UNIVERSIDADE FEDERAL DE MINAS GERAIS
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
Departamento de Botânica
Programa de Pós-Graduação em Biologia Vegetal

GABRIEL FELIPE PEÑALOZA BOJACÁ

Dendroceros Nees (Dendrocerotaceae, Anthocerotophyta): revisão sub-genérica, tolerância à dessecação e interação com *Nostoc* sp.

Belo Horizonte, MG, Brasil
Agosto 2021

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GABRIEL FELIPE PEÑALOZA BOJACÁ

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RESUMO

Os filos Marchantiophyta (hepáticas), Bryophyta (musgos) e Anthocerotophyta (antóceros) compreendem o que comumente chamamos de briófitas. Este grupo de plantas apresenta cerca de 25.000 espécies descritas e corresponde ao segundo maior representante das plantas terrestres. As briófitas conservam características ancestrais que ajudaram no estabelecimento das primeiras plantas à vida terrestre, como a poiquiloidria e a tolerância à dessecação. Os antóceros são um grupo monofilético, com cinco famílias, 12 gêneros e 200 a 250 espécies no mundo; com hábitos terrícolas, saxícolas e epífitas. O gametófito dessas plantas, que é taloso, pode abrigar colônias de cianobácterias (*Nostoc*), gerando uma importante simbiose. Seu esporófito difere dos outros filos de briófitas por crescer a partir de um meristema basal, que desenvolve esporos com germinação exospórica na maioria deles. O gênero *Dendroceros* conta com uma alta diversidade, por ter cerca de 40 espécies, das quais 13 estão presentes no Neotrópico, presentes principalmente em regiões temperadas, subtemperadas e tropicais. Este gênero é o único exclusivamente epifítico, com esporos grandes, multicelulares e de germinação endospórica. Na última atualização da *checklist* mundial para antóceros e hepáticas, foram apresentadas as espécies de *Dendroceros*, onde mais de 60% das espécies evidenciavam grandes vazios de informação. Este fato torna-se ainda mais preocupante em nível de espécie. Há poucas coletas, determinações e nomes duvidosos, ou descrições confusas e/ou do Neotrópico, pois das espécies reportadas 80% precisam de uma revisão taxonômica e estudos de filogenia. Também faltam dados sobre a história natural, fisiologia e ecologia das espécies, deixando muito clara a urgência e a necessidade de estudos detalhados sobre *Dendroceros* no Neotrópico. Neste contexto, os principais objetivos deste projeto de tese foram: 1) Recircunscrever taxonômica e filogeneticamente os subgêneros de *Dendroceros* (Capítulo I); 2) Avaliar os efeitos da dessecação nos gametófitos e esporófitos de *Dendroceros* Nees, o único gênero com um nicho epifítico entre os Anthocerotophyta (Capítulo II); e 3) Investigar e descrever a influência de *Nostoc* sp. nas fases iniciais de desenvolvimento dos esporos de *Dendroceros crispus* (Capítulo III). Assim, no primeiro capítulo aqui apresentado, esclarecemos as principais dúvidas em nível de subgênero, e propomos uma nova circunscrição para *Dendroceros* com quatro subgêneros: **subg. *Apoceros*** (oito espécies), **subg. *Dendroceros*** (catorze espécies), **subg. *Nodulosus* (**cinco espécies) e o **subg. *Cichoraceus*** (monotípico). No segundo capítulo, descrevemos demandas conflitantes entre gametófitos e esporos dos antóceros em resposta à dessecação. Evidenciamos estratégias de vida contrastantes, que estão ligadas com o nicho nos gêneros *Dendroceros*, *Nothoceros*, *Phaeoceros* e *Anthoceros*. Finalmente, no terceiro capítulo, apresentamos os efeitos da cianobactéria *Nostoc* sp. sobre esporos e gametófitos juvenis de *Dendroceros crispus* e *Nothoceros vincentianus*. Os dados reunidos neste trabalho evidenciam a importância de continuar estudando os antóceros, e assim aumentar os esforços de conservação das áreas naturais que estes habitam, como as florestas nebulares na Colômbia e a Mata atlântica do Brasil. Ao mesmo tempo, este estudo foi relevante para preencher importantes lacunas (taxonômicas, ecológicas e geográficas) sobre o conhecimento dos *Dendroceros* no Neotrópico.

Palavras claves: Antóceros; Biodiversidade; Classificação; Ciclo de vida; Especificidade planta-cianobactéria; Esporos verdes; Florestas alto montanas; Fluorescência da clorofila; Germinação; Neotrópicos; Nicho; Simbioses; Taxas de crescimento; Taxonomia de briófitas; Tolerância à dessecação.

ABSTRACT

Marchantiophyta (liverworts), Bryophyta (mosses) and Anthocerotophyta (hornworts) phyla, comprise what we commonly call bryophytes. This group of plants has about 25,000 described species and corresponds to the second largest representative of terrestrial plants. Bryophytes retain ancestral characteristics that helped establish the first plants for land life, such as poikiloidy and desiccation tolerance. Hornworts are a monophyletic group, with five families, 12 genera and 200 to 250 species in the world: with terricolous, saxicolous and epiphytic habits. The gametophyte of these plants can have cyanobacterial colonies (*Nostoc*), generating an important symbiosis. Its sporophyte differs from other bryophyte phyla in that it grows from a basal meristem, which develops spores with exosporic germination in most of them. The genus *Dendroceros* has a high diversity, having about 40 species, of which 13 are present in the Neotropics, present mainly in temperate, subtemperate and tropical regions. This genus is the only one exclusively epiphytic, with large multicellular spores and endosporic germination. In the latest update of the world checklist for hornworts and liverworts, the species of *Dendroceros* were presented, where more than 60% of the species showed large gaps in information. This fact becomes even more worrying at the Neotropical level, as 80% of the reported species need a taxonomic review and phylogeny studies. There are few collections, doubtful determinations, and names or confusing and/or incomplete descriptions, making very clear the urgency and need for a revision of the genus *Dendroceros*. In this context, the main objectives of this thesis were: 1) Taxonomically and phylogenetically circumscription the genus *Dendroceros* (Chapter I); 2) Evaluate the effects of desiccation on gametophytes and sporophytes of *Dendroceros* Nees, the only genus with an epiphytic niche among the Anthocerotophyta (Chapter II); and 3) Investigate and describe the influence of *Nostoc* sp. in the early stages of development of *Dendroceros crispus* spores (Chapter III). Thus, in the first chapter presented here, we clarify the main doubts at the subgenus level, and we propose a new circumscription for *Dendroceros* with four subgenera: ***subg. Apoceros*** (eight species), ***subg. Dendroceros*** (fourteen species), ***subg. Nodulusus*** (five species) and ***subg. Cichoraceus*** (monotypic). In the second chapter, we describe conflicting demands between gametophytes and hornworts spores in response to desiccation. Evidencing contrasting life strategies, which are linked with the niche in the *Dendroceros*, *Nothoceros*, *Phaeoceros* and *Anthoceros* genera. Finally, in the third chapter, we present the effects of the cyanobacterium *Nostoc* sp. on spores and juvenile gametophytes of *Dendroceros* crispus and *Nothoceros vincentianus*. The data gathered in this work show the importance of continuing to study the hornworts, thus increasing efforts to conserve the natural areas they inhabit, such as cloud forests in Colombia and the Atlantic Forest in Brazil. At the same time, this study was relevant to fill important gaps (taxonomic, ecological, and environmental) about the knowledge of *Dendroceros* in the Neotropics.

Key words: Biodiversity; Bryophyte’s taxonomy; Chlorophyll fluorescence; Classification; Desiccation tolerance; Germination; Green spores; Growth rates; high mountain forest; Hornworts; Interaction; Life cycle; Neotropics; Niche; Plant-cyanobacteria specificity; Symbiosis.

Sumário

INTRODUÇÃO GERAL

Briófitas são o segundo maior grupo de plantas terrestres, constituído por três filos distintos: Marchantiophyta (hepáticas), Bryophyta (musgos) e Anthocerotophyta (antóceros), que conjuntamente correspondem a cerca de 25.000 espécies (Goffinet, Buck, & Shaw, 2009; Gradstein, Churchill, & Salazar-Allen, 2001). São plantas que conservam características evolutivamente ancestrais como a poiquiloidria e a tolerância à dessecação (Pardow & Lakatos, 2013; Stark, Oliver, Mishler, & McLetchie, 2007; Stark, Mishler, & McLetchie, 2000). Estas características contribuíram sobremaneira para a colonização e estabelecimento das plantas à vida terrestre.

Baseado em filogenias recentes com dados moleculares e morfológicos (Goffinet & Shaw, 2009; Puttick et al., 2018; Villarreal & Renzaglia, 2015), pode-se destacar a importância do estudo das diferentes linhagens de "briófitas" para a compreensão da evolução das plantas terrestres. Atualmente, a maioria das filogenias propõe que as briófitas são monofiléticas, com os antóceros sendo o grupo irmão de musgos e hepáticas (Leebens-Mack et al., 2019; Wickett et al., 2014). Dessa forma, os antóceros constituem uma linhagem essencial para a compreensão do processo evolutivo das plantas terrestres, em especial no que diz respeito à transição dos vegetais para habitats com menor disponibilidade hídrica.

Antóceros geralmente crescem em nichos diferentes, no solo (ex. *Anthoceros* L. e *Phaeoceros* Prosk.), rochas e troncos mortos nas margens de rios e riachos (a maioria das espécies de *Nothoceros* (R. M. Schust.) J. Haseg.) e epífitas em troncos vivos, galhos e folhas (o gênero *Dendroceros* Nees; Gradstein 2001; Duff et al. 2007; Villarreal and Renzaglia 2015). As plantas com um nicho epífita são comumente relacionadas com à tolerância à dessecação, porque a maioria das plantas epífitas tem adaptações para lidar com a seca (Hosokawa & Kubota, 1957; Leon-Vargas, Engwald, & Proctor, 2006; Schuette & Renzaglia, 2010) e muitas briófitas epífitas tendem a enrolar-se para dentro em estado seco (McLetchie & Stark, 2019; Stark, Greenwood, Brinda, & Oliver, 2013).

O gametófito dos antóceros é taloso, podendo ser facilmente confundido com uma hepática talosa em campo quando não há esporófitos (Glime, 2013; Gradstein et al., 2001; Gradstein & Costa, 2003; Villarreal & Renzaglia, 2015). Seu gametófito pode ter a forma de roseta (ex., *Anthoceros, Phaeoceros* e *Notothylas* Sull. ex A.Gray) ou se apresentar como um talo comprido com uma nervura central (ex., *Dendroceros;* Goffinet et al. 2009). O talo geralmente é formado por várias camadas de células, sem muita diferenciação. Internamente, pode apresentar câmaras de mucilagem e câmaras que abrigam colônias de cianobactérias do gênero *Nostoc,* podendo estar ocasionalmente ausentes (ex., *Notothylas*). A simbiose com *Nostoc* é essencial para o desenvolvimento de muitas espécies de antóceros, sendo responsável principalmente pela fixação do nitrogênio (Glime, 2013; Gradstein et al., 2001; Gradstein & Costa, 2003; Renzaglia, Villarreal, Duff, & Goffinet, 2009).

Por outro lado, o gametófito de antóceros apresenta células com um único até poucos cloroplastos (ex., *Megaceros* Campb. pode ter 2 – 4), com pirenoides presentes ou ausentes (Villarreal and Renner, 2012). O pirenoide é uma região diferenciada do cloroplasto, contendo grande concentração da enzima RuBisCO, somente encontrado em alguns grupos de algas verdes (ex., *Chlamydomonas* e *Coleochaete*) e nos antóceros (Meyer, Seibt, & Griffiths, 2008; Smith & Griffiths, 1996; Vaughn & Hasegawa, 1993).

Outra característica interessante dos antóceros é seu esporófito, que difere das outras linhagens de briófitas devido a seu crescimento a partir de um meristema basal. Isso significa que esporos podem ser continuamente produzidos, sendo liberados progressivamente do ápice até a base do esporófito (Gradstein et al., 2001; Gradstein & Costa, 2003; Longton & Schuster, 1983). Em todos os gêneros de antóceros está presente um esporófito ereto, que possui maturação sincrônica dos esporos, com exceção de *Notothylas*. Neste último gênero, ocorre um esporófito reduzido e horizontal, cujos esporos são produzidos simultaneamente. Os esporos de antóceros são geralmente muito ornamentados, exceto em *Leiosporoceros* Hässel cuja parede é lisa. A germinação dos esporos geralmente se dá de forma exospórica, mas a endosporia parece ter evoluído pelo menos duas vezes independentemente em espécies de *Dendroceros* e *Nothoceros* (Villarreal and Renzaglia 2006ª; Renzaglia et al. 2009; Glime 2013; Villarreal et al. 2014).

O filo Anthocerotophyta não foi registrado como um grupo consolidado inicialmente. Ele foi descrito e ilustrado primeiramente por Micheli (1729), que falou do gênero *Anthoceros*, o qual foi reconhecido por Linnaeus, em sua Species Plantarum de 1753 com três espécies (*A. punctatus* L., *A. laevis* L. e *A. multifidus* L.). Posteriormente, foi integrado o gênero *Notothylas*, reconhecido pelo esporófito reduzido, por Sullivant (1845). Em seguida, Nees von Esenbeck (1846) descreveu o gênero *Dendroceros*, um grupo epifítico. Em 1907, Campbell definiu *Megaceros*, tendo como base os cloroplastos múltiplos e a ausência de pirenoides. Da mesma forma, Proskauer em 1951 dividiu o gênero *Anthoceros* em dois, *Anthoceros* e *Phaeoceros*. Em 1971 no Sul da Ásia, Bharadwaj denominou o gênero *Folioceros* D.C. Bharadwaj. Logo depois, os antóceros deixaram de ser incluídos nas hepáticas e foram elevados a uma divisão própria por Stotler e Crandall-Stotler em 1977.

Em 1986, Hässel de Menéndez definiu o gênero *Leiosporoceros*, tendo como base seus pequenos esporos lisos. Em 1988, realizaram a primeira análise cladística dos antóceros, com base em caracteres morfológicos, ajudando com a nomeação do gênero *Sphaerosporoceros*, um taxon intimamente relacionado a *Anthoceros*, e que atualmente foi dissolvido. Posteriormente, os antóceros começaram a ser estudados sob o enfoque de análises filogenéticas com dados moleculares e morfológicos, resultando no estudo de Piippo (1993), que denominou o gênero *Mesoceros* Piippo. Este gênero apresenta caracteres dos esporos similares a *Anthoceros* e *Phaeoceros*. Duff et al. (2004) realizaram análises filogenéticas mais completas dos antóceros, posicionando o gênero *Leiosporoceros* como um grupo basal dos antóceros, separado dos gêneros *Anthoceros* e *Folioceros* em um clado, e *Dendroceros*, *Megaceros*, *Nothoceros*, *Notothylas* e *Phaeoceros* em outro clado. Da mesma forma, reconheceram a monofilia de *Dendroceros* e de

Megaceros; as divergências entre os gêneros *Anthoceros* e *Phaeoceros*; a proximidade filogenética entre *Notothylas* e *Phaeoceros*; e o gênero *Phaeomegaceros* R.J.Duf. Cargill et al. (2005) fizeram um estudo com oito gêneros de antóceros, onde mantiveram a posição de *Leiosporoceros* e o reconhecimento de apenas cinco gêneros (*Anthoceros*, *Notothylas*, *Phaeoceros*, *Megaceros* e *Dendroceros*) como grupos bem estabelecidos. Paralelamente, Stotler e Crandall-Stotler (2005) definiram o gênero *Phymatoceros* Stotler, W.T. Doyle & Crand.-Stotl. baseado em caracteres morfológicos. Finalmente, Duff et al. (2007) organizaram os antóceros em cinco clados: 1) *Leiosporoceros*; 2) *Anthoceros, Folioceros* e *Sphaerosporoceros*; 3) *Notothylas e Phaeoceros*; 4) *Phymatoceros*; e 5) *Megaceros, Dendroceros, Nothoceros* e *Phaeomegaceros*, agrupando os clados 4 e 5 dentro da subclasse Dendrocerotidae.

Na atualidade, os antóceros são classificados em cinco famílias, 12 gêneros e 200 a 250 espécies distribuídas no mundo (Söderström et al., 2016; Villarreal et al., 2014). A maioria das espécies apresenta hábito terrícola ou saxícola, e poucas são epífitas e epíxilas. (Glime, 2013; Gradstein et al., 2001; Gradstein & Costa, 2003; Villarreal & Renzaglia, 2015).

A família Dendrocerotaceae está consistentemente resolvida como um dos clados mais derivado dos antóceros, contendo quatro gêneros: *Dendroceros*, *Megaceros*, *Nothoceros e Phaeomegaceros.* Segundo Villarreal et al. (2010c) o gênero *Megaceros* mostrou-se polifilético, por apresentar duas linhagens filogeneticamente distintas: as espécies do novo mundo estão incluídas em *Nothoceros* e as do velho mundo em *Megaceros*. O gênero *Nothoceros* foi estudado por Villarreal e Renner (2014), onde, com base em suas análises, redistribuíram duas espécies de *Megaceros* para *Nothoceros* esclarecendo problemas taxonômicos e filogenéticos deste grupo. *Phaeomegaceros* é um grupo provisoriamente designado com duas espécies, que precisa de revisão (Cargill et al., 2005; Duff et al., 2007; Renzaglia et al., 2009; Villarreal, Cargill, & Goffinet, 2010; Villarreal & Renzaglia, 2006a). Já o gênero *Dendroceros* conta com uma alta diversidade entre os antóceros, com 41 espécies distribuídas na África, China, ilhas do Pacífico e no Neotrópico (13 espécies neste último; Villarreal and Renner 2014; Söderström *et al.* 2016).

Espécies de *Dendroceros* estão presentes principalmente sobre casca de árvores, lianas, galhos e folhas em regiões temperadas, subtemperadas e tropicais. É o único gênero de antóceros com caracteres diagnósticos estáveis, como o habito epífito, com esporos grandes multicelulares e a germinação endospórica, que ajudam na delimitação das espécies (E. Campbell, 1986; Garcia, Sérgio, Villarreal, Sim-Sim, & Lara, 2012; Gradstein et al., 2001; Hasegawa, 1980, 1983; Renzaglia et al., 2009; Vaughn & Hasegawa, 1993; Villarreal et al., 2014). Os poucos trabalhos existentes sobre o gênero foram realizados na Ásia (Hasegawa, 1980), Nova Zelândia (E. Campbell, 1986), na África Ocidental (Infante, 2010) e no Golfo da Guiné (Garcia et al., 2012). No Brasil são reconhecidas três espécies, *Dendroceros breutelii* Nees, *Dendroceros crispatus* (Hook.) Nees e *Dendroceros crispus* (Sw.) Nees (Peñaloza-Bojacá et al., 2020). Na última atualização do *checklist* mundial de antóceros, fica notório que mais de 60% das espécies de Dendroceros no mundo apresentam falta de informação, poucas coletas, determinações duvidosas, ou descrições confusas e incompletas, transformando alguns desses táxons duvidosos. Isso é ainda mais acentuado em nível do Neotrópico, pois 80% das espécies registradas precisam de uma revisão taxonômica e análise filogenética (Söderström et al., 2016). Adicionalmente, a falta de conhecimentos sobre a ecologia das plantas, como a resposta à dessecação e os mecanismos envolvidos na interação com cianobactérias em *Dendroceros*, como outros antóceros, são chaves para compreender melhor o processo evolutivo deste grupo e das plantas terrestres.

Desta forma, neste trabalho enfocamos nossos esforços em analisar desde diferentes perspectivas (filogenética, fisiológica e de desenvolvimento) o gênero *Dendrocero*s. Visto que com as informações existentes não é possível explicar as relações internas do grupo, e tampouco reconhecer sua verdadeira diversidade (Renzaglia et al., 2009; Villarreal et al., 2014; Villarreal & Renner, 2014). Desta forma, nossos principais objetivos foram 1) Recircunscrever taxonômica e filogeneticamente os subgêneros de *Dendroceros* (**Capítulo I**); 2) Avaliar os efeitos da dessecação nos gametófitos e esporófitos de *Dendroceros* Nees, o único gênero com um nicho epífita entre os Anthocerotophyta (**Capítulo II**); e 3) Investigar e descrever a influência de *Nostoc* sp. nas fases iniciais de desenvolvimento de esporos gametófitos juvenis de *Dendroceros crispus* (**Capítulo III**).

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CAPÍTULO I: Classificação taxonômica e filogenética dos subgêneros de *Dendroceros* Nees

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Phylogenetic and morphological circumscription of the genus *Dendroceros* Nees (Dendrocerotaceae; Anthocerotophyta), with the addition of two new subgenera

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Abstract

Dendroceros is one of the most diverse genera of hornworts, with 41 species and a widespread distribution. The genus is highly distinctive by its epiphytic habitat, gametophytes with perforated wings, multicellular spores due to endosporic germination, highly modified pyrenoid, and desiccation tolerance. The genus is monophyletic and, until now, was divided into two subgenera (***subg. Apoceros*** and ***subg. Dendroceros***). This classification has not been tested within a phylogenetic framework. Using published phylogenetic data from four molecular markers (*rbcL, trnK* including *matK*, *rps4,* and *nad5*) as well as morphological observations of over 80 specimens. We chose thirty-eight morphological characters, which were mapped in the best maximum likelihood tree and optimized for reconstruction of ancestral character. We circumscribe the genus *Dendroceros* and propose 4 subgenera: ***subg. Apoceros*** (eight species), characterized for having schizogenous cavities in the midrib; ***subg. Dendroceros*** (fourteen species) with undulate-crispate gametophyte wings, and spore distal surface covered by aculeate papilla; ***subg. Nodulosus*** (five species) with rectangular cells in the capsule with nodular wall thickenings; and the monotypic ***subg. Cichoraceus*** with a single species that has granular spore micro-ornamentations, and a macro-ornamentation formed by pustulate papillae.

Key words:
Biodiversity; Bryophytes; Classification; Hornworts; Neotropics; Taxonomy.

Introduction

Molecular data have changed the way researchers view plant phylogenetic relationships, especially in character-poor lineages such as bryophytes (Duff, Villarreal, Cargill, & Renzaglia, 2007; Puttick et al., 2018). There are three main groups within the bryophytes, with highly disparate morphological features: liverworts (Marchantiophyta), mosses (Bryophyta), and hornworts (Anthocerotophyta). The evolutionary ties between the mosses and liverworts (setaphytes; Renzaglia, Villarreal, & Garbary, 2018) have been addressed multiple times and we are on the verge of a consensus about their relationship. The position of hornworts remains uncertain in recent molecular phylogenies. Hornworts, are either part of the bryophyte monophylum or the sister group to all land plants (Puttick et al., 2018; Wickett et al., 2014). Within hornworts, the generic relationships within the hornworts have been firmly established (Duff et al., 2007; Renzaglia, Villarreal, Duff, & Goffinet, 2009; Villarreal, Cusimano, & Renner, 2015), while their infrageneric (subgenera and species) have only been addressed in two studies. One study, within the family Dendrocerotaceae, reconstructed the phylogeny of the mostly Neotropical genus *Nothoceros* (Villarreal & Renner, 2014); the second study focused on several species of *Megaceros* from Australasia (C. Cargill, Vella, Sharma, & Miller, 2013). Thus, the family Dendrocerotaceae seems to be the focus of the most recent phylogenetic studies in hornworts.

The other two genera within the Dendrocerotaceae, *Phaeomegaceros* and *Dendroceros*, remain largely under studied from a systematic point of view. The genus *Dendroceros* is highly distinctive by its: 1) epiphytic habitat, 2) gametophytes with perforated wings; 3) multicellular spores due to endosporic germination; 4) highly modified pyrenoids (i.e., specialized compartments inside chloroplasts essential for carbon concentration mechanism); and, 5) desiccation tolerance (Cargill, Renzaglia, Villarreal, & Duff, 2005; Duff, Cargill, Villarreal, & Renzaglia, 2004; Duff et al., 2007; Li, Villarreal, & Szövényi, 2017; Schuette & Renzaglia, 2010; Villarreal et al., 2015; Villarreal, Goffinet, Duff, & Cargill, 2010). *Dendroceros* is, also, one of the most diverse genera among the hornworts, with 41 species and a widespread distribution (Söderström et al., 2016; Villarreal & Renner, 2014). *Dendroceros* species grow mainly on tree bark, branches, and leaves in temperate and tropical forests, between 800-2000 m a.s.l. (Campbell, 1986; Garcia et al., 2012; Gradstein et al., 2001; Hasegawa, 1980, 1983; Renzaglia et al., 2009; Villarreal et al., 2014).

Dendroceros is unambiguously monophyletic (Duff et al., 2007). However, more than 60% of the described species lack clear morphological descriptions, and ca. 34% have sequence data from 3-4 molecular markers, especially plastid markers (Söderström et al., 2016; Villarreal et al., 2014). Schuster (1987) subdivided the genus into two subgenera (**subg**.): **subg. *Apoceros*** R. M. Schust., which is characterized by schizogenous cavities in the midrib; and the **subg. *Dendroceros***, which possesses a solid midrib (Hasegawa, 1980; Piippo, 1993; Schuster & Engel, 1987; Stephani, 1917). Currently, only seventeen species have sub-generic designations (Tab. S1; Söderström et al., 2016). From the discussion, is evident that the lack of morphological and molecular studies of most of the *Dendroceros* species hamper any systematic evaluation of the subgenera and test character evolution of the subgenera and test character evolution within the genus (Renzaglia et al., 2009; Villarreal et al., 2014; Villarreal & Renner, 2014). Based on the above, we tested the monophyly of whether both subgenera proposed by Schuster (1987) and we revised the current infrageneric classification of the genus using all available data and observations from over 80 specimens. The information presented here fills important gaps in our knowledge of hornworts and establishes a robust phylogenetic framework to test character evolution within *Dendroceros*.

Materials and methods

Taxon sampling

We studied ca. 80 specimens (including type material) assigned to *Dendroceros* held in public herbaria were studied. We examined specimens from the CANB, CHR, CONC, CONN, DUKE, G, NY, S herbaria (acronyms according to *Index Herbariorum*, Thiers, 2017). The specimens were examined under dissecting and compound microscopes, and by scanning electron microscopy, using standard protocols (Villarreal & Renzaglia, 2006a). Images were taken using a Zeiss Axio Lab A1 light microscope equipped with an Axion Erc5 digital camera, as well as a FEG – Quanta 200 FEI Scanning Electron Microscope, at the Microscopy Center of the Federal University of Minas Gerais, Brazil. Spore ornamentation nomenclature follows Lellinger (2002). To improve and complete the morphological information of many of the species, we used previously published taxonomic descriptions and their protologues, as well as the Plant Science photographic database from type specimens available at JSTOR (http:// plants.jstor.org).

Sampling for phylogenetic analyses

We downloaded GenBank sequences of three plastid regions (*rbcL, trnK* including *matK*, and *rps4*) and the mitochondrial *nad5* exon2 (Duff et al., 2004, 2007; Villarreal & Renner, 2012, 2013),. from fourteen species of *Dendroceros* (Tab. S1), and six other members of the family Dendrocerotaceae: *Megaceros flagellaris* (Mitt.) Steph.; *Megaceros leptohymenius* (Hook., F. & Taylr.) Steph.; *Nothoceros giganteus* (Lehm. & Lindenb.) J. Haseg; *Nothoceros renzagliensis* J.C. Villarreal et al.; *Phaeomegaceros fimbriatus* (Gottsche) Duff et al.; and *Phaeomegaceros hirticalyx* (Steph.) Duff et al. We chose *Phymatoceros phymatodes* (M. Howe) Duff et al. as the outgroup taxon, following published studies (Duff et al., 2007). All sequences used in this study are available on the GenBank public platform (https://www.ncbi.nlm.nih.gov/nuccore/?term=Dendroceros). Voucher information and GenBank accession numbers are provided in Table S2.

Phylogenetic analyses

The GenBank sequences were edited using the software MEGA 6.0 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013) and aligned using Muscle (Edgar, 2004), followed by manual adjustments. The alignment of the mitochondrial *nad5* gene and the plastid regions *trnK, rbcL, rps4* were concatenated using Mesquite 3.2 (Maddison & Maddison, 2017). The matrix consisted of 4,170 nucleotides. A maximum parsimony (MP) analysis was performed in PAUP* (Swofford, 2002), using a heuristic search with 1000 repetitions employing Tree-bisection and reconnection (TBR). The statistical support was obtained through non-parametric bootstrapping (Felsenstein, 1985) with 5,000 replicates.

We obtained the best partition for our data and the appropriate nucleotide substitution using Partitionfinder2 (Lanfear, Frandsen, Wright, Senfeld, & Calcott, 2016). The best partition scheme was by locus: *nad5* (1-1106, TRN+ Γ), *rbcL* (1107-2441, GTR + I+ Γ), *rps4* (2442-3024, TVM+I), and *trnK-matK* (3025-4170, K81UF+ Γ). Bayesian analyses (BI) were carried out with two simultaneous and independent runs with four chains (one cold and three heated chains) and 1,000,000 generations; posterior probabilities of tree topologies were estimated from both partitions and the trees were sampled every 100th generation using a Markov Chain Monte Carlo method implemented in MrBayes version 3.2 (Ronquist et al., 2012). The first 25% of sampled trees were discarded for burnin to make the analyzes converge conservatively, and a majority rules consensus of the trees excluding the burnin was produced with posterior probabilities above 0.95 as supported. The alignments and trees have been deposited on TreeBASE (https://treebase.org/treebase-web/home.html; accession no. 23631). We used the software FigTree to view and edit trees (Rambaut, 2017).

We performed maximum likelihood (ML) analyses on RAxML-HPC BlackBox v. 8.2 (Stamatakis, 2014) using Cipres Science Gateway (https://www.phylo.org). The substitution model GTR+CAT was used with four unlinked partitions and the statistical support was evaluated using 1000 ML start replicas using the same substitution model.

Character optimization

We chose thirty-eight morphological variables of categorical characters based on the analyzed material and taxonomic descriptions (Garcia et al., 2012; Hasegawa, 1980; Infante, 2010; Piippo, 1993; Proskauer, 1951, 1953). The characters related to the gametophytes were recorded from mature portions of each plant (e.g., mid region), the apical and basal regions of the plants were not taken into consideration, because were undeveloped or deteriorated. On the sporophyte, the dehisced mature capsules were studied, especially in the middle region of the capsule.

A matrix of morphological characters was encoded with binary and multistate characters (Tab. S3) and mapping characters and reconstruction of ancestral character states was performed in WinClada 10.00.08 (Nixon, 2002) and Mesquite v.3.2 (Maddison & Maddison, 2017) . Characters were mapped on the best maximum likelihood tree and optimized to evaluate synapomorphies and homoplasies (Fig. S1, see supplemental material online). We explored delayed optimization (deltran) and rapid optimization (acctran) to identify additional characters that would define certain clades (Agnarsson & Miller, 2008).

Results

Phylogenetic analyses

The phylogenetic analyses resulting from the combination of the coding regions *rbcL; nad5*; *trnK-matK*; *rps4,* Fig. 1.1) show that the genus *Phaeomegaceros* was not well-supported (bootstrap 59% MP). In contrast, the genera *Megaceros* and *Nothoceros* were identified as monophyletic, with high bootstrap (MPB, MLB) and high posterior probability (p.p., Fig. 1.1). The genus *Dendroceros* is monophyletic, with high support values (MPB 99%; MLB 100% and p.p. 1.0). The *Dendroceros* species analyzed were aggregated into four clearly defined groups.

Clade A includes two species, *D. difficilis* and *D. cucullatus,* with good support (MPB 83%, MLB 97% and p.p. 1.0). Clade B is formed by the species *D. crispus, D. javanicus, D. validus, D. tubercularis*, *D. breutelii,* and *D. paivae,* and was highly supported, with values over 98% (MPB, MLB) and above 0.95 (p.p) in all the analyses. Clade C was highly supported (MPB 99%, MLB 100% and 1.0 p.p.) and comprises *D. borbonicus*, *D. africanus*, *D. crispatus,* and *D. granulatus*. Finally, *D. cichoraceus* is sister to the rest of other taxa (Fig. 1.1).

Character evolution

The morphological matrix of thirty-eight characters included twenty-four gametophyte characters, eleven sporophyte characters, and two ecological characters (Tab. S3, Fig. S1). The most informative characters of the genus were:

1) Wing anatomy: the undulate-crispate wings is the ancestral state in *Dendroceros*, with a shift to flat wings in the ancestor shared by *D. tubercularis, D. breutelii* and *D. crispus*, and a reversal in the former species. There is one evolutionary transition to galeate wings in clade C (Fig. 1.2).
2) Transverse section of the midrib: the ancestral state is a solid midrib with a transition to thallus with schizogenous cavities in the clade A (Fig. 1.3).
3) Thickenings of the cell walls in the capsule: the rectangular cell shape is the ancestral state with a shift to nodular capsule cells in clade C (Fig. 1.4).
4) Spore ornamentation on distal surface: the ancestral state is the postulate papilla (macro-ornamentation), with a shift to aculeate papilla observed in clades B and C; and spines are a synapomorphy for the clade A. (papilla shape see Figs. 1.5, 6).

Discussion

We revisited and partly corroborated Schuster's (1987) subgenera treatment and provided a novel assessment of character evolution in the genus *Dendroceros*. Species with schizogenous cavities in the midrib remain gathered into the clade A, with a high phylogenetic support - **subg. *Apoceros***; while species with a solid thallus were grouped into three distinct clades with an also good phylogenetic support: species with galeate gametophyte, wing perforations always present and capsule cells with nodular thickened walls in clade B **- subg. *Nodulosus***; gametophyte wings with pores and spore distal surface with macro-ornamentations as aculeate papilla in clade C - **subg. *Dendroceros*** and gametophyte wings with almost no pores and spore distal surface with macro-ornamentations as pustulate papilla in **subg. *Cichoraceus***. We will highlight the main morphological characters of the genus in light of the phylogenetic results of our study.

Gametophyte

Dendroceros gametophytes have different morphologies in the wings, as seen in **subg. *Dendroceros*** (undulate and crispate) and **subg. *Cichoraceus*** (only crispate), with a state shift to galeate wings in **subg. *Apoceros*** and **subg. *Nodulosus***. Galeate wings are an apomorphy within the genus and a unique character for these two subgenera. The presence of a multistratose midrib and wings have evolved independently in *Dendroceros* and few species of *Nothoceros* (e.g., *N. endiviifolius, N. superbus*; Villarreal, Menéndez, & Salazar-Allen, 2007). The basic body plan of *Dendroceros* (and few species of *Nothoceros*), a midrib with monostromatic wings may be related to the adaptive response to the hydration and dehydration cycles present in an epiphytic environment (Schuette & Renzaglia, 2010; Wood, 2007).

Another character of the genus is the midrib anatomy, already recognized by Stephani (1909, 1917). The solid midrib, present in the **subg. *Dendroceros***, **subg. *Nodulosus*** and **subg. *Cichoraceus***, is commonly found across the genus with a single transition to cavernous thallus (**subg. *Apoceros***). Nevertheless, schizogenous cavities in the middle region of the thallus seem to be a homoplastic trait in hornworts, equally found in the family Anthocerotaceae and some gametophytes of *Leiosporoceros dussii* (Steph.) Hässel (Renzaglia, 1978; Villarreal & Renzaglia, 2006b). Mucilage-filled cavities are ubiquitous in hornworts, especially in the sporophyte of species bearing stomata (Duckett & Pressel, 2018; Renzaglia, 1978). Mucilage-filled cavities harbor antheridia and nitrogen-fixing cyanobacteria (Renzaglia, Duff, Nickrent, & Garbary, 2000; Renzaglia et al., 2009). At a functional level the mucilage-filled schizogenous cavities are thought to be related to desiccation tolerance in hornworts (Renzaglia et al., 2000).

Pores (2-15 µm) are found at the junction of the cells in monostromatic wings of *Dendroceros*. These wing pores are commonly confused with trigones in the literature (Hasegawa, 1980; Infante, 2010; Piippo, 1993). Trigones are collenchymatous thickenings of the cell walls present at the junctions of three or more cells (Glime, 2017). In older *Dendroceros* specimens, the spaces between wing cells resemble cell wall thickenings. However, recently-collected specimens clearly showed these spaces between cells (Fig. 3.2). Those pores still differ from the macro-perforations (40 to over 200 µm) seen on gametophyte wings in several tropical species (e.g., *D. crispatus* and *D. javanicus*; Chantanaorrapint et al., 2014; Hasegawa, 1980, 1982; Infante, 2010). The development and function of pores and macro-perforations remain unknown.

Capsules and spores

Epidermal cells in the *Dendroceros* capsule have a rectangular shape with different types of wall thickenings (nodular and rectangular), which have been used by different authors to separate species (Chantanaorrapint, 2014; Chantanaorrapint et al., 2014; Garcia et al., 2012; Hasegawa, 1980, 1982; Infante, 2010; Pagán, 1942; Stephani, 1909, 1917). In our study, this character was useful to distinguish the species of **subg. *Nodulosus***, which have nodular thickenings in their cell walls; different from other subgenera with rectangular thickenings.

The thickening of the epidermal cells starts in the basal region of the capsule still covered by the involucre (Ligrone & Renzaglia, 1990; Renzaglia, 1978). During these early stages of development, epidermal cells do not show clear thickenings. The thickenings are easily observed as the sporophyte grows and emerges from the involucre (Renzaglia & Garbary, 2001; Renzaglia et al., 2009). In *D. crispatus*, for example, the cells at the base of the capsule do not show nodular thickenings. Functions of these thickenings are not well defined, although we believe they can play role in the capsule dehiscence, such as pseudoelateres with helical thickenings and the spiral columella that facilitate spore separation and dispersal (Renzaglia et al., 2009).

Dendroceros spores germinate still inside the capsule, which results in a range of size and morphologies (Chantanaorrapint, 2014; Garcia et al., 2012; Hasegawa, 1980; Schuette &

Renzaglia, 2010). Measurements of spore length in our study agree with previous observations in literature, with a range of 45-135 μm for species in the **subg. *Apoceros***, 40-112 μm in **subg. *Nodulosus***, and 85 μm in the **subg. *Dendroceros*** and **subg. *Cichoraceus*** (Hasegawa, 1980, 1986; Infante, 2010; Piippo, 1993; Stephani, 1917). However, there is a considerable amount of variability in published records. For instance, in *D. pedunculatus*, there are records of large spores (e.g., 110-135 μm; Hasegawa, 1980; Stephani, 1917) or rather small spores (e.g., 70-83 μm; Piippo, 1993). We suspect the technique used to measure spores may generate this variation (Tab. 1).

Due to endospory there is considerable variation in the number of cells (5 to <20), which depends directly on the state of development of the capsule (Schuette & Renzaglia, 2010). Endospory is only found in all *Dendroceros* species and *Nothoceros renzagliensis* (Villarreal, Campos, Uribe-M., & Goffinet, 2012). It's plausible that endospory is an adaptation to environments under periodic desiccation (Schuette & Renzaglia, 2010; Wood, 2007). Endosporic germination is highly homoplastic in bryophytes, being reported from disparate orders such as *Pelliales*, *Metzgeriales*, *Marchantiales* (liverworts) and *Andreaeales, Bryales*, *Orthotrichales* (mosses, Frey & Stech, 2009; Schuette & Renzaglia, 2010; Villarreal et al., 2012).

An important taxonomic feature across hornworts is the spore ornamentation. *Dendroceros* species have been typically described as having papillate spores and few studies have stressed the importance on this character in the genus. However, we found that micro-ornamentation is useful to define infrageneric taxa in *Dendroceros* (Fig. 6). For example, the spores of **subg. *Apoceros*** have wart-like micro-ornamentations (< 0.2 μm long), as well as macro-ornamentation of spines (1-3 μm long). In the **subg. *Dendroceros***, the micro-ornamentation consists of granules and vermiculates, with the macro-ornamentation consisting of aculeate papilla 0.8-3.3 μm long, with a small bend at the apex. In the **subg. *Nodulosus*** the micro-ornamentation is similar to the species of the **subg. *Dendroceros***, but the macro-ornamentation of aculeate papilla is smaller (0.9-2.2 μm long) with short, fine, and frequently straight apices. In these two subgenera the format of macro-ornamentations evolved from the ancestral state, pustulate papilla, present in *subg. Cichoraceus* and some species of *Nothoceros* and *Megaceros* (Garcia et al., 2012; Villarreal et al., 2010, 2007)
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The present study brings light to the subgenera relationships within the hornwort genus *Dendroceros*. Additionally, we emphasize the unique biological features of *Dendroceros* such as its epiphytic habitat pyrenoid with protein incrustations and desiccation tolerance. Based on the results shown above, we present a new taxonomic treatment for the genus.

Taxonomic treatments

The analyses confirmed the existence of four distinct natural groups, or subgenera, within *Dendroceros* (Hasegawa, 1980; Piippo, 1993; Schuster, 1987; Söderström et al., 2016; Stephani, 1917). To clarify some of the nomenclatural problems within the genus, we propose a new circumscription of the subgenera of *Dendroceros* Nees based on the phylogenetic inferences obtained here and the morphologic characteristics analyzed for the twenty-eight species listed in Table 1.

Key to *Dendroceros* Nees. Subgenera

1a Cross section of the thallus midrib showing schizogenous cavities; cells in the capsule with thickened non-nodular walls ……………………...……………….. **subg. *Apoceros***

1b Cross section of the thallus midrib solid; cells in the capsule with or without thickened non-nodular walls …..………..………………..………………………….…..………… **2**

2a Capsule cells with nodular thickened walls; gametophyte with galeate (hood-like) wings; perforation on wings always present ………….………..…..…… **subg. *Nodulosus***

2b Capsule cells with non-nodular thickened walls; gametophyte plane, undulate, or crispate, wing perforations not present or rare **3**

3a Gametophyte wings with pores, margins entire; spores with less than a total of 20 cells, distal surface with macro-ornamentations of aculeate papillae 0.8-3.3 μm long (under SEM) .... **subg. *Dendroceros***

3b Gametophyte wings with almost no pores, margins crenate, spores with more than 21 cells, distal surface with macro-ornamentations of pustulate papillae 0.9-2.2 μm long (under SEM) ………………………………………..….…..……..…. **subg. *Cichoraceus***

Family **Dendrocerotaceae**

Dendroceros Nees, 1846

Dendroceros Nees ***subg. Apoceros*** Schust R. M. 1987 (Figs. 2, 6.1)

Type species: *Dendroceros cavernosus* by original designation in Schuster (1987).

Diagnosis: Gametophyte light-green when dried, varying from ocher-green and red to orange hued in the midrib region; wings of the gametophyte with macro-perforations; schizogenous cavities visible in cross sections of the midrib; involucre with ornamentation and a rough cuticle; cells of the capsule rectangular, with thickened non-nodular walls; distal spore surfaces with micro-ornamentations of warts and granules, and macro-ornamentation of spines 1-3 μm long.

In 1987 Schuster described this subgenus and included three species (*D. cavernosus*, *D. difficilis*, and *D pedunculatus*). Piippo (1993) later published the species *D. ogeramnangus* and classified it within this group, together with *D. subdifficilis*. The species *D. muelleri* was studied by Stephani (1917), who placed it in the group of species with schizogenous cavities in the midrib. The Asiatic species *D. seramensis* also presents schizogenous cavities in the midrib (Hasegawa, 1986). The last two species, together with *D. cucullatus*, are currently classified among the incertae sedis of the genus, and we included them in **subg. *Apoceros***, which is composed of eight species; two of which were included in our analysis.

Dendroceros cavernosus J. Haseg. 1980

T*ype*: Malaysia, N. Borneo: between Sosopodon and S. Kelinggen, foot of Mt. Kinabalu, 1350-1400 m, on fallen branches, Mizutani M. 3861 (holotype NICH**!**).

Description and/or illustration: p. 308, Fig. 11 in Hasegawa (1980); 106 p. in Hasegawa (2002).

Geographical distribution: Malaysia, Papua New Guinea, Solomon Islands.

Additional material examined: CANB 578715, CHR 502079, CHR 501509, NY 03103379, NY 03103380.

Dendroceros cucullatus Steph. 1923

Type: Philippines, Mindanao, Butuan Subprovince, 15 m, on tree trunk, March-July 1911, Weber C. M. 1341 (holotype G 00061264**!**, isotype G 00061265**!**, isotype NY 253465**!**).

Description and/or illustration: p. 429 Stephani (1923); Figs. 9 b, c, f, h in Hasegawa (1980); p. 34, Figs. 2, 5a-b in Chantanaorrapint (2014); p. 147, Figs. 3-4 in Chantanaorrapint et al. (2014).

Geographical distribution: Philippines and Thailand.

Dendroceros difficilis Steph. 1917

Type: Indonesia. Java. Provo Preanger, infra lacum vulcanicum Telaga bodas, 1.550 m, November 1894, Schiffner V. # 2 (holotype G 19700, not seen; isotype NY 231498**!**).

Description and/or illustration: p. 1009 in Stephani (1917); p. 304, Fig. 9 in Hasegawa (1980); p. 41, Fig. 9 in Piippo (1993); p.106 in Hasegawa (2002); p. 147, Figs. 3-4 in Chantanaorrapint et al. (2014).

Geographical distribution: Indonesia, Malaysia, Philippines.

Additional material examined: G 00048022, NY 03103374.

Dendroceros muelleri Steph. 1889

Type: Australia, Bellender Ker Range, Coll. Herb. melbourne 1886, Froggatt, W. & Sayer W. # s.n. (holotype G 00060899**!**).

Description: p. 133 in Stephani (1889); p. 1009 in Stephani (1917).

Geographical distribution: Australia.

Dendroceros ogeramnangus Piippo 1993

Type: Papua New Guinea. "Morobe Prov.: Rawlinson Range, near Ogeramnang airstrip. In extensively cultivated garden area with scattered trees along trail from Selimbeng (Serembeng) down toward Rulum River", 1200-1450 m, 29'S, 1421'E, collection site 10k." May 1981, Norris 59226 (holotype H; isotype JE, not seen).

Description and/or illustration: p. 40, Figs. 7, 10 in Piippo (1993).

Geographical distribution: Papua New Guinea.

Dendroceros pedunculatus Steph. 1909

Type: Indonesia, Amboina, Karsten G. (holotype G 00061209, not seen).

Description and/or illustration: p. 1009 in Stephani (1917); p. 306, Fig. 10 in Hasegawa (1980); p. 43, Figs. 7, 11 in Piippo (1993).

Geographical distribution: Indonesia.

Dendroceros seramensis J. Haseg. 1986

Type: Indonesia. Seram. Kecamatan, Tehoru, between Wolu and Wae Waya, in a secondary lowland forest, on trunk of tree, 0-360 m, Akiyama, H. 10269 (holotype KYO, not seen).

Description and/or illustration: p. 10, Fig 1 in Hasegawa (1986).

Geographical distribution: Indonesia.

Dendroceros subdifficilis S. Hatt. 1951

Type: Indonesia, West Irian, Manokwari: Prafi, Bivouae, febrary 1943, Tsuyama 1178 (holotype TNS; isotype NICH, not seen).

Description and/or illustration: Fig. 2 in Hasegawa (1986); p. 44, Fig. 15 in Piippo (1993).

Geographical distribution: New Guinea.

Dendroceros* Nees *subg. Dendroceros (Figs. 3, 6.2)

Type species: *Dendroceros crispus* by original designation in Schuster (1987).

Diagnosis: Gametophyte light-green, dark-green when dry; planar, undulate, or crispate; wings of the gametophyte with macro-perforations; pores mainly irregular; midrib solid; rectangular cells in the capsule with wall thickenings; pseudoelaters papillose or smooth; multicellular spores, proximal surface coarsely vermiculate, distal surface with granular and vermiculate micro-ornamentations, with macro-ornamentations of the aculeate papilla 0.8-3.3 μm long.

Dendroceros crispus, D. javanicus, D. tubercularis, and *D. validus* were placed by Schuster (1987) and Piippo (1993) within this subgenus. Here we include *D. adglutinatus, D. allionii, D. breutelii, D. crassinervis, D. herasii, D. rigidus,* and *D. paivae*, which were classified as incertae sedis by Söderström et al. (2016; Fig. 1). The **subg. *Dendroceros*** therefore now consists of fourteen species.

Dendroceros acutilobus Steph. 1909

Type: Indonesia, Amboina, Ambon X, 1889, Karsten G. s.n. (holotype G, not seen).

Description and/or illustration: p. 298, Fig. 6 in Hasegawa (1980); p. 44, Figs 12,13,14 in Piippo (1993); p. 106 in Hasegawa (2002).

Geographical distribution: Indonesia, Papua New Guinea.

Additional material examined: G 00067713, NY 3103376.

Dendroceros adglutinatus (Hook.f. & Taylor) 1846

Bas.: Monoclea adglutinata Hook.f. et Taylor 1846.

Type: Africa, Saint Helena. ‘On trees on Diana’s Peak, at an elevation of 2000 feet. 1844 Hooker D.J (holotype not seen, isotype NY 920051**).**

Description and/or illustration: p. 580 in Gottsche et al., (1846); p. 1, Fig. 1G in Infante (2010).*Geographical distribution*: Saint Helena.

Additional material examined: S 165088.

Dendroceros allionii Steph. 1917

Type: Ecuador, Morona-Santiago - Bomboiza, Gualaquiza/Provincia de Morona Santiago, 900 m. Coll. Bryotheca Ecuadorensis 1901. Allioni M. 319. (holotype G 00060907**!**).

Description: p. 1014 in Stephani (1917).

Geographical distribution: Ecuador.

Dendroceros breutelii Nees 1846

Type: ST. KITTS. Breutel s.n. (G- 00115617, not seen).

Description: p. 581 in Gottsche et al. (1846); p. 1015 in Stephani (1917).

Geographical distribution: Brazil, Cuba, Ecuador, Montserrat, Puerto Rico,
Saint Kitts and Nevis.

Additional material examined: G 00060906, G 00115614, NY 00544177, NY 00544178, NY 00544179, NY 00544180, DUKE 0263664.

Dendroceros crassinervis (Nees) Gottsche, 1858.

Bas.: *Anthoceros crassinervis* Nees, Syn. Hepat. 4: 589, 1846.

Type : Indonesia, *Java. "Inter Lichenes Indiae occidentalis legit Hampe, Vidi in hb. Hp."* (J. Gottsche et al., 1846).

Description: p. 589 in Gottsche et al. (1846).

Geographical distribution: Indonesia.

Additional material examined: NY 03103378, NY 3103377.

Dendroceros crispus (Sw.) Nees 1844

Bas.: *Anthoceros crispus* Sw., Prodr. (Swartz): 146, 1788.

Type: Jamaica, " In montibus altis 'Liguaneis " ("in the mountains of New Liguanee" - P. Browne; meaning apparently the mountains closely NW of Kingston, rather than the whole of the Blue Mountain range). Swartz, O. s.n (lectotype S-B20564 selected by Proskauer 1960, not seen).

Description: p. 146 in Swartz (1788); p. 581 in Gottsche et al. (1846); p. 1015 in Stephani (1917); p 207 in Howe (1934).

Geographical distribution: Brazil, Colombia, Costa Rica, Cuba, Dominica, Ecuador
Guadeloupe, Jamaica, Martinique, Panama, Peru, Puerto Rico, Saint Helena, Suriname, Trinidad and Tobago, Uruguay, Venezuela.

Additional material examined: G 00115645, G 00115607, NY 231499, NY 01319034, NY 01319035, NY 01319036, NY 01319037, NY 01319038, NY 00544185, NY00544186, NY 00544187, NY 00544188, NY 00544189, NY 00544190, NY 00544191, NY 00544193, NY 00544194, NY 00544195, NY 00544196, NY 00544197, NY 00544199.

Dendroceros foliicola J.Haseg. 1980

Type: East Borneo. Munukan, N. of Tarakan, inner forest in N. part of the island near brooklet, on leaves of Salacca-palm, leg. W. Meijer B 5018 (NICH, not seen).

Description and/or illustration: p. 295, Fig 5 in Hasegawa (1980).

Geographical distribution: East Borneo.

Dendroceros herasii M. Infante 2010

Type: Guinea Ecuatorial, Annobo´n, Mte. Quioveo, Bosque neblinoso de Schefflera mannii (Hook.f.) Harms en la cumbre, muy musgoso. Colgante en liana fina. 590 m. October 2000. Infante M & Heras P, 27 (holotype VIT 25366, not see).

Description and/or illustration: p. 285, Fig. 1 in Infante (2010).

Geographical distribution: Equatorial Guinea (Annobon) and Island of São Tomé.

Dendroceros javanicus (Nees) Nees 1844

Bas: *Anthoceros javanicus* Nees, Enum. Hep. Jav. (1830).

Type: Indonesia, Java,Nees (isotype G 19704, not seen).

Description and/or illustration: p. 582 in Gottsche et al. (1846); p. 1010 in Stephani (1917); p. 301, Fig. 8 in Hasegawa (1980); p. 46, Figs. 12,14,15 in Piippo (1993); p. 119, Fig. 4 in Hasegawa (1995).

Geographical distribution: Federated States of Micronesia, French Polynesia, Indonesia, Papua New Guinea.

Additional material examined: NY 3103366, NY 3103367, NY 3103368, NY3103370.

Dendroceros paivae C. A. Garcia, Sérgio & J. C. Villarreal 2012

Type: São Tomé e Príncipe. Ilha de São Tomé. Caminho para a Roça Trás-os-Montes. NKF3129. 1038 m. 19-07-2007. Epiphyllous on leaves of *Syzygium jambos*. Alston. C. Garcia S. 125 (holotype LISU 237201**!**).

Description and/or illustration: p. 5, Figs. 1-9; 26; 51-53 in Garcia et al. (2012).

Geographical distribution: Island of São Tomé.

Dendroceros rigidus Steph. 1917

Type: Brazil, São Paulo. May 1901. Schiffner V. 992 (holotype not seen; Isotype S B22423**!**).

Description: p. 1017 in Stephani (1917).

Geographical distribution: Brazil.

Dendroceros subplanus Steph. 1909

Type: Java, s.d. Solms 26c (holotype G, not seen).

Description and/or illustration: p. 294, Fig. 4 in Hasegawa (1980); p. 48 in Piippo (1993).

Geographical distribution: Java, Sumatra.

Dendroceros tubercularis S. Hatt. 1944

Type: Japan, Bonin Islands, Hahajima, April 1938, leg. Hattori, S. 3161 (holotype TNS, paratype NY 231509**!**)

Description and/or illustration: p. 6, Fig. 13 in Hattori (1944); p. 291, Fig. 3 in Hasegawa (1980).

Geographical distribution: Japan.

Dendroceros validus Steph. 1917

Type: Indonésia, Sumatra occid., in monte Singalang, in silvis primaevis as decliv. orient., 1760 m, VIII 1894, Schiffner #12 (holotype G 00060897**!**, *i*sotype NY 00231512**!**.

Description and/or illustration: p. 1016 in Stephani (1917); p. 300, Fig. 7 in Hasegawa (1980); p. 108 in Hasegawa (2002).

Geographical distribution: Indonesia and New Zealand.

Additional material examined: CHR 530624, CHR 530665, CANB 788972.

Dendroceros Nees ***subg. Nodulosus*** **Peñaloza-Bojacá G. & Maciel-Silva A.** subg. nov. (Figs 4, 6.3)

Type species: *Dendroceros crispatus* (Hook.) Nees

Diagnosis: Gametophyte galeate or with galeate wings, pale to dark green, but dull brown when dry; gametophyte wings reaching the midrib, with macro-perforations and pores; midrib solid; rectangular cells in the capsule with nodular wall thickenings; pseudoelaters papillose or smooth; multicellular spores, distal surface with granular and vermiculate micro-ornamentations, with macro-ornamentations of aculeate papilla being smaller (0.9-2.2 μm long), with short, fine, and frequently straight apices.

Dendroceros borbonicus and *D. japonicus*, species previously included in **subg. *Dendroceros*** by Schuster (1987), were grouped in a new phylogenetic clade with *D. africanus, D. crispatus,* and D. *granulatus* (previously in uncertain positions based on Söderström et al. 2016). This subgenus therefore comprises a total of five species.

Dendroceros africanus Steph. 1909

Type: Africa, Cameroon, Bibundi. Jungner J. R. #9 1891 (holotype BM, not seen; isotype G 00045214**!**)

Description and/or illustration: p. 1013 in Stephani, (1917); p. 421, Fig. 286 in Jones *et al.* (2004); p. 284 in Infante (2010).

Geographical distribution: Cameroon and Tanzania.

Dendroceros borbonicus Steph. 1893
Type: Africa, Reunion Island, 1875, De l'Isle, (holotype G-00045215**!**)
Description: p. 31 in Stephani (1893); p. 1011 in Stephani (1917).
Geographical distribution: Reunion Island and Mauritius.
Additional material examined: S 7510, BR Theo Arts 153-51.

Dendroceros crispatus (Hook.) Nees 1846
Bas.: *Monoclea crispata* Hook., Bot. Misc. 1: 117, 1829.
Type: Lesser Antilles, Saint Vincent, in ramis arborum. Guilding, L. (lectotype S B1983**!**)
Description and/or illustration: p. 117, Fig. 27 in Hooker (1830); p. 579 in Gottsche et al., (1846); p. 573 in Spruce (1885); p. 8 , Figs 27-36, 51 in Garcia et al., (2012).
Geographical distribution: Australia, Brazil, Cook Islands, Costa Rica, Cuba, Ecuador, Guadeloupe Island, Hawaii, Jamaica, Martinique, Norfolk Island, Nouvelle-Calédonie São Tomé, Saint Vincent Panamá, Peru, Puerto Rico, Tahiti.
Additional material examined: NY 1319033, NY 1319031, NY 1319048, NY 1319049, NY 1319050, NY 1319054.

Dendroceros granulatus Mitt. 1871
Type: Samoa, *s.l.*, component A, Powell, T. 144, (lectotype NY.00231502**!)**
Description: p. 419 in Mitten (1871); p. 1018 in Stephani (1917); p. 98 in Hasegawa (1982);
Geographical distribution: Samoa.
Additional material examined: NY 231538 A (Col: T. Powell 419), NY 00231541, NY 00231542.

Dendroceros japonicus Steph. 1909
Type: Japan, Shikoku - Mont Yokogura, May 1901. Yoshinaga T 26 (holotype *G* 00067715**!**).
Description and/or illustration: p. 288, Fig. 1 in Hasegawa (1980).
Geographical distribution: Japan.
Additional material examined: S B205906, NY 03103373.

Dendroceros Nees ***subg. Cichoraceus*** **Peñaloza-Bojacá G. & Maciel-Silva A.** subg. nov**.** (Figs 5, 6.4).
Type species: *Dendroceros cichoraceus* (Mont.) Gottsche.

Diagnosis: Gametophyte crispate, green to light-yellow when dried; wing cells oval to square-rectangular, without perforations (or rarely a few macro-perforations); wing margins crenulate; cells of the midrib square to rectangular; midrib solid; involucre cells with rough surfaces; capsule cells with rectangular wall thickenings; papillate pseudoelaters; multicellular spores, with more than 21 cells in total, distal surface with micro-ornamentations of granules and macro-ornamentations of pustulate papilla.

D. cichoraceus is newly identified as the sister to all other *Dendroceros* species (Duff et al., 2007; Villarreal et al., 2015; Villarreal & Renner, 2012; Fig. 1), supporting the erection of this new subgenus. The phylogenetic results and the morphological analyses dispel the confusion between this species and *D. crispus*. D. *cichoraceus* is differentiated from *D. crispus* by the lack of pores on the wings almost; having crenulate, margins and spores with macro-ornamentations of pustulate papilla (Howe, 1934; Proskauer, 1953; Stephani, 1917).

Dendroceros cichoraceus (Mont.) Gottsche 1858
Bas.: *Anthoceros cichoraceus* Mont. 1845
Type: Chile australioria ad terram muscosam legit cl. Gay (PC FUSION502 it is possible holotypes, not seen).
Description: p 355 in Montagne (1845); p.1013 in Stephani (1917); p. 69 in Proskauer (1953).
Geographical distribution: Chile.
Additional material examined: CONC Larrain 42330, CONC Larrain 31162.

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Supplemental material

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Figure S1. Tree with the phylogenetic relationships resulting from the analyzes based on the concatenated sequences from the genes *rbcL*, *nad5*, *trnK-matK*, *rps4*, represented by the best tree of posterior probabilities. Optimized morphological characters. Numbers above / below circles indicate character/state (see character list, Tab. S3); **Table S1.** Current taxonomic classification of *Dendroceros* Nees species and their positions within the subgenera (Söderström et al., 2016). Species indicated with "X" are treated in this present study. Phy: included in the phylogenetic analyses; Mor: included in the morphological analyses; Lit: obtained from the literature; **Table S2.** Voucher and GenBank accession numbers (in the sequence *rbcL*, *nad5*, *trnK-matK*, *rps4*) for accessions used in this study. Collector(s), collection number, herbarium and provenance are provided; **Table S3.** Morphological characters used for the optimization analysis in the Dendrocerotaceae family, with emphasis on the genus *Dendroceros* Nees. These characters were based on the material analyzed in the current study and supplemented with published taxonomic descriptions; **Table S4.** Additional information of the examined material of the genus *Dendroceros*.

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Figures

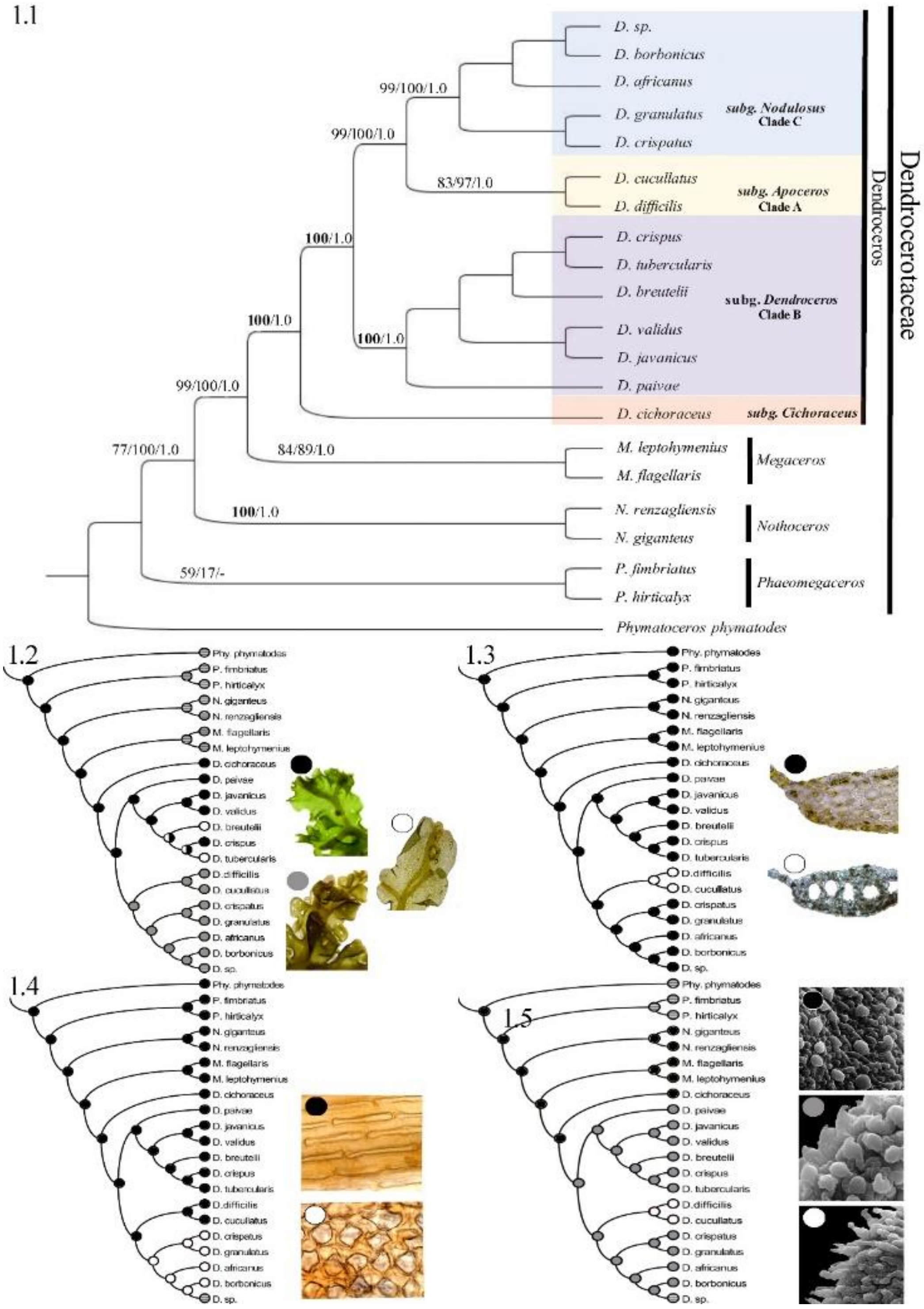

Figure 1. Phylogenetic tree and main morphological characters of *Dendroceros* Nees genus. 1.1. Maximum likelihood Phylogenetic tree resulting from analyses based on the concatenated sequences from the *rbcL nad5*; *trnK-matK*; *rps4* regions. Values in the branches represent MP, ML bootstrap support (as a percentage), and posterior probabilities; when all of the values are equal they are presented in bold. We highlight the new subgenera classification. Key morphological characters used in the ancestral reconstruction analyses (see Tab. S3 and Fig. S1). 1.2. Format of the gametophyte wings undulate to crispate (black), flat (white) and galeate (gray); 1.3. Cross-section of the midrib solid (black) and with schizogenous cavities (white); 1.4. Capsule cell walls thickenings rectangular (black) and nodular (white); 1.5. Spore ornamentation at distal surface postulate papillae (black), aculeate papillae (gray) and spines (white). Striped circles, unknown characteristics.

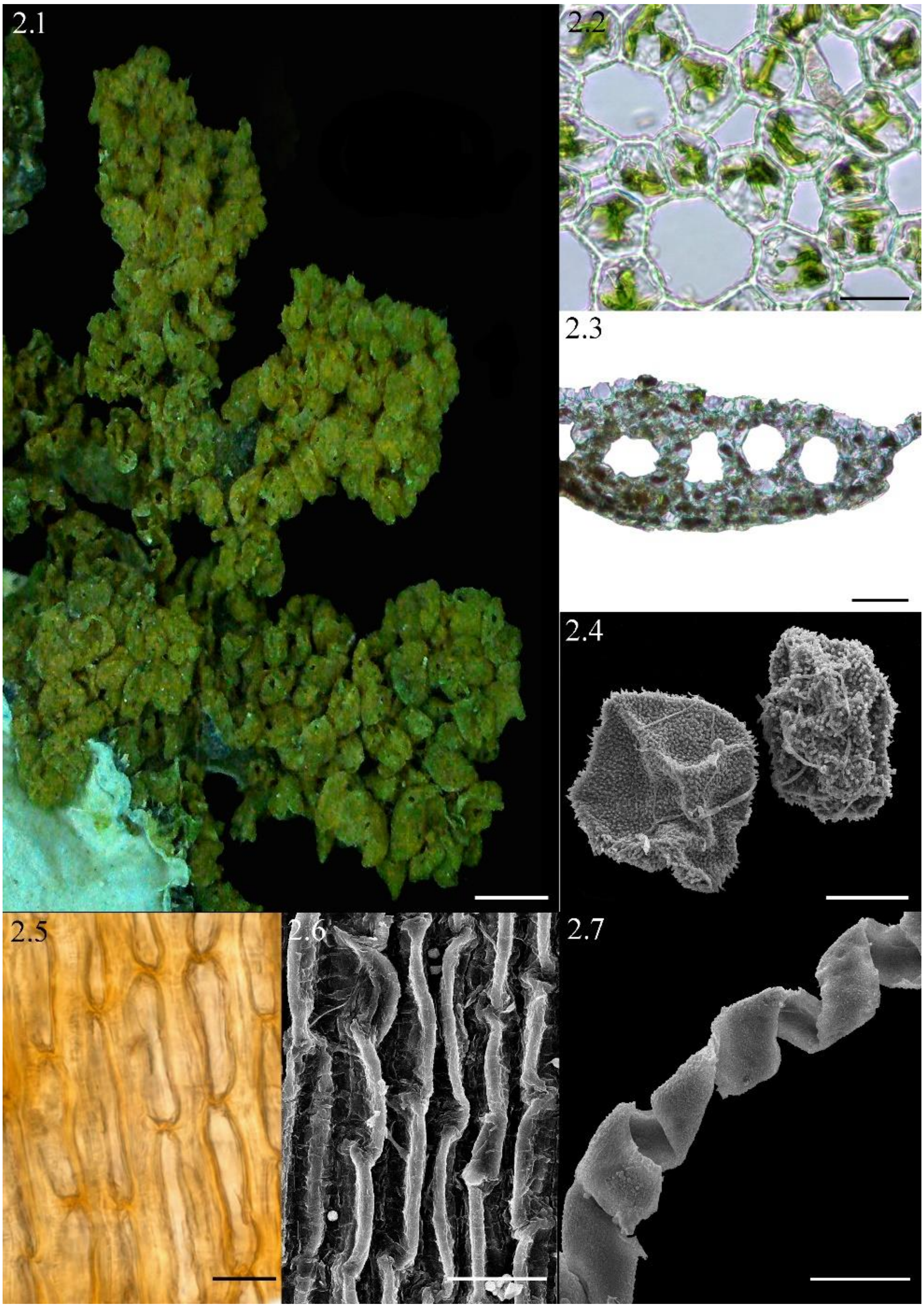

Figure 2. Morphological characteristics of subg. *Apoceros*: 2.1. *D. cucullatus,* gametophyte; 2.2. *D. cucullatus,* gametophyte wing cells; 2.3. *D. cavernosus,* cross-section of the midrib; 2.4. *D. muelleri,* spores proximal and distal (left to right) views; 2.5. *D. cavernosus*, rectangular cells of the capsule with cell wall thickenings (light microscope); 2.6. *D. cucullatus*, rectangular cells of the capsule, with cell wall thickenings (SEM); 2.7. *D. muelleri*, pseudoelaters. Scales: 2.1= 2.1 mm; 2.2, 2.3= 50 μm; 2.4, 2.6= 25 μm; 2.5= 20 μm; 2.7= 10 μm.

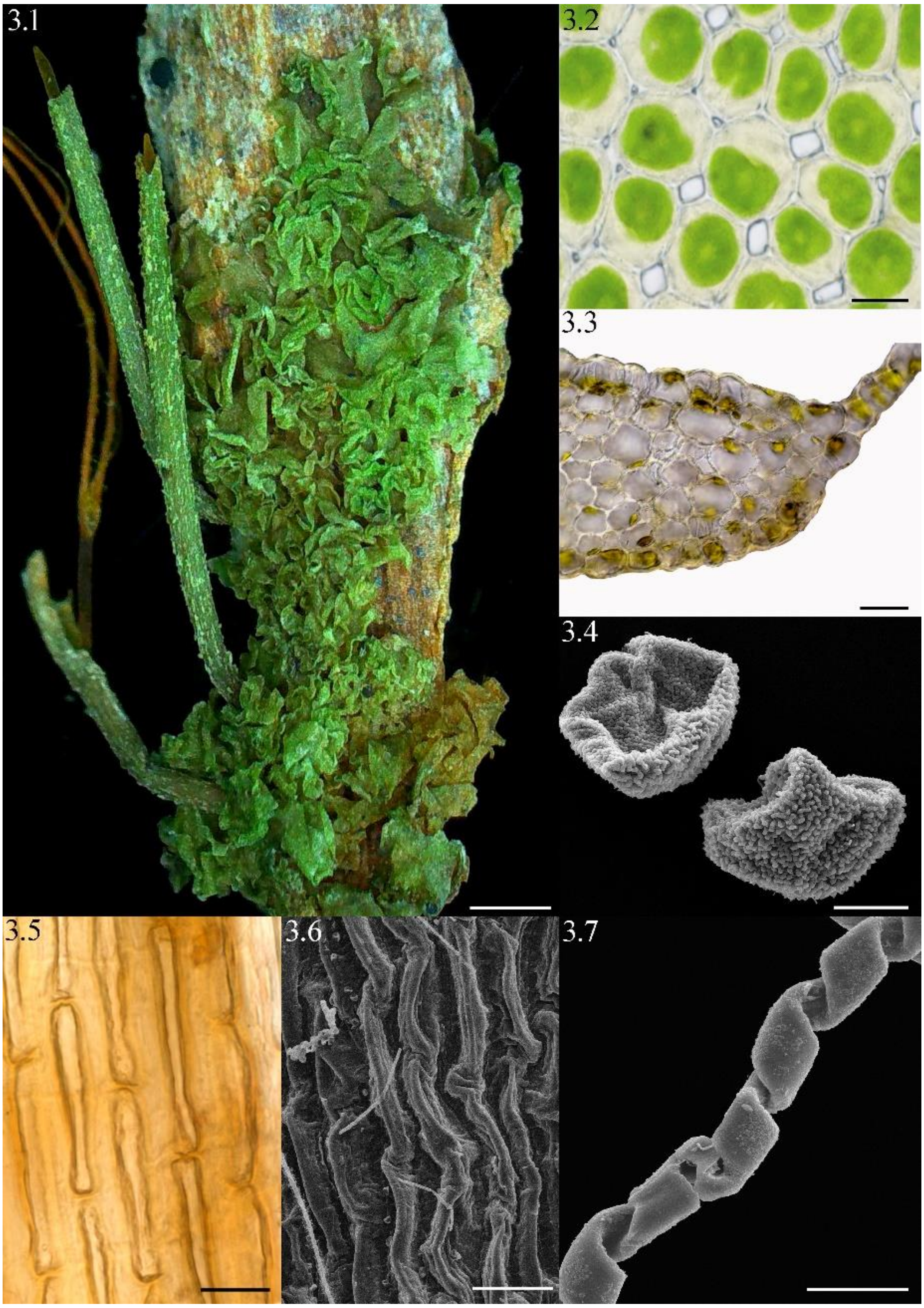

Figure 3. Morphological characteristics of subg. *Dendroceros*: 3.1. *D. crispus,* gametophyte and sporophyte; 3.2. *D. crispus,* gametophyte wing cells with pores; 3.3. *D. crispus,* cross- section of the midrib; 3.4. *D. crispus*, spores, proximal and distal views (left to right); 3.5. *D. crispus*, rectangular cells of the capsule, with cell wall thickenings (light microscope); 3.6. *D. alloni*, rectangular cells of the capsule, with cell wall thickenings (SEM); 3.7. *D. javanicus*, Pseudoelaters. Scales: 3.1= 1 mm; 3.2, 3.4, 3.5= 20 µm; 3.3= 30 µm; 3.6= 25 µm; 3.7= 10 µm.

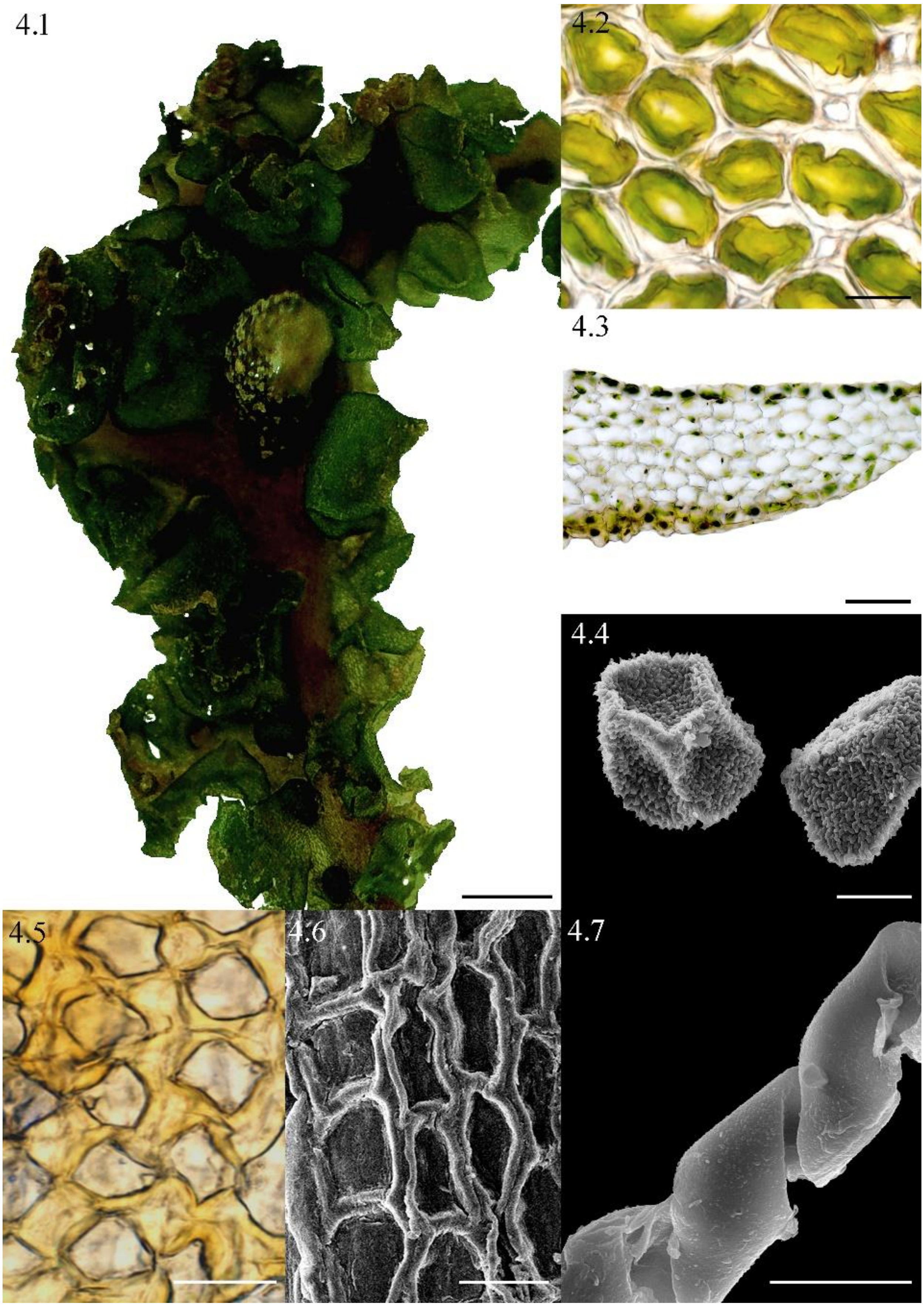

Figure 4. Morphological characteristics of subg. *Nodulosus*: 4.1. *D. crispatus*, gametophyte; 4.2. *D. crispatus*, gametophyte wing cells with irregular pores; 4.3. *D. crispatus*, cross- section of the midrib; 4.4. *D. borbonicus*, spores, distal and proximal view (left to right); 4.5. *D. borbonicus*, rectangular cells of the capsule with thickening of the nodular cell walls (light microscope); 4.6. *D. africanus*, rectangular cells of the capsule, showing thickening of the cell walls (SEM); 4.7. *D. borbonicus*, pseudoelaters. Scales: 4.1= 1 mm; 4.2, 4.5, 4.6= 20 µm; 4.3= 75 µm; 4.4= 30 µm; 4.7= 10 µm.

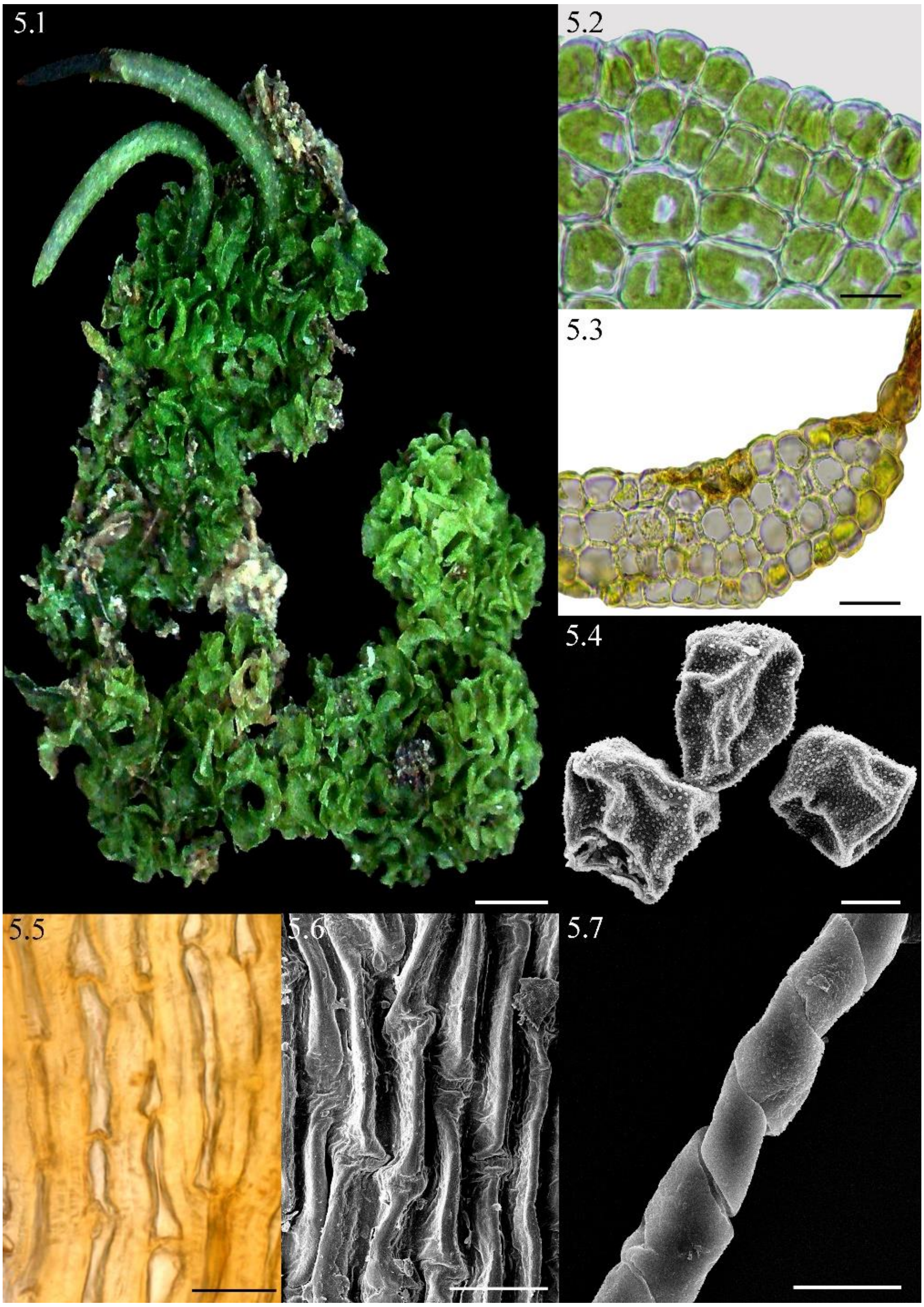

Figure 5. Morphological characteristics of subg. *Cichoraceus*: 5.1-5.7 *D. cichoraceus*: 5.1. Gametophyte and immature sporophyte; 5.2. Margins of the gametophyte wings without pores; 5.3. Cross- section of the midrib; 5.4. Spores; 5.5. Rectangular cells of the capsule, showing thickening of the cell walls (light microscope); 5.6. Rectangular cells of the capsule, showing thickening of the cell walls (SEM); 5.7. Pseudoelaters; Scales: 5.1= 1 mm; 5.2, 5.5= 20 µm; 5.3= 30 µm; 5.4, 5.6= 25 µm; 5.7= 10 µm.

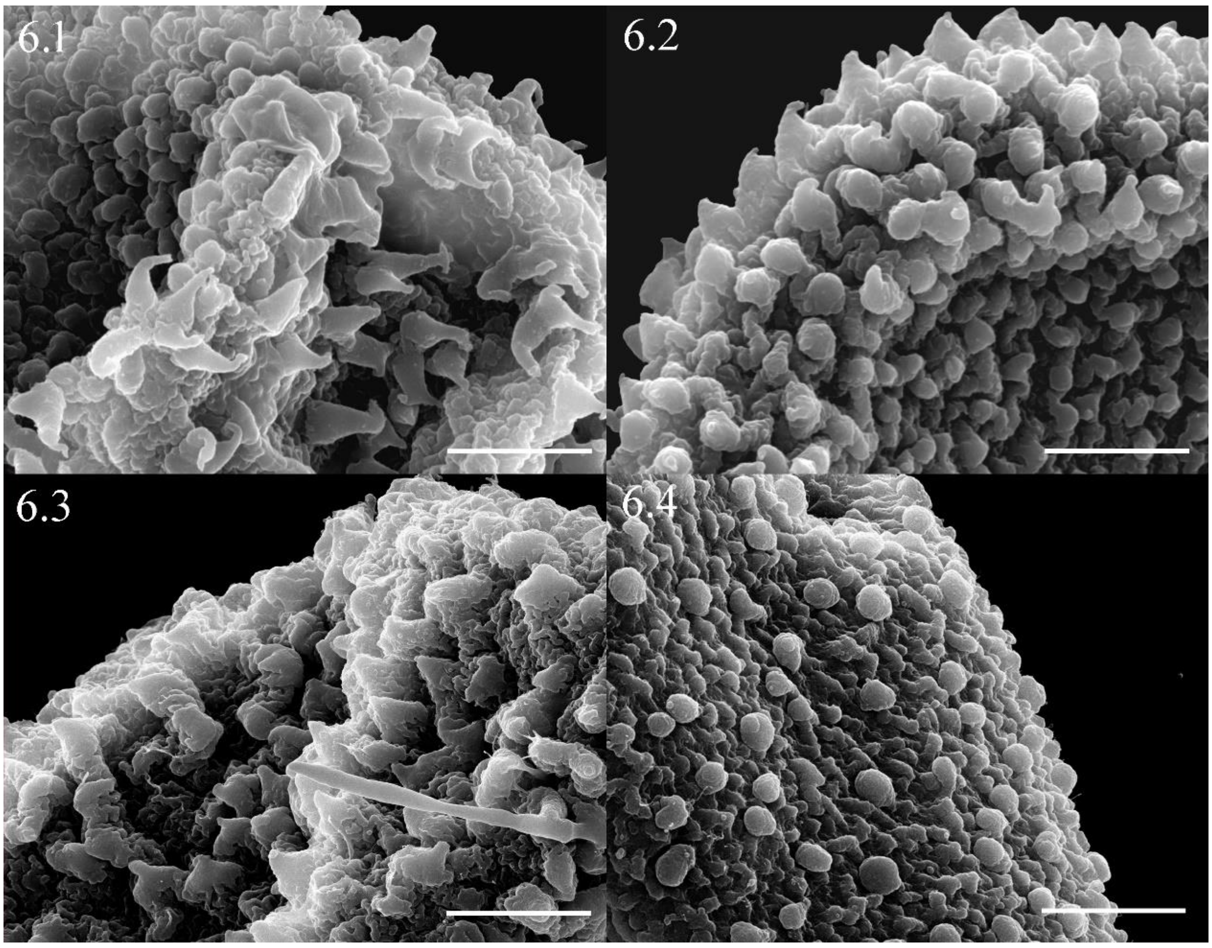

Figure 6. Ornamentation of spores in species of *Dendroceros.* 6.1. *D. cucullatus (*subg. *Apoceros*); 6.2. *D. crassinervis* (subg. *Dendroceros*); 6.3. *D. crispatus* (subg. *Nodulosus*); 6.4. *D. cichoraceus* (subg. *Cichoraceus*). Scales: 6.1-6.4= 5 µm.

Tables

Table 1. Comparison of the sizes of spores in the genus Dendroceros as determined by light and SEM microscopy. Values in µm. Measures were compared and complemented with Chantanaorrapint (2014), Chantanaorrapint et al. (2014), Garcia et al. (2012), Hasegawa (1980, 1982, 1986), Infante (2010), Piippo (1993), and Stephani (1917).

subg.	Species	Light		SEM	
		Length	Width	Length	Width
Apoceros	*Dendroceros difficilis*	40-65	50-85	30-45	42-68
Apoceros	*Dendroceros cavernosus*	45-55	45-60	19-35	35-55
Apoceros	*Dendroceros cucullatus*	---	---	30-60	45-100
Apoceros	*Dendroceros pedunculatus*	100-110	110-135	---	---
Apoceros	*Dendroceros muelleri*	54	---	34-55	59-94
Apoceros	*Dendroceros ogeramnangus*	45-60	63-75	---	---
Apoceros	*Dendroceros subdifficilis*	50-65	60-85	---	---
Apoceros	*Dendroceros seramensis*	45-70	50-85	---	---
Dendroceros	*Dendroceros javanicus*	35-50	35-63	30-40	35-55
Dendroceros	*Dendroceros validus*	25-50	37-60	30-40	40-70
Dendroceros	*Dendroceros breutelii*	40-50	50-70	30-60	40-55
Dendroceros	*Dendroceros tubercularis*	30-55	45-70	24-40	40-60
Dendroceros	*Dendroceros herasii*	52-67	57-75	42-47	40-65
Dendroceros	*Dendroceros foliicola*	45-55	45-65	---	---
Dendroceros	*Dendroceros paivae*	---	---	45-62	50-63
Dendroceros	*Dendroceros rigidus*	50-55	55-77	30-50	50-65
Dendroceros	*Dendroceros crispus*	37-62	45-75	30-45	45-78
Dendroceros	*Dendroceros adglutinatus*	37,6	62,5	---	---
Dendroceros	*Dendroceros subplanus*	45-55	45-65	---	35-60
Dendroceros	*Dendroceros acutilobus*	40-65	40-80	---	---
Dendroceros	*Dendroceros crassinervis*	---	---	27-45	48-75
Dendroceros	*Dendroceros allionii*	---	---	40-45	65-75
Nodulosus	*Dendroceros granulatus*	30-70	40-100	15-33	40-60
Nodulosus	*Dendroceros africanus*	40-62	45-87	30-40	45-60
Nodulosus	*Dendroceros japonicus*	50-100	60-110	45-60	55-75
Nodulosus	*Dendroceros crispatus*	50-80	75-100	35-75	75-90
Nodulosus	*Dendroceros borbonicus*	57-85	50-112	30-50	40-80
Cichoraceus	*Dendroceros cichoraceus*	55-72	62-85	35-70	50-85

CAPÍTULO II: efeitos da dessecação nos gametófitos e esporófitos de *Dendroceros* Nees

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Differential effects of desiccation on gametophytes and spores of hornworts having contrasting life histories

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Abstract

- **Background and Aims** Desiccation tolerance (DT) is the ability of an organism or structure to completely dry and subsequently survive from that air-dry state and may have played an important role during early land colonization by plants. Hornworts are excellent models along bryophyte lineages to examine desiccation effects as they have contrasting life histories (*i.e.*, niches: epiphytes and non-epiphytes; spores: green and non-green) that may be associated with DT. Thus, we tested the following hypotheses: 1) epiphytic species should have more efficient DT responses to drying and *postrehydration* than non-epiphytic species; and 2) "green" spores are more sensitive than non-green spores to extreme drying.
- **Methods** Hornwort species were collected in the Atlantic Forest of Rio de Janeiro, Brazil. We tested desiccation effects on five species (gametophytes and spores: *Dendroceros crispus, Dendroceros crispatus*, *Nothoceros vincentianus*, *Phaeoceros carolinianus*; and only spores of: *Anthoceros lamellatus*), using different *relative humidity*, *Duration dry,* and *postrehydration* treatments.
- **Key Results** All DT treatments affected chlorophyll fluorescence (*Fv/Fm*) of the gametophytes, with species-specific responses: *D. crispatus* and *D. crispus* performed better than *P. carolinianus* and *N. vincentianus*, including the fast recovery of *Fv/Fm* values after *postrehydration.* Spore survival and sporeling size differed among the species examined (in general, with the highest survival in *P. carolinianus* and lowest in *D. crispatus*), with no effect of *Duration dry* on survival. Our data evidenced that the gametophytes of epiphytic species (*D. crispatus* and *D. crispu*s) were more efficient at withstanding drying effects under different conditions. Conversely, the ability of non-green spores of *P. carolinianus*, *A. lamellatus,* and green spores of *D. crispus* to support DT rejected our second hypothesis.
- **Conclusions** DT responses and gametophyte and spore longevities highlight important trade-offs of spore dispersal and fast colonization (*e.g.*, *Dendroceros*), and the spore banks of *Phaeoceros* and *Anthoceros* species.

Key words: Anthocerotophyta, Chlorophyll fluorescence, Growth rates, Green spores, Epiphytic niche, Germination, Desiccation tolerance.

Introduction

Desiccation tolerance (DT) is the ability of an organism or structure to air-dry completely but subsequently survive from that state (Alpert 2000; Wood 2007; Koster *et al.* 2010; Stark and Brinda 2015). This trait occurs mainly in cyanobacteria, lichens, and green algae (Potts *et al.* 2005; Kranner *et al.* 2008; Holzinger and Karsten 2013), and may have played an important role during the colonization of land by plants (Oliver 2005; Proctor *et al.* 2007; Wood 2007; Stark 2017). Desiccation tolerance has become a key factor in plant life cycles, influencing the responses of both their reproductive (e.g., spores, seeds) and vegetative tissues (Proctor *et al.* 2007; Wood 2007; Lughadha *et al.* 2016; Greenwood *et al.* 2019; Oliver *et al.* 2020).

Desiccation tolerance has been reported in less than 0.15% of all vascular plant species in regards to their ability to tolerate water contents (WC) near 30-40% at c. 94-95% relative humidity (RH; Porembski and Barthlott 2000; Rascio and Rocca 2005; Proctor *et al.* 2007). DT in bryophytes (liverworts, mosses, and hornworts), on the other hand, has been characterized by gametophytes surviving 10% WC (or less) at c. 30% RH, and then recovering their metabolic activity even after long periods of time (Bewley 1979; Proctor *et al.* 2007; Wood 2007; Carvalho *et al.* 2011).

Multiple factors are used to assertively assess DT in bryophytes: 1) rate of drying (*RoD*); 2) water content at equilibration (*equilibrating relative humidity*, *RHeq*); 3) *Duration* dry; and 4) rate of rehydration (*RoR*; for details see Wood 2007; Green *et al.* 2011; Brinda *et al.* 2016; Stark 2017). The desert moss *Syntrichia ruralis*, for example, was held dried at ~ 30% RH for 20 years, and when subsequently rehydrated for 28 days it showed regeneration of its shoots and chlorophyll fluorescence of c. 0.35. Although the DT has been investigated in multiple taxa of bryophytes in recent decades (Proctor *et al.* 2007; Wood 2007, and references therein), including the responses of their gametophytes and spores, there is a long-standing question of whether hornworts are desiccation-tolerant species. Wood (2007) classified *Dendroceros granulatus* as a desiccation-tolerant plant based on a personal communication with Proctor, but there is a lack of experimental data focusing on the ability of hornworts to withstand desiccation.

Hornworts are a small group of land plants (c. 250 species; Söderström *et al.* 2016) with a dominant gametophyte phase, and they display various features unique among land plants. All hornwort species, for example, have symbiotic relationships with fungi and/or endophytic nitrogen-fixing bacteria (Villarreal and Renzaglia 2006a; Desirò *et al.* 2013; Nelson *et al.* 2020) and have a pyrenoid-based biophysical carbon-concentrating mechanism (Villarreal and Renner 2012; Li *et al.* 2017). Hornwort phylogeny has recently been resolved, with one of their key morphological traits being spore color and ornamentation (Villarreal and Renzaglia 2006b; Renzaglia *et al.* 2009; Peñaloza-Bojacá *et al.* 2019). Most recent phylogenies propose hornworts as a sister group to mosses and liverworts (Renzaglia *et al.* 2018), as part of the bryophyte clade (Wickett *et al.* 2014; Leebens-Mack *et al.* 2019).

Hornworts grow in many varied niches, such as on soils (*e.g.*, *Anthoceros* and *Phaeoceros*) and rocks, and on rotten logs near rivers and streams (most *Nothoceros* species), and can also be found as epiphytes on live trunks, twigs, and leaves (the genus *Dendroceros;* Gradstein *et al.* 2001; Duff *et al.* 2007; Villarreal and Renzaglia 2015). Epiphytism is commonly related to desiccation tolerance, as most epiphytic plants have adaptations to deal with drying (Hosokawa and Kubota 1957; Leon-Vargas *et al.* 2006; Schuette and Renzaglia 2010) and many bryophyte epiphytes tend to curl inwards when in dry state (Stark *et al.* 2013; McLetchie and Stark 2019).

Hornworts also display an array of spore colors and ornamentations that have been suggested as protection against desiccation. Spores with thick exines (external spore layer) and a yellow to dark color, for example, are more resistant than those with thin exines, colorless spore walls, and a prominent plastid (and thus appearing "green"; Lloyd and Klekowski 1970; Pence 2000; Renzaglia *et al.* 2009; Maciel-Silva *et al.* 2014). Differential rates of metabolism, storage tolerance, and longevity have been recorded for the different spore types (Renzaglia *et al.* 2009; Schuette and Renzaglia 2010; Sundue *et al.* 2011), with "green" spores assumed to germinate faster than darker spores and are typically found in spore banks (Bisang 1996). The spores of *Phaeoceros* and *Anthoceros* are yellow, brown, or black (non-green spores), while *Nothoceros* and *Dendroceros* have "green" spores (Duff et al., 2007; Villarreal, Cargill, Hagborg, Soderstrom, & Renzaglia, 2014), suggesting a contrasting DT response of spores as compared to gametophytes.

Spores have different germination-types, which are also associated with DT strategies in bryophytes. Unicellular spores (*e.g.*, in hornworts as *Nothoceros*, *Anthoceros* and *Phaeoceros*; Renzaglia 1978; Renzaglia and Vaughn 2000; Renzaglia *et al.* 2009) raise gametophytes directly in contact with the substrate, while multicellular spores develop their sporelings inside their walls and protected from desiccation (e.g., *Dendroceros;* Renzaglia *et al.* 2009; Schuette and Renzaglia 2010; Villarreal *et al.* 2012).

Hornworts have contrasting life histories that may be associated with DT – prompting several questions: Are hornworts, in general, desiccation tolerant? Does the species niche influence the DT response? Do spore traits have differential effects on DT responses? Our main goal was to test for DT in different hornwort species. To that end, we investigated the gametophytes and spores of species of the genera *Dendroceros*, *Nothoceros*, *Phaeoceros* and *Anthoceros* (only spores in the latter), and hypothesized that: 1) epiphytic species would have a more efficient DT response to drying and *postrehydration* treatments than non-epiphytic species, and expected the gametophytes of *Dendroceros* species, compared to gametophytes of *Nothoceros* and *Phaeoceros*, to demonstrate higher and/or constant chlorophyll fluorescence during the different *RHeq*, *Duration dry,* and *postrehydration* treatments. Conversely, we also hypothesized that: 2) "green" spores are more sensitive than non-green spores to extreme drying and expected the spores of *Anthoceros* and *Phaeoceros* to have higher survival and growth rates than those of *Dendroceros* and *Nothoceros* under the different desiccation treatments.

Material and methods

Species description

We examined five species of hornworts: 1) *Dendroceros crispatus* (Hook.) Nees: gametophyte with galeate wings; midrib solid; one chloroplast per cell, with pyrenoid; rectangular cells in the capsule with nodular wall thickenings (Peñaloza-Bojacá *et al.* 2019; Fig 1B, F, J); 2) *Dendroceros crispus* (Sw.) Nees: gametophyte flat undulate or crispate; midrib solid; one chloroplast per cell, with pyrenoid; rectangular cells in the capsule with wall thickenings. Both of those epiphytic species produce multicellular green spores, and are found in forests at 800-2000 m a.s.l. (Duff *et al.* 2007; Peñaloza-Bojacá *et al.* 2019); 3) *Nothoceros vincentianus* (Lehm.) J.C. Villarreal:

gametophyte pinnately branched; chloroplasts 1–2 per cell, pyrenoid absent or present; rectangular cells in the capsule, unicellular spores green at maturity. This species is terricolous or saxicolous in Neotropical forests above 400 m a.s.l, along streams (Renzaglia *et al.* 2009; Villarreal and Renner 2014; Fig 1C, G, K); 4) *Phaeoceros carolinianus* (Michx.) Prosk.: gametophyte in rosette with smooth margin; one chloroplast per cell, with pyrenoid; rectangular cells in the capsule with stomata, unicellular spores yellow at maturity. It has worldwide distribution, growing on soils and exposed areas above 300 m a.s.l (Proskauer 1957; Duff *et al.* 2007; Penjor *et al.* 2016; Fig 1D, H, L); 5) *Anthoceros lamellatus* Steph. A.: gametophyte in rosette, margins crenulate, dorsal lamellae abundant, one chloroplast per cell, pyrenoid present, rectangular cells in the capsule with stomata, unicellular spores dark brown to blackish at maturity. It has a neotropical distribution, growing on soils and exposed areas above 1000 m a.s.l. (Renzaglia *et al.* 2009; Ibarra-Morales *et al.* 2015; Fig 1E, I, M).

Field sampling

We carried out field collections in January and February/2020 in the Atlantic Forest, in Rio de Janeiro State, Brazil. We collected samples (c. 8 cm^2) of the hornwort species (except *A. lamellatus*) in the forest located in the Serra da Bocaina National Park (22º 46' 04'' S and 44º 36' 34'' W), 1237-1524 m a.s.l, 17-23 C°, mean rainfall from 1700-3000 mm/year (IBAMA 2003). *Anthoceros lamellatus* samples were collected along the roadside on the way to the Três Picos State Park (22º 20' 04'' S, 42º 42' 04'' W), 1250-1368 m a.s.l, 17-21 C°, mean rainfall from 1000-2000 mm/year (INEA, 2013; Supplementary Date Table S1). The samples were transported in hermetically sealed plastic pots to the laboratory, thus maintaining a high internal relative humidity (> 98% RH) of the plants and preventing their acclimation (acclimation to desiccation occurs during a dry-down or partial drying event; Stark, 2017).

Deacclimation

To assess desiccation tolerance and avoid variations due to unknown field hardening effects, we deacclimated plants for 15 days (effective time in *sphagnum* species; (Hájek and Vicherová 2014; Stark 2017) using a 12h photoperiod, at 21° C, and RH > 98% in a culture room. Deacclimation is a process in which plants collected in field are subjected to uninterrupted hydration under non-stressful conditions, overcoming any physiological hardening to desiccation that may have been obtained in the field (Stark 2017). This process ensured suprasaturation conditions for gametophytes and spores before the experiments began. Twenty-four hours before the end of the deacclimation period, we selected adult gametophytes from each species (here defined as plants with: a) male and female gametangia; b) cyanobacteria colonies; c) lengths greater than 2 cm; and d) mature sporophytes (with signs of apical dehiscence). Those plants were cleaned while viewing under a stereomicroscope and kept in deionized water until completing the deacclimation period.

To control the *rate of drying*, we followed the methodology proposed by Wood (2007), Stark (2017) and Greenwood et al. (2019), with modifications, using cell culture well-plates with two sheets of filter paper (18 mm^2, 3 µm porosity) and 20 µL of sterilized water and a digital thermohygrometer (Htc-2A) to monitor the temperature and internal humidity of the desiccation chambers. We standardize the *rate of drying* in 24h, that is, from full turgor (blotted) to plant curling (the volume of water and the *rate of drying* period had been previously tested in the laboratory, unpublished data). Deacclimated gametophytes and sporophytes were gently passed through filter paper to remove excess surface water, before being placed into the well-plates. We used a total of 27 gametophytes per species, with one individual plant per well. We used a total of 81 sporophytes per species, with three mature sporophytes per well (Supplementary Date Table S1). Plates were kept covered for an additional 24h under the different relative humidity *RHeq* treatments (see below). Once they attained the equilibrium time period, the covers were removed.

Experimental design and desiccation

Deacclimated gametophytes and sporophytes were used in all our assays. During experimental for DT, the plants were maintained under constant temperature and light conditions (21°C, 74 µmol. of photons m^{-2}. s^{-1}) in a culture room with a 12h photoperiod. To evaluate the effects of desiccation times and intensities for each species (described above), we prepared desiccation chambers (airtight plastic boxes 38 x 56 x 37 cm), with saturated salt solution in petri dishes to provide constant internal *RH*s. Equilibrating relative humidity treatments (*RHeq*s) were achieved using saturated saline solutions, as: *RHeq* 56% ($Ca [NO_3]_2$), *RHeq* 74% (NaCl), and *RHeq* ~99% with deionized water. Likewise, a period of 12h was applied so that the plants reached equilibrium with ambient air (Stark 2017) and before the *Duration dry*.

Our experimental design for DT in gametophytes was as follow: four species x three *RHeqs* x three *Duration dry* x three *postrehydration time* and for spores: five species x three *RHeqs* x three *Duration dry* x one *postrehydration time*. We used three different plants and spore sets in each treatment. Treatments of *Duration dry* were: 1 day (1D); 3 days (3D); 9 days (9D); *Postrehydration* were applied for three different times: 24h for gametophytes and spores; 72h and 216h for gametophytes. One period of 8 h of pre-hydration (desiccated plants were submitted to a relative humidity of ~99% *RHeq*) was applied in all *postrehydration* treatments before adding liquid water (Beckett, 1999; Stark, 2017). The plants and spores were subsequently rehydrated by adding 2 ml of deionized water directly onto the filter paper, keeping them immersed until completing the different *postrehydration* times (Stark 2017; Greenwood *et al.* 2019).

To assess gametophyte responses to DT we measured the maximum photochemical quantum yield of PSII (F_v/F_m), using the equation: $F_v/F_m = (F_m - F_o)/F_m$, where F_m is the maximum fluorescence, and F_o is the fluorescence in the absence of actinic light (Butler and Kitajima 1975; Genty *et al.* 1989). F_v/F_m measurements were taken in the morning, before the start of the light period, so that the plants had been under 12 hours of dark adaptation. The latter is related to PSII reaction centers, which are open after dark adaptation and decrease under dehydration stress, thus being used as a fitNees measure in bryophytes (Carvalho *et al.* 2011; Marks *et al.* 2016; Cruz de Carvalho *et al.* 2019). Chlorophyll fluorescence emission was assessed using a pulse-amplitude modulation fluorometer (model MINI-PAM, WALZ, Effeltrich, Germany) with a light saturation pulse of ~5,000 μmol photons m^{-2} s^{-1}.

To assess DT in spores, we collected spores from the capsule apex (sporogenesis is asynchronous in these hornworts and the capsule apex contains mature spores). Spores from three capsules were mixed and cleaned with deionized and sterilized water, and plated for 30 days in petri dishes on solid culture medium (Knop II with 0.5% agar, pH 5.6; Nakazato et al., 1999). We measured spore survival (100) and sporeling size (50) per replicate. Survival was measured as viable spores or germinated when they show signs of germination after 30 days (i.e., development of a rhizoid or germ tube; or several green cells inside the spore wall) or, to the contrary, non-viable spores after 30 days (no signs of development, being hyaline or dark brown, with no green chloroplasts, no rhizoid or germ tube). Sporeling size was analysed using BEL Capture Application software (400x magnification), assessing their lengths (mm). Additionally, before beginning our experiments, we assessed *Fv/Fm* reference values for the gametophytes, and survival and growth for spores of the study species at *RHeq* 56%, 74%, and *RHeq* ~99% (Supplementary Data Fig. S1A).

Statistical analyses

We generated generalized linear models (GLMs) to test our hypotheses and verify our predictions. The effects of "*species*", "*RHeq*", "*Duration dry*" and "*postrehydration*" treatments on the "*Fv/Fm*" values were tested with GLM (Gaussian distribution and identity link function; model details in Supplementary Date Table S2). The effects of "*species*", "*RHeq*", and "*Duration dry*" on "*spore survival*" and "*sporeling size*" were analyzed with a model equivalent to the one above. Because the response variable "*sporeling size*" had spores as a random factor nested in each replicate, we attempted to include that factor in our previous model. Because of singularity effects, however, we preferred to exclude it and to use the simplest and most powerful (Akaike's information criteria, AIC) model for our analyses. Additionally, analyses of deviance for GLMs and *Post hoc* comparisons (Tukey test) were applied. The latter test was used to evaluate all pairwise differences at $\alpha = 0.05$. All analyzes and graphs were performed in R (R Core Team 2020) using RStudio V.3.6.2 software (RStudio Team 2020), with the ggplot2 (Wickham and Chang 2009) and MASS packages (Kemp 2003).

Results

Desiccation effects on hornwort gametophytes

All DT treatments (*RHeq, Duration dry, postrehydration*) had significant effects on the *Fv/Fm* of gametophytes of the study species; significant interactions of these treatments with "*species*" indicated that the *Fv/Fm* in hornwort species had very distinct behaviors under different desiccation treatments (Tab. 1, Supplementary Data Table S3; Fig. 2). In general, the species with the highest *Fv/Fm* values were *D. crispatus* (mean ± standard error: 0.515 ± 0.012) and *D. crispus* (0.443 ± 0.018), as compared to *P. carolinianus* (0.188 ± 0.025) and *N. vincentianus* (0.144 ± 0.020; Supplementary Data Fig. S2). *RHeq* treatments of 56% and 74% had similar and negative effects on the gametophytes, compared to 99% *RHeq*, generating damage mostly in the gametophytes of *P. carolinianus* and *N. vincentianus* (Fig. 3). We observed signs of regeneration in the gametophytes of *P. carolinianus* at 99% *RHeq* after three days of desiccation (Fig. 3). Although several plants recovered their photosynthetic capacities, the chlorophyll fluorescence of the gametophytes in our experiment did not reach the reference values of recently deacclimated plants (Supplementary Data Fig. S1A).

Duration dry decreased *Fv/Fm* values of hornwort gametophytes from 1D (0.360 ± 0.022) and 3D (0.371 ± 0.023) to 9D (0.023 ± 0.020). Significant interactions of "*Duration dry*" with "*species*" and "*RHeq*" were mostly the result of the gametophytes of *D. crispus* and *D crispatus* being less affected by drying than those of *P. carolinianus* and *N. vincentianus*. These latter species had the lowest *Fv/Fm* values among the different treatments (Fig. 2).

We observed a tendency of the gametophytes to recover their *Fv/Fm* values after all tested *postrehydration* times, with better recovery rates recorded after the *postrehydration* times: 24h (0.249 ± 0.021), 72h (0.318 ± 0.024), and 216h (0.40 ± 0.020). *Fv/Fm* values in *D. crispatus* and *D crispus* increased similarly after 24h and 72h *postrehydration* in all *RHeqs,* and after 1D and 3D *Duration dry*. At 216h, the *Fv/Fm* of both species increased for all *RHeqs* only under 1D drying, however, indicating an effect of *Duration dry* on species responses after *postrehydration*. Chlorophyll fluorescence in *N. vincentianus* and *P. carolinianus* had an inverse pattern, with low values at 24h and 72h that increased after 216h for all *Duration dry* and *RHeqs* treatments (Fig. 2).

Desiccation effects on hornwort spores

RHeq and *Duration dry* treatments had no significant effects on spore survival in hornworts, although the species differed significantly in terms of their survival rates (Tab. 1 and Fig. 4A). In general, spore survival differed among hornwort species, as: *Phaeoceros carolinianus* (84.5% ± 1.56) followed by *D. crispus* (82.0% ± 1.02), *A. lamellatus* (69.4% ± 3.67), *N. vincentianus* (61.3 % ± 4.55), and *D. crispatus* (53.0% ± 3.68). We observed significant interactions of "*species*" with "*RHeq"* (Tab. 1, Supplementary Data Table S4) in *A. lamellatus* and *P. carolinianus,* with better spore survival in lower *RHeqs* treatments. In contrast, *D. crispu*s, *D. crispatus,* and *N. vincentianus* had their best spore survival at higher *RHeqs* (Fig. 4A). *P. carolinianus* and *A. lamellatus* spores had similar survival rates after desiccation treatments as compared to recently collected plants (Supplementary Data Fig. S1B).

Sporeling sizes varied in response to *RHeq* treatments, with species having decreasing sizes from 56% *RHeq* (0.40 mm ± 0.007), followed by 74% (0.296 mm ± 0.005), and 99% (0.298 mm ± 0.005; Fig. 4B). *Phaeoceros carolinianus, A. lamellatus,* and *D. crispus* had smaller sporelings in 74% and 99% *RHeqs*. *Dendroceros crispatus* and *N. vincentianus* produced small sporelings in all treatments. Significant interactions were observed between "*Duration dry*" and "*species*" (Tab. 1, Supplementary Data Table S5), with the longer the *Duration dry* the larger the sporelings of *P. carolinianus, A. lamellatus* and *D. crispus*; *D. crispatus* and *N. vincentianus* had the smallest sporelings at 3D as compared to 1D and 9D (Fig. 4B). The spores of *P. carolinianus*, *A. lamellatus* and *D. crispus* reached reference values of sporeling size after the 9D desiccation treatment (Supplementary Data Fig. S1C).

Discussion

We corroborated our first hypothesis: the gametophytes of epiphytic species (*D. crispatus* and *D. crispus*) were more tolerant of desiccation than the non-epiphytic species, and *Phaeoceros carolinianus* and *N. vincentianus* gametophytes suffered greater physiological and morphological damage. Our second hypothesis was only partially verified, as *P. carolinianus* and *A. lamellatus* (non-green spores) and *D. crispus* (green spores) showed better spore survival and sporeling size in the desiccation treatments, distinct from *D. crispatus* and *N. vincentianus* (green spores) in which the same parameters were the lowest.

Desiccation tolerance of hornwort gametophytes

The gametophytes of the epiphytic species studied here experienced less morphological injury and damage to photosystem II, as was suggested earlier for *Dendroceros* (Wood 2007; Schuette and Renzaglia 2010). Epiphytism, unique plastid traits, and star-shaped chloroplast with a complex pyrenoid are assumed to provide protection for the photosynthetic apparatus of *Dendroceros* during drying periods (Renzaglia *et al.* 2007; Wood 2007; Schuette and Renzaglia 2010). A relationship between DT and epiphytism has been widely observed in other tropical plants, including in the mosses *Prionodon densus* and *Dicranoloma fragiliforme*, the liverwort *Frullania peruviana*, and the fern *Microgramma reptans*, whose gametophytes do not show morphological or physiological damage after drying periods with *RHeqs* lower than 50% (Hosokawa and Kubota 1957; Leon-Vargas *et al.* 2006; Wood 2007; Watkins *et al.* 2007).

Additionally, the failure of *N. vincentianus* and *P. carolinianus* gametophytes to restore normal morphological and physiological parameters after prolonged drying regimes at *RHeqs* <99% show low DT capacities. The niches of both species may be linked to their DT responses, as *N. vincentianus* is commonly found in very humid and shady areas (e.g., along rivers, streams, or inside forests) and *P. carolinianus* usually grows on moist soil in exposed sites (e.g., roadsides, ravines). Those niches are very contrasting, with the latter usually being colonized by bryophytes resistant to occasional dry periods (Abel 1956; Proctor *et al.* 2007). We assume, therefore, that the lower DTs of *N. vincentianus* and *P. carolinianus* gametophytes for only short periods of time are related to protective mechanisms, such as their sugar contents or constitutive cellular protection by abscisic acid (ABA; Proctor *et al.* 2007; Wood 2007; Lakatos 2011; Stark 2017).

Postrehydration effects

Epiphytic species responded more effectively to short postrehydration periods of 24h and 72h (with a pre-hydration of 8h for all of the treatments used here) than did non-epiphytic species, which can be explained by the rate at which bryophytes rehydrate – as the highest performance plants commonly have high surface areas and low weights (Larson 1981; Stark 2017). *Phaeoceros* and *Nothoceros* plants are thick, with several layers of cells in their thalli, while *Dendroceros* species are thinner and have uniseriate wings. We observed different responses among the gametophytes of *Dendroceros* species at a *postrehydration* of 216h; *Dendroceros crispus* responded better than *D. crispatus* under longer rehydration. While *D. crispatus* has a galeate gametophyte with many macro-perforations and pores in its wings, *D. crispus* has crispate gametophytes (higher surface areas) with few and irregular pores (Peñaloza-Bojacá *et al.* 2019).

The responses of *N. vincentianus* and *P. carolinianus* gametophytes, with better recovery after 216h of *postrehydration*, suggest the need for longer rehydration times. The desiccated cell membrane in those species may require greater exposure to a saturated atmosphere and free water to recover and mitigate the injuries caused by drying than *Dendroceros* species (Hoekstra *et al.* 2001; Stark 2017). Another important and unprecedented factor in hornworts is the strategy used by *P. carolinianus* at *RHeq* 99% with a *postrehydration* of 216h for all drying treatments (mainly 1D and 3D) for gametophyte regeneration (Fig. 3). This strategy has been reported for other groups of bryophytes (e.g., *Octoblepharum*, *Orthodontium,* and *Dicranoweisia*), with asexual propagule or vegetative regeneration being associated with habitat drying and directly influencing dispersal and establishment processes in these species (Duckett and Ligrone 1992; Duckett *et al.* 2001; Zhang *et al.* 2003; Proctor *et al.* 2007).

Desiccation tolerance of hornwort spores

Phaeoceros carolinianus and *A. lamellatus* had higher spore survival and larger sporeling sizes under the different desiccation treatments, as was expected. Both species have thicker spore walls than do green spores, which apparently provides protection against desiccation (Mogensen 1981; Renzaglia *et al.* 2009). Additionally, the inner spore walls in *Phaeoceros* and *Anthoceros* contain callose (Renzaglia *et al.* 2020), an important polymer for the construction and maintenance of wall integrity (Ellinger and Voigt 2014). Spores with callose may deal well with desiccation as that polymer provides a protective matrix (Johri 1984; Yim and Bradford 1998; Renzaglia *et al.* 2020); although that trait alone is not enough to explain all of the differences observed in our study (e.g., *Nothoceros* vs. *Dendroceros*).

Phaeoceros and *Anthoceros* spores also contain oils, lipids, and starch as storage compounds, which can help provide greater longevity and drought resistance (Renzaglia *et al.* 2009; Schuette and Renzaglia 2010) – but different from green spores, they require longer times to mobilize their storage compounds and to develop protonema (e.g., early gametophytes; (Mogensen 1983; Renzaglia *et al.* 2009; Sundue *et al.* 2011; Maciel-Silva *et al.* 2014). *Phaeoceros* and *Anthoceros*, as well as other hornworts with non-green spores (Oliveira *et al.* 2017), produce gametophytes with short life spans that are able to resist dry seasons as spores in soil banks (Bisang 1996).

The DT responses of green and multicellular spores (e.g., endosporic germination) of *Dendroceros* species are likely associated with endospory. This trait is present in other bryophytes and assumed to be an adaptation to environments that experience periodic desiccation (Schuette and Renzaglia 2010; Maciel-Silva and Pôrto 2014; López-Pozo *et al.* 2018). The differential responses of the spores of both *Dendroceros* species could explain why *D. crispus* is more abundant than *D. crispatus* (Peñaloza-Bojacá *et al.* 2020). The spores of *N. vincentianus* demonstrated the lowest performance due to their exosporic germination, which exposes a delicate protonema to drought conditions. The green spores of *Dendroceros crispus*, *D. crispatus* and *N. vincentianus* also lack oil and lipid storage (Ligrone and Renzaglia 1990; Renzaglia *et al.* 2009, 2020),

Ecological strategies of DT in hornworts

We observed complex trade-offs of DT responses among the different life phases of hornworts, with contrasting life strategies linked to niche requirements, where: 1) gametophytes are sensitive and spores are strongly tolerant of drying (*P. carolinianus* and *A. lamellatus*); 2) gametophytes and spores tolerant of dry periods (*D. crispus*); 3) tolerant gametophytes and sensitive spores (*D. crispatus*); and 4) both gametophytes and spores sensitive to drying (*N. vincentianus*). The above responses are similar to the *DT continuum hypothesis* proposed for bryophytes (Stark & Brinda, 2015 and references), which proposes that plant life history phases (e.g., protonemata, juvenile shoots, adult shoots, spores, and others) can have different DT strategies, even within the same species and along an inducible or constitutive tolerance gradient (Testo and Watkins 2012; Stark and Brinda 2015).

This is the first study to highlight these trade-offs in hornworts, in line with similar differential DT strategies reported in both vascular and non-vascular plants. Sporophytes of the desert moss *Tortula inermis*, for example, were found to be more sensitive to rapid drying than their gametophytes (Stark *et al.* 2007); the protonemata *vs.* juvenile shoots in *Bryum argenteum* (Greenwood *et al.* 2019), and the asexual propagules *vs*. shoots of *Syntrichia pagorum* also have different DT responses. Equally, the gametophytes (cold or dry) of some fern species (e.g., *Asplenium auritum* and *Polystichum retroso-paleaceum*) are more tolerant than their sporophytes (Sato and Sakai 1980; Watkins *et al.* 2007; Testo and Watkins 2012). The differential life strategies of hornworts may be especially important evolutionary triggers for spore dispersal and the maintenance of gametophytes in the field.

Finally, we highlight the importance of hornworts in cloud forests, especially *Dendroceros* species as potential indicators of climate change. This genus presents unique ecological characteristics, such as the altitudinal distribution range (700-1000 m in Brazilian forests and up to 2300 in Colombian forests), the epiphytic niche, the height in the phorophyte (20-200 cm) and the degree of DT (gametophytes *vs* multicellular spores). The aforementioned characteristics make this plant group particularly sensitive to climate change, as reported in vascular epiphytic species (Laube and Zotz 2006; Zotz *et al.* 2010; Mendieta-Leiva *et al.* 2020). In addition, these unique characteristics of *Dendroceros* can be related to the seasonality and conservation status of cloud forests, and indicate possible alterations in their ecosystems (Benzing 1998). Climate change may directly affect the climatic conditions (radiation, temperature, relative humidity and wind) of the forest, together with the diversity, distribution, establishment and spread of *Dendroceros* species (Jump and Penuelas 2005; Pardow and Lakatos 2013; Walsworth *et al.* 2019).

Conclusion

Our study highlights a *DT continuum* among hornwort species, with different strategies associated with different life history phases (spores *vs*. gametophytes), niches, genera, and species. We corroborate the observed DT of *Dendroceros* gametophytes, which is linked to their epiphytic niche in montane forests. The endospory of *Dendroceros* confers a moderate DT response, especially in *D. crispus*, although the high performances of the non-green spores of *Phaeoceros* and *Anthoceros* indicate their excellent potential to form soil banks in open habitats. The high desiccation sensitivities of both the spores and gametophytes of *N. vicentianus* are likely associated with the very humid niches in which that species lives. Differential DT strategies may increase the chances of spore dispersal and fast colonization (e.g., *Dendroceros*), or even spore bank formation by *Phaeoceros* and *Anthoceros* species. Finally, rehydrating cycles suggest that long-term studies will evidence a more complex *DT continuum* across many species.

Supplementary information

Supplementary data are available online at https://academic.oup.com/aob and consist of the following. Fig S1: Reference values for DT experiments with hornworts in this study, before drying and with deacclimation for 15 days. **A**: Means of the *Fv/Fm* chlorophyll fluorescence parameters of hornwort gametophytes. **B-C**: hornworts cultured for 30 days. **B**: Percentage spore survival. **C**: Sporeling reference sizes. Error bars represent ± 1 SE. Fig. S2: Means of the Fv/Fm chlorophyll fluorescence parameters of hornwort gametophytes. **A**: balanced relative humidity treatments; **B**: *Duration* dry; **C**: *postrehydration* periods. Error bars represent ±1 SE. Table S1: Descriptions of the materials used in the desiccation tolerance tests of five hornwort species. Table S2: Summary of the generalized linear models of the desiccation tolerances of the gametophytes and spores of the hornwort species tested. Table S3: *Post hoc* Tukey honest significant difference (HSD) test of chlorophyll fluorescence parameters of hornwort gametophytes. Table S4: *Post hoc* Tukey honest significant difference (HSD) test of spore survival percentages in hornworts. Table S5: *Post hoc* Tukey honest significant difference (HSD) test of sporeling sizes in hornworts.

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Figures

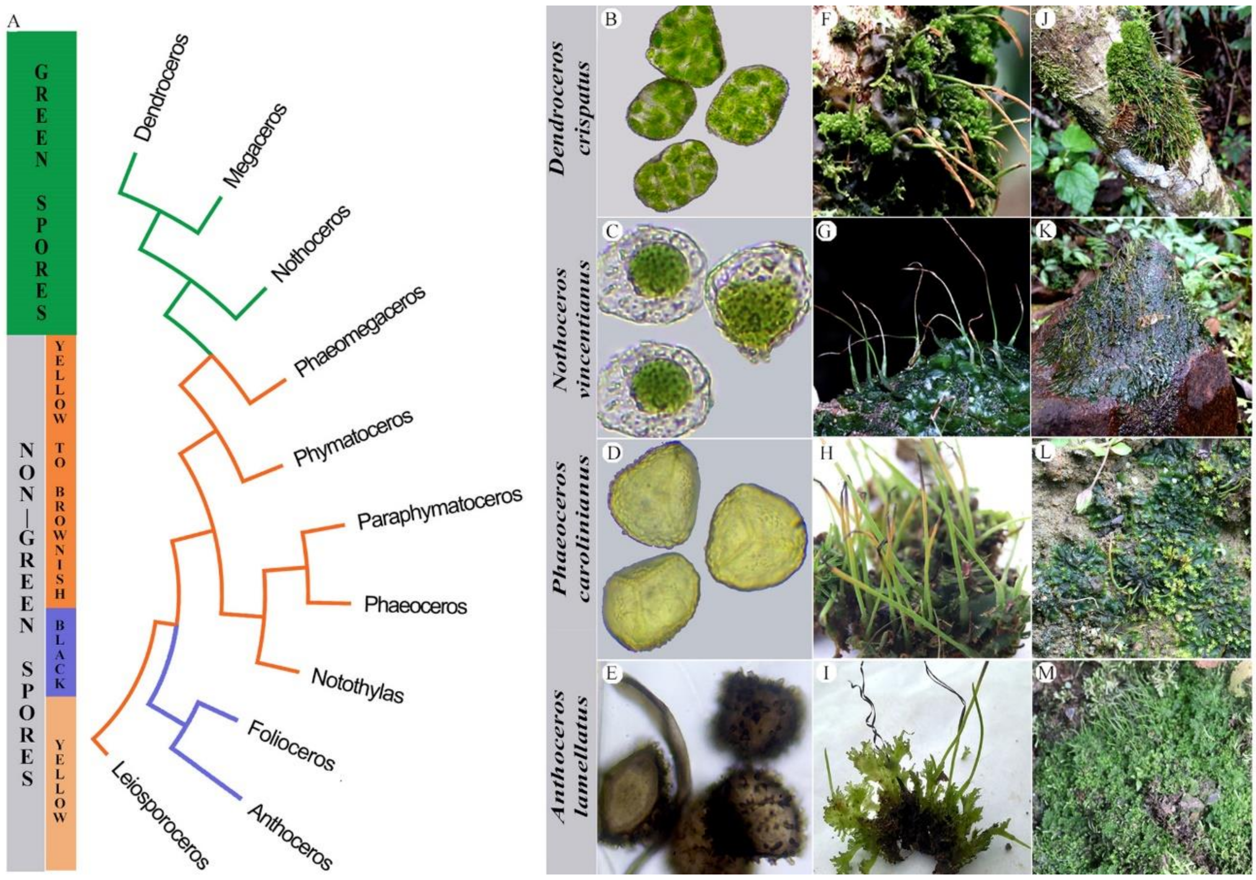

Fig. 1. Diversity of the spores and niches of hornwort species. **A**: Diagram of the hornwort genera with green and non-green spores; **B-C,** types of green spores, **B**: Multicellular spores; **C**: Unicellular spores; **D-E**: Unicellular non-green spores; **F-I**: Hornwort gametophytes and sporophytes; **J-M**: Niches among hornwort species, **J**: Epiphytic (on the bark of live trees and shrubs), *Dendroceros* genus; **K**: Saxicolous (directly on rock surfaces), *Nothoceros vincentianus*; **L-M**: Terricolous (on soils), **L**: *Phaeoceros carolinianus*; **M**: *Anthoceros lamellatus.*

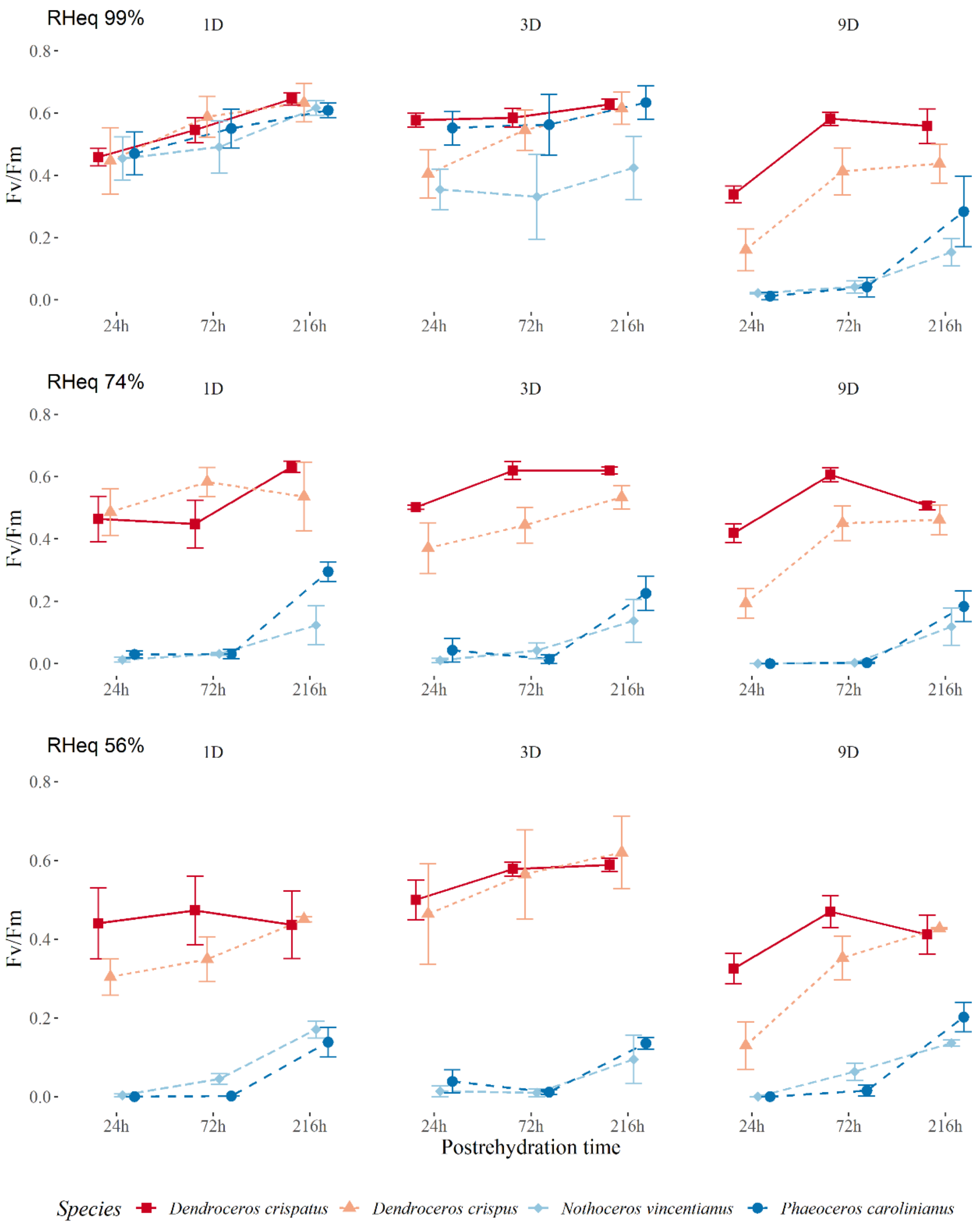

Fig. 2. Means of the *Fv/Fm* chlorophyll fluorescence parameters of hornwort gametophytes in the equilibrating relative humidity treatments (*RHeq*) 99%, 74% and 56%. With the species in the different *Duration dry* treatments in days (1D, 3D and 9D) and *postrehydration time* in hours (24h, 74h, 216h), error bars represent ± 1 SE.

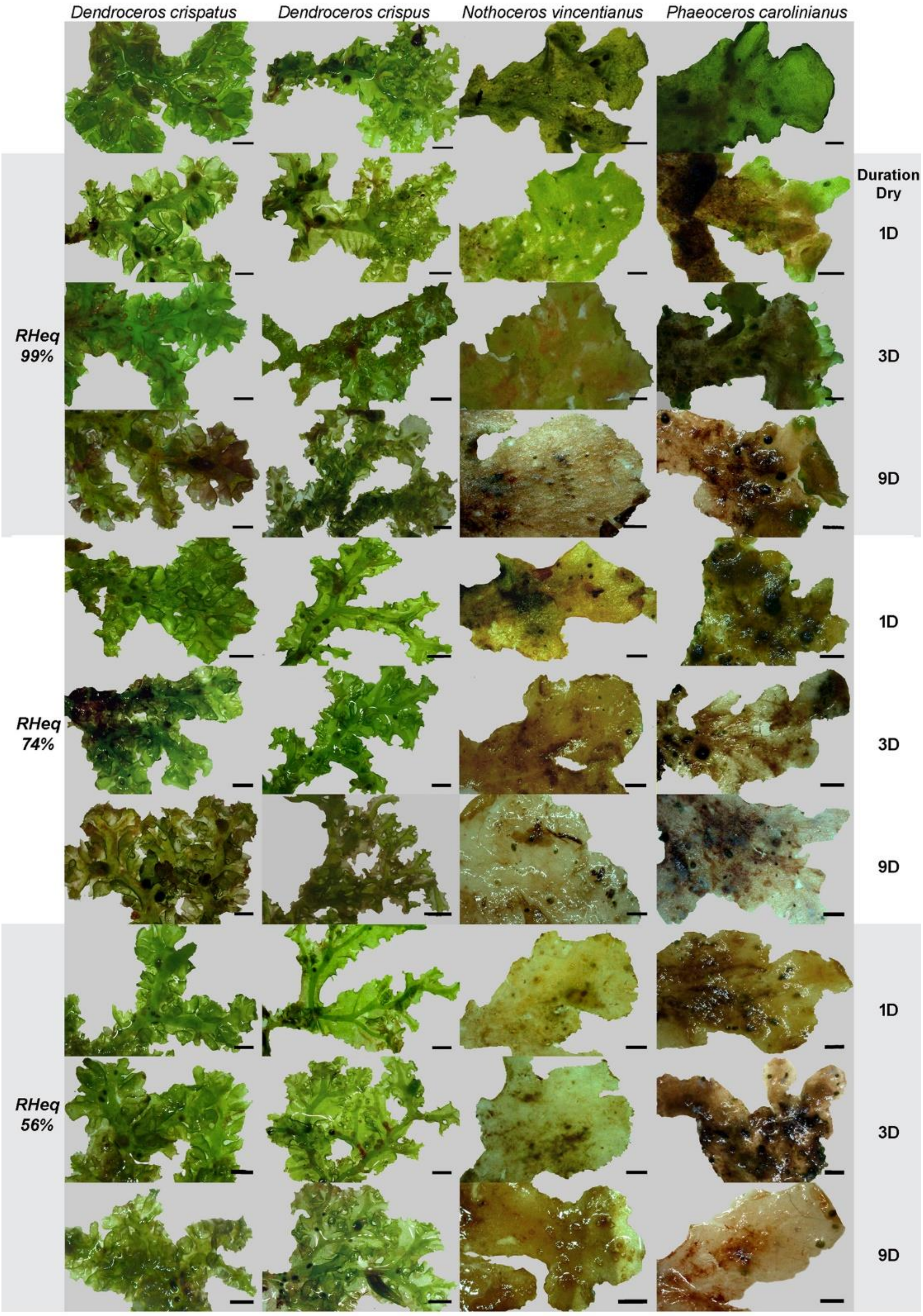

Fig. 3. Effects of drying on hornwort gametophytes in the three treatments of *RHeq* (Left) and *Duration dry* (right) with a *postrehydration* of 216h. First line of gametophytes with deacclimation for 15 days and without drying effects. Scales 1 mm.

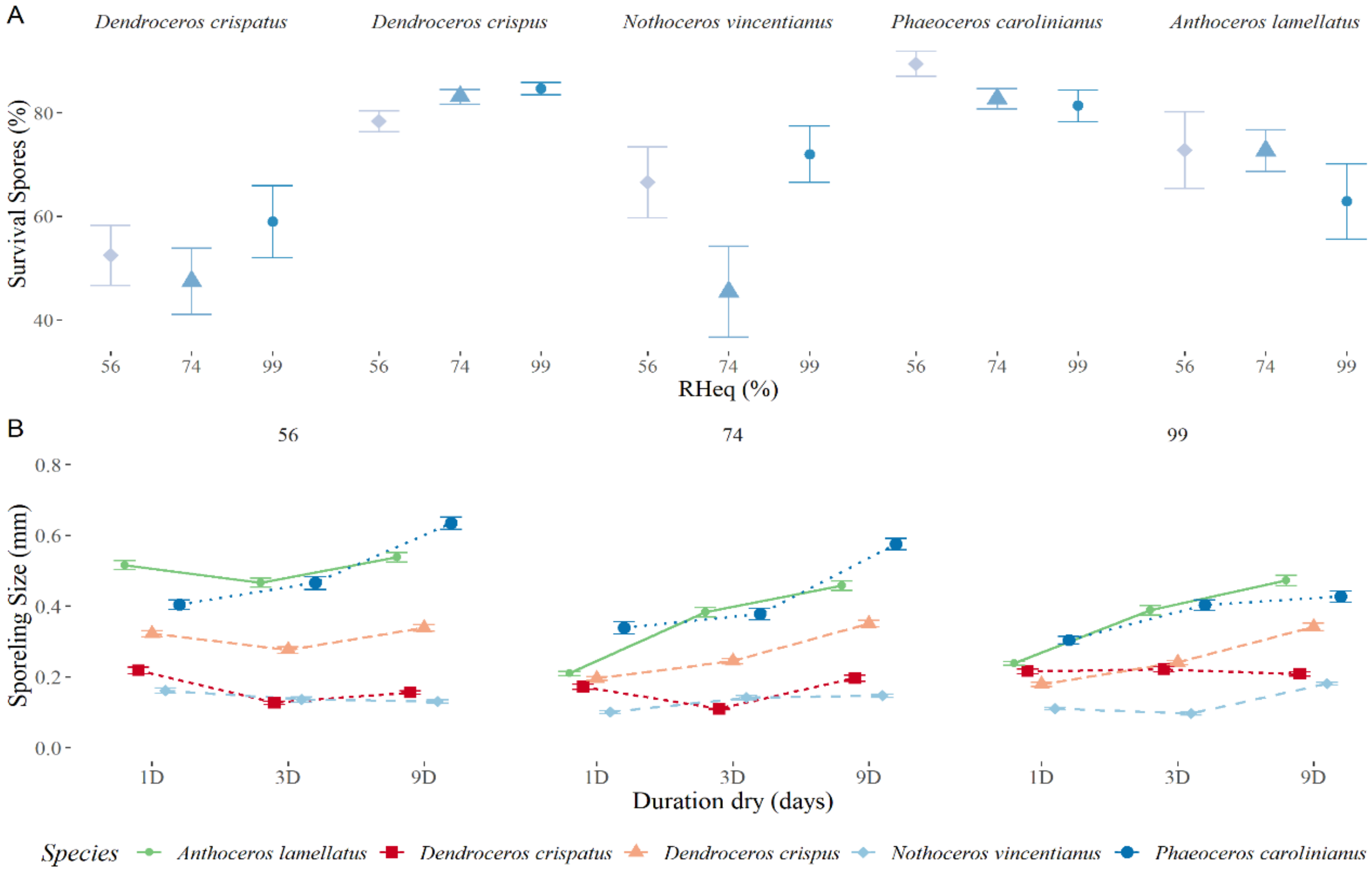

Fig. 4. Spore survival percentages and sporeling sizes of hornworts cultured for 30 days. **A-B**: after *RHeq* and 24-hour *postrehydration* before cultivation; **A**: Spore survival with *RHeq* treatment; **B**: Sporeling size with *RHeq* and *Duration dry* treatments. Error bars represent ± 1 SE.

Tables

Table 1. Generalized linear model of the effects of desiccation tolerance in gametophytes and spores of hornworts species. (GLM with Gaussian distribution and identity link function; **Bold** indicates statistical significance).

Source	*Degree of freedom (d.f.)*	*Deviance*	*Resid. Df*	*Resid.Dev*	*F*	*Pr(>F)*
Chlorophyll fluorescence parameters for hornwort gametophytes						
Null model			323	184.989		
Species	3	82.696	320	102.293	2.130.616	**<0.001**
RHeq	2	21.851	318	80.443	844.457	**<0.001**
Duration Dry	2	12.203	316	68.240	471.616	**<0.001**
Postrehydration	2	12.289	314	55.951	474.911	**<0.001**
Species:RHeq	6	12.782	308	43.169	164.665	**<0.001**
Species:*Duration* Dry	6	0.2100	302	41.069	27.054	**0.014**
Species:postrehydration	6	0.2773	296	38.296	35.723	**<0.001**
Model: AIC: -460.44; Residual deviance: 3.8296; 296 degrees of freedom (df)						
Survival spores						
Null model			134	55441		
Species	4	19447.3	130	35993	190.645	**<0.001**
RHeq	2	967.2	128	35026	18.963	0.154
Duration Dry	2	9.7	126	35016	0.0189	0.981
Species:RHeq	8	4295.7	118	30721	21.056	**0.041**
Species:*Duration* Dry	8	2668.6	110	28052	13.080	0.246
Model: AIC: 1155.5; Residual deviance: 28052; 110 degrees of freedom (df)						
Sporeling Size						
Null model			6749	580.75		
Species	4	198.547	6745	382.21	1.132.689	**<0.001**
RHeq	2	17.994	6743	364.21	205.306	**<0.001**
Duration Dry	2	22.908	6741	341.30	261.377	**<0.001**
Species:RHeq	8	26.078	6733	315.23	74.386	**<0.001**
Species:*Duration* Dry	8	20.523	6725	294.70	58.540	**<0.001**
Model: AIC: -1928.8; Residual deviance: 294.70; 6725 degrees of freedom (df)						

CAPÍTULO III: Influência de *Nostoc* sp. no desenvolvimento dos esporos de *Dendroceros crispus*

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Effect of the cyanobacteria *Nostoc* sp. on sporelings of *Nothoceros vincentianus* and *Dendroceros crispus* (Dendrocerotaceae, Anthocerotophyta)

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Abstract

In mutualistic associations between Bryophytes and microorganisms, the genus *Nostoc* is the most representative cyanobacteria, living inside liverworts and hornworts thallus. In hornworts, the infection process by *Nostoc* originates in the early stages of gametophyte development, and multiple cyanobacterial strains may be present in a same thallus, suggesting a lack of selectivity and specificity in symbiosis of cyanobacteria-hornwort. Considering this low specificity of endosymbiosis between taxa of hornworts and cyanobacteria, we tested the effect of a cyanobacterial strain of *Nostoc* on the sporeling development of *Nothoceros vincentianus* and *Dendroceros crispus.* Both species belong to Dendrocerotaceae family and differs regarding spore type (multicellular in *Dendroceros* vs. unicellular in *Nothoceros*) and niche (epiphyte vs. saxicolous). We isolated *Nostoc* sp. from *N. Vincentianus*, and spores were cultured at *Nostoc* and non-*Nostoc* treatments. They were observed regarding survival and sporeling growth for 154 days. Moreover, we associated the sporeling ontogeny with life cycle phases of *Nostoc*. *Dendroceros crispus* had a higher performance compared to *N. vincentianus* at both treatments. *N. vincentianus* growing with *Nostoc* had low survival and growth compared to the non-*Nostoc* treatment. Additionally, a weak correlation between sporeling ontogeny and *Nostoc* stages was found for both species. Our data corroborates, at least partially, the early expectations on the low specificity between *Nostoc* and hornwort taxa. However, the low performance of *N. vincentianus* suggests the association with *Nostoc* should occurs late along sporeling development, avoiding plant-cyanobacteria competition. Although we may not discard completely plant-cyanobacteria specificity, our findings highlight the complexity of the *Nostoc*-hornwort symbiosis.

Keywords:
Hornwort; Interaction; Life cycle; Niche; Specificity; Symbiosis

Introduction

Symbiosis between cyanobacteria and plants are supposed to exist since 450 million years ago (Raven 2002; Delaux and Schornack 2021). This relationship may have facilitated the transition of plants to land, along with multiple processes of evolution and diversification of species (Bidartondo et al. 2011; Lutzoni et al. 2018; Genre et al. 2020; Nelson et al. 2020). This symbiotic interaction promotes the development of the host, since cyanobacteria provide fixed nitrogen for it (Rasmussen and Nilsson 2002; Adams and Duggan 2008; Bustos-Díaz et al. 2019). In bryophytes, two types of symbiosis have been detailly recorded: 1) cyanobacteria growing epiphytically in mosses (in the species *Pleurozium schreberi* and *Hylocomium splendens)* and in two genera of liverworts (*Marchantia* and *Porella)* (Solheim and Zielke 2002; Gentili et al. 2005; Adams and Duggan 2008; Rousk et al. 2013); and 2) endophytic associations in two genera of liverworts (*Blasia* and

Cavicularia) and all species of hornworts (Adams 2000; Renzaglia et al. 2000; Meeks 2005; Renzaglia et al. 2009; Bustos-Díaz et al. 2019).

Several genera of cyanobacteria (e.g. *Nostoc*, *Stigonema*, *Calothrix* and *Cylindrospermum*) have been identified in symbiosis with bryophytes (Adams and Duggan 2008), with *Nostoc* being the dominant in terrestrial symbiotic systems (Adams 2002; Meeks 2007; Warshan et al. 2018). This cyanobacteria is distinguished by a complex life cycle, with different stages of development, including gelatinous colonies containing many filaments, akinetes and hormogones (for more details, see Meeks, 2007; Paulsrud, 2001). The differentiation of life stages is important for the establishment of symbiosis, since hormogones and mobile filaments are responsible for infecting the host (Rasmussen and Nilsson 2002). Other structure that can be found in *Nostoc* are the heterocysts, which are specialized nitrogen-fixing cells important to endosymbiosis relationships (for reviews see (Golden and Yoon 2003; Meeks 2003; Zhang et al. 2006; Adams and Duggan 2008). The heterocyst frequency in *Nostoc* increases when it is in symbiosis with plants, indicating that N_2 fixation is the major role of the cyanobacteria in this interaction, while plant gives to it a more protected environment in relation to extreme conditions and predation (Adams et al. 2013)

The infection process in hornworts by *Nostoc* originates in the early stages of gametophyte development (i.e., from sporeling to early thallus).Two different forms of cyanobacteria colonies are observed in hornworts: an elongate central strand colony (only in *Leiosporoceros*); and a dark, small and globose colony in all the others genera (Villarreal and Renzaglia 2006; Renzaglia et al. 2007; Renzaglia et al. 2009). Morphology of the hornwort gametophytes also enables the infection process. Commonly, mucilaginous cracks appear in the ventral gametophyte surface, with two cells like stomatal guard cells around a pore. With no ability to close or open, these structures provide an entry point for endosymbionts (Frangedak is et al., 2021; Renzaglia et al., 2009). As several pores may be present in the gametophyte, a single plant can host different strains of cyanobacteria; although this assumption has only been detailly explored in three hornwort species (*Anthoceros fusiformis*, *Phaeoceros laevis* and *Leiosporoceros dusii*) from a genetic framework based on cyanobacteria diversity (West and Adams 1997; Costa et al. 2001; Bouchard et al. 2020).

Sympatric hornwort species as *Notothylas orbicularis*, *Phaeoceros carolinanus* and *Anthoceros agrestis* show similarity regarding their cyanobionts, although with low specificity (Nelson et al. 2020). Therefore, multiple cyanobacterial strains may be present in a same hornwort, suggesting a lack of selectivity and specificity (by the plants) in the symbiosis of cyanobacteria – hornwort (Villarreal and Renzaglia 2006; Bouchard et al. 2020). This low specificity of cyanobacteria – hornwort is similar for mosses and liverworts, contrasting with the high specificity observed in lichens, *Azolla – Anabaena*, and cyanobacteria – diatom symbiosis (Adams 2002; Rasmussen and Nilsson 2002; Magain et al. 2016).

Considering a low specificity of the endosymbiosis between taxa of hornworts and cyanobacteria, we tested the effect of a cyanobacterial strain of *Nostoc* sp. (isolated from a population of *Nothoceros vincentianus*) on the sporeling development of *N. vincentianus* and *Dendroceros* *crispus*. We expect sporelings of both species survive and develop better with cyanobacteria than with none. Simultaneously, we expect to see a relationship between the cyanobacterial cycle and the plant life stages.

Material and methods

Sampling and cultures

We studied two species of hornworts: *Dendroceros crispus* (Sw.) Nees, an epiphytic species with multicellular green spores, found in forests at 800-2000 m a.s.l. (Duff et al. 2007; Peñaloza-Bojacá et al. 2019); and *Nothoceros vincentianus* (Lehm.) J.C. Villarreal, a terricolous or saxicolous species with unicellular green spores, found in Neotropical forests above 400 m a.s.l (Renzaglia et al. 2009; Villarreal and Renner 2014). We carried out field collections in October/2018 in the Atlantic Forest, in São Paulo state, Brazil. We collected samples (c. 8 cm^2) of the hornwort species in the forest located in the Núcleo Santa Virgínia, Parque Estadual da Serra do Mar (23°20' - 23°25'S and 45°08 - 45°15' W), 800-1000 m a.s.l, 20-24 C°, mean rainfall from 1500-4000 mm/year (Fundação Florestal 2008). Plants were maintained hydrated at culture room (21°C, 74 µmol. of photons m^{-2}. s^{-1}) until laboratory essays.

Considering that sporogenesis is asynchronous in hornworts, we collected only the completely developed and mature spores, present in the capsule apex. Spores from ten capsules of each species were mixed and cleaned with 0.05% calcium hypochlorite for 40-50 seconds, then rinsed with autoclaved ionized water for 15 seconds three times. A 0.5 mL spores' solution was plated in Petri dishes (30 mm diameter) on solid culture (0.5% agar) medium 50% KnopII (Nakazato et al. 1999). Spore density varied from 160.000 mL^{-1} in *D. crispus* to 405.000 mL^{-1} in *N. vincentianus*. Spores were cultured under constant temperature and light conditions (21°C, 74 µmol of photons m^{-2}. s^{-1}) in a culture room with a 12 h photoperiod.

Experimental design

Spores of *D. crispus* and *N. vincentianus* were cultured as above described to non-*Nostoc* and *Nostoc* treatments. Fourteen independent observations with three replicates each were performed along 154 culture days. For *Nostoc* treatment, the Petri dishes containing spores were also inoculated with previously cultivated (see below), colonies of *Nostoc* that were passed through a mesh with porosity of 1 µm. An autoclaved glass syringe was used to break down the mucilage of colonies to release the filaments and 0.5 mL of this *Nostoc* solution was added to each plate. *Nostoc* addition occurred simultaneously to hornwort spores in media.

Survival was measured as germinated and viable spores, i.e., spores remaining the capacity to progress with mitosis, showing size increasing, green chloroplasts, development of rhizoid or germ tube, oppositely to dead spores (no signs of development, being hyaline or dark brown spores, no green chloroplasts, no rhizoids). Sporeling growth (*length* and *width* in micrometers) was analyzed using the BEL Capture Application software (400x magnification). 100 hornwort spores and sporelings regarding survival and 50 to growth were measured at each observation time and in each replicate. Survival was recorded as percentage of viable and germinated spores in each replicate.

Isolation and culture of the cyanobacteria

A clonal culture of *Nostoc* sp. was obtained by traditional micropipette isolation method (Andersen and Kawachi 2005) from a *N. vincentianus* gametophyte collected in May/2017 in Atlantic Forest in Itatiaia National Park (22°27'06" S and 44°36'44" W), 837 m a.s.l. Rio de Janeiro state, Brazil. This strain was maintained in Erlenmeyer flasks containing 300 mL of CHU10 medium at a 12:12 h light/dark regime. At the culture chamber, the light intensity was 30 µmol photons m^{-2} s^{-1} and the temperature was 20(±1) °C. The experiments were performed with cultures in exponential growth.

Ontogeny of hornworts and Nostoc sp.

We recorded ontogenetic phases for plants and cyanobacteria. We classified the sporeling ontogeny into seven phases to *D. crispus* and *N. vincentianus* (Table 1; Fig. 1), based on previous studies on hornwort sporelings (Campbell 1907; Renzaglia 1978; Schuette and Renzaglia 2010; Oliveira et al. 2017). We also described seven phases for *Nostoc* life cycle (Table 1; Fig. 1; (Paulsrud 2001; Becerra-Absalón and Tavera 2009). By considering N_2 fixation as the major role of the cyanobionts, we additionally recorded the presence of heterocysts along the experiment.

Statistical analyses

To verify our early prediction of low hornwort-cyanobacteria specificity, we performed generalized linear models (GLMs) to test the influence of cyanobacteria on survival and growth of spores and sporelings of the two hornwort species. Additionally, to the "*Nostoc*" effect, we also included in our models the main and interaction effects of *"hornwort species*" and "*observation time*". GLMs with gaussian distribution and identity link function were used for "*survival*" and "*growth*" response variables (model details in Supplementary Data Table S1). Additionally, when necessary, *post hoc* comparisons (Tukey test, α = 0.05) were applied. Furthermore, we tested a potential relationship between sporeling ontogeny and *Nostoc* phases with Spearman correlations for both species. Since we recorded hornwort sporelings at different phases at each observation time, we used the formula: *phase: (n1.C1+n2.C2+…n7.C7)/(C1+C2+…C7),* where *n* = number of sporelings at a phase and *C* = ontogenetic phase for each replicate. For cyanobacteria, since we were not able to count individuals in each phase, we recorded the presence of each phase as 1 (with no heterocysts) or 2 (with heterocysts). We applied a similar formula to quantify a phase single-value in each replicate. All analyses and graphs were performed in R v.4.0.4(R Core Team 2020) using RStudio v.1.3.959 software (RStudio Team 2020).

Results

Nostoc significantly affected the *survival* and *growth* of sporelings of both species, but especially benefiting *D. crispus* compared to *N. vincentianus*. This later species had a low performance growing with *Nostoc*. Additionally, time did not have a negative effect on *D. crispus* unlike *N. vincentianus*, which decreased in sporeling *survival* and *growth* after 48 days mainly at *Nostoc* treatment (Tab. 2, Supplementary Data Table S1). Averages from the whole data set are higher for *Dendroceros crispus* with *survival* of sporelings (mean ± standard error: 63.84% ± 1.20), *length* (463.51µm ± 25.80) and *width* (156.91 ± 6.73), as compared to *N. vincentianus* (54.77% ± 3.21; 238.88 ± 26.24; 113.48 ± 9.24 respectively; Fig. 2).

Only weak positive correlations between hornworts and *Nostoc* phases were detected (*D. crispus:* r_s= 0.37; p-value = 0.015; *N. vincentianus*: r_s= 0.38; p-value = 0.013; Fig. S1). *Nostoc* phases developed similarly in both hornwort species (Fig. 3A). Nevertheless, we observed more heterocysts in the *Nostoc* treatment among sporelings of *N. vincentianus* than in the treatments with *D. crispus* (Fig. 3B). *Dendroceros crispus* sporelings developed very well at both *Nostoc* and non-*Nostoc* treatments along the 154 culture days. Conversely, different phases of sporelings in *N. vincentianus* were only found at the non-*Nostoc* treatment (Fig. 4).

Discussion

Our data corroborates, at least partially, the early expectations on the low specificity between *Nostoc* and hornwort taxa. *Dendroceros crispus* performed better than *N. vincentianus* regarding both spore survival and sporeling growth, with a similar behavior between the cyanobacteria treatments (*Nostoc* and *non-Nostoc*). Although other studies mention that *Nostoc* infection processes have been recorded in the early stages of development in hornworts (Renzaglia et al., 2009), the lack of infection during our experiment (154 days) raises more questions about the factors that influence the cyanobacteria-hornworts interaction, especially for *Dendroceros crispus* that developed vigorous gametophytes including pores (i.e. gateways for cyanobionts).

Previous studies have demonstrated a low specificity between *Nostoc* sp. and *Anthoceros punctatus*. For instance, a successful association was reconstituted between this hornwort species and colonies of *Nostoc punctiforme* isolated from the cycads *Macrozamia* sp. (Meeks 2003; Meeks 2007). Furthermore, we cannot rule out the influence of the soil and its microbiome as important factors in this symbiosis. Even sympatric hornwort species as *Notothylas orbicularis*, *Phaeoceros carolinanus* and *Anthoceros agrestis* have similarly low specificity, but different preferences toward cyanobionts present in the soil (Nelson et al. 2020). In our study, the *Nostoc* colonies isolated from *N. vincentianus* were collected from a different population than that used for culture experiments, and this could be related to the lower performance of this species when growing with *Nostoc* sp. at laboratory.

Low performance of *N. vincentianus*' spores and sporelings along with *Nostoc* suggests this association should occur later during the sporeling development. Furthermore, the presence of *Nostoc* in the culture medium during the early stages of the hornwort development (i.e., unicellular spore germination) may have generated competition between plant and cyanobacteria, negatively affecting the survival and growth in *N. vincentianus*. Conversely, *D. crispus*, which started as multicellular spores, apparently suffered no negative effect of competition with *Nostoc* colonies. Since the studied species present distinct spore traits (Ligrone and Renzaglia 1990; Renzaglia et al. 2009; Renzaglia et al. 2020), germination and establishment could be differently sensitive to environmental factors (multicellular sporelings of *D. crispus* more resistant than unicellular sporelings of *N. vincentianus*), including cyanobacteria in the culture media.

The high presence of *Nostoc* heterocysts - nitrogen fixation cells (Meeks 2007) in plates of both hornwort species, together with the weak correlation between the life phases of plants and cyanobacteria, leads us to think about an independent development of hornworts and *Nostoc* in our study. This possibility is reinforced when we consider that functional symbiotic association is established when *Nostoc* filaments are fragmented and lose their heterocysts, generating hormogonia that serve as infectious units, upon entering the hornwort host (Adams 2002; Meeks 2007; Adams and Duggan 2008). Unfortunately, we did not observe this synchronicity between cyanobionts and plants. Finally, we may not completely rule out plant-cyanobacteria specificity. Likewise, the divergent responses between *D. crispus* and *N. vincentianus* to the cyanobacteria highlight the complexity of the *Nostoc*-hornwort symbiosis.

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Figures

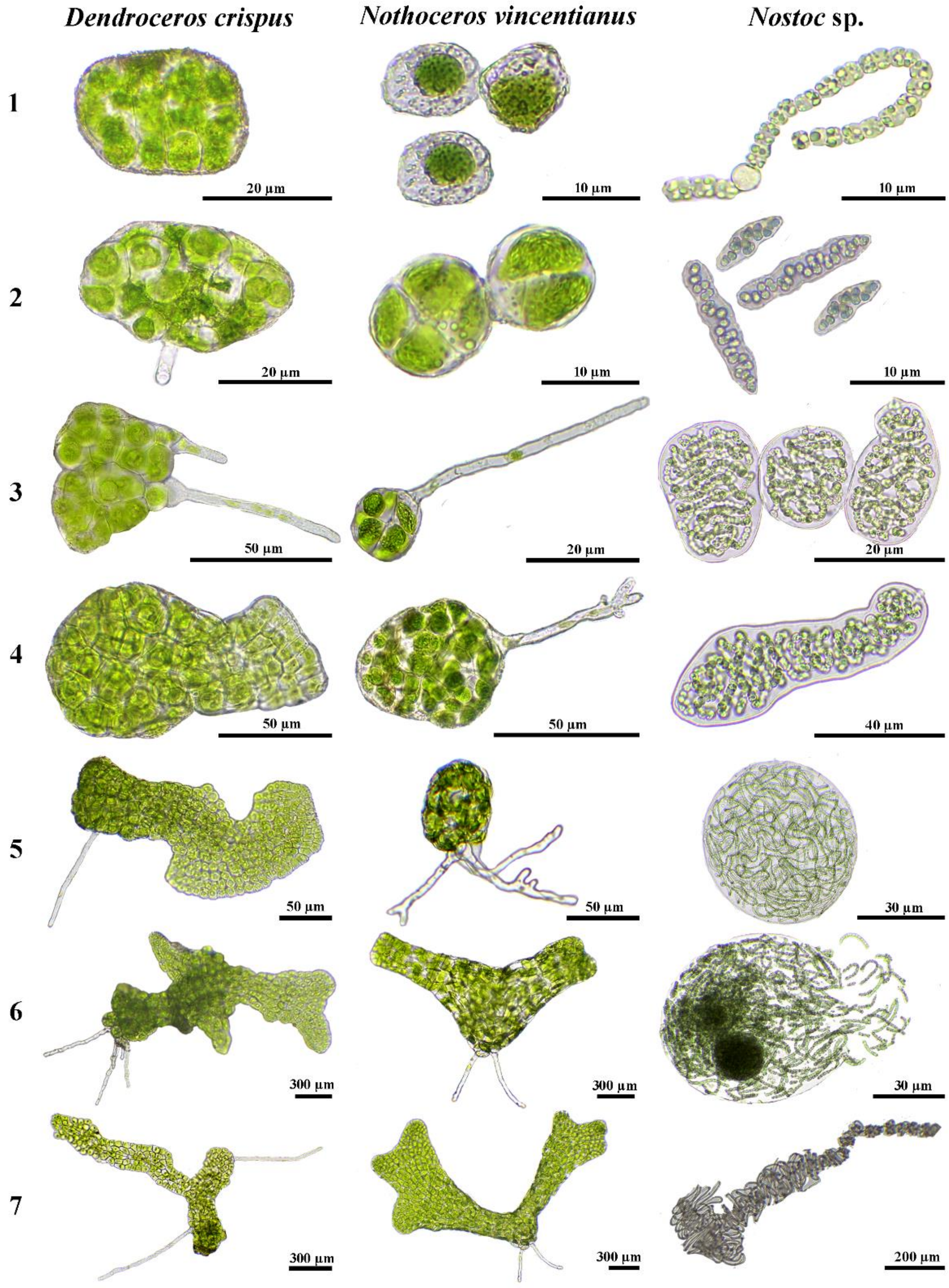

Figure 1. Representation of the ontogeny sporelings in seven phases for *Dendroceros crispus*, *Nothoceros vincentianus* and the life cycle of *Nostoc* sp.

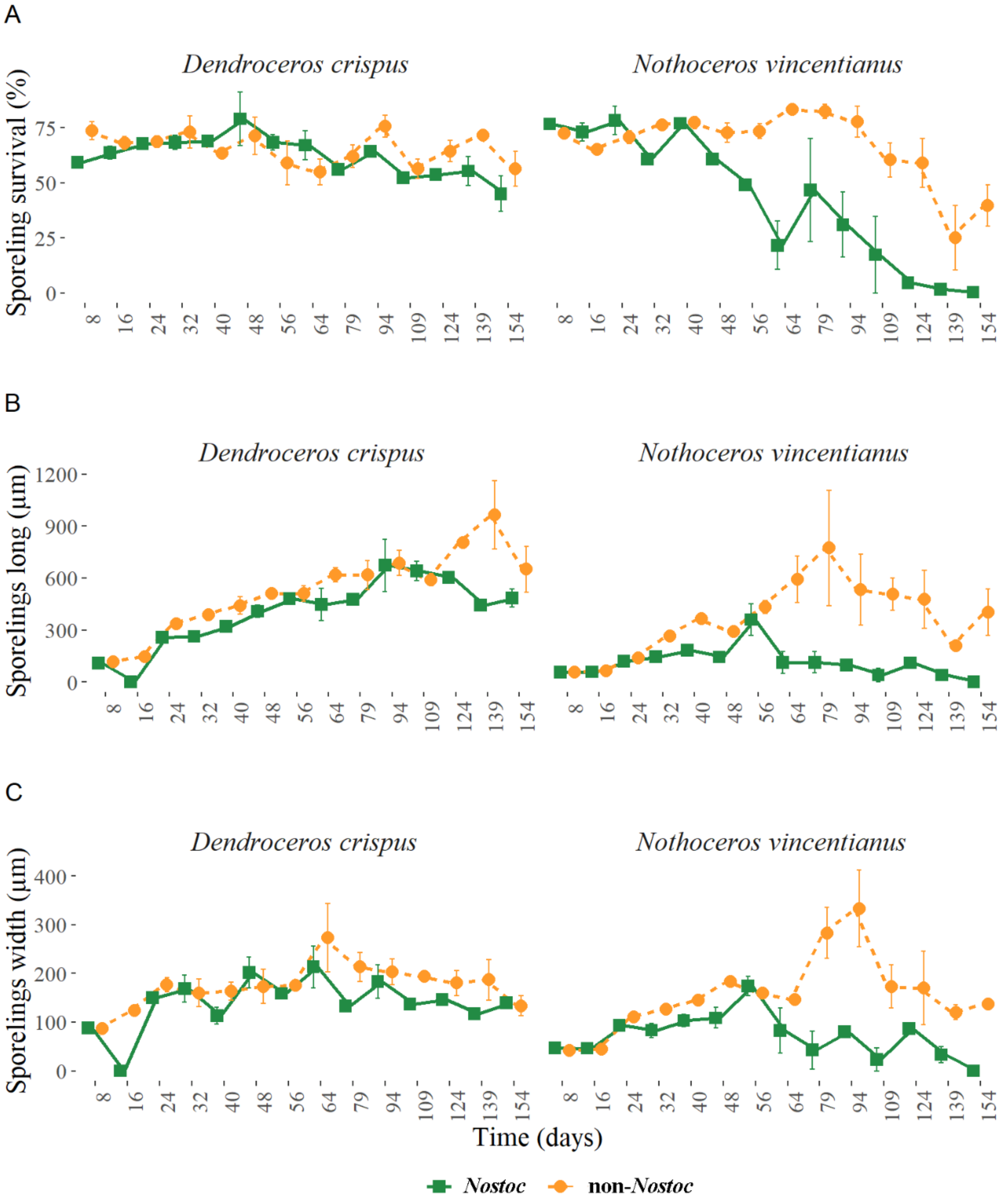

Figure 2. Spore survival percentages and sporeling growth of *Dendroceros crispus* and *Nothoceros vincentianus* overtime. A: Survival; B: growth in long and C: growth in width. Green square: treatment with sporelings and *Nostoc*; Orange circles: treatment only with sporelings (non-*Nostoc*). Error bars represent ± 1 SE.

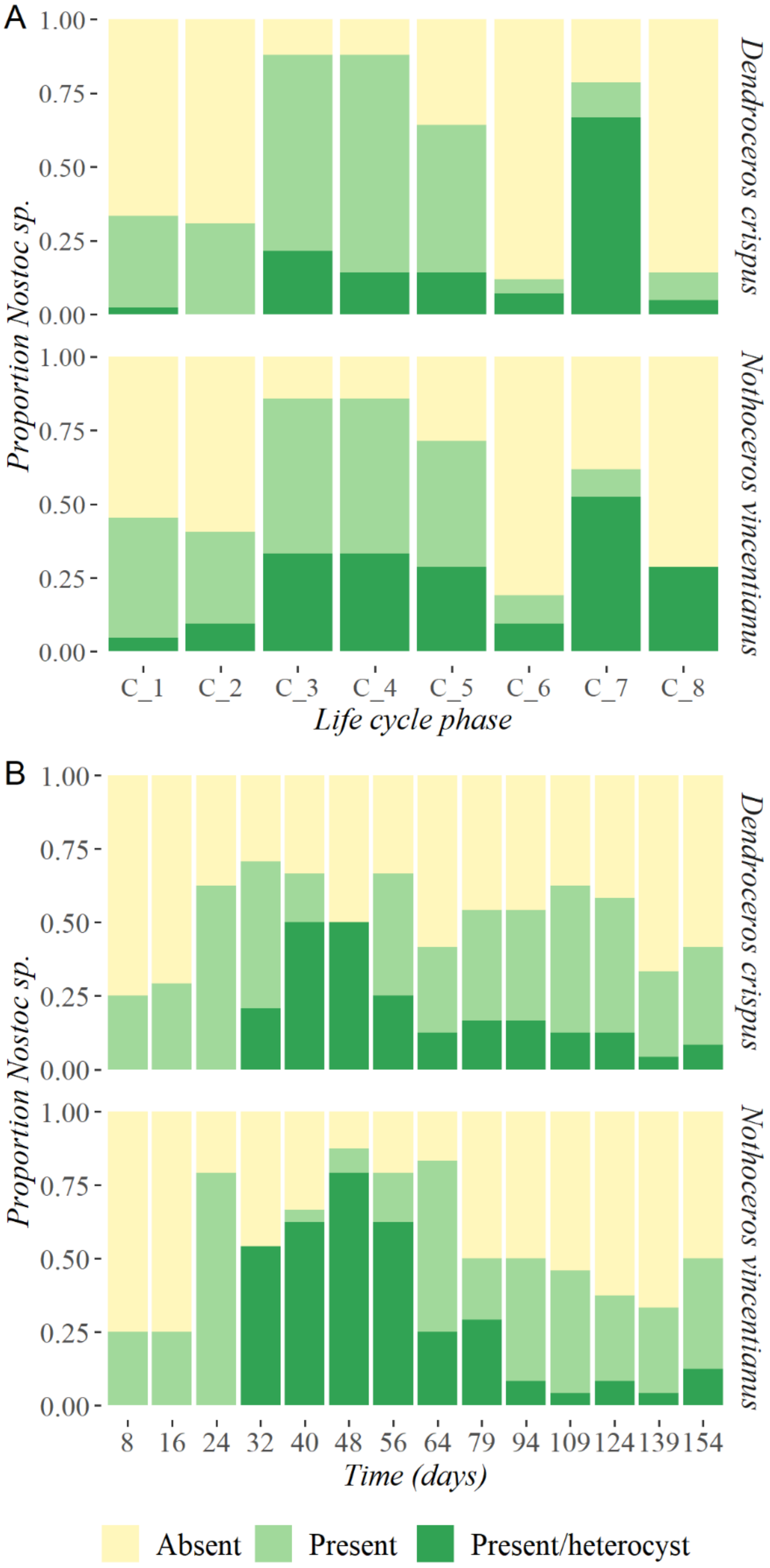

Figure 3. Proportion of the phases of the life cycle of *Nostoc* sp. in the treatments with spores of *Dendroceros crispus* and *Nothoceros vincentianus*. A: Life Cycle *Nostoc* sp. in seven phases (C1-C7; details in Tab. 1); B: *Nostoc* throughout 154 days of observation.

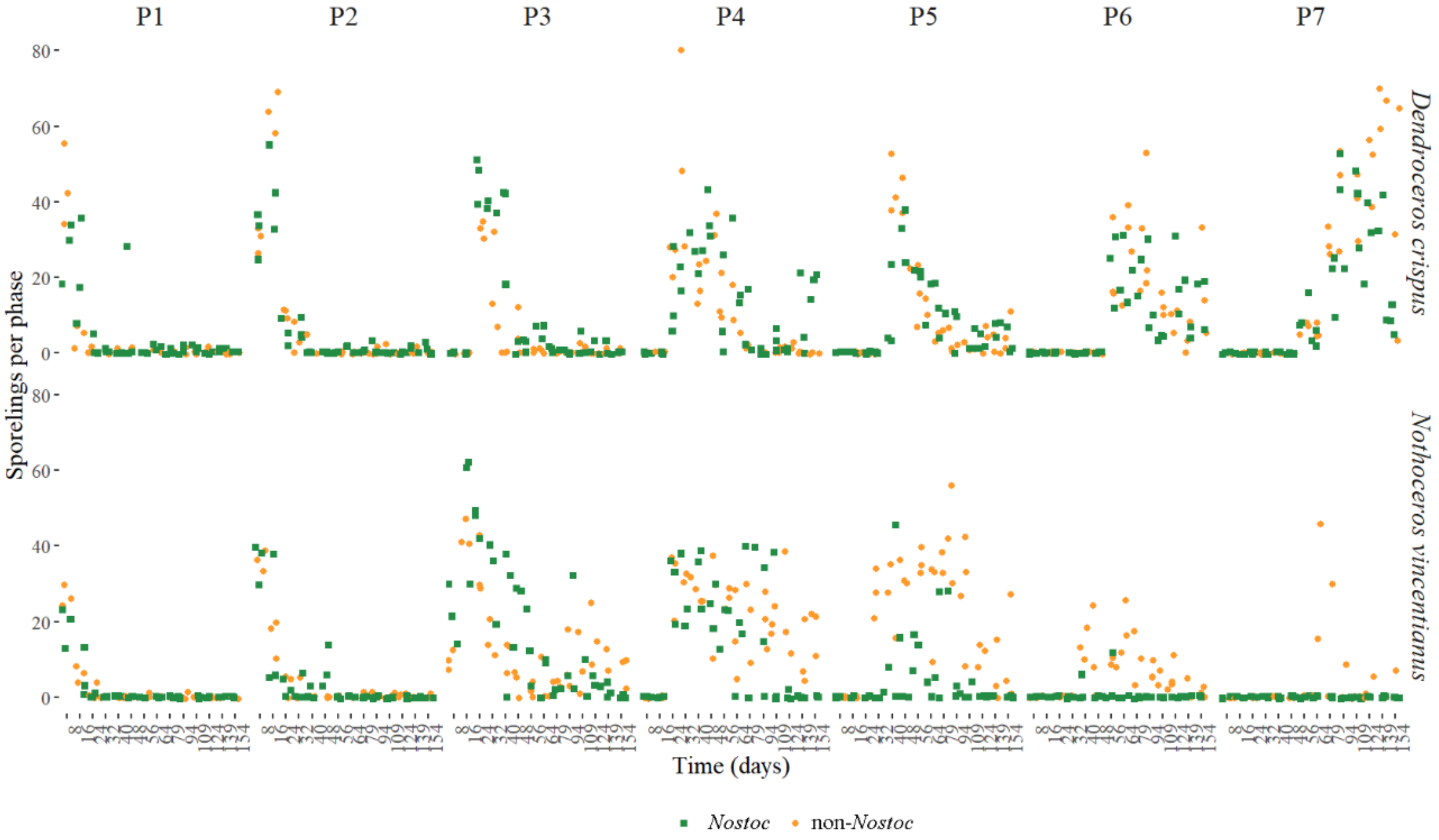

Figure 4. Sporelings development into seven phases (P1-P7; details in Tab. 1) to *Dendroceros crispus* and *Nothoceros vincentianus* along the 14 observations in 154 days. Green square: treatment with sporelings and *Nostoc*; Orange circles: treatment only with sporelings (non-*Nostoc*).

Tables

Table 1 Gametophyte development phases in hornworts species (*Dendroceros crispus* and *Nothoceros vincentianus*) and development phases in *Nostoc* life cycle

Phase	*Dendroceros crispus*	*Nothoceros vincentianus*	Phase	*Nostoc* sp.
P1	Multicellular spores	Unicellular spores	C1	Hormogonia that are the motile filaments
P2	Sporelings are small globose mass two or three times larger than phase one and with a rhizoid beginning its development	Sporelings are small globose mass with more than two cells	C2	Hormogonia with enlargement of the cells and formation of a sheath around the filament
P3	Globose masses and large rhizoid filled with pieces of the chloroplast	Globose masses and large rhizoid filled with pieces of the chloroplast	C3	Vegetative filament long, contorted and wrinkled in a sheath
P4	Globose masses with develop wedge-shaped apical cells and pore	Globose mass two to three times larger than the previous phase; with one or two bifurcated rhizoids	C4	Abundant development of vegetative fragments that break up in the sheath and begin to form colonies and proliferate
P5	Globose masses elongate, differentiation of cells with hemidiscoid shape (apical growth) and beginning of the formation of the midrib	Globose mass with oval shape and several rhizoids; start of differentiation of wedge-shaped apical cells	C5	Colonies larger than the previous phase and a hardened sheath of mucilage
P6	Globose masses well elongate (two to three times longer than wide) and beginning of the sporeling bifurcation	Globose mass bifurcating and wedge-shaped apical cells dividing longitudinally and transversely	C6	Colonies with several vegetative filaments emerging from the broken sheath
P7	Distinction of the wing cells of the gametophyte with lateral expansion and bifurcations present a midrib	Sporeling two or three times larger than the previous phase, with elongated bifurcations and several long rhizoids	C7	Long vegetative filaments extremely contorted and wrinkled. That later can be divided forming the phase 1

Table 2. Generalized linear model of the Survival and Growth in sporelings of *Dendroceros crispus* and *Nothoceros vincentianus*. (GLM with Gaussian distribution and identity link function; **Bold** indicates statistical significance).

Source	*Degree of freedom (d.f.)*	*Deviance*	*Resid. Df*	*Resid.Dev*	*F*	*Pr(>F)*
Survival parameters of Hornworts Sporelings						
Null model			167	81584		
Species	1	3447.1	166	78136	139.983	**<0.001**
Treatments	1	7968.1	165	70168	323.574	**<0.001**
Time	13	25501.9	152	44666	79.661	**<0.001**
Species:Time	1	4412.6	151	40254	179.190	**<0.001**
Treatments:Time	13	6270.6	138	33983	19.588	**<0.01**
Model: AIC: 1430.8; Residual deviance: 33983; 138 degrees of freedom (df)						
Growth (length) parameters of Hornworts Sporelings						
Null model			164	11187176		
Species	1	2410938	163	8776238	883.651	**<0.001**
Treatments	1	1268946	162	7507292	465.091	**<0.001**
Time	13	2887826	149	4619466	81.418	**<0.001**
Species:Time	1	193403	148	4426063	70.886	**<0.001**
Treatments:Time	13	742747	135	3683315	20.941	**<0.01**
Model: AIC: 2182.5; Residual deviance: 3683315; 135 degrees of freedom (df)						
Growth (width) parameters of Hornworts Sporelings						
Null model			164	935395		
Species	1	99982	163	835413	374.804	**<0.001**
Treatments	1	122582	162	712831	459.524	**<0.001**
Time	13	225120	149	487712	64.916	**<0.001**
Species:Time	1	30015	148	457697	112.517	**<0.001**
Treatments:Time	13	97574	135	360123	28.137	**<0.001**
Model: AIC: 1798.8; Residual deviance: 360123; 135 degrees of freedom (df)						

CONCLUSÕES

I. As florestas nebularas da Colômbia e a Mata atlântica do Brasil, abrigam uma alta diversidade de antóceros, que normalmente são omitidos ou ignorados nas coletas biológicas. Nossos resultados ressaltam a diversidade dos antóceros nestes locais, pois essas áreas podem sustentar múltiplas espécies de antóceros pouco conhecidas, e a importância de conservar estes ambientes (**Capítulos I, II e III**);

II. As espéceis de *Dendroceros* (*D. crispus* e *D. crispatus*) conseguem se estabelecer e sobreviver melhor que outros antóceros sob dessecação. Seu nicho epífito e características morfológicas, lhes conferem uma melhor tolerância às condições ambientais adversas presentes nos ecossistemas onde vivem (**Capítulos I, II e III**);

III. A classificação subgenérica aqui proposta com dados moleculares e as análises de mais de 80 espécimes (coletados no Neotrópico, como de coleções de herbários brasileiros e internacionais) ajudou no preenchimento de lacunas de informação taxonômica, como também auxiliou no avanço da compreensão da diversidade de *Dendroceros* **(Capítulo I)**;

IV. Características morfológicas chaves de *Dendroceros* como: formato das asas do gametófito, perfurações das asas do gametófito, engrossamento das paredes da cápsula e as ornamentações dos esporos foram esclarecidas e contribuíram na separação dos quatro subgêneros: *subg. Apoceros* (oito espécies), *subg. Dendroceros* (14 espécies), *subg. Nodulosus* (15 espécies) e *subg. Chicoraceus* (uma espécie). Assim, 68% (28 spp) das espécies do grupo foram reclassificadas em nível de subgênero (**Capítulo I**);

V. Foram evidenciadas estratégias de vida contrastantes que estão ligadas com nicho em *Dendroceros*. As espécies epífitas, *D. crispus* e *D. crispatus*, apresentaram diferentes respostas à dessecação, com gametófitos tolerantes aos períodos de seca, diferente dos gametófitos de *Phaeoceros carolinianus* e *Nothoceros vincentianus*. Da mesma forma, com resultados divergentes nos esporos, onde só *D. crispus* não apresentou efeitos negativos com a dessecação. Este tema precisa ser investigado em detalhes, devido a sua complexidade e importância ecológica (**Capítulo II**);

VI. Uma complexa demanda conflitante foi encontrada nos gametófitos e esporos dos antóceros em resposta à dessecação. Esta resposta pode ser comparada com a hipóteses do "*continuum DT*", onde cada fase da história de vida de uma planta pode apresentar diferentes estratégias na dessecação. Este fato permite uma melhor adaptação aos diferentes nichos colonizados pelos antóceros (**Capítulo II**);

VII. Os esporos e gametófitos juvenis de *Dendroceros crispus*, nas etapas iniciais do desenvolvimento, não apresentaram efeitos negativos sob a presença de *Nostoc* (colônias de *Nothoceros vincentianus*), alcançando taxas de sobrevivência e crescimento melhores que *N. vincentianus*. Ao menos parcialmente, observamos uma baixa especificidade entre *Nostoc* e táxons de antóceros (**Capítulo III**);

VIII. Em *N. vincentianus* foi observado um efeito negativo do *Nostoc* ao longo do desenvolvimento dos esporos. Isto pode ser explicado pela possível exigência de uma simbiose tardia, onde seria evitada a competição planta-cianobactéria nos estágios iniciais da germinação dos esporos. Desta forma, nossos resultados destacam a complexidade da simbiose *Nostoc*-antóceros (**Capítulo III**).