally plane perichaetial leaves, and smooth calyptra; Fig. 2 – A, B and D) match *S. gracilescens,* a species restricted to South Brazil. Instead, clade F is defined as true *S. rugifolia,* due to the combination of rugose vegetative and perichaetial leaves, and a smooth calyptra (Fig. 2 – A, B and D). The character state of rugose vegetative leaves furthermore occurs in clades D, E, J and K, which can be easily confused with *S. rugifolia* when the plants are sterile, but are distinguished by their perichaetial leaves and calyptra (Fig. 2 – A, B and D). Clade D comprises specimens identified as *S. merkelii, S. rugifolia* and *S. jamesonii.* However, they all show a combination of character states that corresponds to *S. merkelii*, namely rugose vegetative leaves, distally undulate perichaetial leaves, and a rugose calyptra. The specimens of clade E were identified as *S. rugifolia* or *S. torquata*, respectively, but the distally undulate perichaetial leaves and spinose calyptra with foliose trichomes do not fit with either species. The latter character is diagnostic for the recently described *S. spinomitria* (Peralta & Ristow, 2017) and distinguishes it, together with the ovoid, larger capsule, from the closely related *S. merkelii*. The species *S. torquata* (clade J) and *S. trichomitria* (clade K) are characterised by the diagnostic character states of acuminate perichaetial leaves and a pilose calyptra, respectively.

The specimens of clades G (*S. jamesonii)* and H (*S. elata)* were initially all identified as *S. jamesonii.* Both species are morphologically similar, but *S. jamesonii* has ascendant, short

and erect branches, and lanceolate vegetative leaves, whereas S. *elata* has long and erect branches, and oblong-lanceolate vegetative leaves (Appendix S2 – characters 4 and 5). Finally, *S. pseudoaffinis* (clade I) differs from all other species by vegetative leaves with a long-aristate apex.

The present results showed the importance of molecular information to better understand species diversity and for the reliable identification of *Schlotheimia* plants, in particular when sporophytic characters are absent, a common situation when collecting specimens of this genus in the field.

3.6 Acknowledgments

3.7 References

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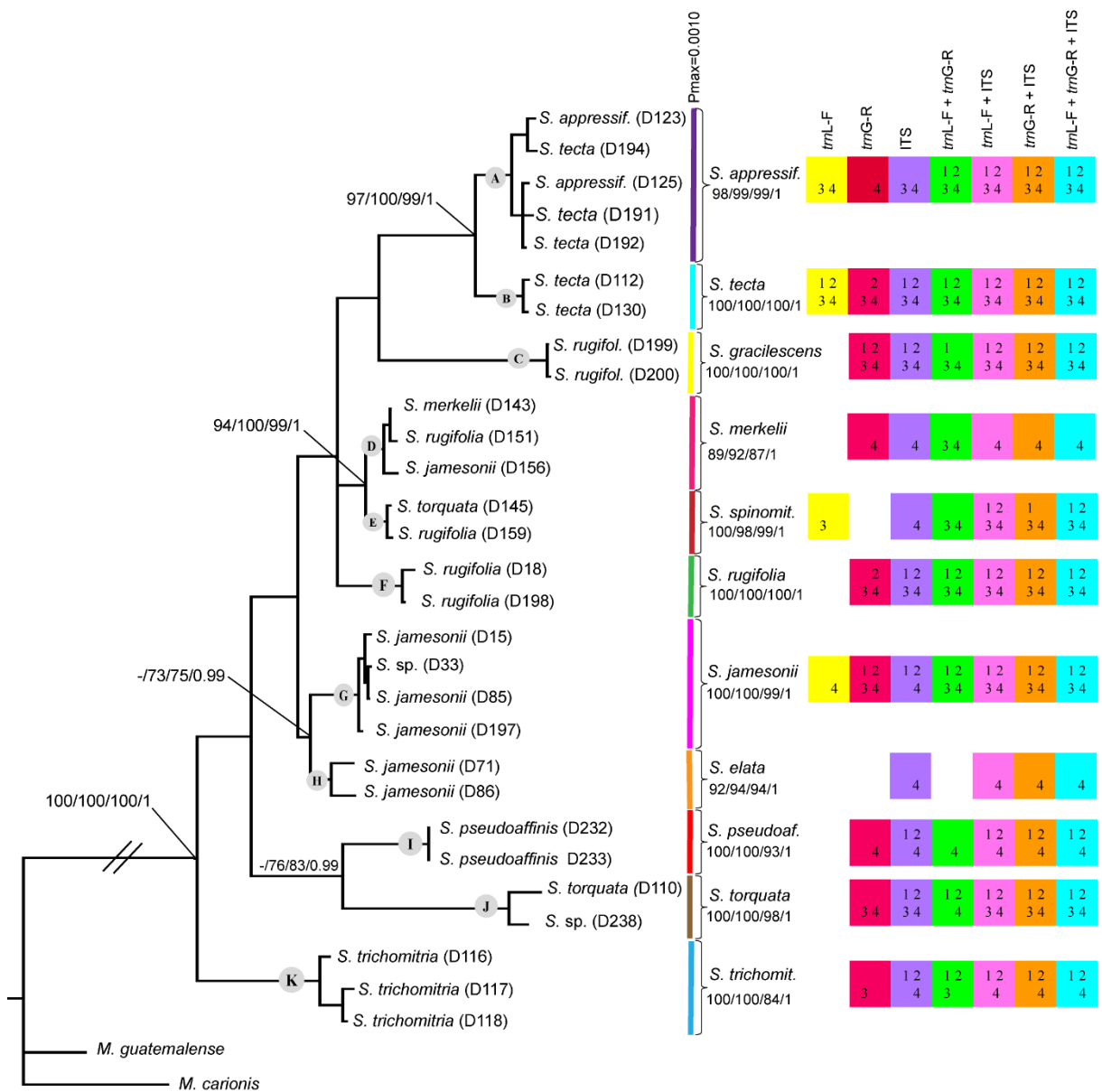
Fig. 1. Phylogram obtained from Bayesian inference (BI) based on combined *trn*L-F + *trn*G-R + ITS sequences of 29 specimens of *Schlotheimia*, including indels coded by simple indel coding. Bootstrap support (BS) values for neighbour-joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) as well as posterior probabilities (PP) for Bayesian inference are shown at the branches. Results of analyses of single markers and combinations are summarized by coloured squares. The absence of a coloured square means no support or clade not resolved for that marker. Numbers inside them represent BS ≥ 95% for **1** - (NJ), **2** - (MP) and **3** - (ML), and PP ≥ 0.95 for **4** - (BI). ABGD species clusters (coloured lines) and Pmax-values are shown next to the species names. // indicates a clade length of 50% of its original size.

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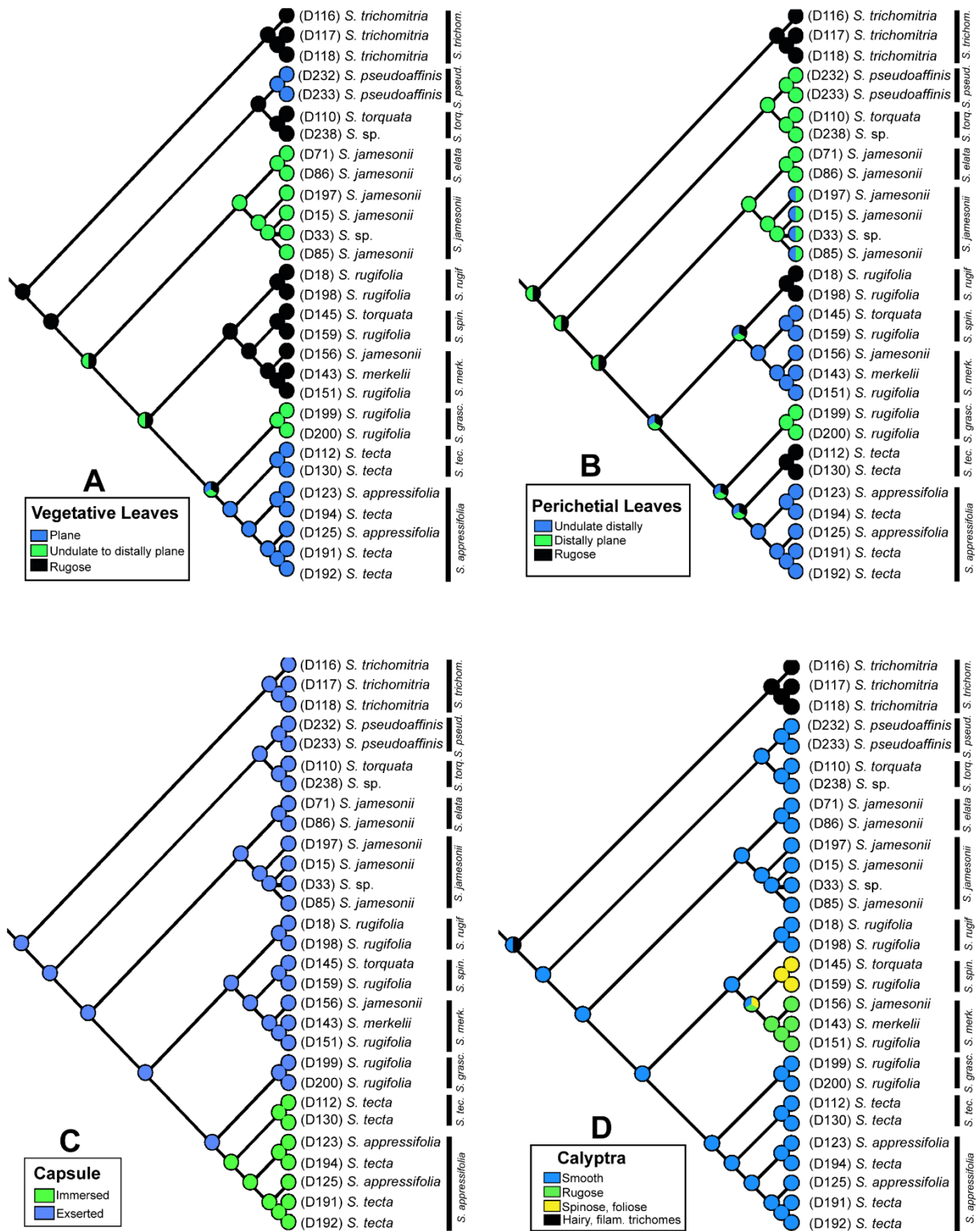
Fig. 2. Ancestral character state reconstruction of four morphological characters based on combined *trn*L-F + *trn*G-R + ITS sequences of 29 specimens of *Schlotheimia.* **A)** vegetative leaves lamina; **B)** perichaetial leaves lamina; **C)** capsule exposure; **D)** calyptra surface.

Table 1. Molecular markers tested or employed in selected DNA barcoding studies of bryophytes. The use of parts of the respective markers (e.g. only *trn*L intron or ITS2) in some of the listed studies is not indicated separately.

Taxon/study	*atp*F-*atp*H	*mat*K	*psb*K-*psb*I	*rbc*L	*rpl*16	*rpo*B	*rpo*C1	*rps*4(-*trn*T)	*rps*19-*rpl*2	*trn*G	*trn*H-*psb*A	*trn*L-*trn*F	ITS	Reference
Dutch bryophytes												x	x	Stech & Sparrius, 2011
German bryophytes (GBOL)		(x)		x								x	x	Geiger et al., 2016
Selected boreal/arctic bryophytes	x			x							x		x	Hassel et al., 2013
Selected British bryophytes		x		x							x		x	Bell et al., 2013
Selected Chinese bryophytes	x	x	x	x									x	Liu et al., 2010
Selected Finnish bryophytes		(x)		x	x	x					x			Cräutlein et al., 2011
Aneura		x		x			x				x	x	x	Bączkiewicz et al., 2017
Herbertus		x		x							x		x	Bell et al., 2012
Schistidium		x		x							x		x	Hofbauer et al., 2016
Arctic *Dicranum*						x		x	x		x	x	x	Lang et al., 2014
Chinese Grimmiaceae								x			x	x		Liu et al., 2011
Chinese *Macromitrium*										x		x		Li et al., 2013

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Table 2. Characterization of the dataset of three molecular markers used for DNA barcoding of Brazilian *Schlotheimia*, and two samples of *Macromitrium* as outgroup representatives. Numbers of conserved sites, variable sites, parsimony informative sites, indels, and medium pairwise distances were calculated from the ingroup sequences only.

Marker	Sequence length	Alignment length outgroup	Alignment withlength without outgroup	Conserved sites (%)	Variable sites (%)	Parsimony-informative sites (%)	indels	Parsimony-informative indels	Medium pairwise distance
*trn*L-F	369−397	435	397	384 (96.7)	3.3	3	3	2	0.008
*trn*G-R	694−727	755	728	689 (94.6)	5.4	4.5	4	4	0.010
ITS	853−1049	1581	1407	1218 (86.5)	13.5	11.9	239	192	0.038

Table 3. Intra- versus interspecific pairwise Kimura 2-parameter (K2P) distances of individual markers (*trn*L-F, *trn*G-R and ITS) and combinations in the analysed *Schlotheimia* dataset. The last row indicates the overlap between the maximum intraspecific and minimum interspecific distances.

	*trn*L-F	*trn*G-R	ITS	*trn*L-F + *trn*G-R	*trn*L-F + ITS	*trn*G-R + ITS	*trn*L-F + *trn*G-R + ITS
Intra	0 – 0.005	0 – 0.007	0 – 0.013	0 – 0.006	0 – 0.011	0 – 0.009	0 – 006
Inter	0 – 0.022	0.003 – 0.019	0.004 – 0.087	0.003 – 0.015	0.004 – 0.055	0.004 – 0.049	0.003 – 0.039
Overlap	0.005	0.004	0.009	0.003	0.007	0.005	0.003

3.8 Appendix

Appendix S1. Voucher information and GenBank accession numbers for the analysed *Schlotheimia* specimens. Samples marked by an asterisk could not be sequenced for all markers and were used in the analyses of individual markers and possible combinations, but excluded from the combined analysis (*trn*L-F + *trn*G-R + ITS) presented in Fig. 1.

DNA Code	Species names according to morphological identification, before barcoding analysis	Species names after barcoding analysis	Sporophyte	Herbarium	Voucher no.	Locality (Brazilian state)	Year of collection	Acc. no. *trn*L-F	Acc. no. *trn*G-R	Acc. no. ITS
D104	*Macromitrium carionis* Müll. Hal	*Macromitrium carionis* Müll. Hal	Absent	UB	Valente, D.V 2626	GO	2017	MN245768	MN259735	MN077383
D172	*Macromitrium guatemalense* Müll. Hal.	*Macromitrium guatemalense* Müll. Hal.	Absent	UB	Valente, D.V. 1346	RS	2017	MN245767	MN259734	MN077384
D123	*Schlotheimia appressifolia* Mitt	*Schlotheimia appressifolia* Mitt	Present	SP464442	Peralta, D.F. 19055	MG	2016	MN245769	MN259736	MN077385
D125	*Schlotheimia appressifolia* Mitt	*Schlotheimia appressifolia* Mitt	Present	SP439346	Peralta, D.F. 14829	SP	2013	MN245770	MN259737	MK991784
D191	*Schlotheimia tecta* Hook. f. & Wilson	*Schlotheimia appressifolia* Mitt	Absent	UB	Valente, D.V. 998	RS	2017	MN245771	MN259738	MK991785
D192	*Schlotheimia tecta* Hook. f. & Wilson	*Schlotheimia appressifolia* Mitt	Absent	UB	Valente, D.V. 1003	RS	2017	MN245772	MN259739	MK991786
D194	*Schlotheimia tecta* Hook. f. & Wilson	*Schlotheimia appressifolia* Mitt	Absent	UB	Valente, D.V. 1116	RS	2017	MN245773	MN259740	MK991787
D121	*Schlotheimia pseudoaffinis* Müll. Hal.	*Schlotheimia capillaris* Hampe*	Present	SP462545	Peralta, D.F. 18094	MG	2016	MN259730	MN259741	−
D232	*Schlotheimia pseudoaffinis* Müll. Hal.	*Schlotheimia capillaris* Hampe	Present	SP483229	Peralta, D.F. 21854	SP	2017	MN245776	MN259742	MN077386
D233	*Schlotheimia pseudoaffinis* Müll. Hal.	*Schlotheimia capillaris* Hampe	Present	SP483465	Peralta, D.F. 22090	SP	2017	MN245777	MN259743	MN077387
D15	*Schlotheimia jamesonii* (Arn.) Brid.	*Schlotheimia jamesonii* (Arn.) Brid.	Present	UB	Valente, D.V. 2625	−	2013	MN259728	MN259744	MN077388
D27	*Schlotheimia jamesonii* (Arn.) Brid.	*Schlotheimia jamesonii* (Arn.) Brid.*	Absent	UB	Valente, D.V. 291	ES	2016	MN259731	−	MN077389
D33	*Schlotheimia* sp.	*Schlotheimia jamesonii* (Arn.) Brid.	Absent	UB	Valente, D.V. 323	ES	2016	MN245778	MN259745	MN077390
D85	*Schlotheimia jamesonii* (Arn.) Brid.	*Schlotheimia jamesonii* (Arn.) Brid.	Absent	UB	Valente, D.V. 479	ES	2016	MN245779	MN259746	MN077391
D197	*Schlotheimia jamesonii* (Arn.) Brid.	*Schlotheimia jamesonii* (Arn.) Brid.	Present	UB	Valente, D.V. 1044	RS	2017	MN245780	MN259747	MN077392
D143	*Schlotheimia merkelii* Hornsch.	*Schlotheimia merkelii* Hornsch.	Present	UB191266	Faria, J.E.Q. 2414	MG	2012	MN245781	MN259749	MN077393
D151	*Schlotheimia rugifolia* (Hook.) Schwägr.	*Schlotheimia merkelii* Hornsch.	Absent	UB	Valente, D.V. 246	ES	2016	MN245782	MN259748	MN077394
D156	*Schlotheimia jamesonii* (Arn.) Brid.	*Schlotheimia merkelii* Hornsch.	Present	SP419354	Yano, O. 32541	SP	2010	MN245783	MN259750	MN077395
D18	*Schlotheimia rugifolia* (Hook.) Schwägr.	*Schlotheimia rugifolia* (Hook.) Schwägr.	Present	UB	Valente, D.V. 351	ES	2016	MN245784	MN259751	MN077396

D198	*Schlotheimia rugifolia* (Hook.) Schwägr.	*Schlotheimia rugifolia* (Hook.) Schwägr.	Present	UB	Valente, D.V. 902	RS	2017	MN245785	MN259752	MN077397
D145	*Schlotheimia torquata* (Sw. ex Hedw.) Brid.	*Schlotheimia spinomitria* D.F. Peralta & R.Ristow	Present	UB	Camara, P.E.A.S 3828	DF	2016	MN245794	MN259758	MN077403
D159	*Schlotheimia rugifolia* (Hook.) Schwägr.	*Schlotheimia spinomitria* D.F. Peralta & R.Ristow	Present	UB	Bijos, N.R. 101	GO	2016	MN245795	MN259759	MN077404
D112	*Schlotheimia tecta* Hook. f. & Wilson	*Schlotheimia tecta* Hook. f. & Wilson	Present	SP477859	Peralta, D.F. 19208	ES	2016	MN245774	MN259760	MN077405
D130	*Schlotheimia tecta* Hook. f. & Wilson	*Schlotheimia tecta* Hook. f. & Wilson	Absent	UB	Valente, D.V. 346	ES	2016	MN245775	MN259761	MN077406
D110	*Schlotheimia torquata*	*Schlotheimia torquata* (Sw. ex Hedw.) Brid.	Present	SP477917	Peralta, D.F. 19266		2016	MN245788	MN259762	MK993019
D196	*Schlotheimia torquata* (Sw. ex Hedw.) Brid.	*Schlotheimia torquata* (Sw. ex Hedw.) Brid.*	Present	UB	Valente, D.V. 1065	RS	2017	MN259732	MN259763	−
D236	*Schlotheimia torquata* (Sw. ex Hedw.) Brid.	*Schlotheimia torquata* (Sw. ex Hedw.) Brid.*	Present	SP486818	Peralta, D.F. 23020	SP	2018	MN259733	MN259764	−
D238	*Schlotheimia* sp.	*Schlotheimia torquata* (Sw. ex Hedw.) Brid.**	Absent	SP486440	Peralta, D.F. 22642	MG	2018	MN245789	MN259765	MK993020
D116	*Schlotheimia trichomitria* Schwägr	*Schlotheimia trichomitria* Schwägr.	Present	SP477363	Carmo, D.M. 1391	SP	2016	MN245790	MN259766	MN077407
D117	*Schlotheimia trichomitria* Schwägr	*Schlotheimia trichomitria* Schwägr.	Present	SP464461	Peralta, D.F. 19074	SP	2016	MN245791	MN259767	MN077408
D118	*Schlotheimia trichomitria* Schwägr	*Schlotheimia trichomitria* Schwägr.	Present	SP464411	Peralta, D.F. 19034	SP	2016	MN245792	MN259768	MN077409
D71	*Schlotheimia jamesonii* (Arn.) Brid.	*Schlotheimia elata* Mitt.	Absent	UB	Valente, D.V. 496	ES	2016	MN259729	MN259753	MN077398
D86	*Schlotheimia jamesonii* (Arn.) Brid.	*Schlotheimia elata* Mitt.	Absent	UB	Valente, D.V. 506	ES	2016	MN245793	MN259754	MN077399
D87	*Schlotheimia jamesonii* (Arn.) Brid.	*Schlotheimia elata* Mitt.*	Absent	UB	Valente, D.V. 632	ES	2016	−	MN259755	MN077400
D199	*Schlotheimia rugifolia* (Hook.) Schwägr.	*Schlotheimia gracilescens* Broth.	Absent	UB	Valente, D.V. 1245	RS	2017	MN245786	MN259756	MN077401
D200	*Schlotheimia rugifolia* (Hook.) Schwägr.	*Schlotheimia gracilescens* Broth.	Absent	UB	Valente, D.V. 1250	RS	2017	MN245787	MN259757	MN077402

Appendix S2. Matrix used for morphological character reconstruction. **1)** Plant size: (1) robust, (2) medium-sized; **2)** Creeping stem covering: (1) short tomentose, (2) tomentose; **3)** Creeping stem format: (1) similar branches, (2) squarrose, (3) triangular; **4)** Branch length: (1) long, (2) short; **5)** Vegetative leaves (VL) shape: (1) lanceolate, (2) oblong-ovate, (3) oblong-lanceolate; **6)** VL lamina: (1) plane, (2) undulate to distally plane, (3) rugose; **7)** VL apex: (1) acute, (2) aristate, (3) mucronate; **8)** VL margin: (1) dentate, (2) entire; **9)** VL costa: (1) subpercurrent, (2) long excurrent, (3) short excurrent; **10)** Perichetial leaves (PL) lamina: (1) distally undulate, (2) distally plane, (3) rugose; **11)** PL apex: (1) aristate, (2) mucronate, (3) acuminate; **12)** PL costa: (1) long excurrent, (2) short excurrent; **13)** Seta length: (1) short, (2) long; **14)** Capsule exposure: (1) immersed, (2) exserted; **15)** Capsule shape: (1) ovoid, (2) ovoid-cylindrical; **16)** Calyptra shape: (1) campanulate, (2) mitrate-campanulate; **17)** Calyptra surface: (1) smooth, (2) rugose, (3) spinose with foliose trichomes, (4) pilose with filamentose trichomes.

DNA number	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
D123	*S. appressifolia*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
D125	*S. appressifolia*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
D191	*S. appressifolia*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
D192	*S. appressifolia*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
D194	*S. appressifolia*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
D232	*S. pseudoaffinis*	2	2	2	1	1	1	2	2	2	2	1	1	2	2	2	2	1
D233	*S. pseudoaffinis*	2	2	2	1	1	1	2	2	2	2	1	1	2	2	2	2	1
D15	*S. jamesonii*	1.2	2	3	2	1	2	3	2	3	1.2	2	2	2	2	2	2	1
D33	*S. jamesonii*	1.2	2	3	2	1	2	3	2	3	1.2	2	2	2	2	2	2	1
D85	*S. jamesonii*	1.2	2	3	2	1	2	3	2	3	1.2	2	2	2	2	2	2	1
D197	*S. jamesonii*	1.2	2	3	2	1	2	3	2	3	1.2	2	2	2	2	2	2	1
D143	*S. merkelli*	1	2	3	2	2	3	3	2	3	1	2	2	1	2	2	2	2
D151	*S. merkelli*	1	2	3	2	2	3	3	2	3	1	2	2	1	2	2	2	2
D156	*S. merkelli*	1	2	3	2	2	3	3	2	3	1	2	2	1	2	2	2	2
D18	*S. rugifolia*	2	2	3	2	2	3	3	2	3	3	2	2	2	2	2	2	1
D198	*S. rugifolia*	2	2	3	2	2	3	3	2	3	3	2	2	2	2	2	2	1
D71	*S. elata*	1	1	3	1	3	2	3	2	3	2	2	2	2	2	2	2	1
D86	*S. elata*	1	1	3	1	3	2	3	2	3	2	2	2	2	2	2	2	1

D199	*S. gracilescens*	2	2	2	2	2	2	3	2	1	2	2	2	2	2	2	2	1
D200	*S. gracilescens*	2	2	2	2	2	2	3	2	1	2	2	2	2	2	2	2	1
D145	*S. spinomitria*	2	2	3	2	2	3	3	2	3	1	2	2	2	2	1	2	3
D159	*S. spinomitria*	2	2	3	2	2	3	3	2	3	1	2	2	2	2	1	2	3
D112	*S. tecta*	1	1	1	1	1	1	1	2	1	3	1	1	1	1	1	1	1
D130	*S. tecta*	1	1	1	1	1	1	1	2	1	3	1	1	1	1	1	1	1
D110	*S. torquata*	2	2	3	2	3	3	3	2	3	2	3	2	2	2	2	2	1
D238	*S. torquata*	2	2	3	2	3	3	3	2	3	2	3	2	2	2	2	2	1
D116	*S. trichomitria*	2	2	3	2	2	3	3	2	3	3	2	2	2	2	2	2	4
D117	*S. trichomitria*	2	2	3	2	2	3	3	2	3	3	2	2	2	2	2	2	4
D118	*S. trichomitria*	2	2	3	2	2	3	3	2	3	3	2	2	2	2	2	2	4

Appendix S3. Support values for the clades of Brazilian *Schlotheimia* species in different analyses of single markers and combinations, with indels included: Bootstrap support (BS) values (%) for neighbour joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) and posterior probabilities (PP) for Bayesian inference (BI). Values below 70% (BS) and 0.95 (PP) are in red. Respective values for the combined analysis of all three markers are shown in Fig. 1.

Clade	*trn*L-F				*trn*G-R				ITS				*trn*L-F + *trn*G-R				*trn*L-F + ITS				*trn*G-R + ITS			
	NJ	MP	ML	BI	NJ	MP	ML	BI	NJ	MP	ML	BI	NJ	MP	ML	BI	NJ	MP	ML	BI	NJ	MP	ML	BI
A - *S. appressifolia*	84	88	99	1	80	80	90	1	89	93	97	1	99	98	100	1	97	97	99	1	97	97	98	1
B - *S. tecta*	98	99	100	1	93	97	100	1	99	100	100	1	100	100	100	1	100	100	100	1	100	100	100	1
C - *S. gracilescens*	-	-	-	-	96	96	100	1	100	100	100	1	95	94	100	1	100	100	100	1	100	100	100	1
D - *S. merkelii*	-	-	-	-	88	84	92	1	60	67	62	1	85	87	99	1	61	73	66	1	88	92	88	1
E - *S. spinomitria*	-	-	96	0.92	-	-	87	-	94	94	94	1	62	86	100	1	99	98	99	1	96	94	96	1
F - *S. rugifolia*	-	-	-	-	92	97	99	1	100	100	100	1	95	99	99	1	100	100	100	1	100	100	100	1
G - *S. jamesonii*	64	64	77	0.99	98	99	100	1	97	99	87	0.99	100	100	100	1	99	100	96	1	100	100	98	1
H - *S. elata*	-	-	-	-	-	-	-	-	67	64	69	0.99	-	-	40	0.79	93	89	92	1	79	82	86	1
I - *S. pseudoaffinis*	-	-	-	-	63	63	91	0.97	100	100	91	1	62	64	92	0.98	100	100	90	1	100	100	93	1
J - *S. torquata*	-	-	-	-	93	93	95	1	100	100	98	1	93	93	96	1	100	100	96	1	100	100	99	1
K - *S. trichomitria*	-	51	89	0.91	92	92	98	0.93	100	100	92	1	97	96	100	0.93	100	100	89	1	100	100	86	1

Appendix S4. Support values for the clades of Brazilian *Schlotheimia* species in different analyses of single markers and combinations, without indels: Bootstrap support (BS) values (%) for neighbour joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) and posterior probabilities (PP) for Bayesian inference (BI). Values below 70% (BS) and 0.95 (PP) are in red.

Clade	*trn*L-F				*trn*G-R				ITS				*trn*L-F + *trn*G-R				*trn*L-F + ITS				*trn*G-R + ITS				*trn*L-F + *trn*G-R + ITS			
	NJ	MP	ML	BI	NJ	MP	ML	BI	NJ	MP	ML	BI	NJ	MP	ML	BI	NJ	MP	ML	BI	NJ	MP	ML	BI	NJ	MP	ML	BI
A-*S. appressifolia*	84	84	97	0.97	80	81	91	1	89	92	94	1	99	100	99	1	97	97	97	1	97	96	96	1	98	98	99	1
B - *S. tecta*	98	98	100	1	93	93	99	1	99	99	100	1	100	100	100	1	100	100	100	1	100	100	100	1	100	100	100	1
C - *S. gracilescens*	-	-	-	-	96	95	99	1	100	100	100	1	95	95	100	1	100	100	100	1	100	100	100	1	100	100	100	1
D - *S. merkelii*	-	-	-	-	88	85	92	1	60	60	50	0.93	85	88	97	1	61	61	43	0.87	88	88	79	1	89	88	78	1
E - *S. spinomitria*	-	-	68	-	-	-	78	--	94	96	99	1	62	61	96	0.98	99	99	100	1	96	96	100	1	100	99	100	1
F - *S. rugifolia*	-	-	-	-	92	94	97	1	100	100	100	1	95	94	99	1	100	100	100	1	100	100	100	1	100	100	100	1
G - *S. jamesonii*	64	64	79	0.98	98	99	100	1	97	96	86	0.96	100	99	100	1	99	99	98	1	100	100	99	1	100	100	100	1
H - *S. elata*	-	-	-	-	-	-	-	--	67	67	67	0.97	-	-	53	-	93	94	94	1	79	80	79	1	92	91	91	1
I-*S. pseudoaffinis*	-	-	-	-	63	62	89	0.97	100	100	100	1	62	65	90	0.97	100	100	100	1	100	100	100	1	100	100	100	1
J - *S. torquata*	-	-	-	-	93	92	96	1	100	100	100	1	93	93	95	1	100	100	100	1	100	100	100	1	100	100	100	1
K- *S. trichomitria*	-	-	93	0.91	92	92	97	0.95	100	100	100	1	97	96	100	0.97	100	100	99	1	100	100	100	1	100	100	100	1

Appendix S5. Number of character state changes of 17 morphological characters in *Schlotheimia* as inferred from ancestral state reconstruction.

Number	Morphological character	No. of changes
1	Plant size	7
2	Creeping stem covering	2
3	Creeping stem format	3
4	Branch length	3
5	Vegetative leaves (VL) shape	5
6	VL lamina	4
7	VL apex	2
8	VL margin	1
9	VL costa	2
10	Perichetial leaves (PL) lamina	9
11	PL apex	3
12	PL costa	2
13	Seta length	2
14	Capsule exposure	1
15	Capsule shape	2
16	Calyptra shape	1
17	Calyptra surface	3

CAPÍTULO II

Taxonomic Notes and new synonyms on Brazilian *Macromitrium* Brid. (Bryophyta, Orthotrichaceae)

4 Taxonomic Notes and new synonyms on Brazilian *Macromitrium* Brid. (Bryophyta, Orthotrichaceae)

4.1 Abstract

Brazil is a megadiverse country that intends to catalog all its flora by 2020. Therefore, knowledge about taxonomy and the correct identification of species is essential for acessing the real species biodiversity. *Macromitrium* Brid. (Orthotrichaceae), is considered the third largest moss genus in the world and with the majority of its species distributed in tropical and subtropical regions. For Brazil 64 species have been cited, but 44 remains as unknown to the Brazilian flora. The aim of this work was to check all the 64 names of Brazilian *Macromitrium* helping to clarify its validity and correct identification. Type specimens from 13 herbaria were studied using optical microscope. After this analyze 22% are good species, 53% are synonymous of others species; 16% are excluded from the Brazilian Flora and 9% not were possible to check. This work contributed to clarify the diversity of *Macromitrium* in Brazil, contributing to the knowledge of bryophytes and important data for the flora of Brazil 2020.

Key Words: mosses, diversity, taxonomy, Brazilian flora.

4.2 Introduction

Brazil has set an aim to have its flora monographed by 2010 (Flora do Brazil 2020 online), being a mega diverse country, this is an ambitious objective, especially for certain groups like Bryophyta that has, especially when compared with the flowering plants, few experts on its taxonomy.

Good taxonomic practice relies on the study of nomenclatural types (Câmara *et al.* 2016) yet few studies focus on this important subject, exception made by a series of papers by Câmara *et al.* (2014, 2014a, 2016, 2016b, 2017) and Costa *et al.* (2016).

Among the Bryophyte groups that are in need of taxonomic revision is *Macromitrium* Brid. (Orthotrichaceae), considered the third largest moss genus in the world and with the majority of its species distributed in tropical and subtropical regions (Crosby *et al.* 1999, Frey & Stech 2009). According to Vitt & Ramsay (1985a), there are 350 species worldwide but only 128 can be morphologically differentiated (Crosby *et al.* 1999).

For Brazil, Yano (1981) listed 54 species and four varieties and after a careful analyze, Costa *et al.* (2011) considered from this previous list, 18 groups as possible to identify with the current literature to Brazil, and recognized those as putative species, also 30 species and two variety were considered as poorly known and in need of urgent revision (Costa *et al.* 2011). In the total, adding the two lists (Yano 1981; Costa *et al* 2011) 64 species have already been listed to Brazil. Currently, according to the online flora of Brazil (http://floradobrasil.jbrj.gov.br/), 20 species, are considered as good and included in the flora, with six endemics: *Macromitrium adnatum* Müll. Hal. (endemic), *M. argutum* Hampe, *M. brotheri* Müll. Hal. (endemic), *M. carionis* Müll. Hal., *M. catharinense* Paris, *M. cirrosum* (Hedw.) Brid., *M. contextum* Hampe, *M. diversifolium* Broth. (endemic), *M. divortiarum* Sehnem (endemic), *M. guatemalense* Müll. Hal., *M. longifolium* (Hook.) Brid., *M. microstomum* (Hook. & Grev.) Schwägr., *M. pellucidum* Mitt., *M.podocarpi* Müll. Hal., *M. proliferum* Mitt., *M. punctatum* (Hook. & Grev.) Brid., *M. richardii* Schwägr., *M. stellulatum* (Hornsch.) Brid., *M. swainsonii* (Hook.) Brid. and *M. undatum* Müll. Hal. (endemic).

During an ongoing molecular study on DNA barcode for Brazilian *Macromitrium* we realized that ca. 50% of accepted Brazilian species of *Macromitrium* are known only by the type specimens or by very old collections (more than 50 years), actually very little is known about the Brazilian species of *Macromitrium*, and identification of local species are often done with foreign literature like Japan (Noguchi 1967), New Zealand and Australia (Vitt &

Ramsay 1983, 1985a, 1985b, 1986), Africa (Rooy & Wijk 1992, Wilbraham 2015, 2016), Mexico (Sharp *et al.* 1994), Papua New Guinea (Vitt *et al.* 1995), China (Shui-Liang *et al.* 2012) and Central America (Allen 2002).

During the barcode study we had access to several type specimens of this genus. It is our aim to check all the 64 names of Brazilian *Macromitrium* previously cited, helping to clarify its validity and correct identification, contributing to the knowledge of the diversity and correct nomenclature on a very complex group of plants of the Brazilian flora.

4.3 Materials and methods

Extensive research on the protologues were performed in the original literature and, also the papers by Yano (1981), Costa *et al.* (2011), Câmara *et al.* (2016), the online Brazilian Flora (Flora do Brasil 2020). Specimens were loaned by the following herbaria: BM, E, G, GOET, ICN, L, MBM, NY, PACA, PC, R, RB, SP, and UB, and examined using an optical microscope, samples were left back into the packs. When authorized, pictures were taken. We carefully checked data from labels against the protologues to confirm the type status of the specimen. This is not a taxonomic revision and lectotypifications were not performed due to the fact that not all syntypes could be examined. The concept of syntypes follows the botanical nomenclature code (Turland *et al.* 2018) specially to avoid inadvertent lectotypifications (McNeill 2014, Prado *et al.* 2015). When a single collection was mentioned we treated them as "type" only as it was not possible to determine if duplicates exists elsewhere (syntypes).

In this work we compiled a list of 64 species cited for Brazil by Yano (1981), costa *et al.* (2011) and Flora Online (2020). However, for analysis of the type material we focused on the list made by Costa *et al* (2011) and Flora online, because many of the names cited by Yano (1981) have already been revised and excluded from the genus *Macromitrium.* Results are presented in alphabetical order of accepted name, followed by the basionym and all combinations in chronological order. Taxonomic comments are provided when needed. Barcode numbers are cited when they were available in herbaria.

4.4 Results

We are recognizing 14 species of *Macromitrium* in Brazil (being one endemic), other 21 species are new synonyms presented here and 13 previous synonyms are agreed with. Six

species remains as unknown status because it was not possible to check its type material, and 9 species were excluded from the Brazilian flora.

Types for species described by Müller Hallensis (Müll.Hal.) and Hornschuch, have been mostly destroyed during the bombing of Berlin in 1943 (Merrill 1943, Hiepko 1990), but we were able to find 14 of those, representing 94% of all the *Macromitrium* names described by Müller Hallensis that were cited for Brazil.

4.5 Good species for Brazil:

1) ***Macromitrium argutum*** Hampe, Linnaea 22: 581. 1849. Type: [Brazil] Rio de Janeiro, *Glaziou 9241* (lectotype: BM000879980! (designated by Costa *et al.* 2016); isolectotypes: BM000879983, BM000879979!, BM000989823!, PC0741810, PC0741814, PC0741815, PC0709395!).

= *Macromitrium proliferum* Mitt., Journal of the Linnean Society, Botany 12: 217. 1869. Type: [Colombia] Andes Bogotenses, *Weir s.n.* (syntypes: NY01086629!, NY01086631, NY01086632!, BM000873088!, BM000873089!)*;* Brasilia tropica, *Burchell 3959* (syntypes: NY01086630!, BM000873087!), *syn. nov.*

= *Macromitrium nematosum* E.B. Bartram, Journal of the Washington Academy of Sciences 42(6): 181. 1952. Type: [Brazil] Rio Grande do Sul: Estação São Salvador, *A. Sehnem 2774* (syntypes: FH; PACA74227!), *syn. nov.*

= *Macromitrium perserratum* E.B. Bartram, invalid (herbaria name), cited in synonym by Sehnem (1978).

Comments: This species is very common in the Atlantic rainforest.

2) ***Macromitrium catharinense*** Paris, Index Bryologicus, supplementum 1:237. 1900. non. *Macromitrium prolongatum* Müll. Hal., Bull. Herb. Boissier 6: 99. 1898, *hom. illeg.* Type: [Brazil] Prov. S. Catharina, Serra Geral, ad ramos arborum, Apr.1891, *E. Ule 1017*, Bryotheca Brasiliensis 134 (lectotype H2649005 hb Brotherus, designated by Li *et al.* (2019); isolectotypes: FI, GOET012311, JE04006251, JE04006252 PC0137901, PC0137902, PC0137903, PC0137904, MICH525906, NY01202245, L!); Brasilia, Sa. Catharina, Serra Geral, in ramis arborum, Januario 1890, cum fructibus junioribus, *E. Ule 847e* (syntype); idem, ad ramjos arborum marginis Serrae ejusdem, Apr.1891 cum fructu vetusta et ramis

aureis, *E. Ule 1017* (syntype); idem, Serra Itatiaia, 2000 m. alt., Mart.1894 sterile, *E. Ule 1835* (syntype).

= *Macromitrium prolongatum* var. *gracilius* Müll. Hal., Bulletin de l'Herbier Boissier 6: 100. 1898. *Macromitrium catharinense* var. *gracilius* (Müll. Hal.) Paris, Index Bryologicus, supplementum 1;237. 1900. *non. Macromitrium profusum* Müll. Hal., Bulletin de l'Herbier Boissier 6: 100. 1898, *invalid name cited as synonym. non. Teichodontium catharinense* var. *gracilior*, *herbaria name*. Type: Brasilia, Rio de Janeiro, in arboribus Serrae dos Orgaos, c.fr. reluslis et junioribus, Dez.1891, *E. Ule 1242* (lectotype H2649007, hb Brotherus, designated by Li *et al.* (2019)), *syn. acc.* Li *et al.* (2019).

= *Macromitrium schiffneri* Broth., Ergebnisse der Botanischen Expedition nach Südbrasilien, Musci 290. 1924. Type: [Brazil] São Paulo, Campo Grande, *V. Schiffner 558* (syntypes:: H - hb Brotherus, NY01243611, BM000873236!), *syn. nov.*

Comments: *Macromitrium catharinense* was described on the same type of *M. prolongatum* (previous but illegitimate name), it has an Afro-American distribution, and is easily recognized by its big pendent branches, always present in borders of nebular forests.

3) *Macromitrium cirrosum* (Hedw.) Brid., Bryologia Universa 1: 316. 1826. *Anictangium cirrosum* Hedw., Species Muscorum Frondosorum 42. 5 f. 1–3. 1801. *Hedwigia cirrosa* (Hedw.) Brid., Journal für die Botanik 1800(1): 272. 1801. *Neckera cirrosa* (Hedw.) F.Weber & D.Mohr, Index Musei Plantarum Cryptogamarum 3: 1803. *Anoectangium cirrosum* (Hedw.) Schwägr., Species Muscorum Frondosorum, supplementum 1: 38. 1811. *Schlotheimia cirrosa* (Hedw.) Brid., Muscologia Recentiorum, supplementum 2: 19. 1812. *Orthotrichum cirrosum* (Hedw.) Hook. & Grev., Edinburgh Journal of Science 1: 130. 6. 1824. *Ulota cirrosa* (Hedw.) Hook & Grev., Edinburgh Journal of Science 1:130. 1824. *invalid. name*. Type: Jamaica, Montserrat, *s.col. s.n.* (syntypes: G, BM000873245, BM000862650!, E00002459)**.**

= *Macromitrium substrictifolium* Müll. Hal., Bulletin de l'Herbier Boissier 6: 98. 1898. Type: Brasilia, Rio de Janeiro, Tijuca, *E. Ule 1672* (syntypes: US02482517, R00014297!), *syn. nov.*

= *Macromitrium hoehnei* Herzog, Arquivos de Botânica do Estado de São Paulo 1(2): 63. 1924. Type: [BRAZIL] São Paulo, Estação Biológica do Alto da Serra, *s.col. s.n. - hb n. 4340 - hb T. Herzog 7500* (syntypes: SP060004!, PH00003401), *syn. nov.*

Comments: *Macromitrium cirrosum* has an American distribution and is a common species in Brazil, *Macromitrium substrictifolium* is a Müller Hallensis name and the original material used for species description probably was destroyed in Berlin in 1943 during World War II, the only citation of this name was from the original publication and replicated by Yano (1981).

4) *Macromitrium diversifolium* Broth., Hedwigia 34: 126. 1895. Type: [Brazil] Goyaz: an Baumstämmen des Corumbagebietes, *E. Ule 1562* (syntypes: H-BR, R000081780!)

= *Macromitrium divortiarum* Sehnem, Pesquisas, Botânica 32: 24. pl. 7: b (pp. 68–69). 1978. Type: [Brazil] Goiás: Reserva de Águas Emendadas, *A. Sehnem 8605* (type: PACA73150!) *syn. nov.*

Comments: The original material used for the description of *M. diversifolium* is probably deposited in Herbarium H-BR. This endemic species is only known by the type specimens collected in central Brazil in the Cerrado Domain, not registered even since and may be extinct or poorly collected.

5) *Macromitrium eriomitrium* Müll. Hal., Bulletin de l'Herbier Boissier 6: 98. 1898. Type: Brasilia, Serra Itatiaia, *E. Ule 1834* (type: R000081757!).

Comments: This is a Müller Hallensis name and the original material used for species description was probably destroyed in Berlin in 1943 during World War II, the specimens of Ule collections are spread in several herbaria sometimes with his on number and others with Bryotheca Brasiliensis number. Is one of the excluded names from the online flora of Brazil, by Costa et al (2011). The only citation of this name was from the original publication and replicated by Yano (1981). This species, unknown in Brazil even since, and may as well be extinct, or under collected.

6) *Macromitrium guatemalense* Müll. Hal., Synopsis Muscorum Frondosorum omnium hucusque Cognitorum 2: 644. 1851. Type: Guatemala, Friedrichstal, *Kegel s.n.* (syntypes: G00284083!, BM000873192!, BM000873191!, L0060440!).

= *Macromitrium negrense* Mitt., Journal of the Linnean Society, Botany 12: 208. 1869. Type: [Brazil] Fl. Negro, Igarapé da Cachoeira, *R. Spruce 106* (syntypes: NY00435668, BM000720547, BM000720548, PC0137817!, PC0137818!, E00165156), *syn. nov.*

Comments: This is a Neotropical species, common in Brazil in Amazon, Cerrado and Atlantic rainforest domain.

7) *Macromitrium longifolium* (Hook.) Brid., Bryologia Universa 1: 309, 738. 1826. *Orthotrichum longifolium* Hook., Musci Exotici 1: 44. 1818. *Schlotheimia longifolia* (Hook.) Schwägr., Species muscorum Frondosorum, supplementum 2(2): 147. 1827. Type: [Venezuela] Caracas, *Humboldt et Bonpland s.n.* (syntypes; E00289633, BM000873190, BM000720648, BM000720656!; PC0137790!, PC0137789!, JE04008699).

= *Macromitrium perfragile* E.B. Bartram, Journal of the Washington Academy of Sciences 42(6): 181. 1952. Type: [Brazil] Rio Grande do Sul: S. Leopoldo, *A. Sehnem 432* (syntype PACA74217!); idem Aparados, Bom Jesús, *Sehnem 576* (syntype PACA74218!); idem, Campestre Montenegro, *A. Sehnem 2175* (syntype PACA74219!); idem Vila Oliva, S. Francisco, *A. Sehnem 2630* (syntype PACA74215!), *syn. nov.*

Comments: *Macromitrium longifolium* is a Neotropical species, common in Brazilian Atlantic rainforest.

8) *Macromitrium microstomum* (Hook. & Grev.) Schwägr., Species Muscorum Frondosorum, supplementum 2(2): 130. 1827. *Orthotrichum microstomum* Hook. & Grev., Edinburgh Journal of Science 1: 114. 4. 1824. *Leiotheca microstoma* (Hook. & Grev.) Brid., Bryologia Universa 1: 729. 1826. Type: [Tasmania] Van Dieman's Land; *Dr. Spence* [*s.n.*] (lectotype E00011665 designated by Vitt & Ramsay (1985), isolectotype BM000873897!).

= *Macromitrium nitidum* Hook. f. & Wilson, London Journal of Botany 3: 156. 1844. Type: [Brazil] Serra de Jaquari, *G. Gardner s.n.* (lectotype BM000878023! designed by Wilbraham (2007); isolectotypes BM000878033!, BM000878034!, BM000878038!; E0002986!)*, syn. acc.* Wilbraham (2007).

= *Macromitrium hornschuchii* Müll. Hal., Botanische Zeitung (Berlin) 3: 526. 1845. Type: [Brazil] Minas Gerais: Serra da Piedade, *J. Warming s.n.* (syntypes: H, BM000873099!, BM000873100, BM000873101), s*yn. acc.* Wijk *et al.* (1964).

= *Macromitrium filicaule* Müll. Hal., Synopsis Muscorum Frondosorum Omnium Hucusque Cognitorum 1: 745, 1849. Type: Brasilia: *Gardner 53b* (lectotype BM000873118! designated by Yu *et al.* (2018), isolectotypes BM000873119, NY01201996), *syn. acc.* Yu *et al.* (2018)

Comments: This is a good cosmopolitan species, common in Brazilian Atlantic rainforest.

9) *Macromitrium pseudofimbriatum* Hampe, Videnskabelige Meddelelser fra Dansk Naturhistorisk Forening i Kjøbenhavn ser. 3, 6: 144. 1874. Type: [Brazil] Rio de Janeiro: *A. Glaziou 7053* (lectotype BM000873081 designated by Costa *et al.* (2016), isolectotypes BM000873080!, BM000873082, BM000873083, PC0107942, PC0709374!).

=*Macromitrium doeringianum* Hampe, Videnskabelige Meddelelser fra Dansk Naturhistorisk Forening i Kjøbenhavn ser. 4, 1: 96. 1879. Type: [Brazil, Rio de Janeiro], Petropolis, *Döring s.n.* (type: BM000873123!), *syn. nov.*

=*Macromitrium podocarpi* Müll. Hal., Bulletin de l'Herbier Boissier 6: 96. 1898. Type*:* Brasilia, Minas Gerais, Serra Itabira do Campo, *E. Ule 1066* (syntypes: NY01086626!, NY01086627!, GOET012309!, G00265988!, G00265989!, BM000873094!, PC0137891), *syn. nov.*

= *Macromitrium podocarpi* Müll. Hal. var. *falcifolium* Müll. Hal., Bulletin de l'Herbier Boissier 6: 96. 1898. Type: [Brazil] Minas Gerais: Caraça, *E. Ule 1408* (Type: R000014299!), *syn. nov.*

Comments: *Macromitrium pseudofimbriatum* is known only from the original publication and replicated by Yano (1981), but after analyzing the type material of *M. podocarpi* and *M. podocarpi* var. *falcifolium.,* these are synonyms of *M. pseudofimbriatum. Macromitrium podocarpi* is a common species in Brazil, but the oldest name *M. pseudofimbriatum* has priority. *Macromitrium podocarpi* and *M. podocarpi* var. *falcifolium* are Müller Hallensis names and the original material used for species description was probably destroyed in Berlin in 1943 during World War II.

10) *Macromitrium punctatum* (Hook. & Grev.) Brid., Bryologia Universa 1: 739. 1826. *Orthotrichum punctatum* Hook. & Grev., Edinburgh Journal of Science 1: 119. 5. 1824. Type: Brazil, *Raddi s.n.* (syntypes: E00011666!, E00011667!).

= *Macromitrium pentastichum* Müll. Hal., Linnaea 21: 186. 1848. Type: Suriname, bei Mariepaston am Saramacca - strome, Mai, 1846. Hb. *Kegel. No. 1405* (Syntypes: GOET013590, GOET013591, NY01086514) *syn. acc.* Vitt (1979).

Comments: *Macromitrium punctatum* is a neotropical species, common in the Brazilian Atlantic rainforest.

11) *Macromitrium regnellii* Hampe, Synopsis Muscorum Frondosorum omnium hucusque Cognitorum 1: 738. 1849. Type: [Brazil] Minas Gerais: Caldas, *A. Regnell 497* (Syntypes: BM000873240! E00011668, E00011669).

= *Macromitrium contextum* Hampe, Annales des Sciences Naturelles, Botanique, sér. 5, 4: 331. 1865. Type: [Brazil] Rio Negro, *A. Lindig s.n.* (lectotype BM000873181! designated by Vitt (1979), isolectotypes: NY00518303!, NY00518304!, NY00518305!, NY00518306!, BM000873180!, BM000873182!, L0060432!, L0060433!, PC0137645, PC0137646, GOET013589), *syn. nov.*

Comments: After investigating into the type of *M. contextum* (synonymized with *M. punctatum* by Grout in 1944), we found a mixed collection with specimens that match exactly with the description of *M. contextum* mixed with *M. richardii*, and specimens of *M. punctatum*, therefore we do not adopt the synonymization of Grout (1944) and we are proposing this new synonym.

12) *Macromitrium swainsonii* (Hook.) Brid., Bryologia Universa 1: 318. 1826.. *Orthotrichum swainsonii* Hook., Musci Exotici 2: 127. 1819. *Leiotheca swainsonii* (Hook.) Brid., Bryologia Universa 2: 730. 1827 Type: [Brazil] Rio Janeiro, *D. Swainson s.n.* (type: BM000873214!).

= *Macromitrium stellulatum* (Hornsch.) Brid., Bryologia Universa 1: 314. 1826. *Schlotheimia stellulata* Hornsch., Horae Physicae Berolinenses 61. 12 f. 1–6. 1820. *Orthotrichum stellulatum* (Hornsch.) Hook. & Grev., Edinburgh Journal of Science 1: 119. 1824. Edinburgh Journal of Science 1: 119. 1824. Type: [Venezuela] sylvae ad *Orinoco* fluminis ripas, [*s.col. s.n.*] (syntypes: E00428901 - hb Wildenow; BM000873227!, NY01272723), *syn. nov.*

= *Macromitrium carionis* Müll. Hal., Bulletin de l'Herbier Boissier 5: 199. 1897. Type: [Guatemala] Caesta de Lovio, *Bernoulli et Cario 48* (syntypes: GOET011888!, GOET012471, GOET012472, GOET011887!, PC0137621), *syn. nov.*

= *Macromitrium brotheri* Müll. Hal., Bulletin de l'Herbier Boissier 6: 97. 1898. Type: Brasilia, Goyaz, Serra Dourada, *E. Ule s.n.* (syntype: R000081764)*;* idem, Goyaz, Mossamedes, *E. Ule 1560, 1561* (syntype: R000081763); idem, Goyaz, Passa Tempo, *E. Ule 1564* (syntypes: PC0137611!, R000081774). *syn. nov.*

Comments: This is a Neotropical species, common in Brazil and seems to be related with dry areas.

13) *Macromitrium undatum* Müll. Hal., Bulletin de l'Herbier Boissier 6: 97. 1898. Type: Brasilia, Serra Itatiaia, *E. Ule 1832* (type: R000081758!).

Comments: This is a Müller Hallensis name and the original material used for species description probably was destroyed in Berlin in 1943 during World War II, this name is only known by the type material, not registered even since and may be extinct or poorly collected.

14) *Macromitrium viticulosum* (Raddi) Brid. Bryologia Universa 1: 738. 1826. *Schlotheimia viticulosa* Raddi, Crittogame Brasiliane 4. 1822. Type: [Brazil: Rio de Janeiro] Corcovado, *Raddi s.n.* (syntypes: PI, PC0695930!), s*yn. nov.*

= *Macromitrium richardii* Schwägr., Species Muscorum Frondosorum, supplementum 2(1): 70–71, pl. 173. 1826. Type: French Guiana, *Richard s.n.* (type herbarium not designated).

= *Macromitrium intortifolium* Hampe, Botanische Zeitung (Berlin) 20: 362. 1862. Type: [Brazil] Sta. Catharina *Blumenau s.n.* (syntypes: BM000873097!, BM000873098, NY01202032), *syn. acc.* Grout (1944).

= *Macromitrium glaziovii* Hampe, Videnskabelige Meddelelser fra Dansk Naturhistorisk Forening i Kjøbenhavn ser. 3, 6: 143. 1874. Type: Brazil, Rio de Janeiro, *Glaziou 6385* p.p. (lectotype BM000873106! designated by Costa et al. (2016); isolectotypes BM000873107!, PC0107940, PC0709381!), *syn. nov.*

Comments: It was not possible to locate the type specimen of *M. richardii.* However, we analyzed the type material of *Macromitrium didymodon* Schwägr (BM000873317!,

BM000873318!) and *Macromitrium goniopodium* Mitt. (BM000873319!), both synonyms of *M. richardii* according to Grout (1944) and according to illustrations and original description of the species, characterized by having 3-4 papillae in each cell at the apex, we concluded that the species *M. richardii M. glaziovii,* and *M. intortifolium* are synonyms of and *M. viticulosum. Macromitrium viticulosum* is a Neotropical species, common in Brazilian Atlantic forest.

4.6 Species of *Macromitrium* transferred to another genera

1) *Cardotiella quinquefari*a (Hornsch.) Vitt, Journal of the Hattori Botanical Laboratory 49: 102. 1981. *Macromitrium quinquefarium* Hornsch., Flora Brasiliensis 1(2): 26. 1840. Type: [Brazil, Bahia state] Habitat in Montibus ad Lages, Sincora, et ad Rio de Contas, rel.in Mediterraneis prov. bahiensis: M. 4 (type: BM000879986), *comb. acc.* Vitt (1981).

Comments: *Macromitrium quinquefarium* was cited for Brazil by Yano (1981). After that, it was placed in the genus *cardotiella* by Vitt (1981)

2) *Groutiella chimborazensis* (Spruce ex Mitt.) Florsch., Flora of Suriname 6(1): 215. 1964. *Macromitrium chimborazense* Spruce ex Mitt., Journal of the Linnean Society, Botany 12: 218. 1869.

Micromitrium chimborazense (Spruce ex Mitt.) A. Jaeger, Bericht über die Thätigkeit der St. Gallischen Naturwissenschaftlichen Gesellschaft 1872–73: 157 (Gen. Sp. Musc. 1: 435). 1874. Type: Andes Quitenses, ad radices occidentalis montis Chimborazo (3500 ped.), *Spruce 110.* (syntypes: NY00518284, E00209742, BM000720680, BM000720679, PC0137625).

= *Macromitrium adnatum* Müll. Hal., Bulletin de l'Herbier Boissier 6: 96. 1898. Type: Brasilia, Goyaz, Serra Dourada, *E. Ule 1558* (type: R000081779!), s*yn. nov.*

Comments: *Macromitrium adnatum* is a Müller Hallensis name and the original material used for species description was probably destroyed in Berlin in 1943 during World War II, and after careful study of the type of *M. adnatum* we concluded that it is a synonym of *Groutiella chimborazensis*.

3) *Macrocoma orthotrichoides* (Raddi) Wijk & Margad., Taxon 11: 221. 1962. *Lasia orthotrichoides* Raddi, Crittogame Brasiliane 6. 1822. Type: Type: Brazil, Rio de Janeiro, Mage ´County, 'Trovasi questo Musco sopra i tronchi degli Alberi nelle vicinanze di Mandioca', *Raddi s.n.* (Lectotype designated by Costa (2009): PI!; Isolectotypes: BM (Hooker), E (Greville), PI!, FI!).

= *Macromitrium chrysomitrium* Müll. Hal., Bulletin de l'Herbier Boissier 6: 101. 1898. *Macrocoma chrysomitria* (Müll. Hal.) Sehnem, Bulletin de l'Herbier Boissier 6: 101. 1898. Type: Brasilia, Serra Itatiaia, *E. Ule 1836* (Lectotype H - hb Brotherus, designated by Vitt (1980) isolectotypes HBG, R000081762!), s*yn. acc.* Vitt (1980).

=*Macromitrium subpycnangium* Müll. Hal., Bulletin de l'Herbier Boissier 6: 100. 1898. Type: Brasilia, Rio de Janeiro, Tijuca ad ramos arborum sylvestrium, Dec. 1893: *E. Ule 1671* (Lectotype HBG, designated by Vitt (1980) isolectotypes H - hb Brotherus, R000081778) s*yn. acc.* Vitt (1980).

Comments: These species were cited for Brazil by Yano (1981). After review of the genus *Macrocoma* by Vitt (1980), the species *Macromitrium chrysomitrium* and *Macromitrium subpycnangium* were synonymized as *Macrocoma orthotrichoides.*

4) *Schlotheimia merkelii* Hornsch., Flora Brasiliensis 1(2): 29. 1 f. 4. 1840. Type: Type: Prope Rio de Janeiro in truncis arborum, Julio et Augusto, Merkel s.n., In Brasilia australi, *Sellow 4* (Type: M!).

= *Macromitrium emarginatum* Broth., Hedwigia 45: 273. 1906. Type: [Brazil] Amazonas, Rio Negro, Manaus *E. Ule 2320* (syntypes: H-BR, BM000873122!), s*yn. nov.*

Comments: *Macromitrium emarginatum* was not re-evaluated since its publication and after check the type specimen it is synonym of *Schlotheimia merkelii.*

5) *Macrocoma tenuis* subsp. *sullivantii* (Müll. Hal.) Vitt., The Bryologist 83(4): 413. 1980[1981]. *Macromitrium sullivantii* Müll. Hal., Botanische Zeitung (Berlin) 20(43): 361. 1862. *Macrocoma sullivantii* (Müll. Hal.) Grout, The Bryologist 47: 5. 1944. Type: In summitate mont. Jonah Georgiae americanae ad corticem pinorum vetust. *Lesquereux s.n.* (Type: MICH525919, NY00345364)

= *Macromitrium lampromitrium* Müll. Hal., Bulletin de l'Herbier Boissier 6: 101. 1898. Type: Brasilia, Serra Itatiaia, *E. Ule 1837* (lectotype H - hb Brotherus designated by Vitt (1980), isolectotypes HBG, R000081760!), s*yn. acc.* Vitt (1980).

= *Macromitrium progressum* Hampe in Vidensk. Meddel. Naturhist. Foren. Kjøbenhavn, ser. 3, 10: 259. 1878 – Lectotype (designated by Vitt in Bryologist 83: 413. 1980): Brazil, Rio de Janeiro, *Glaziou 7415* (BM; isolectotypes: BM, PC0741444), s*yn. acc.* Vitt (1980).

= *Macromitrium pycnangium* Müll. Hal. ex Broth., Bulletin de l'Herbier Boissier 6: 102. 1898. *Macrocoma pycnangia* (Müll. Hal. ex Broth.) Sehnem, Pesquisas, Botânica 32: 12. 1978. Type: Brasilia, Sa. Catharina, Serra do Oratorio, in declivibus prope Orleans ad flumen Laranjeiras superius, in ramis arborum, Septbr. 1889 cum fruct. supramaturis et juvenilibus *E. Ulle,* Coll. Nº 721 (syntypes: PC0137915, PC0137916), s*yn. acc.* Vitt (1980).

Comments: *Macromitrium lampromitrium, Macromitrium progressum and Macromitrium pycnangium* were listed by Yano (1981), but these species were synonymized as *Macrocoma tenuis subsp. sullivanti* by Vitt (1980), and we agree with this synonymization.

6) *Schlotheimia trichomitria* Schwägr., Species Muscorum Frondosorum, supplementum 2(1): 55. pl. 169. 1826. Type: Brasilia, Monte Video (Uruguai), *F. Sellow s.n.* (Type: NY1244105!).

= *Macromitrium pellucidum* Mitt., Journal of the Linnean Society, Botany 12: 203. 1869. Type: Hab. Fl. Uaupes, Panuré ad arbores, *Spruce 80* (syntypes: BM000720573!, BM000720574!, BM000720575!, BM000720576!, BM000720579, BM000720580; NY01086499, NY01086500, NY01086501), s*yn. nov.*

Comments: *Macromitrium pellucidum* was not re-evaluated since its publication and after checking the type specimen it is a synonymous of *Schlotheimia trichomitria*, even in the stereoscope is possible to see its characteristic rugose leaves and hairy calyptra.

7) *Groutiella tumidula* (Mitt.) Vitt, The Bryologist 82(1): 9. 1979. *Macromitrium tumidulum* Mitt., Journal of the Linnean Society, Botany 12: 201. 1869. Type: Andes Peruviae, Tarapoto in truncis praecipue Crescentiae, rarius ad rupes; etiam in ripis pl. Huallaga ad Yurimaguas (1000 ped.), *Spruce 101.* (Syntypes: PC0695965, MICH525921, G00260285)

= *Macromitrium subapiculatum* Broth., Hedwigia 45: 271. 1906. Type: [Brazil] Amazonas, Rio Juruá, Juruá Miry, *E. Ule 2273* (syntypes PC148164!; L910.141-96!), s*yn. nov*.

Comments: *Macromitrium subapiculatum* was not re-evaluated since its publication and after studying the type of *M. subapiculatum* it is synonymous of *Groutiela tumidula* especially due to its differentiated margin.

8) *Macrocoma tenuis* (Hook. & Grev.) Vitt, Revue Bryologique et Lichénologique 39(2): 217. 1973. *Orthotrichum tenue* Hook. & Grev., Edinburgh Journal of Science 1: 120, pl. 5 [lower left]. 1824. *Macromitrium tenue* (Hook. & Grev.) Brid., Bryologia Universa 1: 740. 1826. Type: Trees at the Cape of Good Hope; A. Menzies, Esq.; and W. j. Burchell, Esq. (Type: BM000868373).

= *Macromitrium zikanii* Herzog, Arquivos de Botânica do Estado de São Paulo 1(2): 65. 1924. Type: Brazil, Minas Gerais: Passa Quatro, *J.F. Zikán 5803* (syntypes: JE04008699, hb. Herzog 6285 BM000873202!), s*yn. nov*.

Comments: After analysis of the type material of *M. zikanii,* this species is synonym of *Macrocoma tenuis*.

4.7 Types not seen

1) *Macromitrium atratum* Herzog, Repertorium Specierum Novarum Regni Vegetabilis 21: 30. 1925. Type: Brasilia: São Paulo: Campos do Jordão, Serra da Mantiqueira, *F.C. Hoehne s.n. -* hb no. 637a (type: JE04006253).

Comments: Is one of the excluded names from the Brazilian Flora (Costa *et al.* 2011), it was collected in Brazil and is known only from the original publication and the record that was replicated by Yano (1981), its taxonomic status remains unknown because it was not possible to study the type specimen.

2) *Macromitrium caldense* Ångstr., Öfversigt af Förhandlingar Kongl. Svenska Vetenskaps-Akademien 33(4): 12. 1876. Type: [Brazil] Minas Gerais, Caldas*, S. Henschen s.n.* (type: S).

Comments: Is one of the excluded names from the Brazilian Flora, it is cited for Brazil and it is known only from the original publication and the record is replicated by Yano

(1981), unfortunately it was not possible to visit the S herbarium, as it was closed at the time of this study. The original description fit into *Macromitrium* but is not informative enough to allow further conclusions.

3) *Macromitrium cirrosum* var. *stenophyllum* (Mitt.) Grout, The Bryologist 47: 9. 1944. *Macromitrium stenophyllum* Mitt., Journal of the Linnean Society, Botany 12: 215. 1869. Type: Ins. Jamaica, *Wilson* n. *607* in Herb. Hooker; etiam ex India Occidentali, an Jamaica? (syntypes: NY01086602 - hb R. Brown., MICH525912)

Comments: Unfortunately, it was not possible to study the type material for this species, and the original description does not allow further conclusions.

4) *Macromitrium mosenii* Broth., Bihang till Kongliga Svenska Vetenskaps-Akademiens Handlingar 21 Afd. 3(3): 24. 1895. Type: [Brazil] Minas Gerais, Caldas, *M. Mosén 231* (syntypes: H-BR, M, HUH00213651, HUH00213651).

Comments: Is only known by its original description. Unfortunately, it was not possible to study the type material and the original description does not allow any further conclusions.

5) *Macromitrium rugulosum* Ångstr., Öfversigt af Förhandlingar: Kongl. Svenska Vetenskaps-Akademien 33(4): 12. 1876. Type: [Brazil] Minas Gerais, Caldas, *J.F. Widgren s.n.* (type: S).

Comments: Is was known only by its original description, unfortunately it was not possible to visit the S herbarium, as explained above. The original description would fit into *Macromitrium* but is not informative enough to allow further conclusions.

6) *Macromitrium strictifolium* Müll. Hal., Bulletin de l'Herbier Boissier 6: 99. 1898. Type: [Brazil] Rio de Janeiro, Serra dos Órgãos, *H. Schenck s.n.* (type H-BR).

Comments: This is a Müller Hallensis name and the original material used for species description probably was destroyed in Berlin in 1943 during World War II, is yet another species known only by its original description. The original description would fit into *Macromitrium* but is not informative enough to allow further conclusions.

4.8 Excluded names from Brazilian flora

1) ***Macromitrium clavatum*** Schimp. ex Grout., The Bryologist 47: 16. 1944. Type: Guadeloupe, *L'Herminier s.n.* (syntypes: NY, BM000982716, BM000982717, BM000982718, BM000982719, BM000982720).

Comments: This Caribbean species was cited by Yano (2004) to Serra da Esperança, Parana State in Brazil based on an erroneous identification. The specimen *Vitt 21346* (SP) is *Macromitrium viticulosum.*

2) ***Macromitrium filiforme*** var. ***squarrulosum*** Hampe, Videnskabelige Meddelelser fra Dansk Naturhistorisk Forening i Kjøbenhavn ser. 3, 10: 259. 1878, nom. illeg.

Comments: This species was cited by Yano (1981), but is an illegitimate name, and was not mentioned in the list of Costa *et al*. (2011) nor in the Brazilian flora online.

3) ***Macromitrium fimbriatum*** (P. Beauv.) Schwägr., Species Muscorum Frondosorum, supplementum 2: 37. pl. 111. 1823. *Orthotrichum fimbriatum* P. Beauv., Prodrome des Cinquième et Sixième Familles de l'Aethéogamie 80. 1805. Type: Tristan da Cunha *Du Petit-Thouars* (syntypes: G00120670, BM000873857!, BM000873898, PC0137508).

Comments: This African species was cited by Hornschuch (1840) to Rio de Janeiro, but we could not find any specimen or any other citation to confirm the occurrence of this species in Brazil.

4) ***Macromitrium longirostre*** (Hook.) Schwägr., Species Muscorum Frondosorum, supplementum 2(1): 38. pl. 112. 1823. *Orthotrichum longirostre* Hook., Musci Exotici 1: 25. 1818. Type: New Zealand, Dusky Bay dicto, 1791, *D. Menzies 68* (syntypes; BM000982618, BM000982610, BM000982611!, BM000982612, BM000982613!; E00011661, E00246165).

Comments: This species was cited by Hornschuch (1840) to Rio de Janeiro, but we could not find the specimen cited or any other specimen to confirm this record for Brazil.

5) *Macromitrium pallidum* (P. Beauv.) Wijk & Margad., Taxon 9: 190. 1960. *Orthotrichum pallidum* P. Beauv., Prodrome des Cinquième et Sixième Familles de l'Aethéogamie 81. 1805. Type: África, Madagascar, *sine legit* (syntypes: E00428860, E00428861, BM000982423, BM000982424, BM000873859!).

Comments: This African species was cited to Brazil by Müller (1844) based on the specimen *Gardner 53b* with the name *Macromitrium aciculare* Brid., but we could not find this specimen and we do not find any others specimens to confirm this occurrence in Brazil.

6) *Macromitrium paraphysatum* Sehnem, Pesquisas, Botânica 32: 19. pl. 4: b (p. 62–63). 1978. Illegitimate name. Type: Brazil. Rio Grande do Sul: Sao Francisco de Paula, prope urbem, 1000 m *Sehnem 5370.*

Comments: This species was cited by Yano (1981), but is a illegitimate name, (Mitten had already published another species of the later homonym in 1869). This species is not mentioned in the list of Costa et al (2011) nor in the Brazilian flora online.

7) *Macromitrium sharpii* H.A. Crum ex Vitt., The Bryologist 82: 4. f. 10–20, 66. 1979. Type: Mexico, Durango, *A. Sharp 1859* (holotype: ALTA; isotypes: MICH525909, TENN).

Comments: *Macromitrium sharpii* seems to be a Mexican endemic and all specimens cited under this name to Brazil were several other taxa.

8) *Macromitrium sulcatum* (Hook.) Brid., Bryologia Universa 1: 319. 1826. *Schlotheimia sulcata* Hook., Musci Exotici 2: 156. 1819. *Orthotrichum sulcatum* (Hook.) Hook. & Grev., Edinburgh Journal of Science 1: 129. 1824. Type: Nepal, *D. Gardner s.n.* (syntypes: BM000982538, BM000878045, BM000982539, BM000982541, BM000982540).

Comments: This is an Asian species cited by Hornschuch (1840) but we do not find any specimens to confirm its occurrence in Brazil.

9) *Macromitrium urceolatum* (Hook.) Brid., Bryologia Universa 1: 312. 1826. *Orthotrichum urceolatum* Hook., Musci Exotici 2: t. 124, f. 1–8. 1819. *Leiotheca urceolata* (Hook.) Brid.,

Bryologia Universa 1: 730. 1826. Type: In Insula Sancta Helenae, 1795, D. *Menzies s.n.* (syntypes: E00348715, E00011670; BM000982426, BM000982431).

Comments: This species was cited to Brazil by Hooker & Wilson (1844) and Müller (1845) to the Morro Velho in the Bahia state on specimens collected by Gardner, these authors indicate the identification as "affinis" *Macromitrium urceolato*, but specimens cited have no collector number, and we could not find any specimens that would match with the description, making it impossible to check the validity of this name and this way we are excluding the occurrence of this species from Brazil.

4.9 Acknowledgments

4.10 References

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CAPÍTULO III

Macromitrium Brid. (Bryophyta, Orthotrichaceae) in Brazil: A Molecular Approach

5 *MACROMITRIUM* BRID. (BRYOPHYTA, ORTHOTRICHACEAE) IN BRAZIL: A MOLECULAR APPROACH

5.1 Abstract

In Brazil *Macromitrium* Brid. is a complex group to work, due to presenting many taxonomic and systematic problems, with an uncertain phylogenetic position and the morphological characters used for species identification are not well established to the Brazilian taxa. In this paper we aim was to (i) to test of monophyly of brazilian species of *Macromitrium* based on 4 markers from different genomic compartments (*trn*L-F, *rps*4, *nad*5 and 26S) (ii) if not monophyletic group, to delimit wich Brazilian species belong in true *Macromitrium* and, (iii) test the potential of *trn*G-R, *trn*L-F, and ITS markers to resolve the phylogenetic relationships and species delimitations within *Macromitrium* in Brazil. Our data demonstrate that the species of *Macromitrium* is not monophyletic, occurring the formation of 3 different groups: MG1 (true *Macronitrium* genus), MG2 (new genus *Pseudomacromitrium,* description here) and MG3 (the monospecific new genus). Our barcoding data suggests that the best candidate marker for DNA barcoding was *trn*G-R due to its easy amplification and ability to discriminate all the species for both groups. The nuclear marker ITS was easy to amplify and more variable than the plastid markers, but due the alignment difficult or fungal contamination is a potential drawback. *Trn*L-F had a low discrimination potential. Our results provide important data on the phylogeny of the group, serving as a basis for the expansion of the phylogenetic studies for the other species that occur in the world, as well as providing a new tool to solve the current problems of identification of Brazilian species.

Key words: DNA Barcoding, phylogeny, moss, molecular markers, Macromitrioideae

5.2 Introduction

The cosmopolitan moss family Orthotrichaceae Arn. comprises more than 800 species in approximately 20 genera, divided into two subfamilies, the acrocarpous Orthotrichoideae and the cladocarpous Macromitrioideae (Goffinet and Vitt 1998; Frey and Stech 2009). *Macromitrium* Brid. of the subfamily Macromitrioideae is the third largest moss genus in the world, with an estimated 365, mostly tropical and subtropical, species (Crosby et al. 1999; Frey and Stech 2009), of which roughly one third (128) have been thoroughly treated in revisions (Crosby et al. 1999). The number of little known taxa has decreased due to the accomplishment of taxonomic treatments and floras for Japan (Noguchi 1967), New Zealand and Australia (Vitt and Ramsay 1983, 1985a,b, 1986), (southern and eastern) Africa (Rooy and Wijk 1992; Wilbraham 2015, 2016), Papua New Guinea (Vitt et al. 1995), China (Guo et al. 2012), Mexico (Sharp et al. 1994) and Central America (Allen 2002). However, insufficient morphological and taxonomical knowledge, superfluous names, and the absence of good keys still hamper the identification of *Macromitrium* species in several geographic regions, such as Africa (Wilbraham 2016), South America and the Pacific (Yu et al. 2018). In addition, the morphological characters used for species identification are not well established with many overlaps (Allen 2002).

For Brazil, 54 *Macromitrium* species have been recorded, but of these, 34 were considered poorly known by Costa et al. (2011). Of the remaining 20 species (including six species endemic to Brazil) listed in the current Brazilian Online Flora (http://floradobrasil.jbrj.gov.br/), still 50% are known only by the types and/or very ancient collections according to *SpeciesLink* (http://splink.cria.org.br/).

One hypothesis for the taxonomic complexity and problems with interpreting morphological character variation may be that *Macromitrium* is not monophyletic (Goffinet et al. 1998; Frey and Stech 2009). However, the molecular studies involving genera of Orthotrichaceae published to date either do not include species of *Macromitrium* (Cox et al. 2010; Goffinet et al. 2004) or their taxon sampling is insufficient to test this hypothesis (Goffinet et al. 1998; Li et al. 2013). Phylogenetic studies of extended molecular datasets are necessary for a better understanding of the circumscription of *Macromitrium*.

In addition, DNA barcoding (Herbert et al. 2003) should be implemented as a molecular tool to distinguish *Macromitrium* species and to infer the diagnostic value of morphological characters for species identification. DNA barcoding of plants is generally performed based on a combination of two or more genetic loci. Most existing studies on

bryophytes have compared the highly variable nuclear ribosomal ITS regions and various markers from the chloroplast genome (overview in Valente et al. 2019). In the Orthotrichaceae, Li et al. (2013) tested the use of the chloroplast markers *trn*G and *trn*L-F for Chinese *Macromitrium* species, while Valente et al. (2019) tackled species delimitations in Brazilian *Schlotheimia* Brid. based on *trn*L-F, *trn*G-R and ITS sequence data.

The present study employs an extended molecular dataset (four markers from all three genomic compartments) to study the circumscription and species delimitation of *Macromitrium* in Brazil. Specifically, we aim to (i) to test whether Brazilian species of *Macromitrium* form a monophyletic group, (ii) if not a monophyletic group, to delimit if (and which) Brazilian species belong in true *Macromitrium*, and (iii) test the potential of *trn*G-R*, trn*L-F, and ITS markers for molecular identification of the Brazilian *Macromitrium* species.

5.3 Materials and methods

Taxon and molecular marker sampling

Phylogenetic analyses to test the monophyly of *Macromitrium* followed the sampling of Cox et al. (2010). Albeit not including *Macromitrium*, Cox et al. (2010) employed sequences from three markers, nuclear ribosomal 26S, mitochondrial *nad*5, and chloroplast *rps*4, from most other genera of Macromitrioideae. To those we added chloroplast *trn*L-F sequences of the same taxa that were available on GenBank from other studies, and own sequences from all four markers. The resulting dataset 1 included sequences of eight genera of the Macromitrioideae (*Bryomaltaea* Goffinet*, Cardotiella* Vitt*, Desmotheca* Lindb.*, Groutiella* Steere*, Leiomitrium* Mitt.*, Macrocoma* (Hornsch. ex Müll. Hal.) Grout*, Matteria* Goffinet and *Schlotheimia*) from GenBank as well as new sequences (six species of *Macromitrium*, one of *Macrocoma*, and one of *Groutiella)* from Brazil. In addition, we carried out a separate analysis based on all *trn*L-F sequences available (Genbank and own data), including the type species of *Macromitrium*, *M. pallidum* (P. Beauv.) Wijk & Margad. This dataset 2 aimed to delimit which Brazilian species, if any, belonged to true *Macromitrium*, and comprised 14 species of *Macromitrium* and 11 species of eight other genera of the subfamily Macromitrioideae. Two species of different genera of subfamily Orthotrichoideae were used as outgroup representatives following Goffinet et al. (1998). Voucher information and Genbank accession numbers are listed in Appendix S1.

For molecular species discrimination of Brazilian *Macromitrium*, we focused on the three DNA regions (chloroplast *trn*L-F and *trn*G-R as well as nuclear ribosomal ITS) that

were previously tested for *Schlotheimia* in Brazil (Valente et al. 2019). The three potential DNA barcoding markers comprise two non-coding parts each (*trn*L-F: *trn*L group I intron and *trn*L-*trn*F intergenic spacer, *trn*G-R: *trn*G group II intron and *trn*G-*trn*R intergenic spacer, ITS: internal transcribed spacers ITS1 and ITS2).

 After the study of type material of *Macromitrium* species from herbaria BM, E, G, GOET, L, NY, PACA, PC, SP, R, and RB, by the first author, we considered several names currently accepted for Brazil (http://floradobrasil.jbrj.gov.br/) to be synonymous (pers.obs.). Based on morphology we recognized 10 putative species, which are included here to test whether molecular data support the morphological species concept. Depending on availability and sequencing success, we used 2−4 individuals for each putative species, sampled from herbarium collections or from fresh samples collected in the field. Voucher information and Genbank accession numbers are listed in Appendix S2. Since sequences of taxa of subfamily Orthotrichaceae were difficult to align with *Schlotheimia*, especially for the highly variable marker ITS, we used *Macromitrium catharinense* Paris as outgroup representatives based on the topology of the present phylogenetic reconstructions.

DNA extraction, amplification and sequencing

 DNA extraction and PCR amplification (Mullis and Faloona 1987) were performed at the Molecular Biology of Plants lab, Botany Department, University of Brasília. Total genomic DNA was extracted using the mini-CTAB protocol (Doyle and Doyle 1987), with modifications (Câmara 2010). The PCR amplification mixture had a total volume of 50 μl and contained 5 μl of 5× thermophilic buffer, 5 μl of 50 mM $MgCl_2$, 0.5 μl *Taq* polymerase (Promega, Madison, Wisconsin, U.S.A.), 2 μl of BSA (10 mg/ml), 4 μl of 1 mM dNTP, 2.5 μl of each primer (10 μM), 2.0 μl of DNA and 26.5 μl of water. Primer information for all molecular markers is shown in Table 1. The PCR profile for all markers was 95°C (30 sec), 48°C–56°C (45 sec), 72°C (1 min) for 35 cycles, always preceded by an initial melting step of 1 min at 95°C and with a final extension of 72°C for 5 min. PCR products were purified and sequenced by Macrogen (Seoul, Korea).

Phylogenetic and species delimitation analysis

 Sequences were assembled and edited using Geneious v.6.1.6 (Biomatters 2010), initially aligned using ClustalX 2.1 (Larkin et al. 2007), then manually adjusted using PhyDE v.0.9971 (Müller et al. 2006) and exported as NEXUS files.

Phylogenetic analyses of the Macromitrioideae were done under maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI), for each marker separately (26S, *nad*5, *rps*4, *trn*L-F) as well as for combined matrices of 26S, *nad*5, and *rps*4 (as in Cox et al. 2010).

To discriminate Brazilian *Macromitrium* species, we followed a step-wise approach. Firstly, putative species were inferred from tree-based analyses (neighbour-joining [NJ; Saitou and Nei 1987], MP, ML, BI), based on topological congruence between markers (*trn*L-F, *trn*G-R, ITS) and clade support. Analyses were carried out for each marker separately as well as for all combinations of two markers, and all three markers combined.

Secondly, an automated species delimitation approach, Automatic Barcode Gap Discovery (ABGD; Puillandre et al. 2012), which uses a pairwise genetic distance-based method to find non-overlapping intra- and interspecific genetic distance distributions within the sequence dataset, was employed as an alternative to construct hypothetical candidate species from the molecular data.

Maximum parsimony and NJ analyses were performed in PAUP* v.4.0b10 (Swofford 2002). Heuristic searches under MP were performed with 1,000 random addition replicates and tree-bisection-reconnection (TBR) branch swapping, saving a maximum of 10,000 trees. All characters were unordered and equally weighted, and gaps were either treated as missing data or coded as informative by Simple Indel Coding (SIC; Simmons & Ochoterena 2000) as implemented in SeqState (Müller 2005). Neighbor-joining analyses were performed using the "Kimura 2-parameter" (K2P) model (Kimura 1980). Besides, intraspecific and interspecific variation was inferred from the pairwise distances, calculated using the K2P model in MEGA7 (Kumar et al. 2015).

For ML and BI analyses the best-fit model of evolution for each locus was obtained based on the Akaike information criteria using jModeltest 3.06 (Posada 2008). ML analyses were carried out using RAxML v7.2.6 (Silvestro and Michalak 2011). Clade support for MP and ML was assessed from bootstrap analyses with 1000 replicates (Felsenstein 1985).

Bayesian inference analyses were carried out in MrBayes v. 3.2.6 (Ronquist et al. 2012). Two runs with four Markov Chain Monte Carlo chains each were run for 5,000,000 generations. Chains were sampled every 1,000 generations and the respective trees were written to a tree file. Convergence of runs was verified by ensuring that the average standard deviation of split frequencies was <0.01. Tracer 1.5 (Rambaut and Drummond, 2013) was used to determine when the tree sampling stabilized. The first 25% of the trees were discarded

as 'burn-in'. A majority rule consensus tree and posterior probabilities were calculated from the resulting trees.

For species discrimination and phylogenetic analyses we follow Valente et al. (2019) where were considered as high support (bootstrap support [BS] ≥95% and posterior probability [PP] ≥0.95) moderate bootstrap support (BS ≥85−≤94%); and lower support (BS ≤84%) or no support

Automatic Barcode Gap Discovery analyses were carried out on the online web server (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html). The dataset of three markers combined (*trn*L-F + *trn*G-R + ITS) was used with the input file in fasta format and the Kimura-2-parameter model and a range of different settings employed. Since the latter resulted in the same number of initial partitions, the final parameters were set as follows: Pmin = 0.001, Pmax = 0.01, Steps = 50, X = 1.1, Nb bins = 100.

5.4 Results

Phylogenetic analysis

Alignment statistics, best-fitting models of evolution, and tree scores are summarized in Table 2. Trees based on analysis of individual markers and on different analysis methods differed only in degree of resolution but did not show statistically supported conflicting topologies, indicating that the markers of dataset 1 could be combined.

Phylogenetic analysis of dataset 1 (Fig. 1) revealed that the Brazilian *Macromitrium* do not form a monophyletic group but are split into three different clades, here named *Macromitrium* group 1 (MG1), 2 (MG2), and 3 (MG3), respectively. Clade MG1 was not supported in MP, but received moderate bootstrap support in ML (88%) and a posterior probability of 1 in BI. This clade was composed of the species *Macromitrium richardii* Schwägr. and *M. microstomum* (Hook. & Grev.) Schwägr. and was resolved as sister to *Desmotheca* Lindb., with high support in ML (97% BS) and a PP of 1 in BI. Clade MG2 received high support in ML (BS 96%) and BI (PP 1). It contained the species *Macromitrium levatum* Mitt., *M. guatemalense* Müll. Hal., *Macromitrium punctatum* (Hook. & Grev.) Brid. and *M. argutum* Hampe and was resolved as sister to *Groutiella* Steere, with equally high support in ML and BI. Clade MG3, formed by the single species *Macromitrium catharinense* Paris, received maximum support in all analyses. Its sister group relationship to the MG2-*Groutiella* clade received lower support (77% BS) in ML and a PP of 1 in BI.

The analyses of dataset 2 (Fig. 2), based on *trn*L-F alone, corroborated the results of dataset 1 in terms of the splitting of the Brazilian *Macromitrium* species in three clades. In addition, they proved that clade MG1 corresponds to *Macromitrium* s.str., since it included the type species *M. pallidum*. Apart from the Brazilian *M. richardii* and *M. microstomum* and the African *M. pallidum*, all additionally included Asian species (*M. gymnostomum* Sull. & Lesq., *M. taiheizanense* Nog., *M. cavaleriei* Cardot & Thér., *M. japonicum* Dozy & Molk., *M. rhacomitrioides* Nog., and *M. incurvifolium* (Hook. & Grev.) Schwägr.) fell into clade MG1 as well.

Molecular species discrimination (DNA barcoding)

PCR amplification and sequencing success using a single primer pair was high for all three markers. The amplification potential of the *trn*G-R region was 93%, followed by *trn*L-F and ITS (both 89%). All sequences were of good sequencing quality, with 41 *trn*G-R, 39 *trn*L-F and 39 ITS sequences included in the analyzes.

Characteristics of sequence lengths and variability of all three markers are summarized in Tables 3 (clade MG1) and 4 (clade MG2), respectively. The nuclear ITS marker was larger (36/67% in MG1, 46/70% in MG2) and more variable (64/99% in MG1, 62/82% in MG2) than the plastid markers *trn*G-R and *trn*L-F. Sequences were more variable in MG2, presenting 2.6% more informative sites in ITS, 2.7% in *trn*G-R, and 5.6% in *trn*L-F than in MG1.

Phylogenetic trees of each individual marker (*trn*L-F, *trn*G-R and ITS) did not show incongruence in terms of well-supported branches (data not shown), indicating that the markers could be combined. The Bayesian inference tree based on the maximum amount of information (three markers combined with indels coded by SIC), is shown in Figs. 3 and 4 for MG1 and MG2, respectively, together with a summary of the results obtained from all applied phylogenetic and species delimitation methods.

The ITS marker alone as well as the combinations *trn*L-F+*trn*G-R, *trn*L-F+ITS, *trn*G-R+ITS, and all markers combined, were efficient to discriminate 100% of the putative species clades for both groups (MG1: clades A/B in Fig. 3; MG2: clades A−G in Fig. 4), with moderate support (*M. microstomum* clade, ML analysis, ITS and *trn*L-F+ITS markers; *M. longifolium* clade, NJ analysis, *trn*L-F+*trn*G-R) or high support in all analyses (Tables 5 and 6). The *trn*G-R marker alone showed good performance in ML and BI analyses, with high support and 100% discrimination potential of MG2 species, whereas the NJ and MP analysis

of *trn*G-R discriminated only 72% of the species with high support. For MG1, this marker separated both species with maximum support in all analyses. The *trn*L-F marker performed least for both MG1 and MG2. For MG2, percentages of species discriminated with high support were 86% (ML), 72% (BI) and 43% (NJ, MP), respectively. For MG1, ML analysis discriminated both species with maximum support, whereas with NJ, MP and BI analysis received lower support or not support.

The use of indels significantly increased clade support in MP and BI analyses of *trn*G-R for MG2 (Table 6). Maximum parsimony BS for clades B and E raised from 66 to 90% and from 62 to 94%, respectively. Furthermore, both clades obtained maximum Bayesian support. For MG1 the use of indels resulted in a decrease of BS for the ITS marker and the combinations *trn*L-F+ITS in ML analyses (data not shown).

Ranges of intraspecific versus interspecific pairwise nucleotide distances according to the K2P model are shown in Tables 7 and 8. No overlap between the maximum intraspecific and minimum interspecific distance was present in MG1, whereas in MG2 there was small overlap (0.002) between *Macromitrium guatemalense* and *M. podocarpi* in ITS.

The species delimitation method ABGD revealed a clear "barcode gap" in two partitions at Pmax = 0.0010 and Pmax = 0.0016, delimiting seven putative species clusters in MG2 (Fig. 4). For MG1, four partitions with Pmax = 0.0010, 0.0016, 0.0027 and 0.0046, all delimiting two putative species clusters, were found, of which the first and second partitions are shown in Fig. 3.

5.5 Discussion

Circumscription of Macromitrium

According to Sharp et al. (1994) the diagnostic morphological characters of *Macromitrium* are: plants light-to dark green; leaf cells flat or bulging, smooth to papillose-tuberculate below; peristome usually rudimentary, single or, if double, fused to form membranes; calyptra conic, short, with numerous lobes and more or less laciniate, plicate. But there are no characters shared by all *Macromitrium* species. This difficult morphological circumscription may indicate that *Macromitrium* is not monophyletic, which was already suggested by Goffinet et al. (1998) and Frey and Stech (2009). The present phylogenetic data confirm that *Macromitrium* is indeed polyphyletic and split into at least three different clades (MG1, MG2 and MG3) in Brazil. Extended studies of further *Macromitrium* species from

other geographic areas should reveal whether all species can be assigned to one of these three clades, or whether more separate lineages are to be discovered.

Clade MG1 includes the type species, *M. pallidum* (Fig. 2), and is thus considered *Macromitrium* s.str. This group is sister of *Desmotheca* (Fig. 1)*,* as previously reported by Goffinet et al. (1998) for *M. richardii*. We suggest to maintain both groups as separate genera due to their substantial morphological differences, as *Desmotheca* differs from *Macromitrium* by totally lacking a peristome, very short setae, and enlarged, sheathing, ligulate perichaetial leaves. The oblong, apiculate branch leaves with isodiametric cells are also characteristic of *Desmotheca* (Vitt 1990).

The second clade MG2 is formed by the majority of the species recognized as *Macromitrium* in Brazil. Its sister group relationship with *Groutiella* was already shown by Goffinet et al. (1998) for *M. longifolium* (Hook.) Brid., *Groutiella chimborazensis* (Spruce ex Mitt.) Florsch. and *G. apiculata* (Hook.) H.A. Crum & Steere. Despite their close phylogenetic relationship, *Groutiella* differs from *Macromitrium* by the marginal limbidium of hyaline elongate cells and a short calyptra covering only the upper portion of the urn (Goffinet et al. 1998). In addition, the species belonging to MG2 have tuberculate basal cells, whereas *Groutiella* always has smooth basal cells (Gradstein et al. 2001). Based on the morphological differences between the two groups we suggest to keep them as separate genera, and to describe the new genus *Pseudomacromitrium* for the MG2 clade (see Taxonomic treatment).

The MG3 clade, formed only by *M. catharinense*, had maximum support in all analyses. It appeared sister to the *Groutiella - Pseudomacromitrium* (MG2) clade, albeit with high support only under Bayesian inference. *Macromitrium catharinense* (Fig. 5; Table 9) has a very different morphology: leaves larger (up to 4 mm) than the species of *Macromitrium, Groutiella* and *Pseudomacromitrium* (up to 2 mm), papillose upper leaf cells and smooth basal cells. Due the morphological characteristics and molecular results, we describe a new genus *Aureomacromitrium* for *M. catharinense* (see Taxonomic treatment).

Molecular species discrimination (DNA barcoding)

For Brazilian *Macromitrium* (MG1) and *Pseudomacromitrium* (MG2) the *trn*G-R region presented the best results. The *trn*G-R region is easy to amplify, with amplicons ranging from 734−744 base pairs in the sequenced specimens, excellent sequence quality, easy to align and with sufficient variability to identify 100% of the species of MG1 and MG2.

Usually for bryophytes only the *trn*G intron has been used (e.g. Câmara and Shaw 2013; Carter 2010, 2012; Hedenäs 2009; Medina et al. 2013). Li et al. (2013) tested the *trn*G intron for the identification of Chinese *Macromitrium* species, presenting discrimination potential for 86% of the species, 14% less variation compared to the intergenic *trn*G-*trn*R spacer used in this study. For the genus *Schlotheimia*, the *trn*G-R region discriminated 100% of the species, demonstrating that the addition of the *trn*G-*trn*R intergenic spacer significantly increases the discrimination potential (Valente et al. 2019). The *trn*G-R region has also been used successfully for phylogenetic inference (Nagalingum et al. 2007; Leon et al. 2013) and DNA barcoding (Pryer et al. 2010) of ferns, and we suggest to employ the same region in future studies of bryophytes as well, instead of only the *trn*G intron.

The ITS marker was easy to amplify and highly variable, allowing for 100% discrimination of the MG1 and MG2 species. However, due to the large interspecific variation, it was very difficult to perform the sequence alignment for both groups together. Compared to *Schlotheimia* (Valente et al. 2019), the ITS sequences of MG1 and MG2 were more conserved, presenting 5.7 and 2.4% less informative sites, respectively. However, the sequences of MG1 and MG2 presented higher quality in relation to the genus *Schlotheimia* due to the lack of poly-C, -T and -A nucleotide stretches in ITS1, which were prominent in the latter genus (Valente et al. 2019). In addition, ITS sequencing is prone to fungal contamination (e.g. Hollingsworth et al. 2011; Valente et al. 2019).

The *trn*L-F region was also easy to amplify and sequence quality was high, as in earlier DNA barcoding studies of bryophytes (e.g. Liu et al. 2010; Valente et al. 2019). The species discrimination capacity of *trn*L-F, however, varied considerably in different bryophyte genera, from 53% in *Dicranum* (Lang et al. 2014) to 89% in 49 species of mosses (Liu et al. 2010). In Orthotrichaceae, this marker alone was able to discriminate only 57% of the Chinese species of *Macromitrium* (Li et al. 2013), and in Brazilian *Schlotheimia* the discrimination potential was even lower, 45% (Valente et al. 2019). For the Brazilian *Macromitrium* s.l. species, the discrimination capacity was 43%.

With the exception of *trn*L-F, all other markers and combinations were efficient to be used as barcoding markers for the identification of Brazilian *Macromitrium* s.l. species. However, by comparing the characteristics of each marker (amplification rate, variability, sequence quality) costs and laboratory time, we suggest *trn*G-R as core barcoding marker for routine identification of all Brazilian species.

The intraspecific variation of *M. argutum* is greater than the interspecific variation between *M. podocarpi* and *M. guatemalense,* which leads to overlap of intra- and interspecific distances in ITS (Table 8). However, the clades of all species are well supported and the recognition of *M. argutum* is supported by ABGD, indicating that an overall comparison of pairwise distances is less meaningful than the phylogenetic and species delimitation methods. Our results for ABGD analyses suggests the delimitation two species in MG1 and seven species in MG2, corroborate the results of tree-based analyses. The ABGD method presented congruent results with other discrimination methods for the delimitation of *Macromitrium* species and for other groups of bryophytes, like *Aneura* (Metzgeriales) (Bączkiewicz et al. 2017), *Schistidium* (Grimmiaceae) (Biersma et al. 2018) and *Bartramia* Hedw. (Bryophyta) in Antarctica (Câmara et al. 2019).

Diagnostic morphological characters

To corroborate the molecular data, we present a set of morphological characters (Table 9) and illustrative figures to aid in the morphological identification of the Brazilian species of *Aureomacromitrium* (Fig. 5, see discussion above), *Macromitrium* (Fig. 6−7) and *Pseudomacromitrium* (Fig. 8−13).

Brazilian *Macromitrium* species differ from *Pseudoacromitrium* mainly in that they do not have tuberculate cells at the base of the leaves. Both species of *Macromitrium* differ from each other by unipapillose upper lamina cells in *M. microstomum* (Fig. 6) versus pluripapillose cells with 3-4 papillae on each cell in *M. richardii* (Fig. 7; Allen, 2002).

The Brazilian species of *Pseudomacromitrium* can be distinguished from each other by combinations offour morphological characters of the leaf, namely (1) leaf base margins, (2) basal lamina cells, (3) apex shape and (4) apex margins (Table 9).

Pseudomacromitrium carionis (Fig. 8) and *P. guatemalense* (Fig. 9) both have swollen teeth on the basal leaf margins, and strongly tuberculate cells, however the specimens of *P. carionis* has a rounded-obtuse, emarginate to mucronate apex and entire margin, while *P. guatemalense* has an acute or rarely broadly acute apex and serrulate margins.

The other *Pseudomacromitrium* species do not have teeth at the basal margin but present well inflated rectangular and / or quadratic cells, two or three times wider than the inner basal lamina cells. *Pseudomacromitrium podocarpi* (Fig. 10) and *P. cirrosum* (Fig. 11) have evident tubercles on the basal lamina. *Pseudomacromitrium podocarpi* is the smallest plant (leaves 1.5 – 2.2 mm) with acute to obtuse-apiculate apex and entire or crenulate

margins, while *P. cirrosum* is larger (leaves up to 3−5 mm) with lanceolate apex and serrulate to irregular serrulate distal margins.

The species *P. longifolium*, *P. punctatum* and *P. argutum* have few tubercles, which are often difficult to detect. The specimens of *P. argutum* (Fig. 12) show acute or lanceolate apex and strongly serrulate margins The other two species present an acute to acuminate apex and can be distinguished from each other by entire margins at the leaf apex and upper lamina cells of each leaf not bulging (*P. punctatum*, Fig. 13) The clade F present slightly serrulate margin apex and cells bulging upper leaves (*P. argutum*, Fig. 14).

5.6 Taxonomic Treatment

Aureomacromitrium D.V. Valente, P.E.A.S. Câmara & D.F. Peralta **gen. nov. –** Type: *Macromitrium catharinense* Paris

Plants robust, yellow-green, yellow, yellowish brown in old stems. Prostrate caulids with ascending branches. Narrow-lanceolate leaves, contorted or crisped when dry, and erect to undulate when wet; apex acuminate finished in few elongated cells; costae single, percurrent, or excurrent; margins, serrate to serrulate above, entire below; upper cells isodiametric to gradually rectangular in direction at median lamina, with a big tuberculate papilla in each cell. basal cells linear, long- rectangular, incrassate and porose, smooth. Cladocarpous. Capsules oblong, exserted; peristome double, exostome rudimentary, with truncated teeth, endostome of 32 teeth, with fused segments. Spores globular. Calyptra cucullate, with many hairs.

Etymology. – The name is linked with the fact that this species presents a golden coloration in nature.

Distribution. – Occurs in Brazil, Colombia, Ecuador

Aureomacromitrium catharinense (Paris) D.V. Valente, P.E.A.S. Câmara & D.F. Peralta **comb. nov.** ≡ *Macromitrium catharinense* Paris, Index Bryologicus Supplementum Primum 237. 1900. = *M. prolongatum* Müll.Hal., Bull. Herb. Boissier 6: 99 (1898), hom. illeg. — TYPE: 'Brasilia, Sa. Catharina, Serra Geral, in ramis arborum, Januario 1890, cum fructibus junioribus *No 847* e; ad ramjos arborum marginis Serrae ejusdem, Aprili 1891 cum fructu vetusta et ramis aureis *No 1017*; Serra Itatiaia, 2000 m. alt., Martio 1894 sterile, *No 1835'*. (lectotype designated by Li et al. (2019): 'E. Ule. Bryotheca brasiliensis. 134. *Macromitrium prolongatum* C. Müll. *n. sp.*, Prov. S. Catharina, Serra Geral, ad ramos arborum, m. Apr. 1891. *E. Ule 1017'* (H-Brotherus 2649005; isolectotypes: FI, GOET 012311, JE 04006251, JE 04006252 PC barcode PC0137901, PC0137902, PC0137903, PC0137904, MICH barcode MICH525906, NY barcode NY01202245, L!). Other syntype: 'Estado de Sta. Catharina, Serra Geral, Januario 1890. *E. Ule 847'* (FI). (Fig. 5: A-E).

Pseudomacromitrium D.V. Valente, P.E.A.S. Câmara & D.F. Peralta **gen. nov.** – Type: *Macromitrium podocarpi* Müll. Hal.

Plants small, medium-sized to robust, yellow-green, dark-green, olive-green, greenish yellow, reddish yellow, to reddish brown, mats or cushions on trees or rocks. Primary stems creeping, secondary stems erect-ascending, tomentose. Leaves plicate, erect-appressed, keeled, erect below, spirally twisted or crisped when dry, erect to flexuose-spreading when wet, 1.5–5 mm long, lingulate, oblong-lanceolate or lanceolate; apices rounded, obtuse emarginate or short-mucronate, acuminate, acute to obtuse-apiculate; costae strong, subpercurrent, percurrent, or excurrent; margins entire, crenulated, serrate to serrulate, undulate, erect to plane above, recurved or plane below, enlarged basal teeth at leaf insertion present or absent; upper cells 5–15 um, rounded-quadrate, rhombic, rounded-hexagonal, smooth, bulging to mamillose, upper marginal cells not differentiated, basal cells linear, long-rectangular 16–60 μm, incrassate and porose, weakly or strongly tuberculate. Autoicous, dioicous or pseudautoicous. Setae 3–15 mm long, smooth or papillose. Capsules 1–2.5 mm long, ovoid, oblong, cylindrical, plicate or furrowed. operculum conic-rostrate to rostrate, 1–1.5 mm long; annulus non-revoluble, with fragments adhering to capsule mouth; exostome teeth truncate or lanceolate 160–312 um high, papillose or papillose-striate, not fusioned or united and forming an erect membrane; endostome hyaline, papillose to weakly papillose, membrane basal with 50–280 um high, segments rudimentary, present or absent; anisosporous, ornamentation smooth or papillose. Calyptra mitrate, laciniate, naked, with a few hairs or densely hairy, 2.5–4 mm long.

Comments - The Brazilian species of *Pseudomacromitirum* differs from *Macromitrium* by having tuberculate cells at the base of the leaves.

Etymology. – The name is linked with the fact that the species of the genus were so far believed to be part of *Macromitrium*.

Distribution. – Occurs in Mexico, Central America, Caribbean, Western and Northern South America, Brazil.

Pseudomacromitrium argutum **(**Hampe) D.V. Valente, P.E.A.S. Câmara & D.F. Peralta **comb. nov.** ≡ *Macromitrium argutum* Hampe, Linnaea 22: 581. 1849. —TYPE: [Brazil], Rio de Janeiro, *Glaziou 9241* (lectotype designated by Costa et al. (2016) BM000879980!; isolectotypes: BM000879983, BM000879979!

Pseudomacromitrium carionis (Müll. Hal.) D.V. Valente, P.E.A.S. Câmara & D.F. Peralta **comb. nov.** ≡ *Macromitrium carionis* Müll. Hal., Bulletin de l'Herbier Boissier 5: 199. 1897. —TYPE: [Guatemala] Caesta de Lovio, *Bernoulli et Cario 48* (lectotype **designated here:** GOET011888!; isolectotypes: GOET012471, GOET012472, GOET011887!, PC0137621!). (Fig. 8: A-E).

Pseudomacromitrium cirrosum (Hedw.) D.V. Valente, P.E.A.S. Câmara & D.F. Peralta **comb. nov.** ≡ ***Anictangium cirrosum*** Hedw., Species Muscorum Frondosorum 42. 5 f. 1–3. 1801.≡ *Hedwigia cirrosa* (Hedw.) Brid., Journal für die Botanik 1800(1): 272. 1801. ≡ *Neckera cirrosa* (Hedw.) F.Weber & D.Mohr, Index Musei Plantarum Cryptogmarum 3:1803. ≡ *Anoectangium cirrosum* (Hedw.) Schwägr., Species Muscorum Frondosorum suplementum primum 1: 38. 1811.≡ *Schlotheimia cirrosa* (Hedw.) Brid., Muscologia Recentiorum Supplementum 2: 19. 1812. ≡ *Orthotrichum cirrosum* (Hedw.) Hook. & Grev., Edinburgh Journal of Science 1: 130. 6. 1824. ≡ *Ulota cirrosa* (Hedw.) Hook & Grev., Edinburgh Journal of Science 1:130. 1824. invalid. name, ≡ *Macromitrium cirrosum* (Hedw.) Brid., Bryologia Universa 1: 316. 1826. —TYPE: Jamaica, Montserrat, *sine legit* (holotype: G, isotypes: BM barcode BM000873245; E barcode E00002459)**.** (Fig. 11: A-E).

Pseudomacromitrium guatemalense (Müll. Hal.) D.V. Valente, P.E.A.S. Câmara & D.F. Peralta **comb. nov.** ≡ *Macromitrium guatemalense* Müll. Hal., Synopsis Muscorum Frondosorum omnium hucusque Cognitorum 2: 644. 1851. —TYPE: Guatemala, Friedrichstal, *Kegel s.n.* (lectotype **designated here** BM000873191!; isolectotypes: G barcode G00284083!; BM barcodes BM000873192!, L barcode L0060440!). (Fig. 9: A-E).

Pseudomacromitrium podocarpi (Müll. Hal.) D.V. Valente, P.E.A.S. Câmara & D.F. Peralta **comb. nov.** ≡ *Macromitrium podocarpi* Müll. Hal., Bulletin de l'Herbier Boissier 6: 96. 1898. —TYPE: Brasilia, Minas Gerais, Serra Itabira do Campo, *E. Ule 1066* (lectotype **designated here:** GOET012309!; isolectotypes: NY01086626!, NY01086627!, G00265988!, G00265989!**;** BM barcode BM000873094!; PC0137891!). (Fig. 10: A-E).

Pseudomacromitrium longifolium (Hook.) D.V. Valente, P.E.A.S. Câmara & D.F. Peralta **comb. nov.** ≡ *Orthotrichum longifolium* Hook., Musci Exotici 1: 44. 1818 ≡ *Macromitrium*

longifolium (Hook.) Brid., Bryologia Universa 1: 309, 738. 1826 ≡ *Schlotheimia longifolia* (Hook.) Schwägr., Species muscorum Frondosorum, supplementum secundum 2(2): 147. 1827. —TYPE: [Venezuela] Caracas, *Humboldt et Bonpland s.n. (*lectotype designated by Goffinet (1993) BM000873190; isolectotypes: BM000720648, BM000720656!, PC0137790!, PC0137789!, JE04008699, E00289633. (Fig. 13: A-E).

Pseudomacromitrium punctatum (Hook. & Grev.) D.V. Valente, P.E.A.S. Câmara & D.F. Peralta **comb. nov.** ≡ *Orthotrichum punctatum* Hook. & Grev. Edinburgh Journal of Science 1: 119. 5. 1824. ≡ *Macromitrium punctatum* (Hook. & Grev.) Brid. Bryologia Universa 1: 739. 1826. —TYPE: Brazil, Raddi s.n. (isotype: E00011666!, E00011667!). (Fig. 14: A-E).

5.7 Acknowledgments

5.8 References

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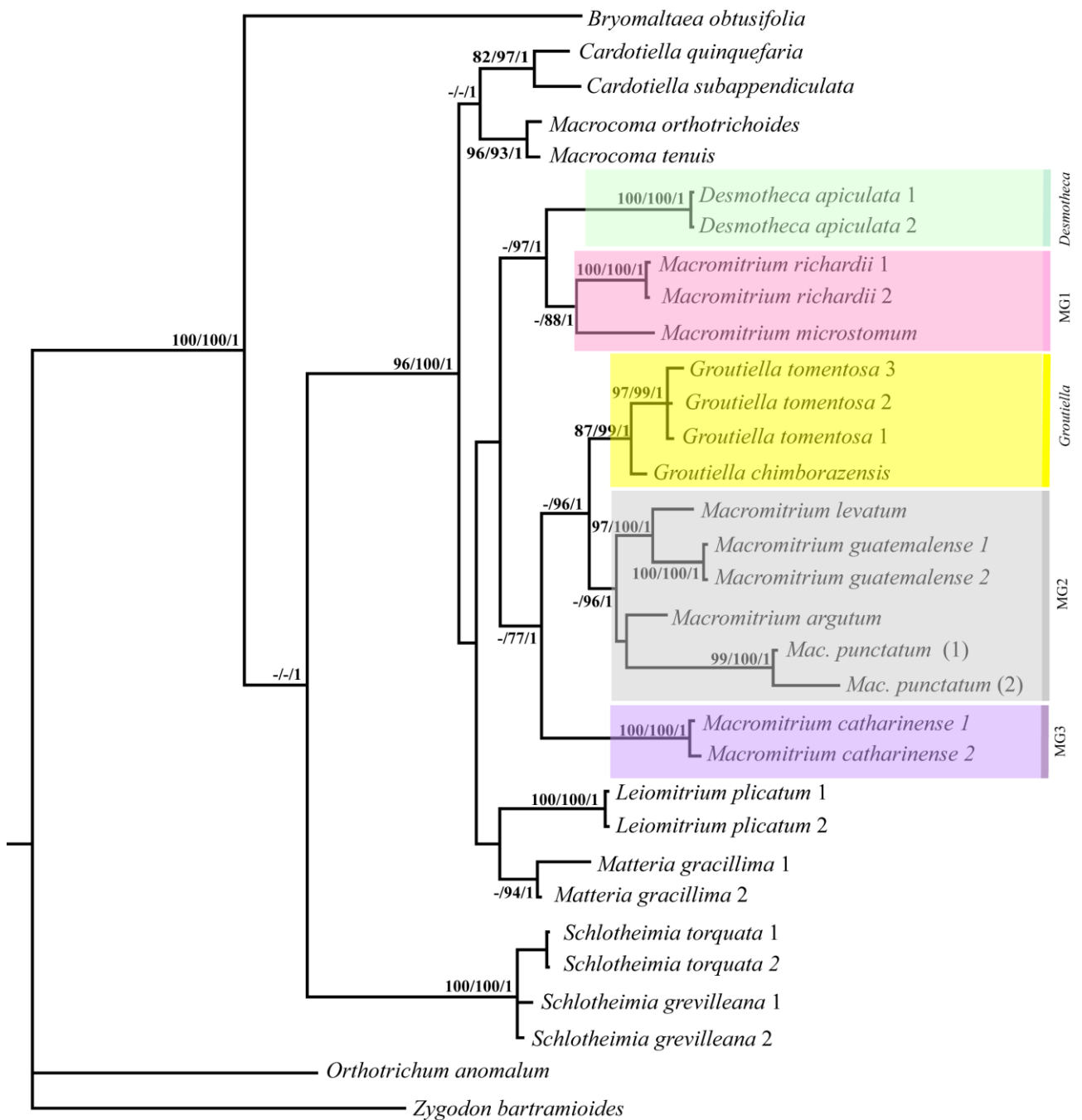
Fig. 1 Phylogram obtained from Bayesian inference (BI) based on combined *trn*L-F + *rps*4 + *nad*5 + 26S sequences of 32 specimens of Orthotrichaceae, including indels coded by simple indel coding. Bootstrap support for Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian posterior probabilities are shown respectively in each clade and node. MG1: *Macromitrium* group 1; MG2: *Macromitrium* group 2; MG3: *Macromitrium* group 3
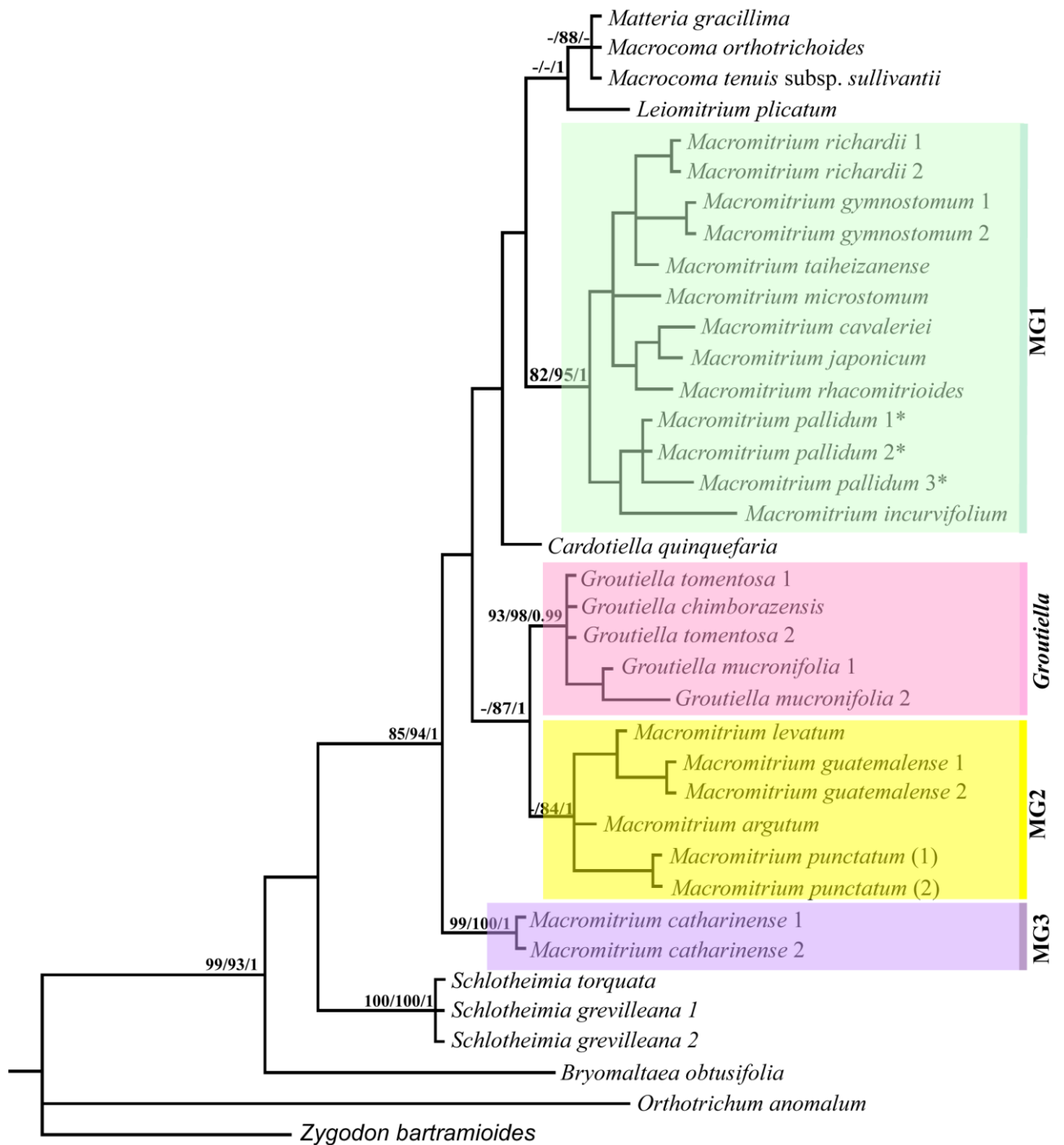
Fig. 2 Phylogram obtained from Bayesian inference (BI) based on *trn*L-F marker sequences of 38 specimens of Orthotrichaceae, including indels coded by simple indel coding. Bootstrap support for Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian posterior probabilities are showed in each clade and node. MG1: *Macromitrium* group 1; MG2: *Macromitrium* group 2; MG3: *Macromitrium* group 3. (*) *types* species
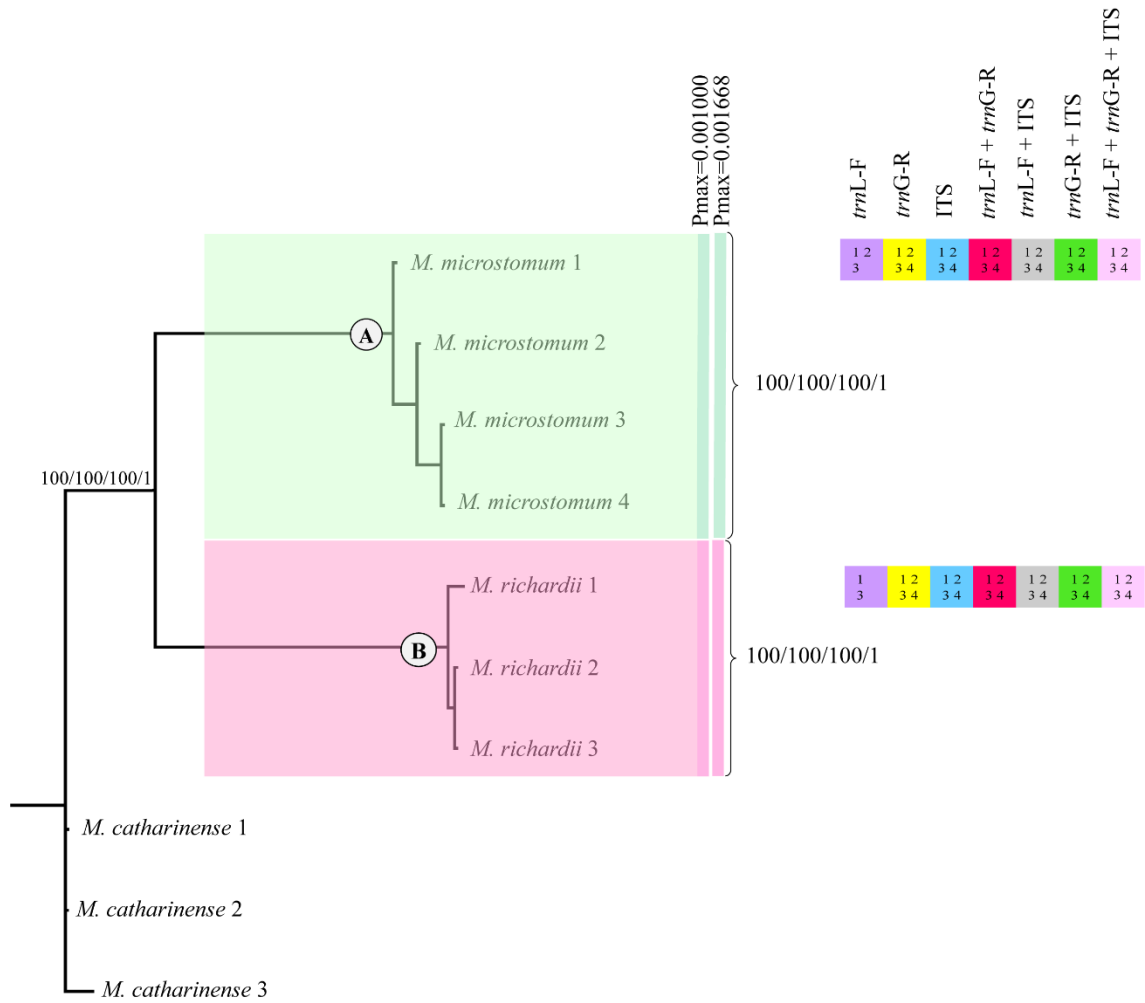
Fig. 3 Phylogram obtained from Bayesian inference (BI) based on combined *trn*L-F + *trn*G-R + ITS sequences of 10 specimens of *Macromitrium* (Group 1), including indels coded by simple indel coding. Bootstrap support for Neighbor-joining (NJ), Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian posterior probabilities are showed in each clade and node. Coloured squares, and numbers inside them represent the clades with bootstrap ≥ 70% for **1** - (NJ), **2** - (MP) and **3** - (ML), and Posterior Probability ≥ 0.95 for **4** - (BI). Each color in square represents an analysis of a single marker or combinations of them. The absence of color means no support or clades not resolved for that marker. ABGD species clusters with different Pmax-values are shown next to the species names. Each color represents one species

Fig. 4 Phylogram obtained from Bayesian inference (BI) based on combined *trn*L-F + *trn*G-R + ITS sequences of 28 specimens *Macromitrium* (Group 2), including indels coded by simple indel coding. Bootstrap support for Neighbor-joining (NJ), Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian posterior probabilities are showed in each clade and node. Coloured squares, and numbers inside them represent the clades with bootstrap ≥ 70% for **1** - (NJ), **2** - (MP) and **3** - (ML), and Posterior Probability ≥ 0.95 for **4** - (BI). Each color in square represents an analysis of a single marker or combinations of them. The absence of color means no support or clades not resolved for that marker. ABGD species clusters with different Pmax-values are shown next to the species names. Each color represents one species
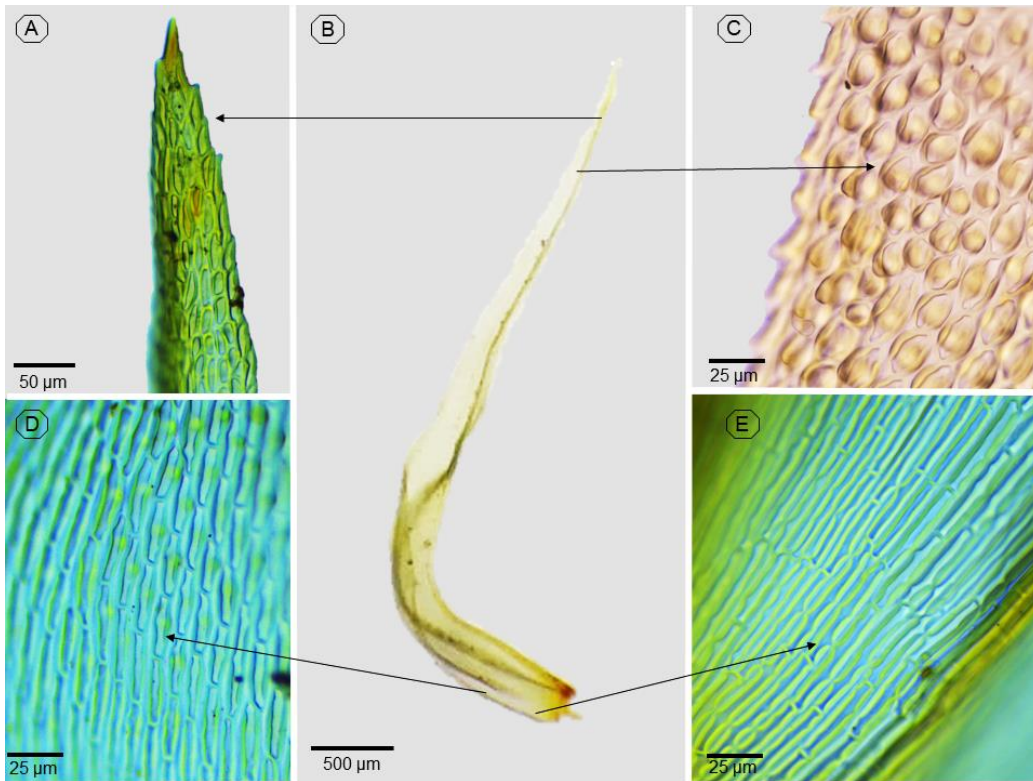
Figure 5: *Macromitrium catharinense:* (A) margin and apex format; (B) leave; (C) pluripapilose cells; (D) pluripapilose base cells; (E) base cells.
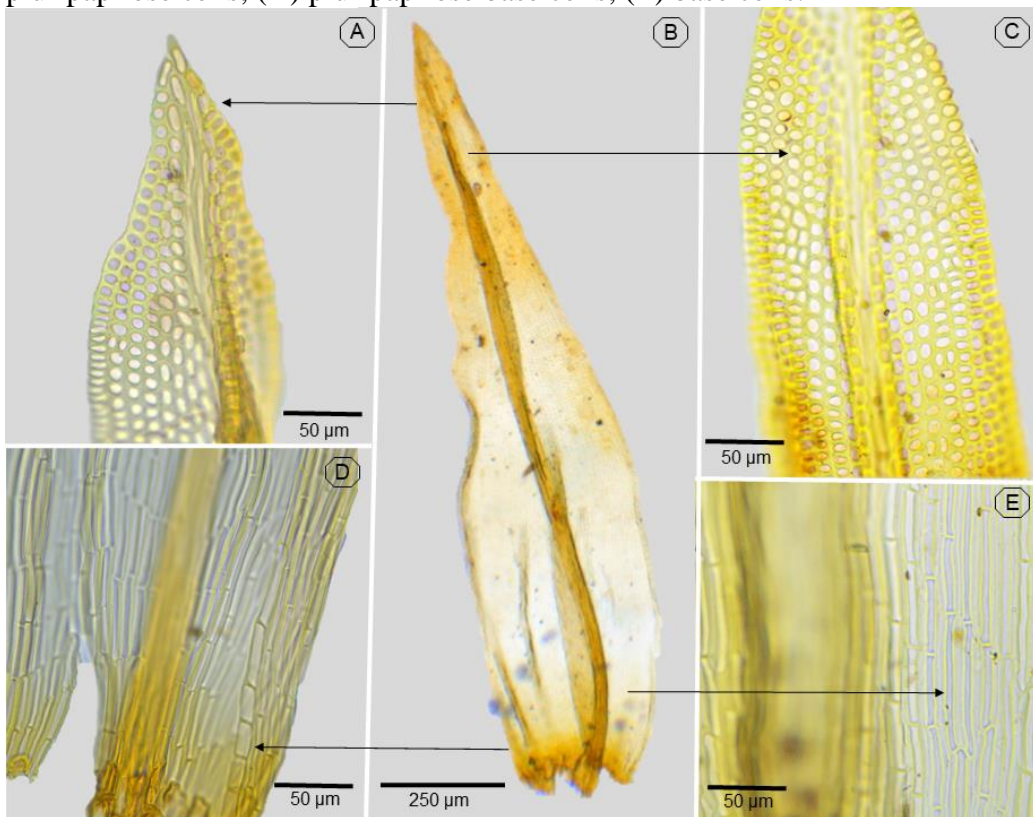
Figure 6: *Macromitrium microstomum;* (A) margin and apex format; (B) leave; (C) pluripapilose apical cells; (D) base margin; (E) base cells.
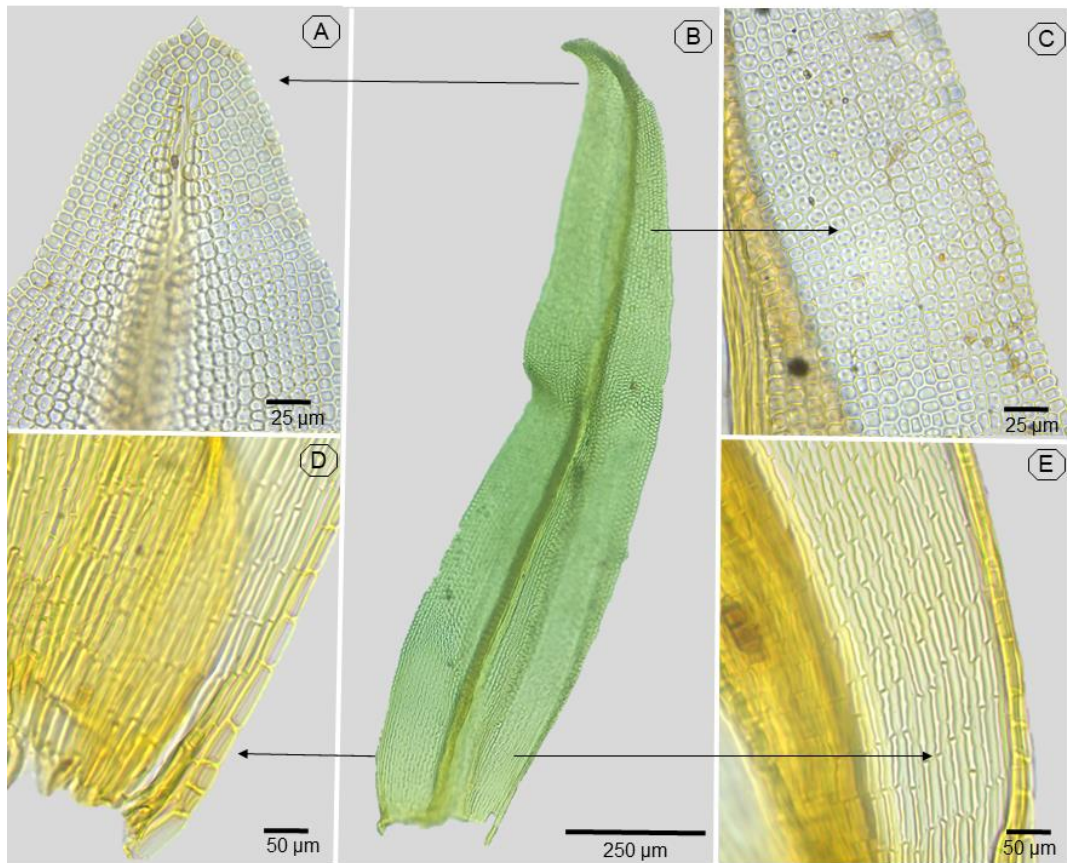
Figure 7: *Macromitrium richardii;* (A) margin and apex format; (B) leave; (C) apex cells; (D) base margin; (E) base cells.
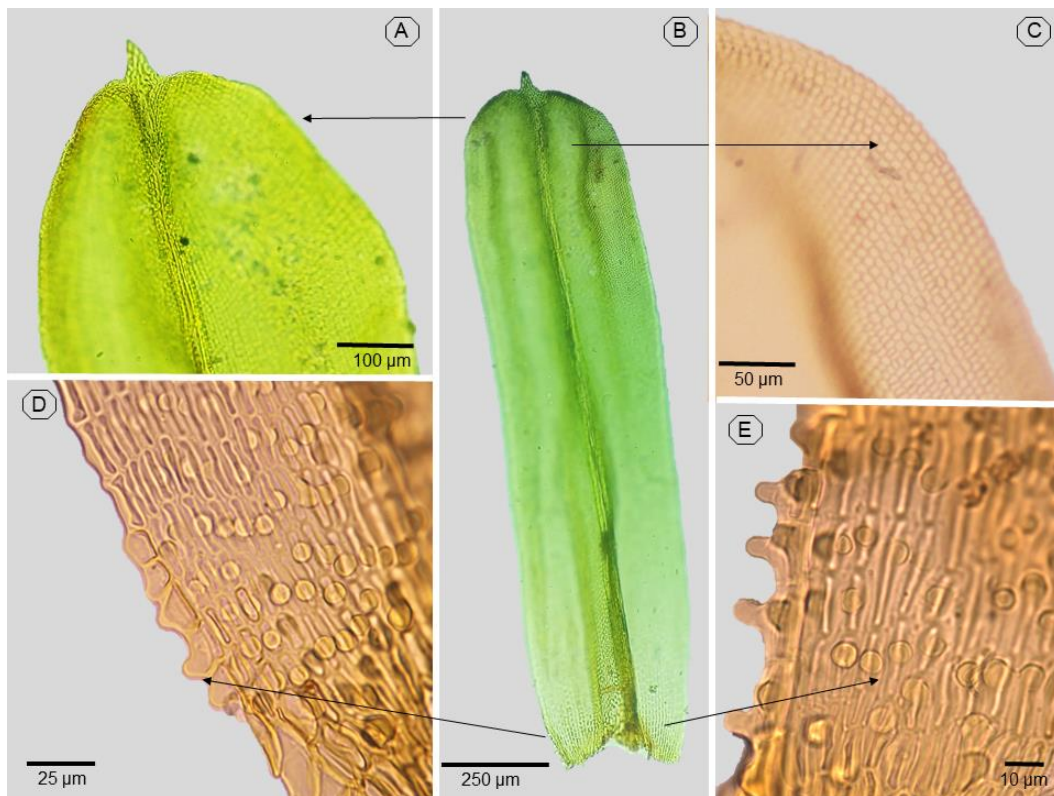
Figure 8: *Macromitrium carionis;* (A) margin and apex format; (B) leave; (C) apex cells; (D) base margin; (E) base cells.
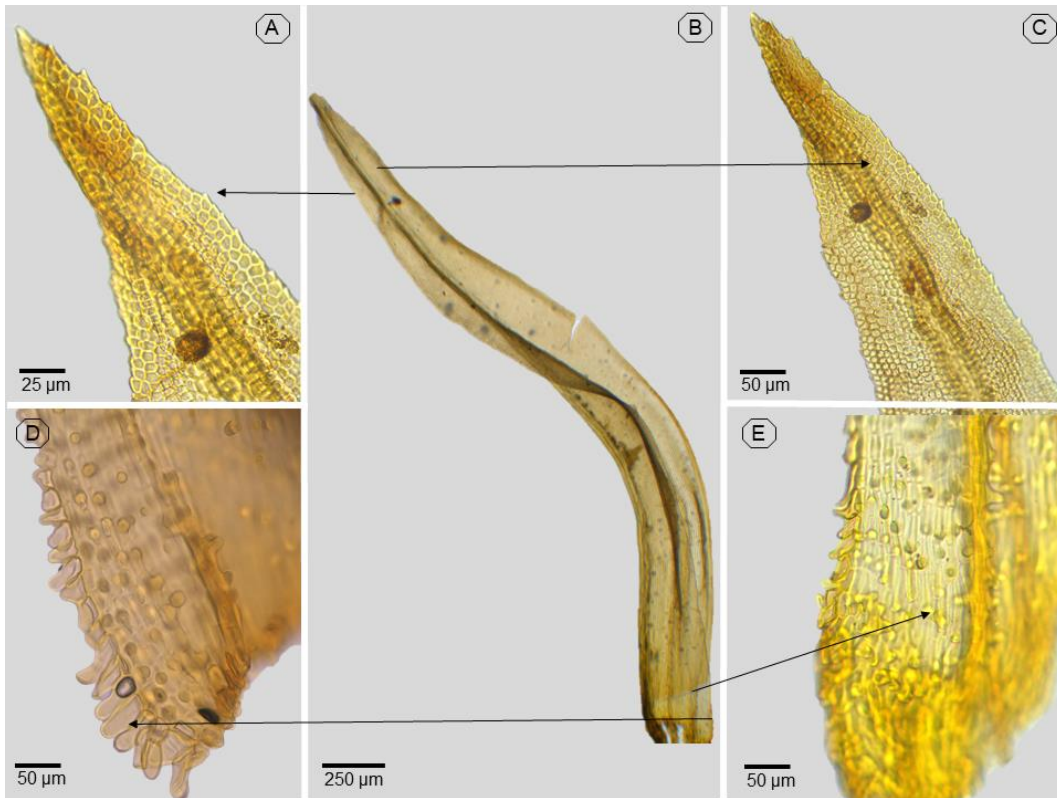
Figure 9: *Macromitrium guatemalense;* (A) margin and apex format; (B) leave; (C) apex cells; (D) base margin; (E) base cells.
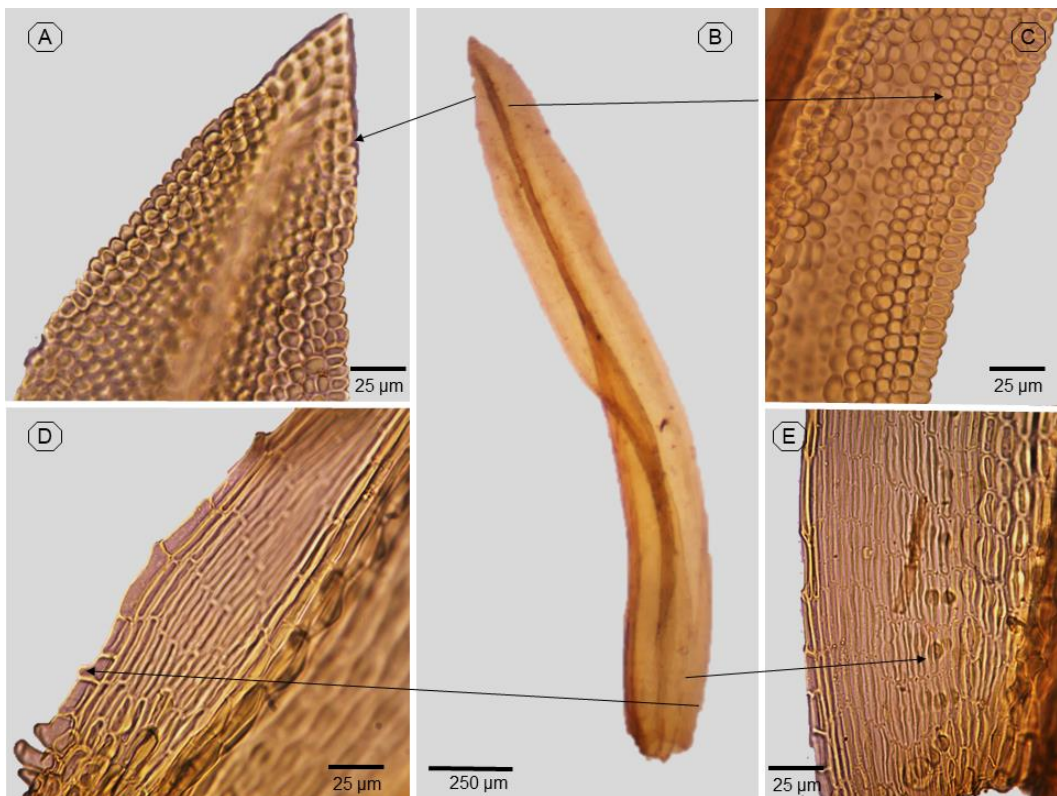
Figure 10: *Macromitrium podocarpi;* (A) margin and apex format; (B) leave; (C) apex cells; (D) base margin; (E) base cells.
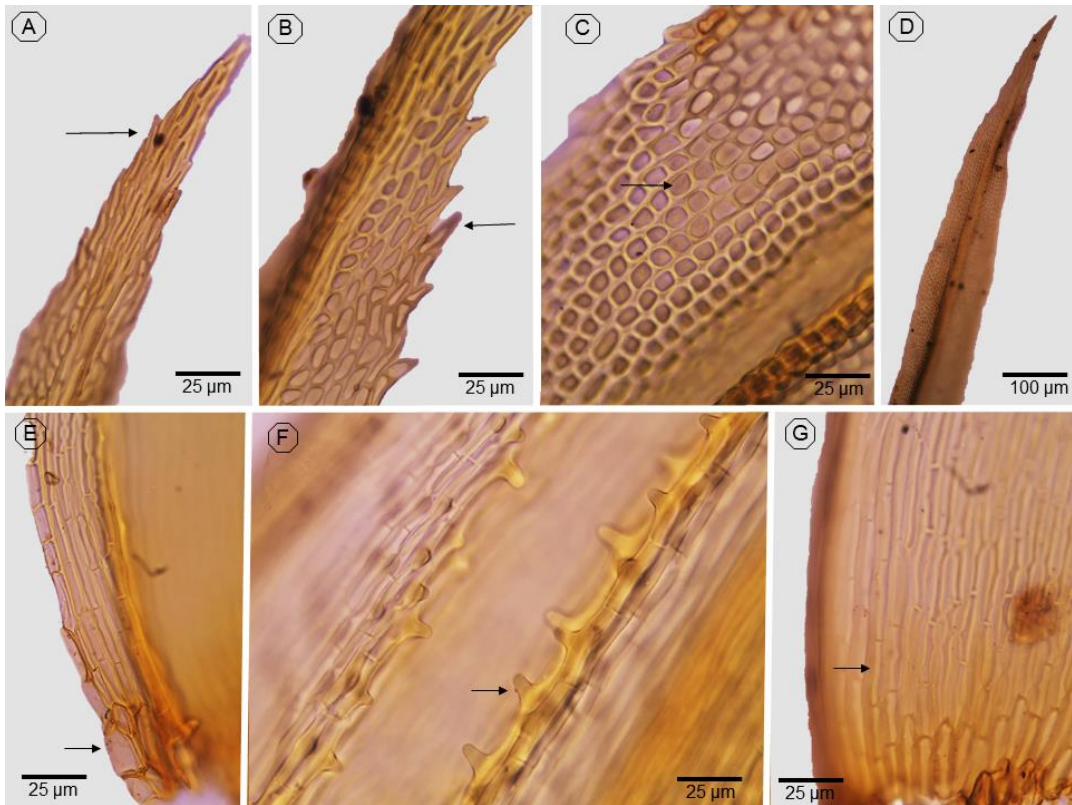
Figure 11: *Macromitrium cirrosum;* (A) apex format; (B) apex margin; (C) apex cells; (D) upper leave; (E) base margin; (F) tubercles; (G) base cells.

Figure 12: *Macromitrium argutum;* (A) margin and apex format; (B) leave; (C) apex cells; (D) base margin; (E) base cells.
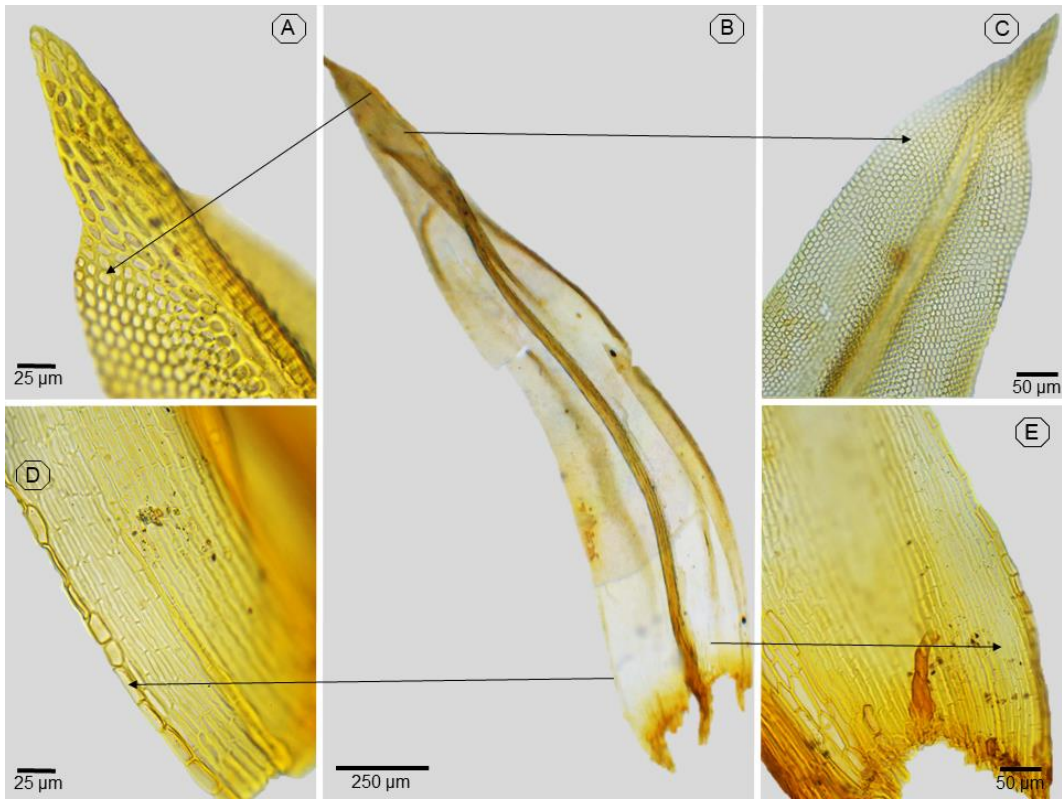
Figure 13: *Macromitrium longifolium;* (A) margin and apex format; (B) leave; (C) apex cells; (D) base margin; (E) base cells.
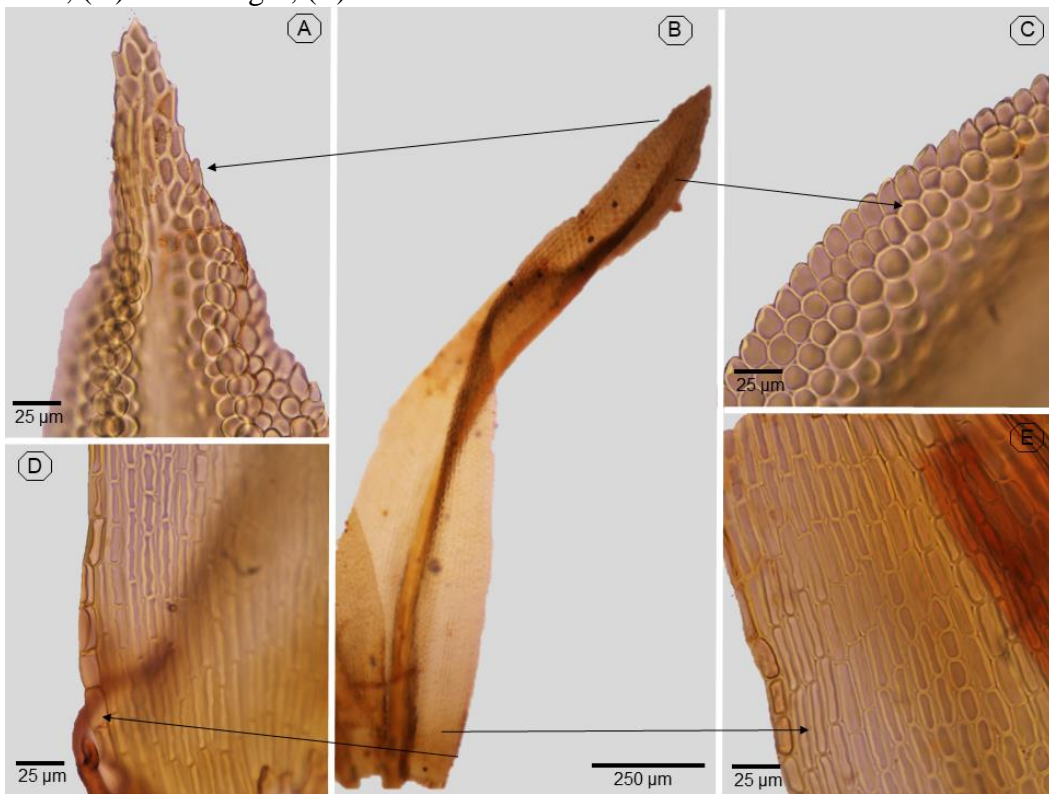
Figure 14: *Macromitrium punctatum;* (A) margin and apex format; (B) leave; (C) apex cells; (D) base margin; (E) base cells.

Table 1: Description of *Primers used* for amplification in phylogenetic and DNA Barcoding analyses.

Primers	Região	Direção	Sequência 5'-3'	Annealing temp. ºC	Referência
Cm	*trn*L-F (Chloroplast)	Forward	CGA AAT TGG TAG ACG CTG CG	56º	Frey et al. 1999
Fm	*trn*L-F (Chloroplast)	Reverse	ATT TGA ACT GGT GAC ACG AG	56º	Frey et al. 1999
Rps5	*rps*4 (Chloroplast)	Forward	ATG TCC CGT TAT CGA GGA CCT	52º	Nadot et al. 1994
***trn*as**	*rps*4 (Chloroplast)	Reverse	TAC CGA GGG TTC GAA TC	52º	Souza-Chies et al. 1997)
***nad*5F4**	*nad*5 (mitochondrial)	Forward	GAA GGA GTA GGT CTC GCT TCA	53º	Shaw et al. 2003
***nad*5R3**	*nad*5 (mitochondrial)	Reverse	AAA ACG CCT GCT GTT ACC AT	53º	Shaw et al. 2003
LS0F	26S (nuclear)	Forward	ACC CGC TGT TTA AGC ATA T	48º	Shaw 2000ª)
zLS12R	26S (nuclear)	Reverse	ATC GCC AGT TCT GCT TAC CA	48º	Shaw 2000ª)
B	*trn*G-R (Chloroplast)	Forward	GCG GGT ATA GTT TAG TGG	53º	Pacak et al. 2000
TRNR22R	*trn*G-R (Chloroplast)	Reverse	CTA TCC ATT AGA CGA TGG ACG	53º	Nagalingum et al. 2007
18F	ITS (nuclear)	Forward	GGA AAG AGA AGT CGT AAC AAG G	48º	Stech and Frahm 1999
25R	ITS (nuclear)	Reverse	TCC TCC GCT TAG TGA TAT GC	48º	Stech and Frahm 1999

Table 2: Alignment statistics, best-fitting models of evolution, and tree scores for the phylogenetic datasets 1 and 2.

DNA Region	*trn*L-F	*rps*4	*nad*5	26S	Combined	*trn*L-F
Dataset	1	1	1	1	1	2
Taxa included	25	33	25	19	32	37
Variable sites	135	186	158	118	594	160
Parsimony informative sites	75	100	91	56	321	82
Number of trees retained	36	1200	2760	72	9	1560
Number of best trees	181	255	180	182	816	222
Tree lenght	193	280	203	212	844	232
CI	0.777	0.721	0.798	0.646	0.763	0.776
RI	0.794	0.760	0.747	0.522	0.764	0.835
Model	TIM1+I+G	TVM+G	TPM3uf+G	TIM2+I+G	TVM+I+G	TrN+G
Log Likelihood	− 1350.1358	−2053.5605	−3390.1241	−2157.4561	−9469.3062	− 1566.0043

Table 3. *Macromitrium* (Group 1). Characterization of each marker. The data for conserved sites, variable sites, parsimony informative sites, parsimony informative indels and medium pairwise distance were performed of matrix without outgroup.

Marker	Fragment lenght	Alignment lenght with outgroup	Aligment lenght without outgroup	Conserved sites (%)	Variable sites (%)	Parsimony informative sites (%)	Number Indels without outgroup	Parsimony informative indels	Medium pairwise distance
*trn*L-F	390−403	407	390	386(99)	4(1)	4(1)	0	0	0.006
*trn*G-R	739−744	753	752	725(96,4)	27(3,6)	27(3,6)	6	4	0.020
ITS	898−947	1474	1163	1063(91,4)	100(8,6)	75(6,5)	78	68	0.062

Table 4. *Macromitrium* (Group 2). Characterization of each marker. The data for conserved sites, variable sites, parsimony informative sites, parsimony informative indels and medium pairwise distance were performed of matrix without outgroup.

Marker	Fragment lenght	Alignment lenght with outgroup	Alignment lenght without outgroup	Conserved sites (%)	Variable sites (%)	Parsimony informative sites (%)	Number Indels without outgroup	Parsimony informative indels	Medium pairwise distance
*trn*L-F	403−412	413	412	388(94,2)	24(5,8)	23(5,6)	3	2	0.013
*trn*G-R	734−744	747	747	698(93,4)	49(6,6)	47(6,3)	11	8	0.019
ITS	678−898	1681	1379	1211(87,8)	168(12,2)	125(9,1)	265	196	0.064

Table 5. *Macromitrium* (Group 1). Bootstrap values (%) for analysis Neighbor joining (NJ) using 2-parameter (K2P) model, Maximum Parsimony (MP), and Maximum Likelihood (ML). For Bayesian Inference (BI) are showed Posterior probability. Every analysis were performed for individual and combined markers. For the combined analysis of all three markers the support is showed in Fig. 4. To NJ, MP and ML were considered bootstrap ≥ 70% and ≥ 0.95 to BI. MP, ML and BI without IndelCoder.

Clado	*trn*L-F				*trn*G				ITS				*trn*L-F + *trn*G				*trn*L-F + ITS				*trn*G + ITS				*trn*L-F + *trn*G + ITS			
	NJ	MP	ML	BI	NJ	MP	ML	BI	NJ	MP	ML	BI	NJ	MP	ML	BI	NJ	MP	ML	BI	NJ	MP	ML	BI	NJ	MP	ML	BI
M. microstomum	73	71	100	−	100	100	100	1	100	100	90	1	100	100	100	1	100	100	92	1	100	100	100	1	100	100	100	1
M. richardii	70	69	100	−	100	100	100	1	100	100	100	1	100	100	100	1	100	100	100	1	100	100	100	1	100	100	100	1

Table 6. *Macromitrium* (Group 2). Bootstrap values (%) for analysis Neighbor joining (NJ) using 2-parameter (K2P) model, Maximum Parsimony (MP), and Maximum Likelihood (ML). For Bayesian Inference (BI) are showed Posterior probability. Every analysis were performed for individual and combined markers. For the combined analysis of all three markers the support is showed in Fig. 3. To NJ, MP and ML were considered bootstrap ≥ 70% and ≥ 0.95 to BI. P, ML and BI with IndelCoder.

Clado	*trn*L-F				*trn*G				ITS				*trn*L-F + *trn*G				*trn*L-F + ITS				*trn*G + ITS				*trn*L-F + *trn*G + ITS			
	NJ	MP	ML	BI	NJ	MP	ML	BI	NJ	MP	ML	BI	NJ	MP	ML	BI	NJ	MP	ML	BI	NJ	MP	ML	BI	NJ	MP	ML	BI
M. *argutum*	63	62	100	0.97	100	100	100	1	100	100	100	1	100	100	100	1	100	100	100	1	100	100	100	1	100	100	100	1
M. *carionis*	−	−	−	−	96	98	99	1	100	100	99	1	96	98	98	1	97	100	100	1	100	100	100	1	100	100	100	1
M. *cirrosum*	98	97	100	1	100	100	99	1	?	?	?	?	100	100	100	1	?	?	?	?	?	?	?	?	?	?	?	?
M. *guatemalense*	99	100	100	1	100	100	100	1	100	100	100	1	100	100	100	1	100	100	100	1	100	100	100	1	100	100	100	1
M. *longifolium*	62	62	100	−	63	94	99	1	100	100	95	1	86	98	99	1	100	100	99	1	100	100	100	1	100	100	100	1
M. *podocarpii*	93	93	100	1	64	90	99	1	100	100	100	1	96	99	100	1	100	100	100	1	100	100	100	1	100	100	100	1
M. *punctatum*	98	99	100	1	100	100	100	1	100	100	100	1	100	100	100	1	100	100	100	1	100	100	100	1	100	100	100	1

Table 7. Intra- versus interspecific pairwise distances of individual markers (*trn*L-F, *trnG-R* and ITS) and combined markers (*trn*L-F + *trnG-R*, *trn*L-F + ITS, *trnG-R* + ITS and *trn*L-F + *trnG-R* + ITS) in 2 species *Macromitrium* (Group 1).

	*trn*L-F	*trnG-R*	ITS	*trn*L-F + *trnG-R*	*trn*L-F + ITS	*trnG-R* + ITS	*trn*L-F + *trnG-R* +ITS)
Intra	0−0.003	0−0.004	0−0.022	0−0.004	0−0.006	0−0.006	0−0.005
Inter	0.008−0.010	0.034−0.038	0.109−0.114	0.025−0.028	0.072−0.075	0.070−0.074	0.056−0.060
Overlap	−	−	−	−	−	−	−

Table 8. Intra- versus interspecific pairwise distances of individual markers (*trn*L-F, *trnG-R* and ITS) and combined markers (*trn*L-F + *trnG-R*, *trn*L-F + ITS, *trnG-R* + ITS and *trn*L-F + *trnG-R* + ITS) in 7 species *Macromitrium* (Group 2).

	*trn*L-F	*trnG-R*	ITS	*trn*L-F + *trnG-R*	*trn*L-F + ITS	*trnG-R* + ITS	*trn*L-F + *trnG-R* +ITS)
Intra	0−0.003	0−0.003	0−0.019	0−0.002	0−0.003	0−0.003	0−0.002
Inter	0.005−0.029	0.010−0.035	0.017−0.120	0.011−0.031	0.005−0.029	0.010−0.034	0.011−0.031
Overlap	−	−	0.002	−	−	−	−

Table 9: Morphological characteres used for discriminate the species of *Macromitrium* and *Pseudomacromitrium.*

Caracteres	*Macromitrium*		*Pseudomacromitrium*							*Aureomacromitrium*
	A *M. microstomum*	**B** *M. richardi*	**A** *M. carionis*	**B** *M. podocarpi*	**C** *M. guatemalense*	**D** *M. cirrosum*	**E** *M. longifolium*	**F** *M. punctatum*	**G** *M. argutum*	**A** *M. catharinense*
Leaves format	Lanceolate to ligulate-lanceolate	Lanceolate to ligulate-lanceolate	Lingulate to ovate-lingulate	Lanceolate	Lanceolate to ovate-lanceolate	Linear-lanceolate	Lanceolate	Lanceolate	Lanceolate	Narrow-lanceolate
Apex margin	Entire	Crenulate	Entire	Entire or crenulate	Serrulate	Serrulate to irregularly serrulate above	Entire	Slightly serrulate	Strongly serrulate	Serrulate above
Apex format	Acute or short-acuminate	Acute, obtuse, or obtusely mucronate	Rounded-obtuse, emarginate to mucronate	Acute to obtusely-apiculate	Acute or rarely broadly acute	Lanceolate	Acute to acuminate	Acute to acuminate	Acute	Acuminate finished in few elongated cells
Apex cells	Isodiametric smooth	Isodiametric pluripapilosas (3-4) papillas	Isodiametric bulging	Isodiametric bulging	Isodiametric bulging	Elliptic to rhomboidal	Isodiametric	Isodiametric bulging	Isodiametric	Isodiametric with a big papilla in each cell
Basal cells format	Linear-rectangular not tuberculate	Smooth, rectangular to long-linear, with thick, unevenly thickened walls	Linear, strongly tuberculate	Linear, strongly tuberculate	Linear, strongly tuberculate	Elongate-rectangular, very thick-walled, tuberculate cells	Slightly tuberculated	Slightly tuberculated	Slightly tuberculated	Linear, long-rectangular, incrassate and porose, smooth
Base margin	Inflated long-linear, non-tuberculate	Inflated long-linear, non-tuberculate	Tuberculate projections	Inflated long-linear, non-tuberculate	Cells tuberculate, with up to 20 thin-walled cells at basal margins with ends projecting as blunt teeth	Thin-walled, hyaline, rectangular cells forming an entire basal border	Inflated long-linear, non-tuberculate	Inflated long-linear, non-tuberculate	Inflated long-linear, non-tuberculate	Inflated long-linear, non-tuberculate

Appendix S1 Voucher information and GenBank accession numbers for the analysed Orthotrichaceae specimens in phylogeny

Taxon	Voucher	*trn*L-F	*rps*4	*nad*5	26S
Bryomaltaea obtusifolia (Hook.) Goffinet	Allen 12284 (DUKE)(rps4)/Schofield 98451 (DUKE) *(trn*L-F, 26S, nad5?)*	AY636004	AY908006	AY908948	AY621513
Cardotiella quinquefaria (Hornsch.) Vitt	Schofield 98451, Duke	AY636032	AY618362	AY618414	AY621538
Cardotiella subappendiculata (Broth.) Vitt	Arts 18/01 (herb. Goffinet)	–	AY908615	AY908937	AY621538
Desmotheca apiculata (Dozy & Molk.) Lindb. 1	Vinas 96-4 (herb. Goffinet)	–	AY908614	AY908942	HM751703
Desmotheca apiculata (Dozy & Molk.) Lindb. 2	Schofield 98451, Duke	–	AY618379	AY618410	AY621534
Groutiella chimborazensis (Spruce ex Mitt.) Florsch.	Goffinet 823, herb. Goffinet	AY636033	AY618380	AY618415	–
Groutiella mucronifolia (Hook. & Grev.) H.A. Crum & Steere 1	4012536009_L0255223	MN962632	–	–	–
Groutiella mucronifolia (Hook. & Grev.) H.A. Crum & Steere 2	4012536010_L0255224	MN962633	–	–	–
Groutiella tomentosa (Hornsch.) Wijk & Margad. 1	D243 SP 481100 Peralta, D. F. 20645 2017	MN962634	OK	–	–
Groutiella tomentosa (Hornsch.) Wijk & Margad. 2	D244 SP 486364 Peralta, D. F. 22566 2018	MN962635	OK	–	–
Groutiella tomentosa (Hornsch.) Wijk & Margad.3	Allen B. 18062 Dec 7 1996 (MO)	–	AY908001	AY908943	–
Leiomitrium plicatum (P. Beauv.) Mitt.	Arts (1120:nad5) (1121: rps4) (herb. Goffinet); Schofield 98451, Duke *(trn*L-F, 26S)	AY636029	AY908003	AY908939	AY621535
Leiomitrium plicatum (P. Beauv.) Mitt.	Schofield 98451, Duke	–	AY618359	AY618411	–
Macrocoma orthotrichoides (Raddi) Wijk & Margad	(D201) Valente, D.V. 588, (UB)	MN962636	OK	OK	OK
Macrocoma tenuis (Hook. & Grev.) Vitt	Schofield 98451, Duke, 61513" *(trn*L-F), Arts 04/04 (herb. Goffinet) (rps4, nad5, 26S)	AY636030	AY908004	AY908940	HM751658
Macrocoma tenuis subsp. *sullivantii* (Müll. Hal.) Vitt	100404315	JX827003	–	–	–
Macromitrium catharinense Paris 1	(D169) Valente, D.V. 992, (UB)	MN962595	OK	OK	–
Macromitrium catharinense Paris 2	(D170) Valente, D.V. 993 (UB)	MN962596	OK	–	OK
Macromitrium cavaleriei Cardot & Thér.	110619518	JX826992	–	–	–
Macromitrium guatemalense Müll. Hal. 1	(D172) Valente, D.V. 1346, (UB)	MN245767	OK	OK	–
Macromitrium guatemalense Müll. Hal. 2	(D174) Valente, D.V. 1339, (UB)	MN962619	OK	OK	OK
M*acromitrium gymnostomum* Sull. & Lesq. 1	110619145	JX826995	–	–	–
M*acromitrium gymnostomum* Sull. & Lesq. 2	110619312	JX826999	–	–	–
Macromitrium incurvifolium (Hook. & Grev.) Schwägr.	?	AF231162	–	–	–
Macromitrium japonicum Dozy & Molk.	110619234	JX826998	–	–	–
Macromitrium levatum Mitt.	taxon:66996 *(trn*L-F/rps4); Pocs & al. 88110/BC (RNG)(nad5)	AF023725	AF023813	AY908944	–
Macromitrium microstomum (Hook. & Grev.) Schwägr.	(D181) Valente, D.V. 1013, (UB)	MN962628	OK	OK	OK
Macromitrium pallidum (P. Beauv.) Wijk & Margad. 1	Arts,T, 153/21, MEISE (J12a)	MN967049	–	–	–
Macromitrium pallidum (P. Beauv.) Wijk & Margad. 2	Ah-Peng, C., R08-128, BM (S17)	MN967050	–	–	–

Species	Voucher				
Macromitrium pallidum (P. Beauv.) Wijk & Margad. 3	Ellis, L. & Wilbraham, J., R08-103, BM (S16)	MN967051	–	–	–
Macromitrium rhacomitrioides Nog.	110619643	JX826997	–	–	–
Macromitrium richardii Schwägr.	(D182) Valente, D.V. 1035, (UB)	MN962630	OK	–	OK
Macromitrium richardii Schwägr.	(D184) Valente, D.V. 1026, (UB)	MN962631	OK	OK	OK
Macromitrium punctatum (Hook. & Grev.) Brid. 1	(D177) Valente, D.V. 1127, (UB)	MN962603	OK	OK	–
Macromitrium punctatum (Hook. & Grev.) Brid. 2	(D188) Valente, D.V. 1111, (UB)	MN962604	OK	–	OK
Macromitrium argutum	(D179) Valente, D.V. 1129, (UB)	MN962621	OK	OK	–
Macromitrium taiheizanense Nog.	31317	JX827006	–	–	–
Matteria gracillima (Besch.) Goffinet	Goffinet 7197a (herb. Goffinet);	AY636031	AY907947	AY908621	AY621537
Matteria gracillima (Besch.) Goffinet	Goffinet 7197a, herb. Goffinet	–	AY618360	AY618413	–
Orthotrichum anomalum Hedw.	Vitt Orth. Exs. 43, Duke	AY636019	AF306970	AY908979	AY621525
Schlotheimia grevilleana Mitt. 1	101126003	JX827004	JX827039	–	–
Schlotheimia grevilleana Mitt. 2	100612476	JX827002	JX827002	–	–
Schlotheimia torquata (Sw. ex Hedw.) Brid. 1	Schofield 98451, Duke	–	AY618378	AY618409	AY621533
Schlotheimia torquata (Sw. ex Hedw.) Brid. 2	Allen 11979,MO (rps4/nad5:);Schofield 98451, Duke (26S/*trn*L-F)	AY636027	AY908005	AY908935	HM751702
Zygodon bartramioides Malta	Goffinet 5476, Duke (rps4/nad5/26S?); Schofield 98451, Duke (*trnL*-F)	AY636007	AY908169	AY908978	HM751662

Appendix S2 Voucher information and GenBank accession numbers for the analysed *Macromitrium* specimens in DNA Barcoding

Code	Species	Herbaria Voucher	Coletor/N°	State	Year	Voucher ITS	Voucher *trn*L-F	Voucher *trn*G-R
D66	*Macromitrium argutum* Hampe	UB	Valente, D.V. 569	ES	2016	MN959834	MN962620	MN956556
D164	*Macromitrium argutum* Hampe.	UB	Sousa, R.V. 443	DF	2012	MN959835	MN962621	MN956560
D179	*Macromitrium argutum* Hampe	UB	Valente, D.V. 1129	RS	2017	MN959836	MN962622	MN956557
D215	*Macromitrium argutum* Hampe	SP	Peralta, D.F. 21723	SP	2017	MN959837	MN962623	MN956558
D218	*Macromitrium argutum* Hampe	SP	Peralta, D.F. 21943	SP	2017	MN959838	MN962624	MN956559
D104	*Macromitrium carionis* Müll. Hal.	UB	Valente, D.V. 2626	GO	2017	MN077383	MN245768	MN259735
D105	*Macromitrium carionis* Müll. Hal.	UB	Valente, D.V. 2627	GO	2017	–	MN962600	MN956548
D128	*Macromitrium carionis* Müll. Hal.	UB	Valente, D.V. 2628	GO	2017	MN959840	MN962601	MN956549
D129	*Macromitrium carionis* Müll. Hal.	UB	Valente, D.V. 2629	GO	2017	MN959841	MN962602	MN956550
D148	*Macromitrium carionis* Müll. Hal.	UB180679	Janssen, L. 7	GO	2012	MN959842	–	–
D169	*Macromitrium catharinense* Paris	UB	Valente, D.V. 992	RS	2017	MN950975	MN962595	MN956543
D170	*Macromitrium catharinense* Paris	UB	Valente, D.V. 993	RS	2017	MN950977	MN962596	MN956544
D171	*Macromitrium catharinense* Paris	UB	Valente, D.V. 994	RS	2017	–	MN962597	MN956546
D208	*Macromitrium catharinense* Paris	SP	Peralta, D.F. 22057	SP	2017	MN950978	MN962598	MN956545
D211	*Macromitrium catharinense* Paris	SP	Peralta, D.F. 22086	SP	2017	–	MN962599	MN956547
D239	*Macromitrium cirrosum* (Hedw.) Brid.	SP	Canestraro, B.K. 1163	PR	2017	MN959839	MN962610	MN962583
D242	*Macromitrium cirrosum* (Hedw.) Brid.	SP	Peralta, D.F. 22960	SP	2017	–	MN962611	MN962584
D172	*Macromitrium guatemalense* Müll. Hal.	UB	Valente, D.V. 1346	RS	2017	MN077384	MN245767	MN259734
D173	*Macromitrium guatemalense* Müll. Hal.	UB	Valente, D.V. 1295	RS	2017	MN959843	MN962618	MN956554
D174	*Macromitrium guatemalense* Müll. Hal.	UB	Valente, D.V. 1339	RS	2017	MN959844	MN962619	MN956555
D22	*Macromitrium longifolium* (Hook.) Brid.	UB	Valente, D.V. 350	ES	2016	MN959847	MN962612	MN956569
D79	*Macromitrium longifolium* (Hook.) Brid.	UB	Valente, D.V. 609	ES	2016	MN959848	MN962613	MN956564
D175	*Macromitrium longifolium* (Hook.) Brid.	UB	Valente, D.V. 1176	RS	2017	MN959850	MN962614	MN956565
D176	*Macromitrium longifolium* (Hook.) Brid.	UB	Valente, D.V. 1164	RS	2017	MN959851	MN962615	MN956566
D178	*Macromitrium longifolium* (Hook.) Brid.	UB	Valente, D.V. 1109	RS	2017	MN959849	MN962616	MN956567
D209	*Macromitrium longifolium* (Hook.) Brid.	SP	Peralta, D.F. 21857	SP	2017	MN959852	MN962617	MN956568
D23	*Macromitrium microstomum* (Hook. & Grev.) Schwägr.	UB	Valente, D.V. 278	ES	2016	MN950979	MN962625	MN962590
D74	*Macromitrium microstomum* (Hook. & Grev.) Schwägr.	UB	Valente, D.V. 292	ES	2016	MN950980	MN962626	MN962593
D180	*Macromitrium microstomum* (Hook. & Grev.) Schwägr.	UB	Valente, D.V. 1118	RS	2017	MN950981	MN962627	MN962591
D181	*Macromitrium microstomum* (Hook. & Grev.) Schwägr.	UB	Valente, D.V. 1013	RS	2017	MN950982	MN962628	MN962594
D190	*Macromitrium microstomum* (Hook. & Grev.) Schwägr.	UB	Valente, D.V. 1042	RS	2017	MN963995	–	MN962592
D213	*Macromitrium podocarpi* Müll. Hal.	SP	Peralta, D.F. 21760	SP	2017	MN959845	MN962608	MN956551
D217	*Macromitrium podocarpi* Müll. Hal.	SP	Peralta, D.F. 21702	SP	2017	MN959846	MN962609	MN956552
D222	*Macromitrium podocarpi* Müll. Hal.	SP	Peralta, D.F. 21863	SP	2017	–	–	MN956553
D177	*Macromitrium punctatum* (Hook. & Grev.) Brid.	UB	Valente, D.V. 1127	RS	2017	MN959829	MN962603	MN956561
D188	*Macromitrium punctatum* (Hook. & Grev.) Brid.	UB	Valente, D.V. 1111	RS	2017	MN959830	MN962604	MN956562
D189	*Macromitrium punctatum* (Hook. & Grev.) Brid.	UB	Valente, D.V. 1128	RS	2017	MN959831	MN962605	MN956563
D240	*Macromitrium punctatum* (Hook. & Grev.) Brid.	SP	Peralta, D.F. 22954	SP	2017	MN959832	MN962606	MN962585
D241	*Macromitrium punctatum* (Hook. & Grev.) Brid.	SP	Peralta, D.F. 23029	SP	2017	MN959833	MN962607	MN962586
D17	*Macromitrium richardii* Schwägr.	UB	Valente, D.V. 295	ES	2016	MN950983	MN962629	MN962587

| D182 | *Macromitrium richardii* Schwägr. | UB | Valente, D.V. 1035 | RS | 2017 | MN950984 | MN962630 | MN962588 |
| D184 | *Macromitrium richardii* Schwägr. | UB | Valente, D.V. 1026 | RS | 2017 | MN950985 | MN962631 | MN962589 |

6 CONSIDERAÇÕES FINAIS

a)   Após análise do potencial de discriminação á nível de espécie dos marcadores de DNA utilizados, *trn*G-R demonstrou maior eficiência como um marcador de DNA Barcoding para identificação das espécies devido ser fácil de amplificar, possuir fragmentos curtos 600-700 pares de base, boa qualidade das sequências para discrimimação das espécies de ambos os gêneros *Schlotheimia* e *Macromitrium*. Além disso, foi a primeira vez que o espaçador *trn*G-R foi utilizado para estudo moleculares de musgos. Os demais marcadores não se encaixaram em todos os critérios barcoding, ITS foi o marcador com maior taxa de variação, fácil de amplificar, porém apresentava baixa qualidade das sequências, contaminação por fungos e regiões poli em *Schlotheimia*. *Trn*L-F foi fácil de amplificar, boa qualidade das sequências, mas apresentou baixa taxa de variação para discriminação das espécies.

b)   Nossos dados *trn*G-R fornecem uma eficiente ferramenta para identificação das espécies de *Macromitrium* e *Schlotheimia* (em especial quando as plantas não estão férteis).

c)   Os dados sugerem que *Macromitrium* não é um grupo monifilético, ocorrendo a formação de 3 diferentes grupos (MG1, MG2 e MG3), sendo MG1 o verdadeiro clado de *Macromitrium* representado por duas espécies, MG2 um gênero novo descrito nesse trabalho como *Pseudomacromitrium* e MG3 outro gênero novo, monoespecífico, *Aureomacromitrium.*

d)   Com relação ao gênero *Schlotheimia,* este é um clado monofilético (dados não divulgados), que serão publicados na revisão taxonômica de *Schlotheimia.*

e)   Nossos dados contribuíram para a re-circunscrição do gênero *Macromitrium* e reavaliação do número de espécies com uma redução de 20 espécies previamente aceitas na Flora do Brasil para 14, das quais 3 são conhecidas apenas pela coleta do material original, utilizado na descrição. Após análise molecular e morfológica*,* das cinco espécies de *Macromitrium* consideradas endêmicas para o Brasil, permaneceu apenas uma. As demais são sinônimas de outras espécies já conhecidas. Em relação a *Schlotheimia* esse trabalho forneceu dados para a revisão taxonômica do gênero em parceria com o Instituto de Botânica de São Paulo, lançando um novo olhar para a morfologia á luz de uma abordagem molecular, reduzindo o número de espécies aceitas para o Brasil de 13 para 11. Com relação ao número de espécies endêmicas permance inalterado (5 espécies).

f)   Após a compararação da variação molecular interespecífica x intraespecífica, detectamos um possível caso de especiação críptica em *Schlotheimia apressifolia.*

g)   Foi necessário otimizar protocolos de amplificação de DNA, para os dois gêneros para cada marcador testado, conforme especificado na metodologia dos artigos.

Por fim, foi possível desenvolver novas parcerias de trabalho e analisar o material typus para as 64 espécies de *Macromitrium* já citadas para o Brasil. Dessas, 22% são boas espécies, 53% são sinônimos de outras espécies; 16% foram excluídas da flora brasileira e 9% não foram possíveis verificar.

Esse trabalho trouxe importantes contribuições para a circunscrição do gênero e conhecimento da biodiversidade brasileira, além de uma importante ferramenta para identificação das espécies de *Shlotheimia* e *Macromitrium.*