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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS (BIOLOGIA VEGETAL)

Taxonomia e filogenia dos gêneros *Nitella* e *Chara* (Charales, Charophyceae) baseada em dados moleculares e morfológicos com ênfase nas regiões Sudeste e Centro-Oeste do Brasil

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setembro - 2017

TAXONOMIA E FILOGENIA DOS GÊNEROS *NITELLA* E *CHARA* (CHARALES, CHAROPHYCEAE) BASEADA EM DADOS MOLECULARES E MORFOLÓGICOS COM ÊNFASE NAS REGIÕES SUDESTE E CENTRO-OESTE DO BRASIL

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Tese apresentada ao Instituto de Biociências do Câmpus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Doutor em Biologia Vegetal.

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À minha família: minha mãe, base de tudo;

Minha esposa: companheira e amada em todos os momentos;

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Resumo Geral

A ordem Charales da classe Charophyceae inclui seis gêneros (*Chara* Linnaeus, *Lamprothamnium* J. Grove, *Lychnothamnus* (Ruprecht) Leonhardi, *Nitella* C. Agardh, *Nitellopsis* Hy e *Tolypella* A. Braun) classificados numa única família (Characeae). O sistema de classificação mais amplamente utilizado foi proposto por Wood & Imahori, é baseado em exclusivamente em caracteres morfológicos e considera que as Characeae formam um contínuo de espécies. As 400 espécies do grupo foram reduzidas a 81 com muitas variedades e formas. Muitos autores apontaram inconsistências no sistema de Wood & Imahori e sugeriram estudos com novas ferramentas (especialmente ultraestruturais e moleculares). No Brasil, foram reportados os gêneros *Chara* e *Nitella*, com abordagens essencialmente florísticas. Este trabalho teve por objetivo aplicar marcadores moleculares (sequências dos genes plastidiais que codificam a subunidade grande da enzima ribulose 1-5 bifosfato carboxilase/oxigenase, *rbc*L – o gene que codifica a enzima maturase K, *mat*K; e os espaçadores nucleares 1 e 2 dos transcritos internos do DNA ribossômico, ITS1 e ITS2), bem como morfologia estrutural (todos caracteres morfológicos considerados diagnósticos) e ultraestrutural (microscopia eletrônica de varredura da ornamentação da parede dos oósporos), para: 1) caracterizar as espécies de *Chara* e *Nitella* com ênfase nas regiões Centro-Oeste e Sudeste do Brasil; 2) inferir relações filogenéticas entre as espécies brasileiras de *Chara* e *Nitella* com as de outras regiões do globo; e 3) associar dados morfológicos, ultraestruturais da parede dos oósporos e moleculares, visando detectar os caracteres que permitem definir diferentes espécies reconhecidas, bem como propor eventuais caracteres novos. Foram geradas doze novas sequências para os marcadores *rbc*L e *mat*K, e oito para ITS2 para seis espécies de *Chara*: *Chara braunii*, *Chara foliolosa*, *Chara guairensis*, *Chara haitensis*, *Chara hydropitys* e *Chara rusbyana*. Os dados reforçaram a visão de que alguns táxons infra-genéricos (como subgênero *Charopsis* e subsecção *Willdenowia*) não são naturais, enquanto algumas espécies (*C. foliolosa*, *C. haitensis*, *C. hidropitys* e *C. rusbyana*), anteriormente consideradas como variedades e formas de *C. zeylanica*, mostraram-se distintas nas análises dos três marcadores moleculares. Para *Nitella* geramos quarenta e duas novas sequências de *rbc*L, doze de ITS1 e vinte e três de ITS2 para cinco espécies: *Nitella acuminata*, *Nitella axillaris*, *Nitella elegans*, *N. flagellifera* e *Nitella microcarpa*. Dados sobre a parede oósporo confirmaram estudos prévios e os dados moleculares sugeriram a sinonimização de: *Nitella subglomerata* e *Nitella gollmeriana* com *Nitella acuminata*; e *Nitella axilliformis* com *Nitella axillaris*. A identidade entre as seqüências foi alta para os três marcadores, mesmo entre populações de *Nitella* geograficamente muito distantes, assim como a variação dentro de cada caráter morfológico analisado. Nossa primeira hipótese de que o número de espécies de *Chara* e *Nitella* descritas até o momento para as regiões Sudeste e Centro-Oeste encontra-se equivocado foi parcialmente comprovada, com redução devido à sinonimização de algumas espécies; porém, nenhuma espécie nova foi reconhecida. A segunda hipótese, que previa que algumas espécies de *Chara* e *Nitella* ocorrentes no Brasil apresentariam alta divergência de sequências, devido ao isolamento geográfico em período relativamente remoto, daquelas de outras regiões do mundo foi refutada.

Palavras chave: espaçadores transcritos internos do DNA ribossômico, *mat*K, morfologia, ornamentação da parede do oósporo, *rbc*L.

Abstract

The order Charales of the class Charophyceae includes six genera (Chara Linnaeus, *Lamprothamnium* J. Grove, *Lychnothamnus* (Ruprecht) Leonhardi, *Nitella* C. Agardh, *Nitellopsis* Hy and *Tolypella* A. Braun) classified in a single family (Characeae). The most widely used classification system, proposed by Wood and Imahori, is based exclusively on morphological characters and considers that within Characeae there is a continuum of species. The 400 species of the group were reduced to 81 with many varieties and forms. Many authors pointed out inconsistencies in this system and suggested studies applying new tools (especially ultrastructural and molecular). In Brazil, only the genera *Chara* and *Nitella* were reported, mostly in floristic studies. The aim of this investigation was to apply molecular markers (sequences of the plastid genes coding for the large subunit of the ribulose 1-5 biphosphate carboxylase/oxygenase enzyme, *rbc*L - and the maturase K enzyme coding gene, *mat*K; internal transcriber spacer of nuclar ribosomal DNA, ITS1 and ITS2), as well as morphological (all diagnostic characters currently used) and ultrastructural (scanning electron microscopy of oospore wall ornamentation), to: 1) characterize the *Chara* and *Nitella* species with emphasis in the Midwest and Southeast regions of Brazil; 2) to infer phylogenetic relationships between the Brazilian species of *Chara* and *Nitella* with those from other regions of the globe; and 3) to associate morphological, ultrastructural oospore wall and molecular data, in order to determine characters that can be applied to define species, as well as to propose possible new characters. Twelve new sequences were generated for the *rbc*L and *mat*K markers, and eight for ITS2 for six species of *Chara*: *Chara braunii*, *Chara foliolosa*, *Chara guairensis*, *Chara haitensis*, *Chara hydropitys* and *Chara rusbyana*. The data reinforced the view that some infra-generic taxa (such as *Charopsis* subgenus and *Willdenowia* subsection) are not natural, whereas some species (*C. foliolosa*, *C. haitensis*, *C. hidropitys* and *C. rusbyana*), previously considered as forms of C. zeylanica, were found to be distinct in the analyzes of the three molecular markers. For Nitella we generated forty-two new *rbc*L sequences, twelve of ITS1 and twenty-three of ITS2 for five species: *Nitella acuminata*, *Nitella axillaris*, *Nitella elegans*,*Nitella flagellifera* and *Nitella microcarpa*. Data on the oospore wall confirmed previous studies and the molecular evidences suggested the following synonyms: *Nitella subglomerata* and *Nitella gollmeriana* with *Nitella acuminata*; and *Nitella axilliformis* with *Nitella axillaris*. Identity values among the sequences were high for the three markers, even among populations of *Nitella* geographically very distant, with wide variation for morphological characters. Our first hypothesis that the number of *Chara* and *Nitella* species described so far for the Southeast and Midwest regions is equivocal was partially confirmed, with a reduction due to the proposal of synonyms for some species; however, no new species was recognized, contradicting the second part of this hypothesis. The second hypothesis, which predicted that some species of *Chara* and *Nitella* occurring in Brazil would present high sequence divergence due to ancient geographic isolation, from those of other regions of the world was refuted.

Key words: internal transcribed spacers, *mat*K, morphology, oospore wall ornamentation, *rbc*L.

SUMÁRIO

INTRODUÇÃO GERAL

A ordem Charales da classe Charophyceae inclui seis gêneros: *Chara* Linnaeus, *Lamprothamnium* J. Grove, *Lychnothamnus* (Ruprecht) Leonhardi, *Nitella* C. Agardh, *Nitellopsis* Hy e *Tolypella* (A. Braun) A. Braun; todos são classificados dentro da família Characeae (Wood & Imahori 1965). É um grupo de algas cosmopolita, que habitam principalmente ecossistemas de água doce e, secundariamente, água salobra (Wood & Imahori 1965). Segundo Wood & Imahori (1965) e Sakayama (2008), membros da família Characeae são caracterizados por possuírem talo macroscópico e diferenciado em nós e entrenós que se alternam ao longo de um eixo principal. Estes nós suportam ramos verticilados com crescimento limitado. Os órgãos reprodutivos masculino e feminino (glóbulo e núcula, respectivamente) são formados nos nós dos ramos. As estruturas femininas apresentam um envelope protetor formado por cinco células tubulares espiraladas que se posicionam ao redor da célula ovo. No topo das células tubulares, divisões transversais dão origem a uma ou duas células coronais (coroa). Após a fecundação, a célula ovo produz um zigoto resistente e de parede grossa, denominado oósporo.

Segundo Karol (2004), numerosos sistemas de classificação baseados em dados morfológicos têm sido propostos para as Charales (Halsted 1879; Robinson 1906; Groves & Bullock-Webster 1920; Groves & Bullock-Webster 1924; Wood 1951; Allen 1954). O trabalho de Wood & Imahori (1965) é bastante completo e vem sendo amplamente utilizado como referência para estudos taxonômicos. Na concepção de Wood & Imahori (1965), as Characeae formam um contínuo de espécies, variedades e formas taxonômicas definidas por limites, em geral, pouco e/ou mal definidos, porque supostamente, constituem um grupo de evolução contínua, desde os primórdios do pré-Pleistoceno até hoje. Muitas dessas espécies, variedades e formas taxonômicas foram estabelecidas sem circunscrições bem definidas, com muitas sobreposições entre elas. Ainda segundo Wood & Imahori (1965), melhor seria interpretar esta intergradação de limites como reflexos da miscigenação e infertilidade, de modo que, na prática, mais apropriado seria unir essas ‘espécies’ em outras, com circunscrição mais ampla, porém, melhor definidas e separadas. Wood & Imahori (1965) denominaram as espécies sem circunscrição muito bem definida de microespécies e as de circunscrição mais ampla de macroespécies.

Desta forma, o sistema proposto por Wood & Imahori (1965) sugeriu que as cerca de 400 espécies de Characeae descritas até então fossem reduzidas à níveis taxonômicos infra-específicos (ou seja, variedades ou formas) ou admitidos como sinônimos, resultando em um sistema com apenas 81 espécies.

Chara e *Nitella* são os dois maiores gêneros dentro das Charales (Wood & Imahori 1965), ambos cosmopolitas, sendo representados em todas as regiões temperadas e tropicais, exceto em algumas ilhas isoladas. *Nitella* ocorre preferencialmente em águas neutras ou moderadamente ácidas, sendo mais raras em condições alcalinas (Pal 1932, Zaneveld 1940, Olsen 1944, Imahori 1954, Corillion 1957, Wood & Imahori 1965), enquanto *Chara* ocorre tanto em águas ácidas quanto em alcalinas, mas a grande maioria dos relatos de ocorrência é em hábitats alcalinos (Wood & Imahori 1965). Karol (2004) afirmou em sua monografia, que Wood & Imahori (1965) delimitaram os táxons baseados na "morfologia macro e microscópica" mais do que nas características filogenéticas chave (sinapomorfias). Com isso, reduziram as aproximadamente 180 espécies de *Nitella* conhecidas até o ano de 1959 em níveis infraespecíficos (variedades e formas) ou as agrupou como sinônimos, reduzindo-as a apenas 49 espécies com circunscrição ampla; enquanto que as 116 espécies de *Chara* foram reduzidas a apenas 19 (Wood & Imahori 1965).

Trabalhos taxonômicos clássicos sobre a família Characeae feitos previamente à monografia de Wood & Imahori (1965), foram realizados em diversas partes do mundo, dos quais destacamos: para a Europa as publicações de Migula (1890-1897), Hy (1913, 1914), Groves & Bullock-Webster (1920, 1924), Olsen (1944), Corillion (1957); para a Australásia os trabalhos de Nordstedt (1891), Groves & Allen (1927), Pal (1932), Zaneveld (1940), Imahori (1954); e para a América do Norte, os trabalhos de Allen (1888, 1954), Robinson (1906) e Wood (1948). Entre os estudos executados após 1965, destacam-se para a América do Norte os trabalhos de Wood (1967), Mann (1989), Langangen *et al*. (1996), Mann *et al*. (1999); para a Ásia e Australásia Wood (1972), Blazencic & Temniskova-Topalova (1991), Garcia (1999); e para a Europa os trabalhos de Langangen (1974), Moore (1986), Blazencic *et al.* (1990), Simons & Nat (1996) e Krause (1997).

Com o advento da Sistemática Molecular e o aperfeiçoamento das técnicas de análise filogenética, outros trabalhos têm sido desenvolvidos englobando

espécies de Characeae em estudos mais abrangentes geografica e taxonomicamente. Neste aspecto, merecem destaque os trabalhos de McCourt *et al*. (1996a, 1996b, 1999), Karol (2004) e os estudos de Sakayama e colaboradores (Sakayama 2008, Sakayama *et al*. 2002, 2004a, 2004b, 2005, 2006); Boegle *et al*. (2010), Schneider *et al.* (2015, 2016). Autores como Mandal *et al*. (2002), Casanova (2005), Sakayama *et al*. (2002, 2004a, 2004b, 2005, 2006, 2009), Kato *et al*. (2008, 2010) têm utilizado ainda técnicas de microscopia eletrônica de varredura para descrever a ultraestrutura, tanto de aspectos externos quanto internos, da parede do oósporo. Em alguns casos, correlacionaram esses dados com evidências moleculares para gerarem árvores filogenéticas mais consistentes. Os resultados desses trabalhos demonstraram que o sistema proposto por Wood & Imahori (1965) apresenta sérios problemas, principalmente no que se refere ao agrupamento de diferentes espécies nos níveis infraespecíficos.

Problemas no sistema de Wood & Imahori (1965) já foram apontados em trabalhos mais antigos e baseados exclusivamente na morfologia. Enquanto Wood & Imahori (1965) alegavam que seu sistema era reflexo das relações evolutivas, Proctor (1980) apontou que tal sistema era baseado na conveniência, e não em inferências filogenéticas. Resultados de numerosos experimentos com espécies do gênero *Chara* (Proctor 1970, 1971, 1972, 1975, 1980; Proctor *et al*. 1971; Proctor & Wiman 1971; McCracken *et al*. 1966) também sugerem que existe uma diversidade de espécies maior do que a proposta por Wood & Imahori (1965).

No Brasil, o início efetivo das pesquisas com Characeae foi marcado pelos trabalhos de Bicudo (1968a, 1968b, 1969, 1972, 1974, 1976 1977, 1979) e Bicudo & Yamaoka (1978). Estudos de grande valor taxonômico tiveram continuidade com os trabalhos de Astorino (1983), Picelli-Vicentim (1990), Picelli-Vicentim & Bicudo (1993), Bueno *et al*. (1996, 2009, 2011a, 2011b e 2016), Bueno & Bicudo, (1997), Vieira *et al.* (2002, 2003), Picelli-Vicentim *et al*. (2004) e Meurer & Bueno (2012). Apesar de relativamente numerosos, todos estes trabalhos utilizaram apenas dados morfológicos clássicos para a identificação das espécies e na maioria deles o sistema de Wood & Imahori (1965) foi utilizado para a determinação das espécies e táxons infra-específicos. Nenhum destes trabalhos adotou dados moleculares ou caracterização da parede do oósporo em microscopia eletrônica de varredura.

O trabalho de Picelli-Vicentim *et al*. (2004), corresponde a uma síntese de vários outros trabalhos de vários pesquisadores e pode ser considerado uma das obras de maior relevância para o estudo dos gêneros *Chara* e *Nitella* no Brasil. Os autores relataram a ocorrência de vários espécimes cujas características divergiam das descritas nos trabalhos de Wood & Imahori (1965). Da mesma forma, vários autores (McCracken *et al.* 1966; Proctor 1970, 1971, 1972, 1975, 1980; Proctor *et al.* 1971; Proctor & Wiman 1971; MacCourt *et al.* 1996a, 1999; Karol 2004; Sakayama *et al.* 2004a, 2004b, 2005, 2006; Sakayama 2008;) sugerem fortemente a existência de um número maior de espécies do que o proposto por Wood & Imahori (1965).

Tomando-se como base os aspectos críticos apontados em investigações anteriores em âmbito mundial, bem como a aúsência de dados moleculares e de ultraestrutura da parede do oósporo para espécies de *Chara* e *Nitella* brasileiras, este estudo contempla os seguintes objetivos:

1) Caracterizar as espécies de *Chara* e *Nitella* analisadas com relação às características morfológicas tradicionais e ultraestruturais da parede dos oósporos com ênfase nas amostras das regiões Sudeste e Centro-Oeste do Brasil;

2) Inferir as relações filogenéticas entre as espécies de *Chara* e *Nitella* do Brasil e de outras regiões do globo com base nas sequências de DNA de diferentes marcadores.

3) Associar os dados morfológicos tradicionais, ultraestruturais da parede dos oósporos e moleculares, de forma a detectar os caracteres que permitem definir as espécies reconhecidas, bem como propor eventuais caracteres novos.

Foram testadas as seguintes hipóteses:

1) O número de espécies de *Chara* e *Nitella* descritas para as regiões Sudeste e Centro-Oeste encontra-se equivocado. Por um lado novas características morfológicas e moleculares servirão de subsídio para reconhecimento de um número maior de espécies do que o proposto por Wood & Imahori (1965), o que elevaria o número de espécies. Por outro lado, caracteres que têm sido usados tradicionalmente para delinear espécies (como comprimento dos dactilos e diâmetro do talo, entre outras) serão evidenciados como variáveis dentro e entre populações, o que nos levaria a propor que espécies descritas para o Brasil sejam sinônimos.

2) Algumas espécies de *Chara* e *Nitella* ocorrentes no Brasil apresentarão, devido ao isolamento geográfico em período relativamente remoto, sequências de DNA bastante divergentes daquelas de outras regiões do mundo (especialmente da América do Norte, Europa Ásia e Austrália). Esta hipótese possibilitaria inclusive a proposição de novas espécies, algumas crípticas e outras com variações em caracteres morfológicos não empregados tradicionalmente na identificação em nível específico.

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CAPÍTULO 1

Taxonomy and phylogeny of *Chara* (Charophyceae, Characeae) from Brazil with emphasis on the midwest and southeast regions

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Taxonomy and phylogeny of *Chara* (Charophyceae, Characeae) from Brazil with emphasis on the midwest and southeast regions

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Abstract

South American studies on the genus *Chara* are relatively scarce, most consisting of floristic surveys and using only traditional morphological characters. This study is a first approach to the systematics of the genus *Chara* applying modern techniques (DNA sequences and oospore SEM analyses) in addition to the alpha-taxonomy investigations that have been conducted in Brazil. Twelve populations of *Chara* were analyzed from the midwest and southeast regions of Brazil. Sequences of three molecular markers were applied to infer phylogenies. The ultrastructure of the oospore wall and currently used morphological characters were analyzed for *Chara* populations. Maximum likelihood and Bayesian analyses of sequences of *rbc*L, ITS2, and *mat*K were congruent in that they grouped the species in six clades, each representing one species: *Chara braunii* C.C. Gmelin, *C. foliolosa* C.L.Willdenow, *C. guairensis* R.Bicudo, *C. haitensis* M.P.J.F. Turpin, *C. hydropitys* H. Reichenbach and *C. rusbyana* M. Howe. Morphological characters, including ultrastructure of oospore wall, provided good evidences to characterize each species. Molecular data supported the recent view that some traditional infra-generic taxa (e.g. subgenus *Charopsis* and subsection *Willdenowia*) are not supported in phylogenetic analyses, whereas some species (e.g. *C. foliolosa*, *C. haitensis*, *C. hydropitys* and *C. rusbyana* previously considered as varieties and forms of *C. zeylanica*) were consistently distinguished in the analyses for the three molecular markers.

Introduction

Charophytes, extant and fossil members of the order Charales plus the members of the extinct orders Sycidiales and Moellerinales (Feist *et al.* 2005, Schneider *et al.* 2015a), are algae with a complex morphology (Fritsch 1935, Pickett-Heaps 1975, Sanders *et al.* 2003 Schneider *et al.* 2016). The order consists of six extant genera—*Chara*, *Lamprothamnium*, *Lychnothamnus*, *Nitella*, *Nitellopsis* and *Tolypella* (Wood & Imahori 1965, Karol 2004), and over 700 species (Guiry & Guiry 2016).

According to Nowak *et al.* (2016), the taxonomy of charophyte algae relies on an almost purely phenetic species concept, i.e. the classification is based on overall similarities in the morphological characters of the organisms. They assume that there are two competing concepts at species level for charophytes: a microspecies concept (e.g., Krause 1997) that posits the existence of approximately 30 distinct European *Chara* species, and a macrospecies concept (Wood & Imahori 1965) that recognizes only 18 distinct species of genus *Chara* globally. The macrospecies tend to be polymorphic, with each macrospecies encompassing a number of subspecies, varieties, and forms—most of which are raised to the species level in the microspecies concept. The advocates of both concepts apply almost exclusively vegetative traits as diagnostic characters (especially the number of cortex cells and the number, shape, and length of stipulodes and spines), and differ only in their conclusions regarding the taxonomic relevance of these traits.

In order to contribute to the taxonomy of Characeae, and following the trend found in the other groups of organisms, several phylogenetic studies based on molecular data have been carried out using different molecular markers such as: ITS1 and ITS2 (internal transcribed spacers regions 1 and 2 of the nuclear ribosomal DNA), *mat*K (plastid-encoded protein-coding gene, resident within a group II intron of the plastid encoded lysine tRNA), *rbc*L (plastid-encoded gene encoding the large subunit of ribulose 1, 5 bisphosphate carboxylase/oxygenase), *atp*B (gene encoding the beta sub- unit of ATP synthase), and 18S rDNA (Schneider *et al.* 2015, Norwak *et al.* 2016, Schneider *et al.* 2016). Some of these studies have shown that morphological characters traditionally used to separate clades (such as cortication,

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spines shape or sexual differentiation) do not reflect the phylogeny of the groups. Schneider (2015) pointed out that species delineation of charophytes based only on morphological traits is more complicated than it might seem, because of: (i) there is considerable overlap in morphological characteristics used to discriminate species (Boegle *et al.* 2007), and (ii) phenotypic plasticity in charophytes may be environmentally induced, e.g. by light, water temperature, nutrient concentrations, and salinity (Wood & Imahori 1965, Schneider *et al.* 2015). Such plasticity makes it difficult to know which morphotypes are environmentally induced and which ones are genetically controlled (Boegle *et al.* 2010). On the other hand, Schubert (2014) argues that with new techniques available, it was shown that even microspecies are subdivided by lines of reproductive incompatibility (Proctor 1971, Grant and Proctor 1972) shedding new light on intraspecific diversity, as well as global biogeography (e.g., Proctor 1975, Proctor 1980).

Sakayama *et al.* (2005 and 2008) conciliated the characteristics of the oospore wall ultra-structure of *Nitella* with molecular data, demonstrating that this is an important feature for the separation of taxa within the genus. Similar studies for the *Chara* genus were conducted (Mandal *et al.* 2002, Casanova 2005, Sakayama *et al.* 2009, Kato *et al.* 2010, 2014). However, John *et al.* (1990) stated that surface features are considered to be taxonomically less significant in *Chara* as compared to *Nitella*, because the former genus presents fewer types of ornamentation.

Relevant investigations on Characeae, after the monograph of Wood & Imahori 1965, were carried out in Europe (Krause 1997, Mandal *et al.* 2002, Blume *et al.* 2009, Schneider *et al.* 2015 and 2016, Norwak *et al.* 2016), Australasia (Casanova 2005 and 2014), Asia (Kato *et al.* 2010; Sakayama *et al.* 2002, 2005, 2006, 2008 and 2015) and North America (McCourt *et al.* 1996, Sanders *et al.* 2003; Karol *et al.* 2004,Cartajena & Carmona 2009, Pérez *et al.* 2014). South American studies are relatively scarce, most of them consisting of floristic surveys and not applying modern approaches (molecular evidences or evaluation of the oospore wall by scanning electron microscopy). A few South American species have been included in studies of phylogeny based on molecular data and carried out in other continents, such as three Argentinean specimens used in the investigations of Schneider *et al.* (2015).

Although Brazil is a country of continental dimensions, the studies on Characeae are relatively scarce, and all are based on morphological characters only. Twenty-three species of *Chara* have been reported from Brazil (Bicudo 1972, 1974, 1979, Vieira *et al.* 2003, Picelli-Vicentim *et al.* 2004, Prado *et al.* 2005, Bueno *et al.* 2009, 2011): *Chara angolensis* A.Braun*, Chara braunii* Gmelin, *Chara bulbillifera* (Donterberg) A.Garcia, *Chara compressa* Kunth, *Chara diaphana* (Meyen) R.M.T.Bicudo, *Chara drouetii* R.D.Wood, *Chara fibrosa* C.Agardh *ex* Bruzelius *emend*. R.D.Wood*, Chara foliolosa* Mullenberg *ex* Willd., *Chara formosa* Robinson, *Chara globularis* Thuillier *emend.* R.D.Wood, *Chara guairensis* R.M.T.Bicudo, *Chara haitensis* Turpin, *Chara hydropitys* H.Reichenbach, *Chara hispida* L. *emend*. R.D.Wood, *Chara hornemannii* Wallman *emend.* R.D.Wood*, Chara indica* Bertero *ex* Spreng., *Chara kenoyeri* Howe, *Chara martiana* Wallman, *Chara pseudohydropitys* Imahori, *Chara rusbyana* Howe, *Chara socotrensis* Nordst., *Chara vitalii* R.M.T. Bicudo, *Chara vulgaris* L. *emend.* R.D. Wood, *Chara zeylanica* Klein ex Willd.

Considering the lack of studies on Brazilian Characeae involving modern tools, this study was carried out aiming to: i) conduct a first approach to the systematics of the genus *Chara* applying modern techniques (DNA sequences and oospore SEM analyses) in addition to the alpha-taxonomy investigations that have been made in Brazil; ii) characterize *Chara* species from the Southeast and Mid-west regions of Brazil applying traditional and ultrastructural morphological characters of oospore walls; iii) infer the phylogenetic relationships among species from Brazil and from other regions of the globe based on the *rbc*L, *mat*K and ITS2; iv) correlate the traditional morphological features, the ultrastructural ornamentation of oospores wall and molecular data, in order to propose a more reliable species definition for the species found in Brazil compared to those found in other regions of the world.

Material and methods

We analyzed 12 samples collected in three Brazilian states (Fig. 1). Specimens were collected directly by hand and divided into three sets for distinct purposes: morphology, oospore ultrastructure and molecular.

Morphological methods. Specimens were preserved in formaldehyde 4% and lodged at herbaria SJRP (Thiers *et al.* 2016). The identification at specific and/or infra-specific levels, as well as the selection of the main diagnostic characters, followed the Wood & Imahori (1965) system and Brazilian specific references (Bicudo 1974, Picelli-Vicentim 1990, Bueno & Bicudo 1997, Vieira *et al.* 2002, Bueno *et al.* 2009, 2011). After identification, the currently accepted taxonomic status was checked at Algae Base (Guiry & Guiry 2016) and recent references were consulted.

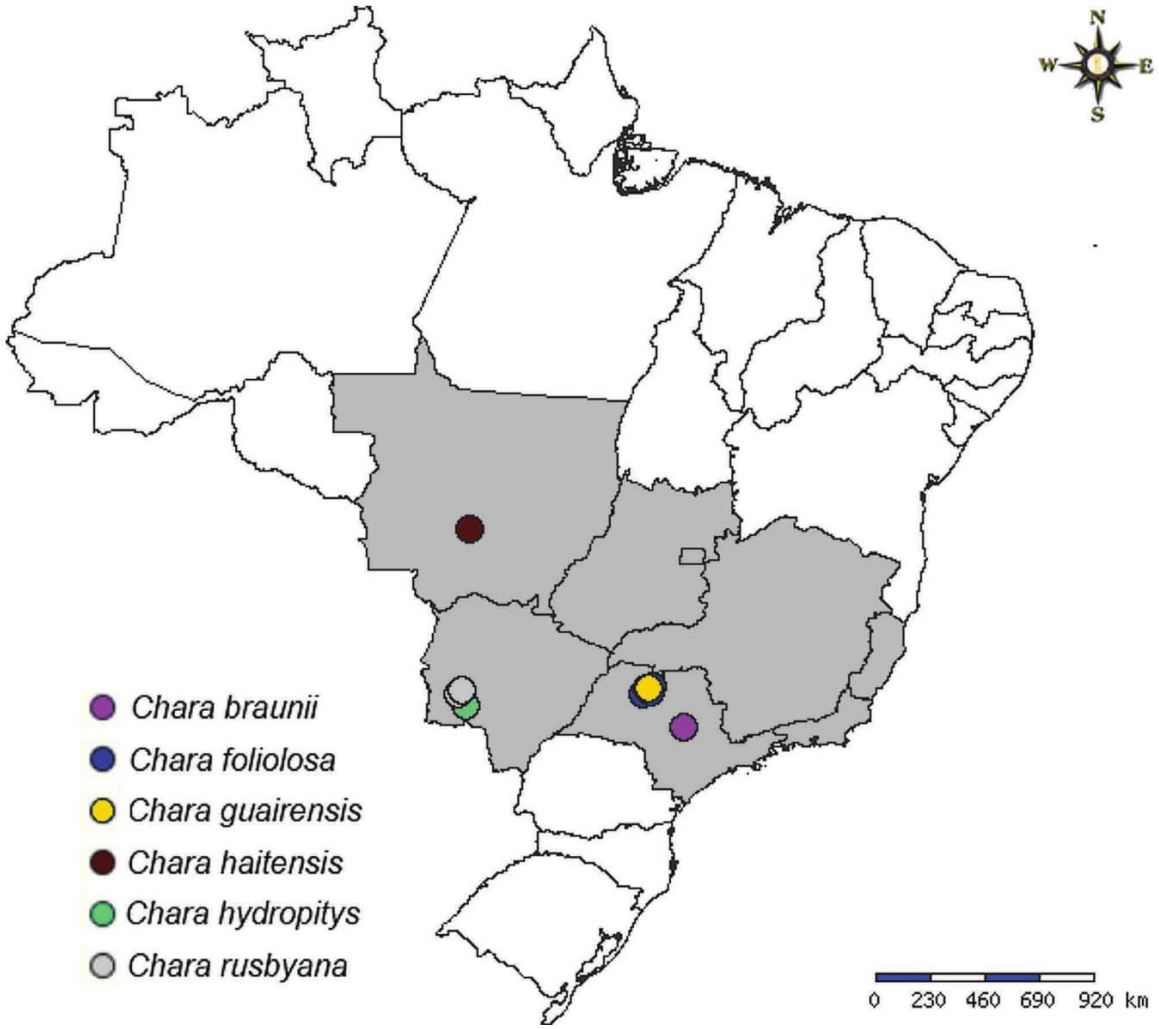


FIGURE 1. Map of Brazil with the location of sampling points. The area highlighted in gray corresponds to the study area.

The following formula was used to determine the minimum number of measurements to be taken for each morphometric character (Southwood & Henderson 2009): $n = (s/Ex)^2$, where s=standard deviation, E= the predetermined standard error (in this case 0.05), x = mean. For the vast majority of the diagnostic characters we have verified the need for less than 25 measurements, so we have adopted this number as the standard for measurements of all characters.

Photomicrographs were prepared using a Leica DFC 320 digital camera using LAS capture and image analysis software coupled to a Leica DM5000 microscope (Leica Microsystems, Wetzlar, Germany).

Ultrastructure of the oospore wall. Mature oospores for ultrastructural analyses could be recovered for tree species only. Oospores were cleaned and placed in bottles containing Carnoy's solution (60% ethanol, 30% chloroform and 10% glacial acetic acid—Grimstone & Skaer 1972). Before examination, oospores were cleaned by incubation in 10% Triton-X 100 at 60°C for at least 12 hours. They were subjected to acetolysis (Erdtman 1960; Faegri & Iversen 1989) and then transferred to a 15ml centrifuge tube containing distilled water to be vigorously vortexed for 3 min. The oospores were then dehydrated in an ethanol series and mounted on aluminium stubs with double-sided adhesive tape, air-dried, sputter-coated with gold and observed with a Zeiss DSM 940A scanning electron microscope (Oberkochen, Germany).

Oospore surface details were described by adapting the terminology adopted by Faegri & Iversen (1964) for pollen grain analysis, and using the terminology used by John & Moore (1987), Leitch *et al*. (1990) and Casanova (1991) for Charophyte oospores.

Molecular phylogenetic analysis. Fresh individuals were cleaned immediately after collection with fine-tipped tweezers and preserved in silica desiccant. The extraction, amplification, purification and sequencing steps followed the protocols described in Necchi *et al.* (2016), except for the primers and thermal cycles. The entire *rbc*L gene was amplified and sequenced in two fragments, using the following primer pairs: RH1-F (Zurawski & Clegg 1987) and *rbc*L-972R (Karol 2004), for the 5'gene fragment; and *rbc*L-295F (Karol 2004) and 1385-R (Manhart 1994) for the 3'fragment. The cycle used was described by McCourt *et al.* (1995) and modified by Manhart (1994). For ITS2, we used the primers ITS3 and ITS4 (White *et al.* 1990) and the cycle described by Sakayama *et al.* (2004). *mat*K was amplified using four pairs of primers (Schneider *et al.* 2016): F-*mat*K-*Chara* and R-*mat*K-*Chara*; *Chara*-*mat*K-BT2F and *Chara*-*mat*k-BT2R; *mat*KF2 and *mat*KR1a; and *Chara_mat*KF2 and *Chara_mat*KR2. PCR for all different primer combinations of *mat*K cited was performed with an initial five-minute 94°C denaturation step and one minute each of denaturation (94°C), annealing (55° C) and polymerization (72°C) for 15 cycles, followed by one minute each of denaturation (94°C), annealing (52°C) and polymerization (72°C) for 20 cycles before the final elongation step (10 min). Sequences from Genbank were used to elucidate the phylogenetic positioning of the Brazilian *Chara* species.

For phylogenetic analyses the best-fit model of sequence evolution by the Akaike Information Criterion was determined using jModelTest 2.1.4 (Darriba *et al.* 2012) for each molecular marker as follows: GTR + I for *rbc*L; HKY + I for ITS2; GTR + G for *mat*K. Separate analyses were conducted for each marker data set. A combined analysis with the three markers was not tried because of the completely different data set of GenBank sequences available for the markers altogether. Maximum likelihood (ML) topologies and bootstrap values from 1,000 replicates were inferred using the Randomized Accelerated Maximum Likelihood graphic user interface (RAxMLGUI version 1.2; Silvestro & Michalak 2011). Bayesian analysis (BA) was performed in MrBayes 3.2 (Ronquist & Huelsenbeck 2003) with three runs of five chains of Metropolis coupled Markov Chain Monte Carlo for 5,000,000 generations. 500,000 chains were removed as burn-in prior to determining the posterior probabilities.

Results and Discussion

Twelve new sequences of *rbc*L and of *mat*K and eigth of ITS2 were generated in this study. The tree topologies obtained with *rbc*L (Fig. 2), ITS2 (Fig. 3) and *mat*K (Fig. 4) data were congruent in that they separated sequences from Brazilian samples into six different clades, with high support, corresponding to six species: *Chara braunii*, *C. haitensis*, *C. rusbyana, C. foliolosa*, *C. hydropitys* and *C. guairensis*.

Clade 1 includes *Chara braunii* specimen (Fig. 2 and 4) and exhibited identity values ranging from 99,7% to 99,8% for *rbc*L marker among our specimen and two Japanese and one New Zealander specimens. The ITS2 marker was not amplified. This species is cited for all continents except Africa and Antarctica (Guiry & Guiry 2016), but Langangen (2015) describes *C. braunii* from Africa. In Brazil this species was described in several studies (Vieira *et al.* 2003, Picelli-Vicentim *et al.* 2004, Bueno *et al*. 2011b, Meurer & Bueno 2012).

Clade 2 was represented by *Chara rusbyana* (Figs. 2–4) and identity values ranged from 99,8% to 100% for *rbc*L among our specimens and 99,8% between our species and that of Genbank (from Argentina). For ITS2, the identity value was 95,8% between our species and that of Genbank (unknown origin). *C. rusbyana* is reported only for South America (Guiry & Guiry 2016), and in Brazil is often reported in several studies (Picelli-Vicentim *et al.* 2004; Bueno *et al*. 2009, 2011b; Prado & Baptista 2005).

Chara haitensis composed Clade 3 (Figs 2–4). Identity values were as follows: *rbc*L—99,7% between our sequence and that of Genbank (from Mexico); ITS2—95,4% with a sequence from Dominican Republic. *C. haitensis* is described for North, Central and South Americas (Guiry & Guiry 2016), with only two records for Brazil (Wood & Imahori 1965, unspecified collection site; and Bicudo 1972 and 1974- same material from Minas Gerais State).

Chara guairensis specimens formed Clade 4 (Figs 2–4). This is an endemic species from Brazil and relatively widespread in some regions (Vieira *et al.* 2003, Picelli-Vicentim *et al.* 2004, Bueno *et al*. 2009, 2011b, 2012). To date, there are no GenBank *rbc*L, ITS2 and *mat*K sequences for this species.

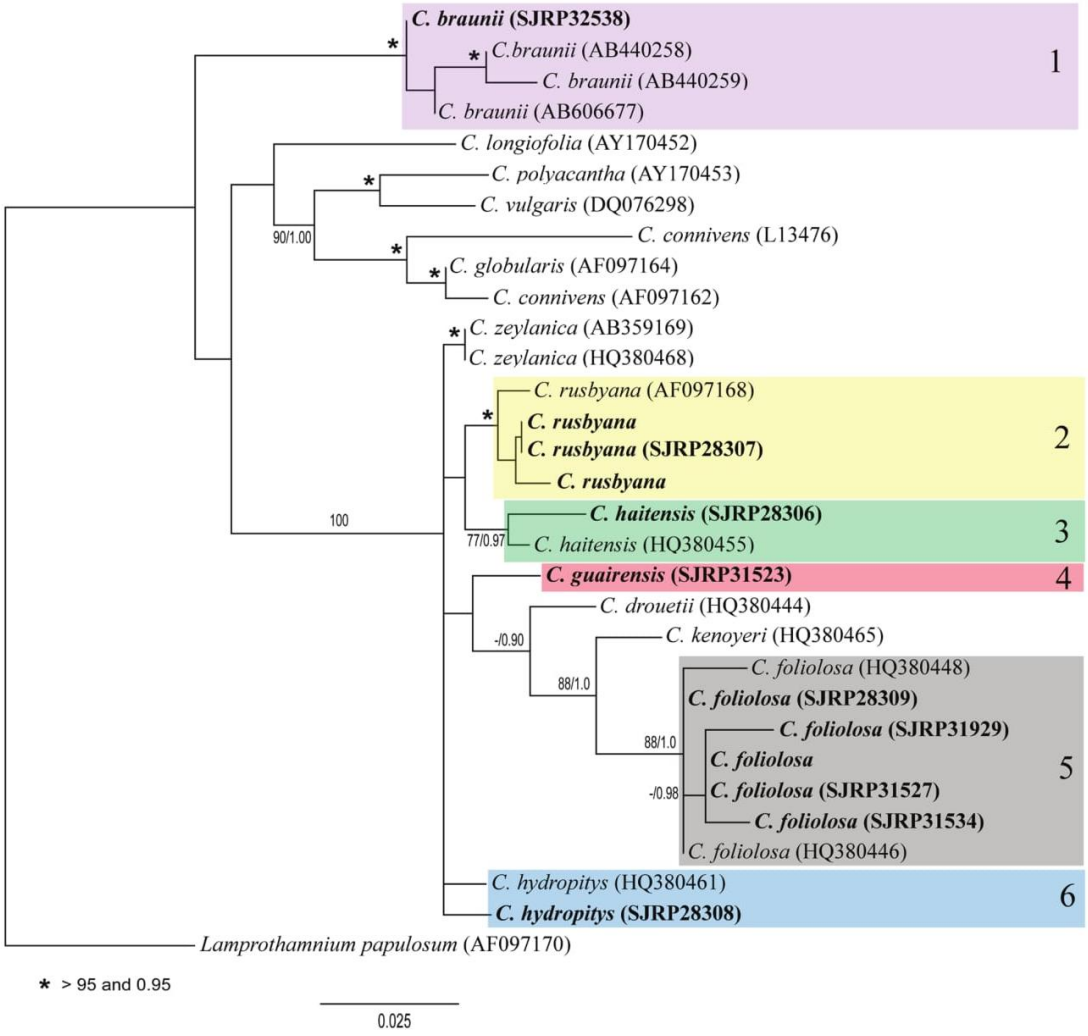


FIGURE 2. ML Phylogenetic tree based on *rbc*L sequences. The numbers associated with the nodes indicate the bootstrap values (BS) for maximum likelihood and posterior probability (PP) for Bayesian analysis; nodes without values indicate BS < 70% and PP <0.70. Sequences in bold are the new sequences generated. The clades formed are: 2—*C. rusbyana*, 3—*C. haitensis*, 4—*C. guairensis*, 5—*C. foliolosa*, 6—*C. hydropitys*.

Specimens of *C. foliolosa* composed Clade 5 (Figs 2–4). Identity values were as follows: *rbc*L—99,5% to 99,9% among the specimens of the clade, including one of Genbank (from U. S. A.); ITS2—96,3% to 99,4% between our sequence and two from Genbank (from Mexico and U. S. A.). The species is described for the whole South-american continent (Guiry & Guiry 2016). Proctor *et al*. (1971) reported the presence of *C. foliolosa* also in Japan and India. The New York Botanical Garden Herbarium has material attributed to this species from North and Central America and Asia (India).

Clade 6 was formed by specimens of *Chara hydropitys* (Figs 2–4). Identity values were: *rbc*L—99,7% between our sequence and that of Genbank (from Mexico); ITS2—97,7% with a sequence from Mexico. *C. hydropitys* is distributed throughout the North, Central and South-american continents and some countries in Asia (Guiry & Guiry 2016), whereas in Brazil it is widely distributed and cited in several studies, often referred to as *Chara fibrosa* var. *hydropitys* (Bueno *et al.* 2009, 2011b, Siqueira & Bueno 2012, Meurer & Bueno 2012).

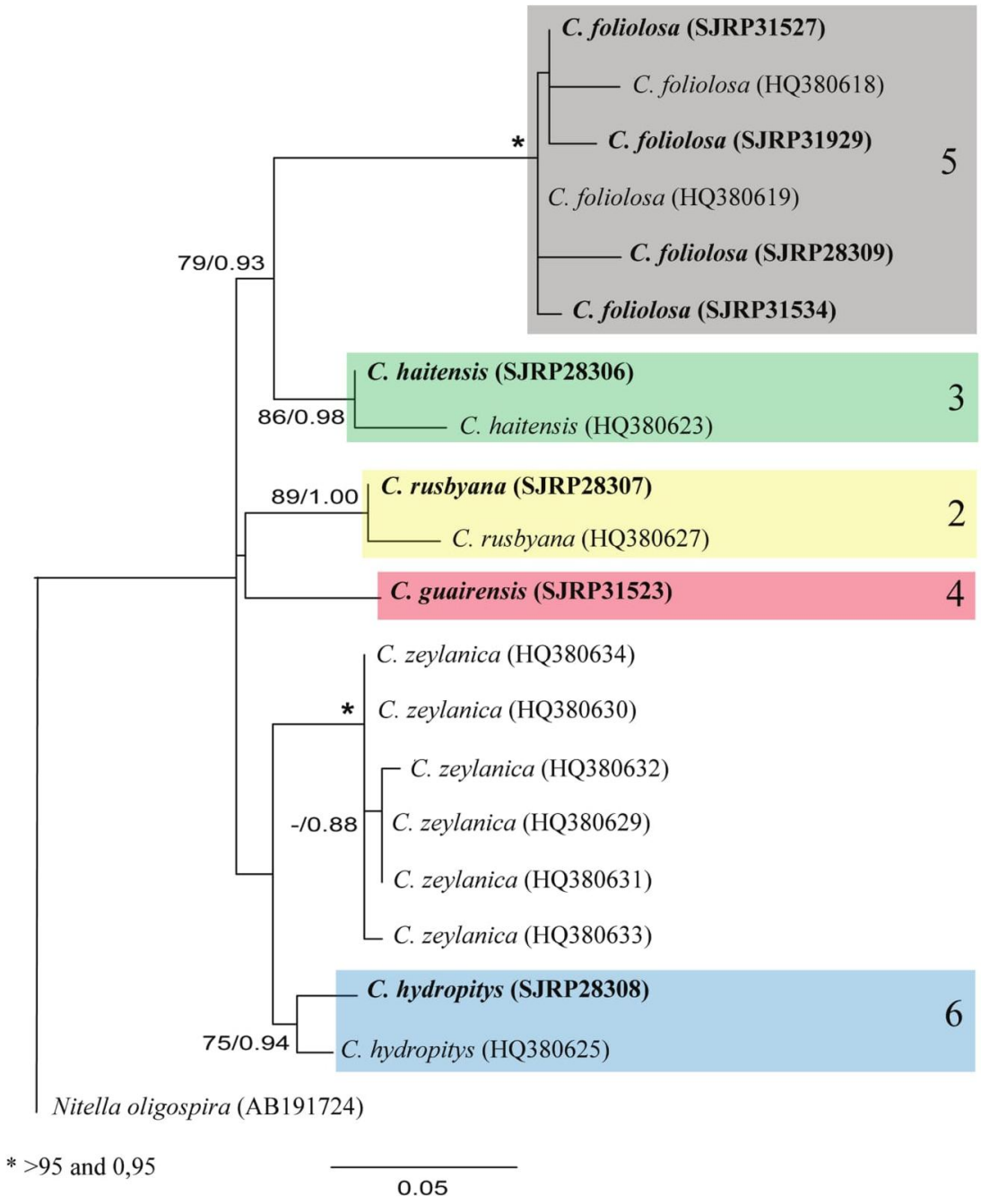


FIGURE 3. ML Phylogenetic tree based on ITS2 sequences. The numbers associated with the nodes indicate the bootstrap values (BS) for maximum likelihood and posterior probability (PP) for Bayesian analysis; nodes without values indicate BS < 70% and PP <0.70. Sequences in bold are the new sequences generated. The clades formed are: 1—*C. braunii*, 2—*C. rusbyana*, 3—*C. haitensis*, 4—*C. guairensis*, 5—*C. foliolosa*, 6—*C. hydropitys*.

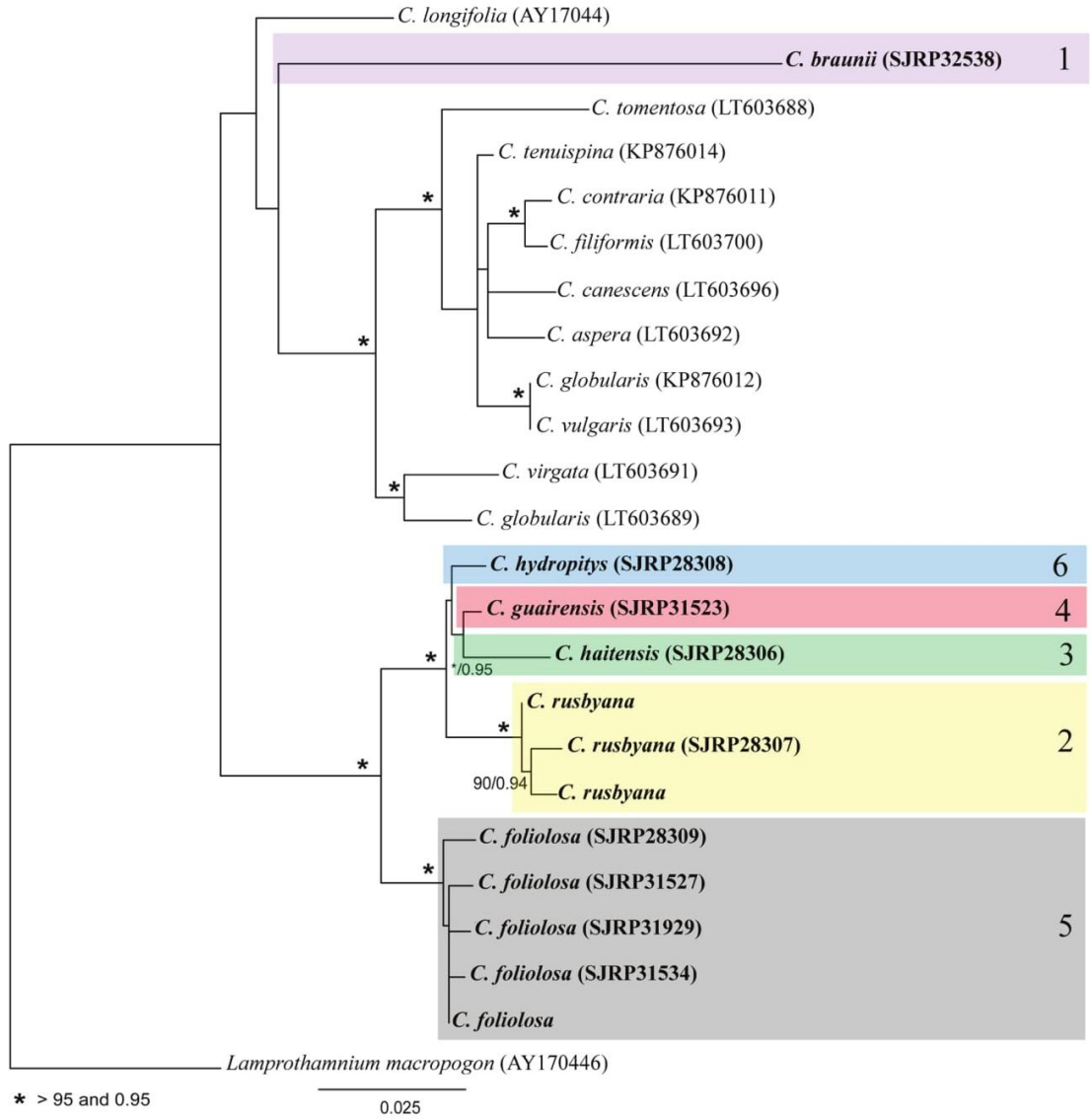


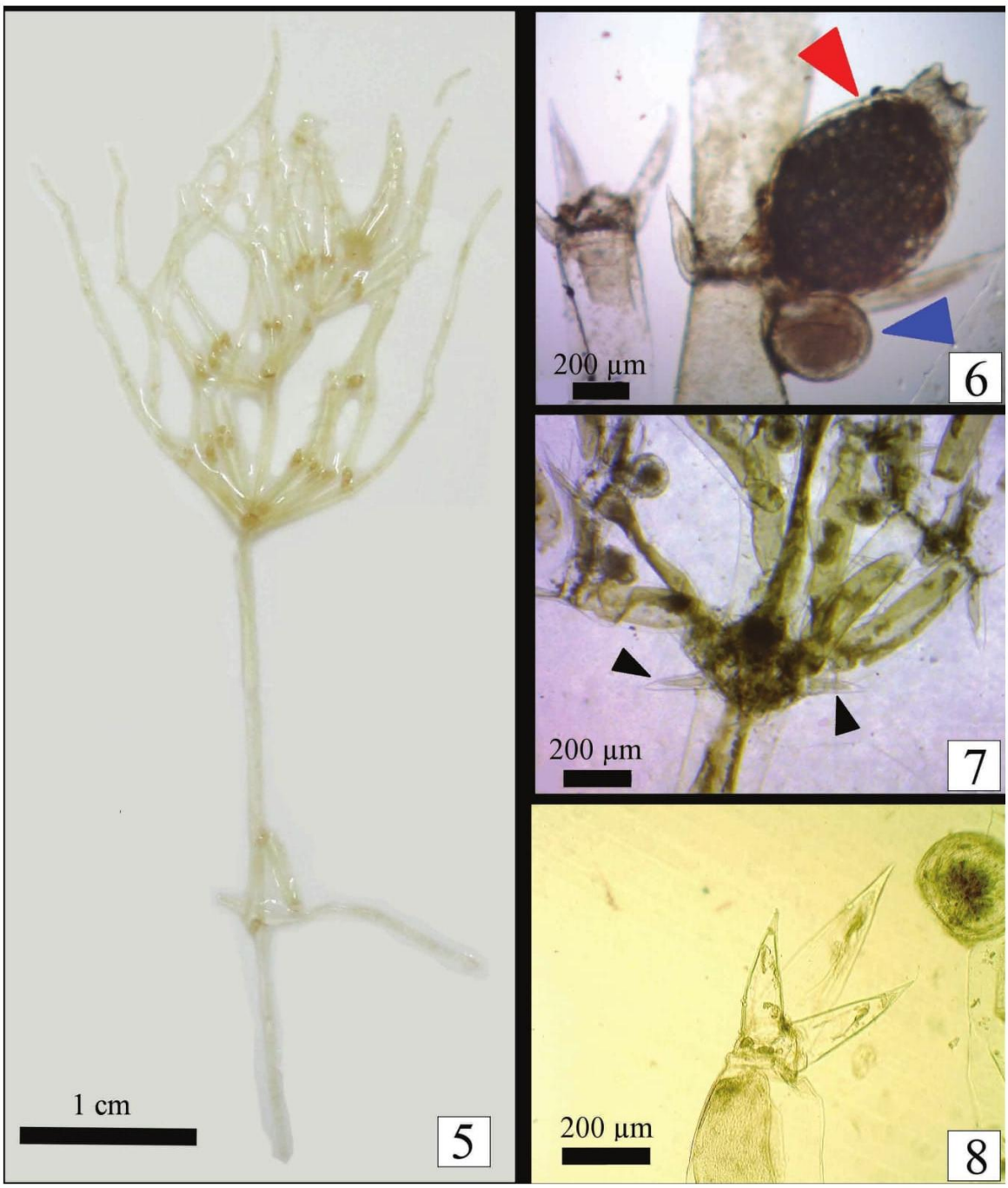
FIGURE 4. ML Phylogenetic tree based on *mat*K sequences. The numbers associated with the nodes indicate the bootstrap values (BS) for maximum likelihood and posterior probability (PP) for Bayesian analysis; nodes without values indicate BS < 70% and PP <0.70. Sequences in bold are the new sequences generated. The clades formed are: 1—*C. braunii*, 2—*C. rusbyana*, 3—*C. haitensis*, 4—*C. guairensis*, 5—*C. foliolosa*, 6—*C. hydropitys*.

Species description

Chara braunii C.C.Gmelin 1826: 646
Fig. 5–8

Plants monoecious, up to 10.1 cm high, without calcium carbonate deposition. Axes 255–649 µm in diameter; internodes 0.1–1.15 times longer than the branchlets, to 1.85 cm length; ecorticated; spine-cells none. Stipulodes in one whorl, one per branchlet, never exceeding basal branchlet segment; 120–437 µm long, 65–152 µm in diameter, acuminate. Branchlets 7–10 forming a whorl, 1.0–2.7 cm long; intercalary segments 3–5, ecorticated, 0.1–0.6 cm long, 183–563 µm in diameter; basal segment ecorticated, 0.1–0.6 cm long, 193–782 µm in diameter; apical segment formed

by 1 ecorticated cell or forming a corona with the similar subtending bract-cells, 130–508 µm long, 73–131 µm in diameter. Bract-cells (2)3–4, forming whorls, the largest ones often directed towards the thallus, 185–637 µm long, 67–145 µm in diameter, acuminate. Bracteoles 2, 465–862 µm long, 70–150 µm in diameter, smaller than the oogonia. Gametangia conjoined, at the 1st and 2nd nodes. Oogonia 584–995 µm long, 330–736 µm in diameter; convolutions 8–11(12); coronula 140–223 µm high, 227–344 µm wide, apex divergent. Oospores dark brown to black, 610–866 µm long, 335–489 µm wide; striae of (9)10–11 low ridges; fossae 65–106 µm across; wall granulate. Antheridia 233–450 µm in diameter, octoscutate.



FIGURES 5–8. *Chara braunii* 5. Habit. 6. Gametangia conjoined. Oogonium (red arrow) and antheridium (blue arrow). 7. Thallus and branchlets ecorticated. The black arrow indicates stipulodes in one tier. 8. Apical segment of branchlet and two bract-cells around.

Specimen examined:—BRAZIL. São Paulo: Parque dos Saltos, Municipality of Brotas, 22º17'30.001"S, 48º7'50.999"W, 9 Oct 2012, coll. *O. Necchi Jr. et al*.

DNA sequences:—*rbc*L-KY656906; *mat*K-KY656917.

Chara foliolosa H.L.Mühlenberg ex C.L.Willdenow 1805: 86
Fig. 9–15

Plants monoecious, up to 45.6 cm high, without calcium carbonate deposition. Axes 305–999 µm in diameter; internodes 0.2–2.5 times longer than the branchlets, to 5.9 cm length; cortex 3 corticate, isostichous to slightly tylacanthous; spine-cells solitary, 56.7–1101 µm long, 26–83 µm in diameter at base. Stipulodes in two whorls, two times as numerous as the branchlets, exceeding basal branchlet segment, except at basal nodes; uppers 275–1337 µm long, 48–141 µm in diameter, acuminate; lowers 212–890 µm long, 49–127 µm in diameter, acuminate. Branchlets 7–15 forming a whorl, 0.6–5.7 cm long; intercalary segments 3–13, corticate, 370.1–9291 µm long, 124–695 µm in diameter; basal segment ecorticate, 195–1967 µm long, 135–620 µm in diameter; apical segment formed by 1 ecorticated cell,, 204–1606 µm long, 31–143 µm in diameter. Bract-cells 3–11, forming whorls, the largest ones often directed towards the thallus, 44–2135 µm long, 35–117 µm in diameter, acuminate. Bracteoles 2, 188–1320 µm long, 53–131 µm in diameter, larger or smaller than the oogonia. Gametangia sejoined, at the same or different branchlet, at the 2nd to 5th nodes. Oogonia 346–955 µm long, 147–637 µm in diameter; convolutions 9–12; coronula 69–271 µm high, 103–293 µm wide, apex divergent. Oospores dark brown, 612–919 µm long, 379–602 µm wide; striae of 10–14 low to prominent ridges; fossae 41–86 µm across; wall granulate. Oospore wall featureless to finely granulate in ultrastructure. Antheridia 247–831 µm in diameter, octoscutate.

Specimens examined:—BRAZIL. Mato Grosso do Sul: entrance to Recanto Ecológico Rio da Prata, Municipality of Jardim, 21°29'21.1''S, 56°26'16.6''W, 15 May 2014, Coll. *F.R. Borges* (SJRP28309). São Paulo: Talhadinho Stream, Municipality of São José do Rio Preto, elev. 502m, 20°43'45.5''S; 49°17'51.3''W, 4 September 2014, colls. *F.R. Borges* & *O. Necchi Jr.* (SJRP31929). Paulicéia, Municipality of José Bonifácio, elev. 440 m, 21°00'44.1''S; 49°41'21.6''W, 4 September 2014, colls. *F.R. Borges* & *O. Necchi Jr.* (SJRP31527). Municipality of Neves Paulista, elev. 530 m, 20°50'54.6''S; 49°34'13.7''W, 4 September 2014, colls. *F.R. Borges* & *O. Necchi Jr.* (SJRP31534). Rio das Pedras, Municipality of São José do Rio Preto, 20º56'1"S, 49º23'28"W, 02 July 2010, coll. *L.H.Z. Branco*

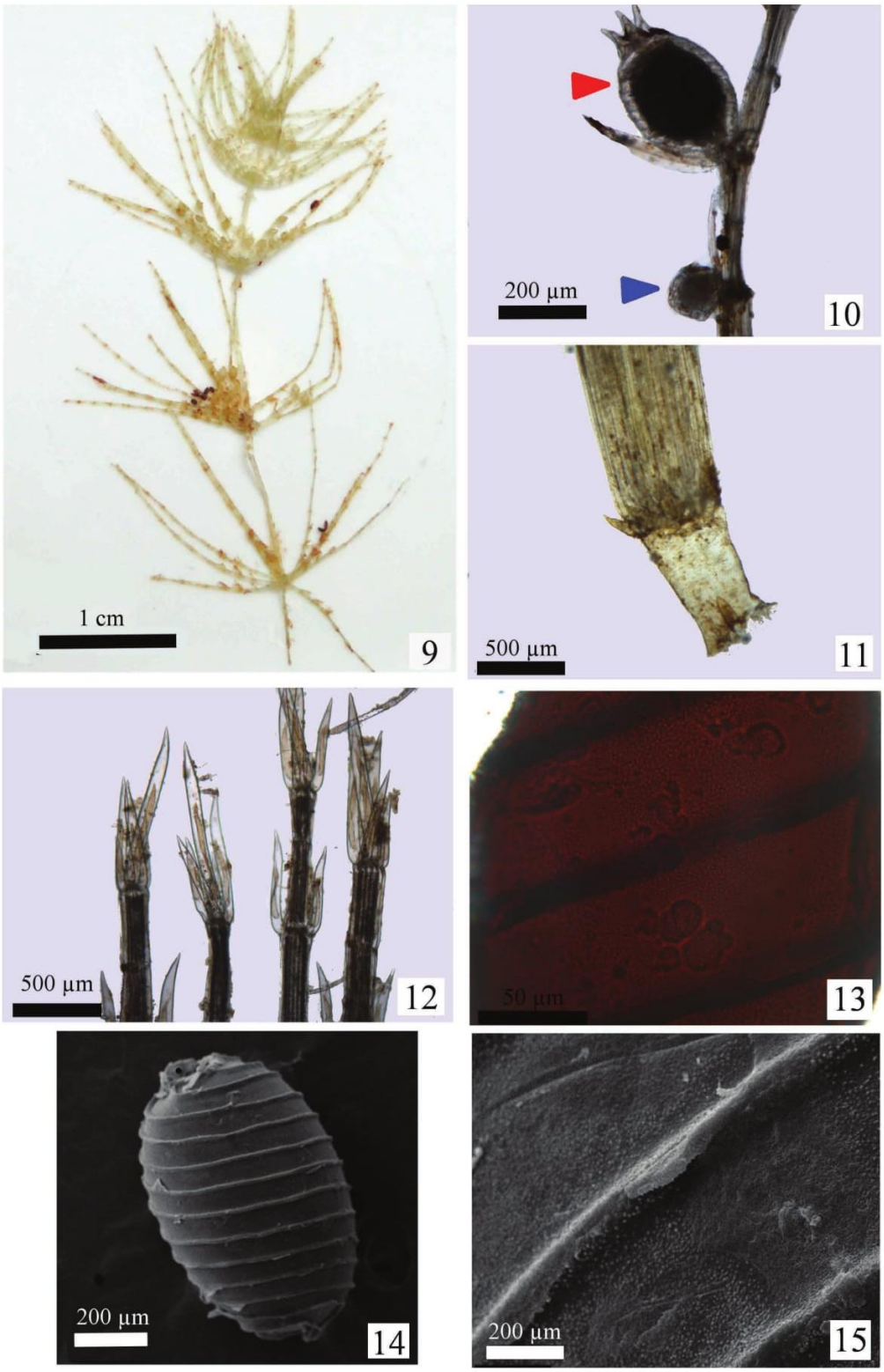
The sample from Rio das Pedras had only few fragments and not enough to perform observations and measurements of the morphological characters. It was included because values of DNA sequence identity revealed close affinity to the other samples. The ITS2 marker was not amplified for this sample.

C. foliolosa was described in older studies (Bicudo 1972, 1974), but not in the more recent investigations on Characeae from Brazil. This is remarkable since it was the most frequently species collected in our expeditions.

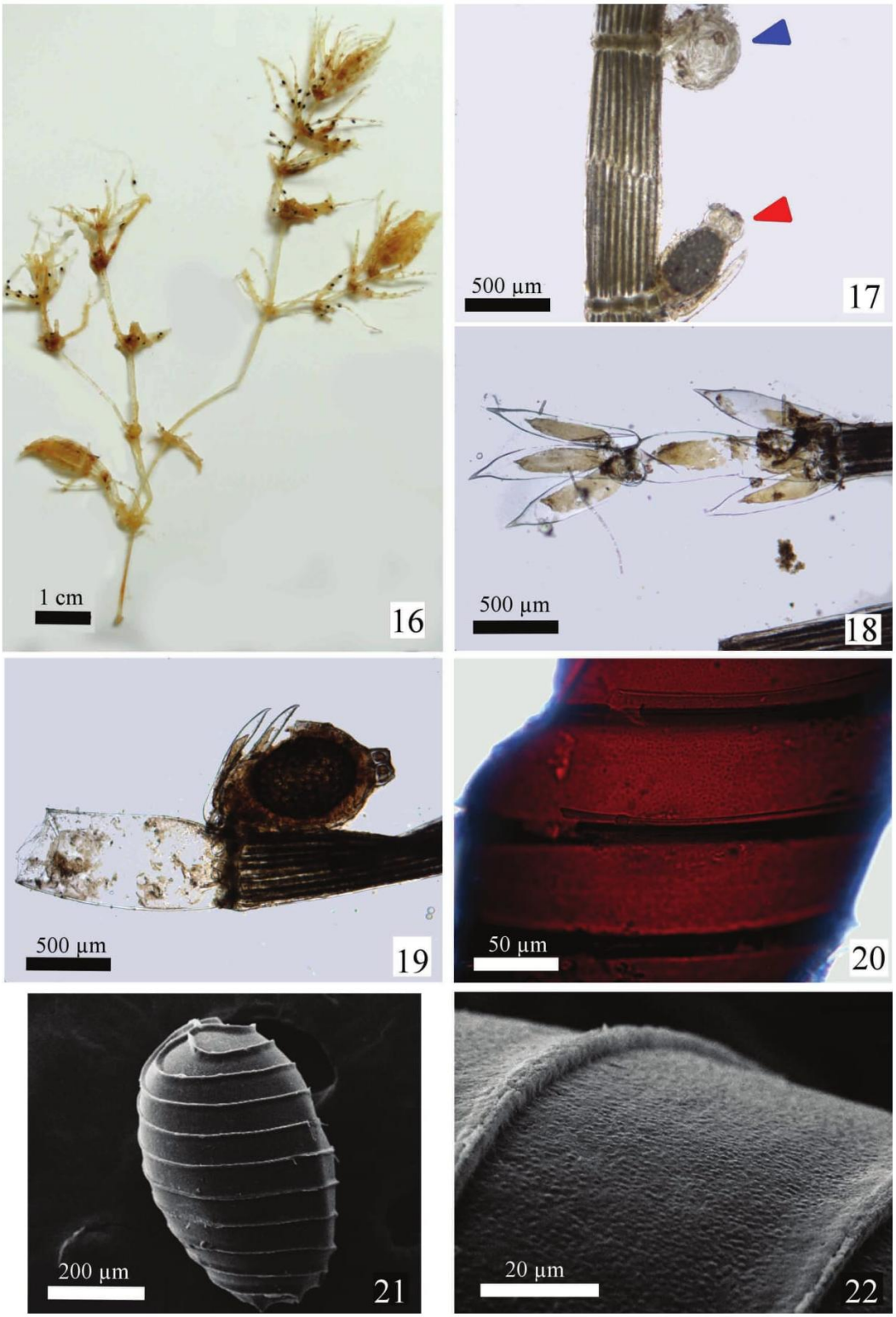
DNA sequences:—*rbc*L-KY656911, KY656912, KY656914, KY656915, KY656907; ITS2-KY656931, KY656932, KY656934, KY656935 *mat*K-KY656922, KY656923, KY656925, KY656926, KY656918.

Chara guairensis R.Bicudo 1974: 145–149
Fig. 16–22

Plants monoecious, up to 10 cm high, with calcium carbonate deposition. Axes 372–877 µm in diameter; internodes 0.12–0.81 times longer than the branchlets, to 0.9 cm length; cortex 3 corticate, isostichous, regular; spine-cells solitary, 43–138 µm long and 37–89 µm in diameter at the base, the basals are smaller or deciduous. Stipulodes in two whorls, two times as numerous as the branchlets; exceeding basal branchlet segment, except at basal nodes, uppers 435–932 µm long, 85–157 µm in diameter, acuminate; lowers 191–391 µm long, 59–131 µm in diameter, acuminate. Branchlets (6) 8–10 forming a whorl, 1.1–2.2 cm long; intercalary segments (3)4–8, corticate, 802–3264 µm long, 226–477 µm in diameter; basal segment ecorticate, 624–1414 µm long, 284–574 µm in diameter; 1–3 apical segments ecorticated, 802–3264 µm long, 226–477 µm in diameter. Bract-cells 4–6, forming whorls, largest ones facing the thallus, 122–843 µm long, 71–145 µm in diameter, acuminate. Bracteoles 2, 371–938 µm long, 93–184 µm in diameter, same size or larger than the madure oogonia. Gametangia sejoined, at the 1st to 5th nodes. Oogonia 549–1034 µm long (excluded coronula), 369–650 µm in diameter; convolutions 9–11(12); coronula 95–162 µm high, 219–266 µm wide, apex divergent. Oospores black, 558–716 µm long, 352–487 µm wide; striae of 8–11 low to prominent ridges; fossae 64–95 µm across; membrane smooth to slightly granulate. Oospore wall featureless to finely granulate in ultrastructure. Antheridia 270–535 µm in diameter, octoscutate.



FIGURES 9–15. *Chara foliolosa.* 9. Habit. 10. Monoecious plant with oogonium (red arrow) and antheridium (blue arrow) at the same branchlet. 11. Ecorticated basal segment of a branchlet. 12. Apical segment with one ecorticated cell and bract-cells around. 13. Granular aspect of the oospore wall under light microscope. 14. Overview of the oospore SEM. 15. Granular aspect of the oospore wall under SEM.



FIGURES 16–22. *Chara guairensis.* 16. Habit. 17. Monoecious plant with sejoined gametangia, with oogonium (red arrow) and antheridium (blue arrow) at the same branchlet. 18. Two ecorticated apical segments. 19. Basal segment of a branchlet and a oogonia. 20. Granular aspect of the osospore wall under light microscope. 21. Overview of the oospore under scanning electron microscopy (SEM). 22. Granular aspect of the oospore wall under SEM.

Specimen examined:—BRAZIL. São Paulo: Estação Ecológica do Noroeste Paulista, Municipality of São José do Rio Preto, elev. 509 m, 20°50'31.5''S, 49°26'17.4''W, 4 September 2014, colls. *F.R. Borges & O. Necchi Jr.* (SJRP31523).

DNA sequences:—*rbc*L-KY656913; ITS2-KY656933; *mat*K-KY656924.

Chara haitensis M.P.J.F.Turpin 1829: pl. s. 229
Fig. 23–27

FIGURES: 23–27. *Chara haitensis.* 23.Habit. 24. Apical ecorticated segment of a branchlet.. 25. Basal segment of a branchlet. The granular aspect is due calcium carbonate deposition. 26. Antheridium at a branchlet node of a male plant. 27. Oogonium at a branchlet node of a female plant.

Plants dioecious, up to 65 cm high, with calcium carbonate deposition. Axes 341–798 µm in diameter; internodes 0.9–3.5 times longer than the branchlets, to 3.8 cm length; cortex 3 corticate, isostichous, regular; spine-cells solitary, well developed, 193– 1011 µm long and 39–82 µm in diameter at the base, restricted to upper internodes. Stipulodes in two whorls, two times as numerous as the branchlets, never exceeding basal branchlet segment; uppers 536–986 µm long, 71–156 µm in diameter, acuminate; lowers 480–863 µm long, 63–138 µm in diameter, acuminate. Branchlets 11–15 forming a whorl, 0.9–3.5 cm long; intercalary segments 6–12, corticate, 928–4382 µm long, 247–478 µm in diameter; basal segment ecorticate, 1402–4565 µm long, 357–613 µm in diameter; apical segment formed by 1 ecorticated cell, 289–546 µm long, 60–150 µm in diameter. Bract-cells 5–8, forming whorls, with two larger and positioned next to the bracteoles, 170–726 µm long, 56–134 µm in diameter, acuminate. Bracteoles 2, 433–1008 µm long, 72–149 µm in diameter, often larger than the madure oogonia. Gametangia at 2nd–6th lowest nodes, never at the base of branchlets. Oogonia 428–819 µm long, 211–747 µm in diameter; convolutions 9–13; coronula 108–240 µm high, 177–233 µm wide, apex divergent. Mature oospores not observed. Antheridia 840–1020 µm in diameter, octoscutate.

Specimen examined:—BRAZIL. Mato Grosso: entrance to Municipality of Nobres, 14°45'11.5"S, 56°20'9.3"W, 11 May 2014, coll. *F.R. Borges*, (SJRP28306).

DNA sequences:—*rbc*L-KY656908; ITS2-KY630508 , *mat*K-KY656919.

Chara hydropitys H.Reichenbach 1829: 1600
Fig. 28–35

Plants monoecious, up to 6.7cm high, without calcium carbonate deposition. Axes 172–326 µm in diameter; internodes 0.6–1.5 times longer than the branchlets, up to 1 cm length; cortex 3 corticate, isostichous to slightly tylacanthous, regular; spine-cells solitary, rudimentary to well developed, 46–1487 µm long and 27–50 µm in diameter at the base. Stipulodes in one whorl, two times as numerous as the branchlets 296–833 µm long, 39–74 µm in diameter, acuminate. Branchlets 6–10 forming a whorl, 0.3–0.8 cm long; intercalary segments 3–4, corticate, 648–1601 µm long, 98–168 µm in diameter; basal segment ecorticate, 380–1891 µm long, 44–88 µm in diameter; apical segment formed by 1 ecorticated cell , 386–974 µm long, 43–88 µm in diameter. Bract-cells 4–7, forming whorls, 206–838 µm long, 41–89 µm in diameter, acute or acuminate. Bracteoles 2, 332–786 µm long, 41–84 µm in diameter, often smaller than the mature oogonia. Gametangia conjoined or sejoined, at 1st–5th lowest nodes. Oogonia 330–541 µm long 231–333 µm in diameter; convolutions 9–11; coronula 51–111 µm high, 125–169 µm wide, apex divergent. Oospores dark brown to black, 331–404 µm long, 208–284 µm wide; striae of 9–11 low ridges; fossae 33–50µm across; membrane opaque granulate. Oospore wall granular in ultrastructure. Antheridia 189–279 µm in diameter, octoscutate.

Specimen examined:—BRAZIL. Mato Grosso do Sul: entrance to Recanto Ecológico Rio da Prata, Municipality of Jardim, 21°29'21.1"S, 56°26'16.6"W,15 May 2014, coll. *F.R. Borges*, (SJRP28308).

DNA sequences:—*rbc*L-KY656910; ITS2-KY656930; *mat*K-KY656921.

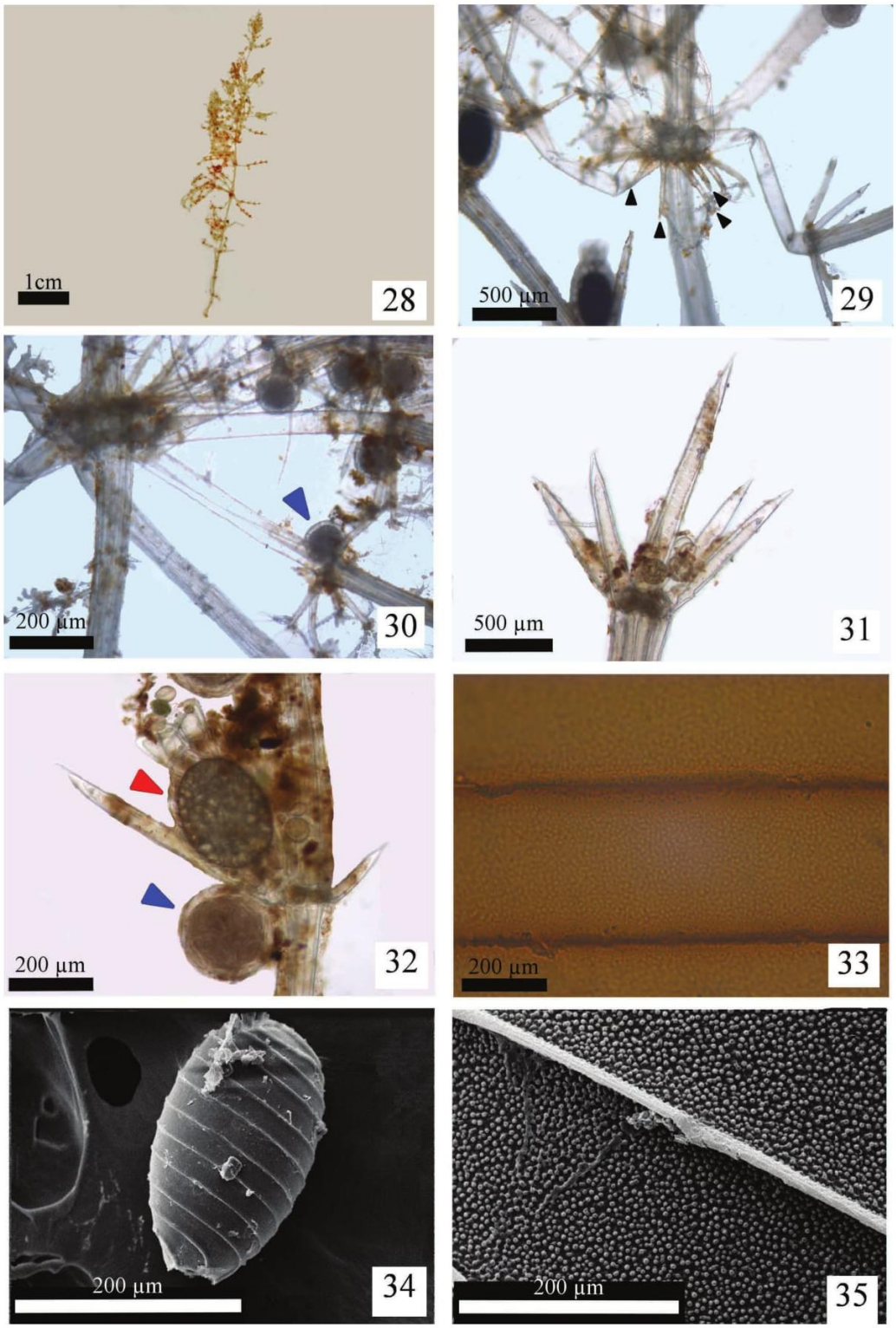
Chara rusbyana M.Howe 1929: 160–161
Fig. 36–39

Plants up to 39 cm high, with strong calcium carbonate deposition. Axes 424–1123 µm in diameter; internodes 0.12–2 times longer than the branchlets, to 3.8 cm length; cortex 3 corticate, isostichous; spine-cells solitary, well developed, 87–435 µm long, 44–96 µm in diameter at base, concentrated at apical internodes. Stipulodes in two whorls, two times as numerous as the branchlets always exceeding basal branchlet segment; uppers 495–824 µm long, 63–123 µm in diameter, acuminate; lowers 190–535 µm long, 66–122 µm in diameter, acuminate. Branchlets 13–16(17) forming a whorl, 0.8–2.4 cm long; intercalary segments 6–11, corticate, 430–3000 µm long, 113–555 µm in diameter; basal segment ecorticate, 248–685 µm long, 198–477 µm in diameter; apical segment formed by 1 ecorticated cell, 417–713 µm long, 67–150 µm in diameter. Bract-cells (4)5–8, forming whorls, 107–348 µm long, 57–86 µm in diameter, acuminate. Oogonia and antheridia not observed.

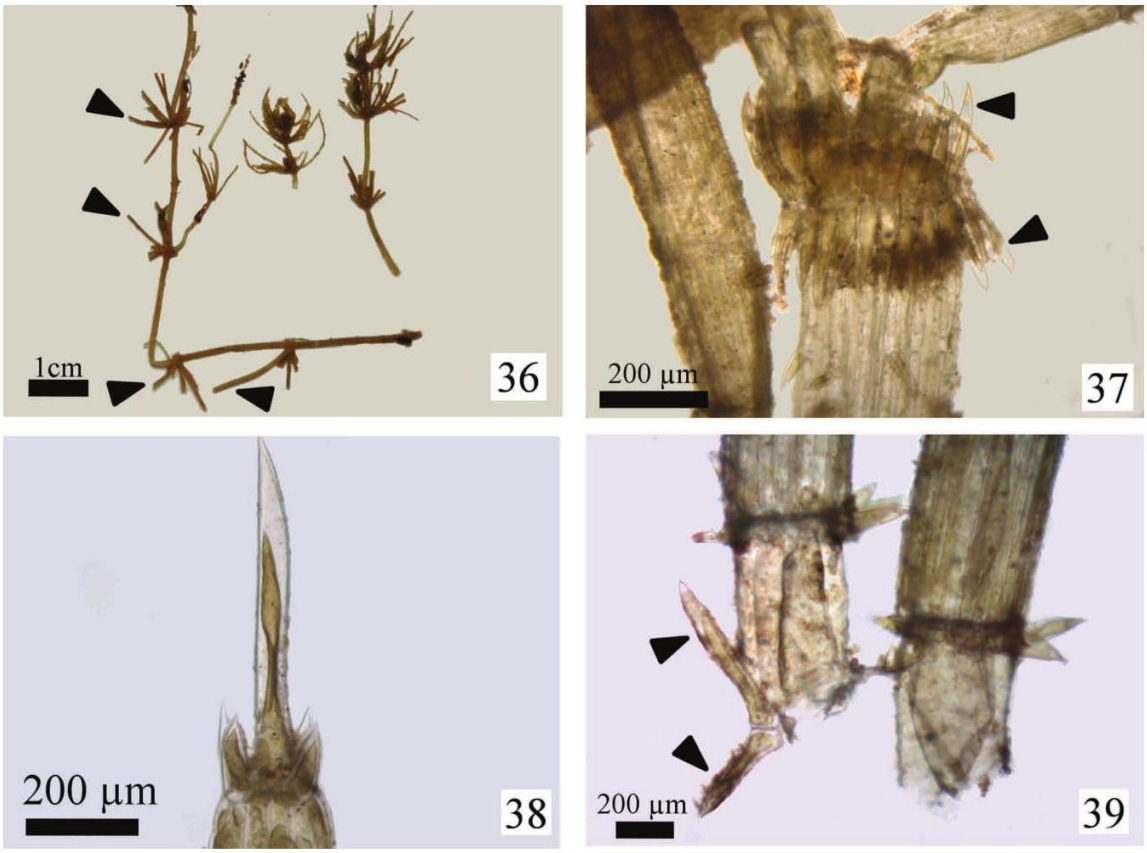
Specimen examined:—BRAZIL. Mato Grosso do Sul: Anhumas Stream, road leading to the grotto Lagoa Azul, near the entrance of the São Miguel Cave, Municipality of Bonito, 21°06'07.8"S; 56°34'17"W, 14 May 2014, coll. *F.R. Borges* (SJRP28307). Seputá Stream, Municipality of Bonito, 21º03'51"S, 56º44'25"W, 14 Aug 2002, coll. *L.H.Z. Branco*. Olaria Stream, Municipality of Bonito, 20º55'02"S, 56º35'01"W, 15 Aug 2002, coll. *L.H.Z. Branco*.

The samples from Seputá and Olaria Streams had only few fragments and not enough to perform observations and measurements of the morphological characters. The ITS2 marker was not amplified for these samples.

DNA sequences:—*rbc*L-KY656909, KY630506, KY656905; ITS2-KY656929; *mat*K-KY656920, KY630507, KY656916.



FIGURES: 28–35. *Chara hydropitys*. 28. Habit. 29. Stipulodes (black arrows) in one tier, and branchlets with ecorticated basal segment. 30. Ecorticated basal segment of a branchlet and an antheridium (blue arrow) at the first node. 31. Apical segment with one ecorticated cell and bract-cells around. 32. Monoecious plant with conjoined gametangia (the red arrow indicates an oogonium and the blue an antheridium). 33. Granular aspect of the osospore wall under light microscope. 34. Overview of the oospore under SEM. 35. Granular aspect of the osospore wall under SEM.



FIGURES: 36–39. *Chara rusbyana* 36. Habit. The arrows indicates some broked branchelets. 37. Node of the thallus. The arrows points two tiers of stipulodes. 38. Ecorticated apical segment of a branchlet. 39. Ecorticated basal segment of two branchlets. The arrows point to stipulodes in two tiers.

General Remarks

According to Wood & Imahori (1965) *C. braunii* and *C. hydropitys* belong to Subgenus *Charopsis*, characteristized by stipulodes in a single row and plants tipically ecorticated or cortication restricted to the main axis. The subgenus is considered polyphyletic (Meiers *et al*. 1999, McCourt *et al*. 1999 and Sakayama *et al*. 2009), which was confirmed in this study based on phylogenetic analyses for the *rbc*L and *mat*K (Figs. 1 and 3, respectively). Schneider *et al*. (2016) discouraged the description of new *Chara* species based solely on morphological differences such as partial or total loss of cortication, sex differentiation, or number and length of spine cells, bract-cells and stipulodes. In our study, the number of rows of stilpulodes proved to be a feature that does not reflect the phylogeny of the species and therefore it was inconsistent to define the Subgenus *Charopsis*.

The occurrence of *Chara braunii* Gmel var. *brasiliensis* R. Bic. is common in Brazil. According to Picelli-Vicentim *et al.* (2004), this variety differs from *Chara braunii* Gmel. var. *braunii* by having larger oospores: 800–830 µm longer and 430–480 µm in diameter for variety *brasiliensis*; 368–750 µm long and 224–400 µm in diameter for variety *braunii*. Oospores had intermediate values between the two varieties (610–866 µm longer and 335–489 µm in diameter) in our samples. Some authors (Casanova 1997, Blume *et al*. 2009, Proctor 1975) attributed variation in size and shape of oospores to be influenced by environmental (effects of temperature, temporary or permanent habitats), as well as developmental factors. Intermediate values between the two varieties were found in this and a previous study (Meurer and Bueno 2012) and the evidences suggest that the distinction is rather artificial.

TABLE 1. List of taxa with GenBank accession numbers used for tree construction.

Marker	Species	GenBank accession	Strain designation and/or collection information	Reference
*rbc*L	*C. braunii*	AB606677	NZL, New Zeland	Unpublished
		AB440258	S019, Japan	Sakayama *et al.* 2009
		AB440259	S036, Japan	Sakayama *et al.* 2009
	C. longifolia	AY170452	MB, Canada	Sanders *et al*. 2003
	C. connivens	L13476	JRM, unknown	Manhart 1994
		AF097162	X214, Israel	Manhart 1994
	C. globularis	AF097164	X692, Australia	McCourt *et al*. 1999
	Chara vulgaris	DQ076298	Taiwan	Unpublished
	C. polyacantha	AY170453	F122, Unknown	Sanders *et al*. 2003
	C. drouetii	HQ380444	KGK0467, Mexico	Hall *et al*. 2010
	C.haitensis	HQ380455	KGK0483, Mexico	Hall *et al*. 2010
	C. zeylanica	AB359169	SK022, Japan	Kato *et al.* 2008
		HQ380468	KGK0055b, China	Hall *et al*. 2010
	C. hydropitys	HQ380461	KGK0213, Mexico	Hall *et al*. 2010
	C. rusbyana	AF097168	X-066, Argentina	McCourt *et al.* 1999
	C. kenoyeri	HQ380465	TP118, Panamá	Hall *et al*. 2010
	C. foliolosa	HQ380446	KGK0061, U. S. A.	Hall *et al*. 2010
		HQ380448	KGK0233, U. S. A.	Hall *et al*. 2010
	Lamprothamnium papulosum	AF097170	F137, France	McCourt *et al.* 1999
ITS2	*C. hydropitys*	HQ380625	KGK0213, Mexico	Hall *et al*. 2010
	C. zeylanica	HQ380634	KGK0709, U. S. A.	Hall *et al*. 2010
		HQ380630	KGK0062, U. S. A.	Hall *et al*. 2010
		HQ380629	KGK0059c, U. S. A.	Hall *et al*. 2010
		HQ380631	KGK0034, U. S. A.	Hall *et al*. 2010
		HQ380632	KGK0389, U. S. A.	Hall *et al*. 2010
		HQ380633	KGK0468, Nicaragua	Hall *et al*. 2010
	C. rusbyana	HQ380627	LG, Unknown.	Hall *et al*. 2010
	C. foliolosa	HQ380619	KGK0341, U. S. A	Hall *et al*. 2010
		HQ380618	KGK0233, Mexico	Hall *et al*. 2010
	C.haitensis	HQ380623	KGK0032, Dominican Republic	Hall *et al*. 2010
	Nitella oligospira	AB191724	S095, New Caledonia,	Sakayama *et al.* 2005
*mat*K	*C. longifolia*	AY170444	MB, Canada	McCourt *et al.* 1999
	C. tomentosa	LT603688	GJ01, Sweden	Nowak *et al.* 2016
	C. tenuispina	KP876014	02, Unknown	Unpublished
	C. contraria	KP876011	02, Unknown	Unpublished
	C. filiformis	LT603700	TK67, Sweden	Nowak *et al.* 2016
	C. canescens	LT603696	SE20, Sweden	Nowak *et al.* 2016
	C. aspera	LT603692	GJ51, Sweden	Nowak *et al.* 2016
	C.globularis	KP876012	Unknown	Unpublished
		LT603689	GJ29, Sweden	Nowak *et al.* 2016
	C.vulgaris	LT603693	GJ75, Sweden	Nowak *et al.* 2016
	C. virgata	LT603691	GJ41, Sweden	Nowak *et al.* 2016
	Lamprothamnium macropogon	AY170446	X695, Australia	Sanders *et al*. 2003

Four of the six species found (*C. rusbyana, C. haitensis, C. foliolosa and C. guairensis*) belong to the Subgenus *Grovesia*, Section *Grovesia*. These algae can be alternatively included in the group called *Gymnobasalia* Zaneveld, or Subsection *Willdenowia* Wood. According to Proctor (1971) this group is characterized by: a- presence of basal internodes of the branch always ecorticated; and b- recurved, depauperate-appearing branches, superficially reminiscent of the prop-roots of mangrove or the rhizopores of *Selaginella*, are produced. The second characteristic is observed only in living material, usually grown in laboratory, since this structure is lost in specimens from the field. According to Wood & Imahori (1965), Subsection *Willdenowia* consisted of only one widely distributed and highly polymorphic species: *Chara zeylanica*. They disregarded the fact that different species are dioecious or monoecious (with conjoined or sejoined gametangia) and extended the circumscription of *Chara zeylanica*, encompassing other species, which were demoted to infraspecific levels, as is the case of *C. rusbyana*, *C. sejuncta* (now *C. foliolosa*) and *C. haitensis*. McCracken *et al*. (1966) demonstrated that *C. rusbyana* and *C. foliolosa* were reproductively isolated. Later on, Proctor & Wiman (1971) and Proctor *et al.* (1971) refuted Wood's decision to broaden the concept of *C. zeylanica*

to encompass many traditionally recognized species. John *et al.* (1990) pointed out that gametangial arrangement and oosporangial characters were already used traditionally to separate these species. Our results corroborate these statements, since these three species form distinct clades with high support for the *rbc*L and ITS2 markers. For *mat*K there are no *C. zeylanica* sequences available in GenBank, but again there is a clear distinction between *C. rusbyana*, *C. foliolosa* and *C. haitensis*.

C. guairensis is considered an endemic species to Brazil. According to original description by Bicudo (1974), it is differentiated from the other species of the Subsection *Willdenowia* Wood by presenting very long bract-cells (2000–4800 μm in length), fertile basal nodes branchlets, gametangia sejoined, antheridia octoscutate and triangular shells. Casanova (2005) pointed out that genetic analysis, breeding studies and information on oospore morphology, not only traditional characters, are needed for taxonomy of the genus *Chara*. In addition Schneider *et al*. (2016) discourage the description of new *Chara* species based exclusively on morphological differences. Our data for molecular markers support the recognition of *C. guairensis* and also that this species should be inserted in the Subsection *Willdenowia*. Regarding the diagnostic characters of *C. guairensis*, we verified that length of bract-cells and the characteristics of the antheridia are similar to those reported for *C. foliolosa*. Once again the fertile basal nodes of the branches can be used as a character to separate these two species.

The ultra-structural studies of the oospore wall have revealed valuable data. John *et al.* (1990) analyzed the oospore wall of *C. foliolosa* and *C. hydropitys*, with slightly different results from ours: *C. foliolosa* is described as featureless except for some low undulations and a few pits or pores based on a sample from Panama. The two Brazilian samples of this species were variable, one having featureless wall, while the other presenting a finely granulated pattern (Fig. 15). The granulated pattern has already been described by Wood & Imahori (1965) in light microscopy studies. For *C. hydropitys* the pattern observed was similar to that described by John *et al.* (1990) with the granular wall of the oospore (Fig.35). However, those authors described the surface as having minute pits or pores, which were not observed in Brazilian specimens, perhaps due to the use of a smaller image increase. Our study is the first to report ultra-structural features for the oospore wall for *C. guairensis*, which was featureless to finely granulate in ultrastructure (Fig. 22), corroborating the light microscopic observations by Bicudo (1974) in the protologue.

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CAPÍTULO 2

Taxonomy and phylogeny of *Nitella* (Charophyceae, Characeae) from Brazil with emphasis on the midwest and southeast regions

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Abstract

The genus *Nitella* is the most species-rich within the Charales. Brazilian studies on the genus are relatively scarce and consist of floristic surveys, lacking modern and more precise information. This investigation applied scanning electron microscopy to analyze the oospore wall and molecular data associated with traditional morphological characters to analyze forty-two populations of *Nitella* from the midwest and southeast regions of Brazil. Forty-two new sequences of *rbc*L, twelve of ITS1 and twenty-three of ITS2 were generated for the five species recognized in this study. Phytlogenetic analyses of sequences of these three markers were congruent in that they grouped the species in five clades, each one representing a distinct species: *Nitella acuminata* A. Braun *ex* Wallman, *Nitella axillaris* A. Braun, *Nitella elegans* B. P. Pal, *N. flagellifera* J. Groves & G. O. Allen and *Nitella microcarpa* A. Braun. Our results on ultrastrucure of the oospore wall were consistent with previous studies for the same species from other regions of the world. The data reinforced the conclusion that the use of ornamentation of oospore wall may be extremely useful for the construction of a natural system for Characeae. Molecular evidence, reinforced by morphological data, support to place *Nitella subglomerata* A. Braun and *Nitella gollmeriana* A. Braun under synonymy of *Nitella acuminata*; and *Nitella axilliformis* K. Imahori under *Nitella axillaris*. We showed that even among geographically distant populations, such as from other continents, of some *Nitella* species, the degree of identity among DNA sequences was high and they exhibited wide variation in morphological characters.

Introduction

The genus *Nitella* is the most species-rich in the Charales (Khan & Sarma 1984) and also the one exhibiting the highest diversity of vegetative and oospore morphology (Wood & Imahori 1965, Sakayama 2008). The genus is characterized by having one or more forked branchlets that are composed of unicellular segments and single- or multiple-celled terminal segments (called "dactyls"), two tiers of coronal cells in the female reproductive organ, and

laterally compressed oospores (Wood & Imahori 1965, Sakayama *et al.* 2002, 2005, Sakayama 2008).

The most comprehensive study of Characeae at global level is the review by Wood & Imahori (1965), in which they delimited taxa based on 'macroscopic and microscopic general morphology' rather than key characters. As a result, they reduced the approximately 204 recognized *Nitella* species to many intra-specific taxa (i.e. varieties or forms) or submerged them in synonymy yielding just 53 broadly defined species (Sakayama *et al.* 2005, Sakayama 2008).

Several phylogenetic studies involving molecular data (McCourt *et al.* 1996; Meiers *et al.* 1997, Sakayama *et al.* 2002, 2004a, 2004b, Sanders *et al.* 2003; Karol 2004, Sakayama 2008, Borges & Necchi 2017), as well as analysis of oospore wall morphology using scanning electron microscopy (SEM, John and Moore 1987, Sakayama *et al.* 2002, 2004a, 2004b, 2005, Sakayama 2008, Casanova 2009), suggested that the taxonomic system of Wood & Imahori (1965) is unnatural. Most of these studies were conducted with European, Asian, North American or Australasian species. South American studies about Characeae are relatively scarce, most of them consisting of floristic surveys applying only traditional morphological approaches, lacking modern and more reliable information (molecular evidences or characters of oospore wall ornamentation by SEM). Some of the few exceptions are the studies by Cáceres (1975), who used SEM techniques to analyze the oospores wall ornamentation of Argentinean Characeae species, and recently Borges & Necchi (2017) presented the first molecular data for Brazilian species of *Chara*, along with traditional morphological characteristics, as well as the ultrastructure of the oospore wall.

Studies on Characeae in Brazil began in the 1960's by Rosa M.T. Bicudo with her long term and sound work on the taxonomy of this group (Bicudo 1968a and b, 1969, 1972, 1974, 1979). Later, other investigators devoted to the taxonomic study of Characeae and among the most important studies on Brazilian Characeae are: Vieira *et al.* (2002, 2003), Picelli-Vicentim *et al.* (2004), Prado *et al*. (2005), Bueno *et al.* (2009, 2011), Bicudo & Bueno (2011), Meurer *et al.* (2012) e Borges & Necchi (2017). With the exception of the latter, all other publications were based only on classical morphological data for the

idendification and description of the species. Taking into account all records of *Nitella*, 27 species were reported for Brazil, four of them were considered as endemic *(*Bueno *et al. 2015)*: *N. arechavaletae* Spegazzini, *N. rosa-mariae* Picelli-Vicentim, *N. microcarpa* var. *sieberi* (A. Braun) J.C. Raam and *N. tolypelloides* Picelli-Vicentim.

The present investigation is the second part of a broader project on Characeae in the midwest and southeast regions of Brazil, initiated by Borges and Necchi (2017), which focused on the genus *Chara*. We applied modern tools to study the Brazilian species of *Nitella*, aiming to: i) conduct a first approach to the systematics of the genus *Nitella* using DNA sequences and oospore SEM analyses in addition to the alpha-taxonomy investigations that have been previously carried out in Brazil; ii) characterize *Nitella* species from these regions of Brazil applying traditional and SEM ultrastructural characters of oospore walls; iii) infer the phylogenetic relationships among species from Brazil and other regions of the globe based on two molecular markers - *rbc*L (plastid-encoded gene encoding the large subunit of ribulose 1, 5 bisphosphate carboxylase/oxygenase), ITS1 and ITS2 (internal transcribed spacers regions 1 and 2 of the nuclear ribosomal DNA); iv) correlate the traditional morphological features, the ultrastructural ornamentation of oospores wall and molecular data, in order to propose a more reliable species definition for the species occurring in these regions of Brazil compared to those found in other regions of the world.

Material and methods

42 samples were collected in 29 sampling sites of five states from midwest and southeast regions of Brazil (Fig. 1). Specimens were collected directly by hand and divided into three sets for distinct purposes: morphology, oospore ultrastructure and molecular. This study is part of a broader project involving the genera *Chara* and *Nitella*, applying the same general methods, which have already been described in a previous paper (Borges & Necchi 2017). Thus, only the different specifications for the genus *Nitella* are here described.

Morphological methods. Specimens were preserved in formaldehyde 4% and lodged at herbaria SJRP (Thiers *et al.* 2017). The identification and selection of the main diagnostic characters followed the Wood & Imahori (1965) system and Brazilian specific references (Bicudo 1974, Picelli-Vicentim 1990, Bueno & Bicudo 1997, Vieira *et al.* 2002, Bueno *et al.* 2009, 2011). After identification, the currently accepted taxonomic status was checked at Algae Base (Guiry & Guiry 2017) and recent references were consulted. For the description of the species, we adopted 25 measurements for each morphometric character (Borges & Necchi 2017).

Ultrastructure of the oospore wall. The conservation and preparation steps of mature oospores for analysis under scanning electron microscope followed the protocols described in Borges & Necchi (2017). Oospore surface details were described by adapting the terminology adopted by Faegri & Iversen (1964) for pollen grain analysis, and using the terminology used by John & Moore (1987), Leitch *et al.* (1990) and Casanova (1991) for Charophyte oospores

Molecular phylogenetic analysis. The extraction, amplification, purification and sequencing steps followed the protocols described in Borges & Necchi (2017). Sequences from Genbank were used to elucidate the phylogenetic positioning of the Brazilian species of *Nitella*. For phylogenetic analyses the best-fit model of sequence evolution by the Akaike Information Criterion was determined using jModelTest 2.1.4 (Darriba *et al.* 2012) for each molecular marker as follows: GTR + I for *rbc*L; HKY + I for ITS1 and ITS2.

Species	Sampling sites
Nitella acuminata	2, 6, 7, 8, 9, 10, 12, 14, 15, 16, 17, 18, 19, 20, 21, 22, 25, 28
Nitella axillaris	11, 23
Nitella elegans	7, 23, 27
Nitella flagellifera	5, 6, 9, 15, 16, 25, 29
Nitella microcarpa	1, 3, 4, 11, 13, 24, 26

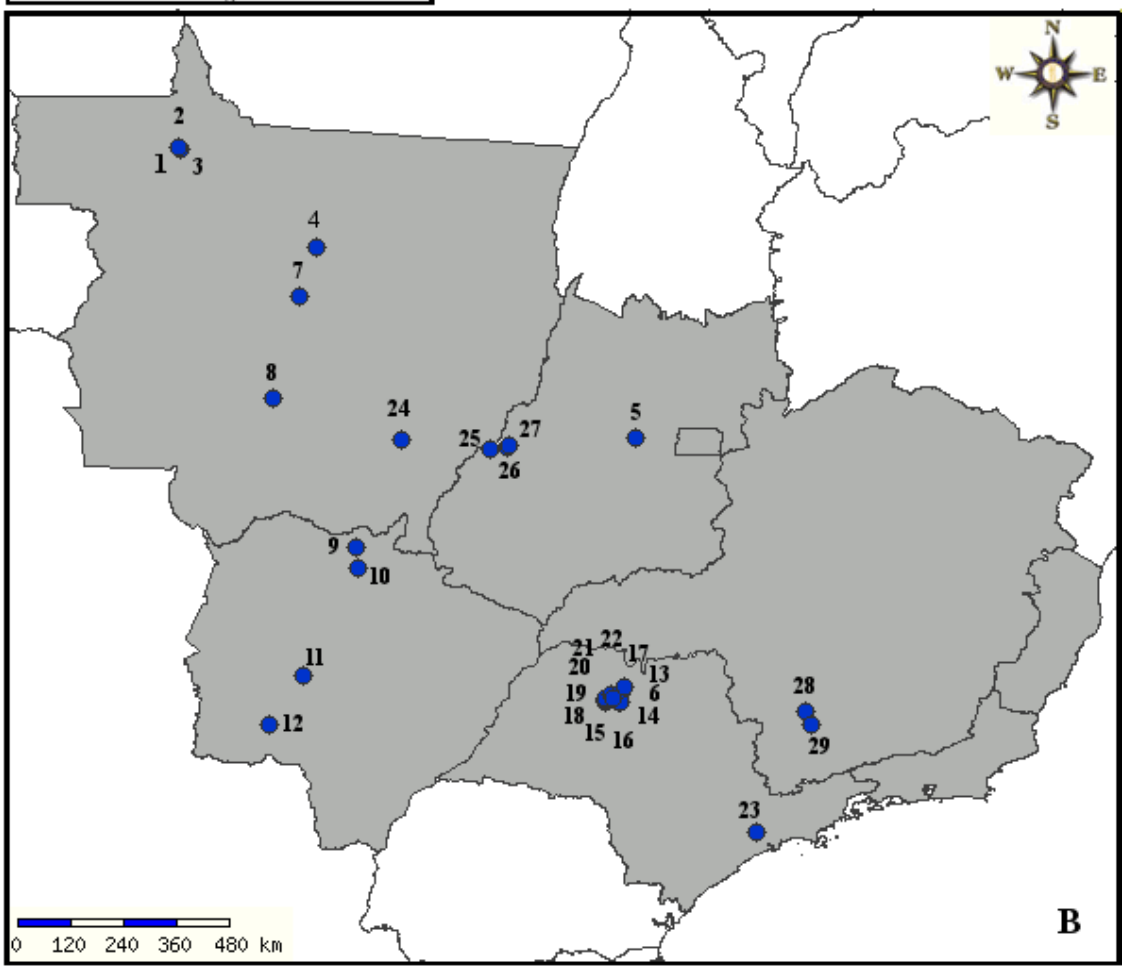


FIGURE 1. A- Map of Brazil. The area highlighted in gray corresponds to the study region. **B-** Area of study with the sampling sites. The table indicates sampling sites for each species collected.

Results and Discussion

Forty-two new sequences of *rbc*L, twelve of ITS1 and twenty-three of ITS2 were generated in this study. We were not able to sequence thirty samples for ITS1 and nineteen for ITS2 despite many attempts with different combinations of primers. *Nitella acuminata* was the only species without sequences for ITS1. The length used in the analyses for each molecular marker were as follows: *rbc*L (1,328 bp, except two samples: SJRP32545 and SJRP32530, with partial sequences of 828-859 bp), ITS1 (373-384 bp, except SJRP32574 with 307 bp) and ITS2 (251-267 bp).

The tree topologies obtained with *rbc*L (Fig. 2), ITS1 (Fig. 3) and ITS2 (Fig. 4) data were congruent in that they separated sequences from Brazilian samples into five different clades, most of them with high support, each one representing a distinct species.

The first clade, named *Nitella pseudoflabellata* (the oldest name among the species of the clade) includes *N. pseudoflabellata* (as well as the *N. pseudoflabellata* var. *comptonii*), *N. megaspora* and our samples of *N. elegans* (Figs. 2-4). For *rbc*L and ITS2 markers we got sequences from three samples and the identity values ranged, respectively, from 98.7% to 99.7% and 95.1 to 99.2% among our samples and some from Japan. For ITS1 were sequenced two samples and the identity values ranged from 92.7 to 99.9% among our sequences and three also from Japan. There are records of *N. pseudoflabellata* for Asia and Australasia (Guiry & Guiry 2017), whereas *N. megaspora* is reported for Africa and Asia (Guiry & Guiry 2017) and there are registers of *N. elegans* for Asia (Guiry & Guiry 2017) and this is the first record for Brazil.

N. flagellifera was the only species that formed the second clade (Figs. 2-4), with eight sequences for *rbc*L, four for ITS1 and four for ITS2. Identity values ranged from 99.4 to 100%, 96.6 to 100% and 99.2 to 100% for each of the markers, respectively. *N. flagellifera* is reported for South America and Asia (Guiry & Guiry 2017).

The clade named *Nitella axillaris* grouped specimens of *N. axillaris* and *N. axilliformis* (Figs. 2-4). The *rbc*L sequence of *N. translucens* (L13482) probably belongs to *N. axillaris*, since when compared to another of *N.*

translucens (AF097745) from a specific study on Characeae (McCourt *et al.* 1999), the identity value between the sequences is only 95.5%, considered too low for this marker to be considered as being of the same species. Two sequences were amplified for each marker and grouped with sequences from North America, Japan and unknown locality. The identity values ranged from 98.4 to 99.8% for the *rbc*L marker, 90 to 94.8% for ITS1 and 94.4 to 97.3% for ITS2. *Nitella axillaris* is reported for Americas (North, Central and South) and Asia (Guiry & Guiry 2017), while *N. axilliformis* is reported only for Asia (Guiry & Guiry 2017).

A fourth clade included taxa identified as *N. furcata*, *N. tumulosa*, *N. inversa, N. japonica* and our samples of *N. microcarpa* named *Nitella furcata* (Figs. 2-4). Sequences obtained from Genbank are from Taiwan, Malaysia and Japan. Seven sequences were amplified for *rbc*L, four for the ITS1 and five for the ITS2. The identity values among the sequences were from 99.0 to 99.7% for *rbc*L, 94.1 to 100% for ITS1 and 95.1 to 100% for ITS2. There are records of *N. furcata* for the Americas (North, Central and South), Europe, Asia and Australasia (Guiry & Guiry 2017), whereas *N. tumulosa* and *N. inversa* are reported for Asia (Guiry & Guiry 2017) and *N. japonica* is recorded for Asia (Guiry & Guiry 2017) and South America (Picelli-Vicentim *et al.* 2004). *Nitella microcarpa* is reported for North and South America (Wood & Imahori 1965), and there are exsicates from Africa, Asia and Central America lodged in NYBG

Nitella acuminata was the only species to compose the clade of the same name (Figs. 2 and 4). We here propose that the morphological characters previously used to delimit *N. gollmeriana* (heads well formed, fairly compact and dactyls abbreviate, tiny and barely visible to naked eye) and *N. subglomerata* (heads weakly formed) be incorporated into *N. acuminata*. Twenty-two sequences were added to the three obtained from GenBank (from Malaysia, Taiwan and an unknown locality) to compose the *rbc*L tree, and nine to compose the ITS2 tree. The identity values for *rbc*L were 98.6 to 100% and for ITS2 from 95.1 to 99.2%. *Nitella acuminata* is reported for Asia, Australasia, Americas (North and South) and Europe (Guiry & Guiry 2017).

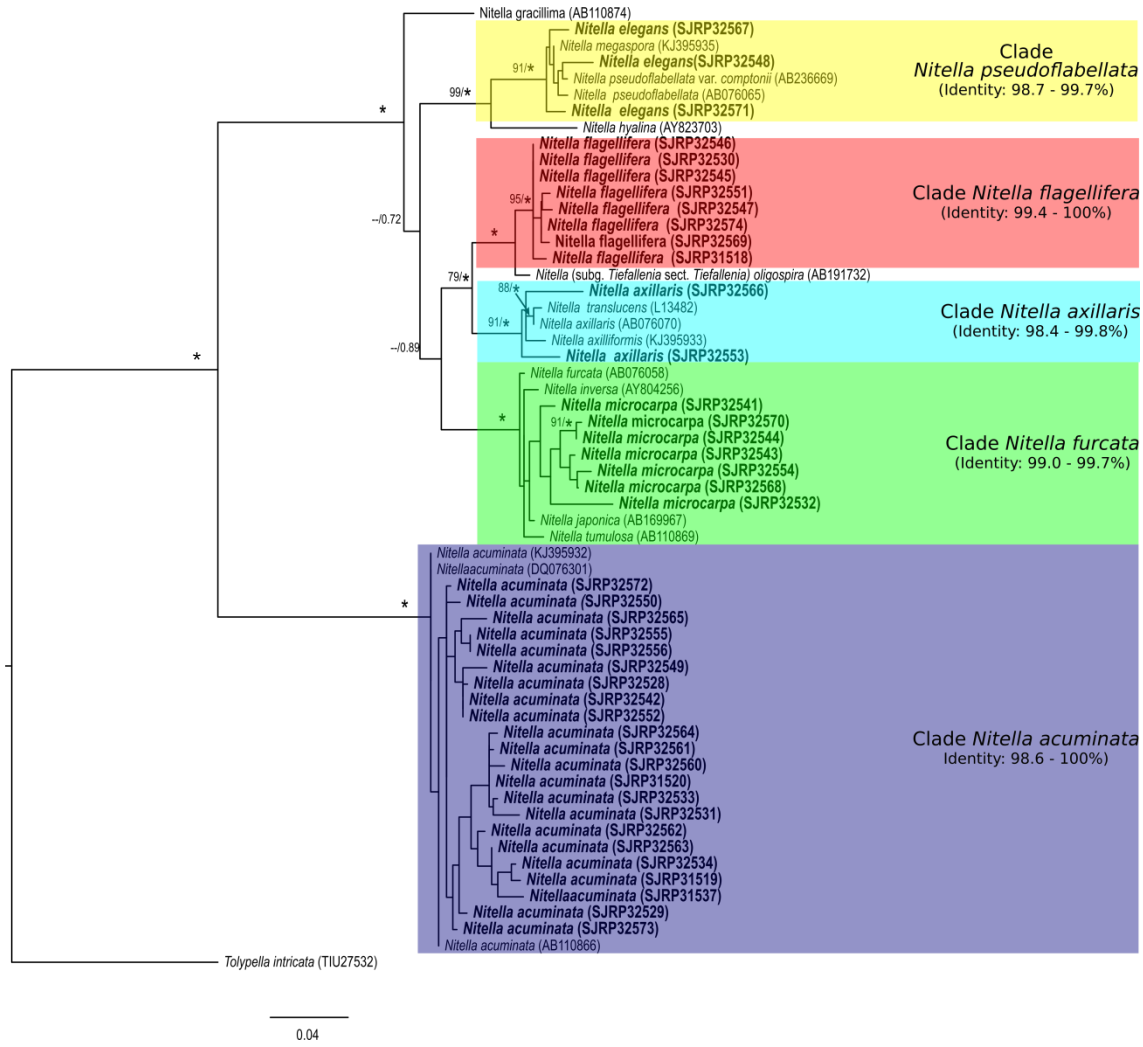


FIGURE 2. Maximum likelihood Phylogenetic tree based on *rbc*L sequences. The numbers associated with the nodes indicate the bootstrap values (BS) for maximum likelihood and posterior probability (PP) for Bayesian analysis; nodes without values indicate BS < 70% and PP <0.70; nodes with * indicate BS > 95% and PP>0.95 Sequences in bold are the new sequences generated in this study.

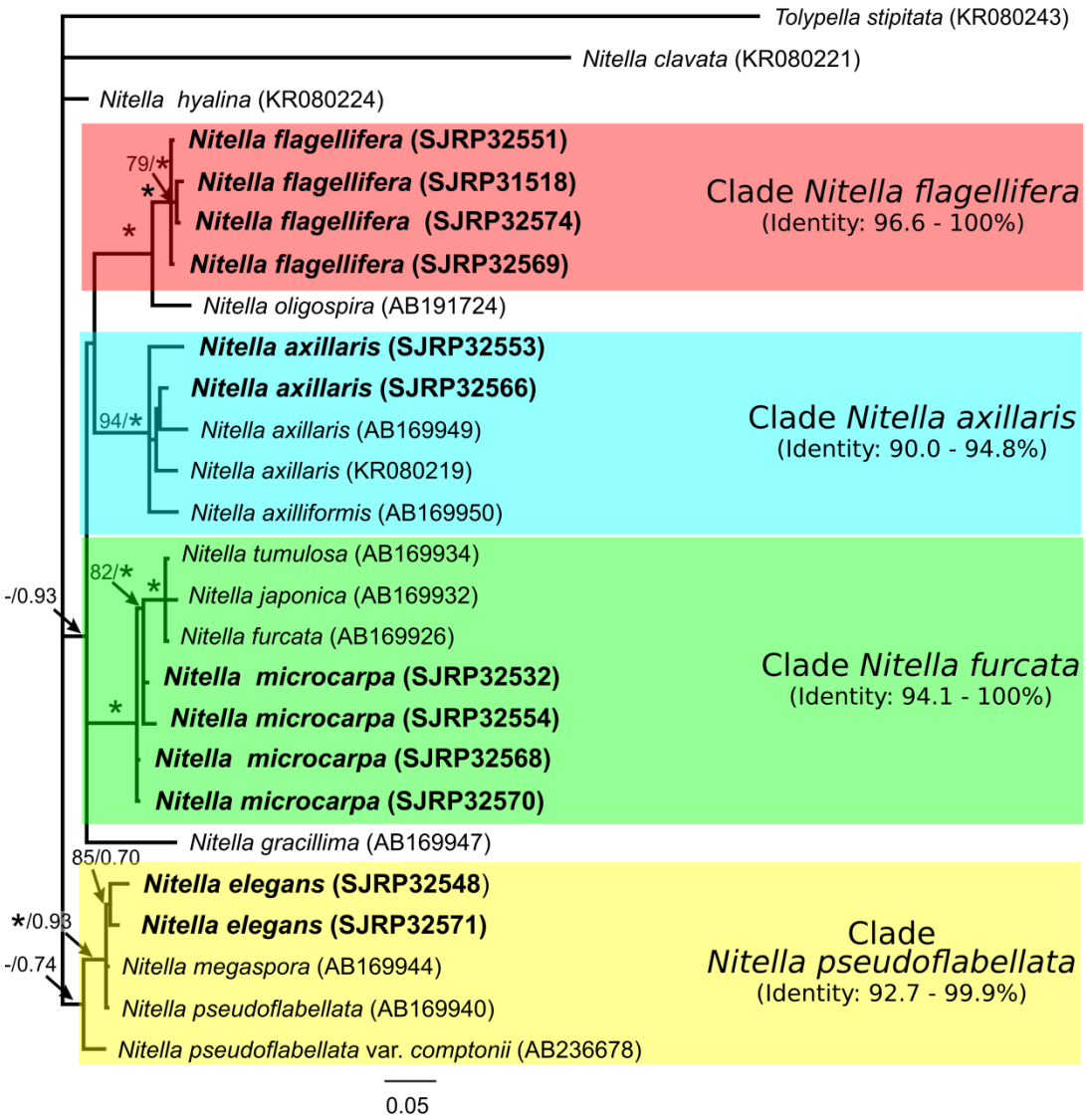


FIGURE 3. Bayesian Phylogenetic tree based on ITS1 sequences. The numbers associated with the nodes indicate the bootstrap values (BS) for maximum likelihood and posterior probability (PP) for Bayesian analysis; nodes without values indicate BS < 70% and PP <0.70; nodes with * indicate BS > 95% and PP>0.95 Sequences in bold are the new sequences generated in this study.

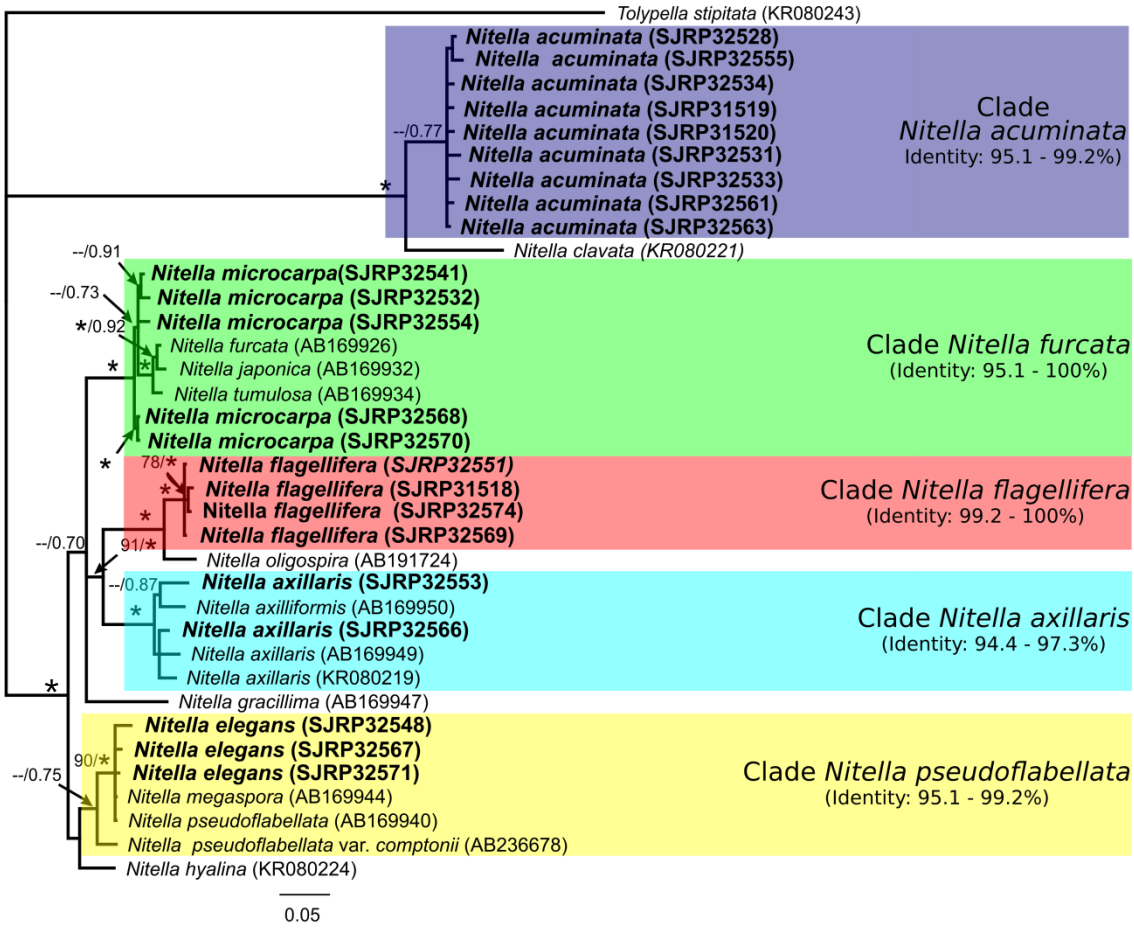


FIGURE 4. Bayesian Phylogenetic tree based on ITS2 sequences. The numbers associated with the nodes indicate the bootstrap values (BS) for maximum likelihood and posterior probability (PP) for Bayesian analysis; nodes without values indicate BS < 70% and PP <0.70; nodes with * indicate BS > 95% and PP>0.95 Sequences in bold are the new sequences generated in this study.

Species description

The species sampled are included in the subgenus *Nitella*, section *Rajia* (*N. acuminata*) and in the subgenus *Tieffallenia*, with representatives of the sections *Gioallenia* (*N. elegans*), *Tieffallenia* (*N. flagellifera* and *N. microcarpa*) and *Persoonia* (*N. axillaris*).

Nitella acuminata A. Braun *ex* Wallman (1853: 35)

Synonyms: *Nitella acuminata* f. *brachyteles* T. F. Allen 1880: 10 (*Nomen nudum*); *Nitella acuminata* f. *brachyteles* A. Braun in A. Braun & C. F. O. Nordstedt 1882: 37; *Nitella subglomerata* A. Braun 1858: 356, *Nitella acuminata* var. *subglomerata* (A. Braun) T. F. Allen 1880: 10; *Nitella gollmeriana* A. Braun 1858: 355-356; *Nitella acuminata* var. *gollmeriana* (A. Braun) Zaneveld ex R. D. Wood 1962: 16.

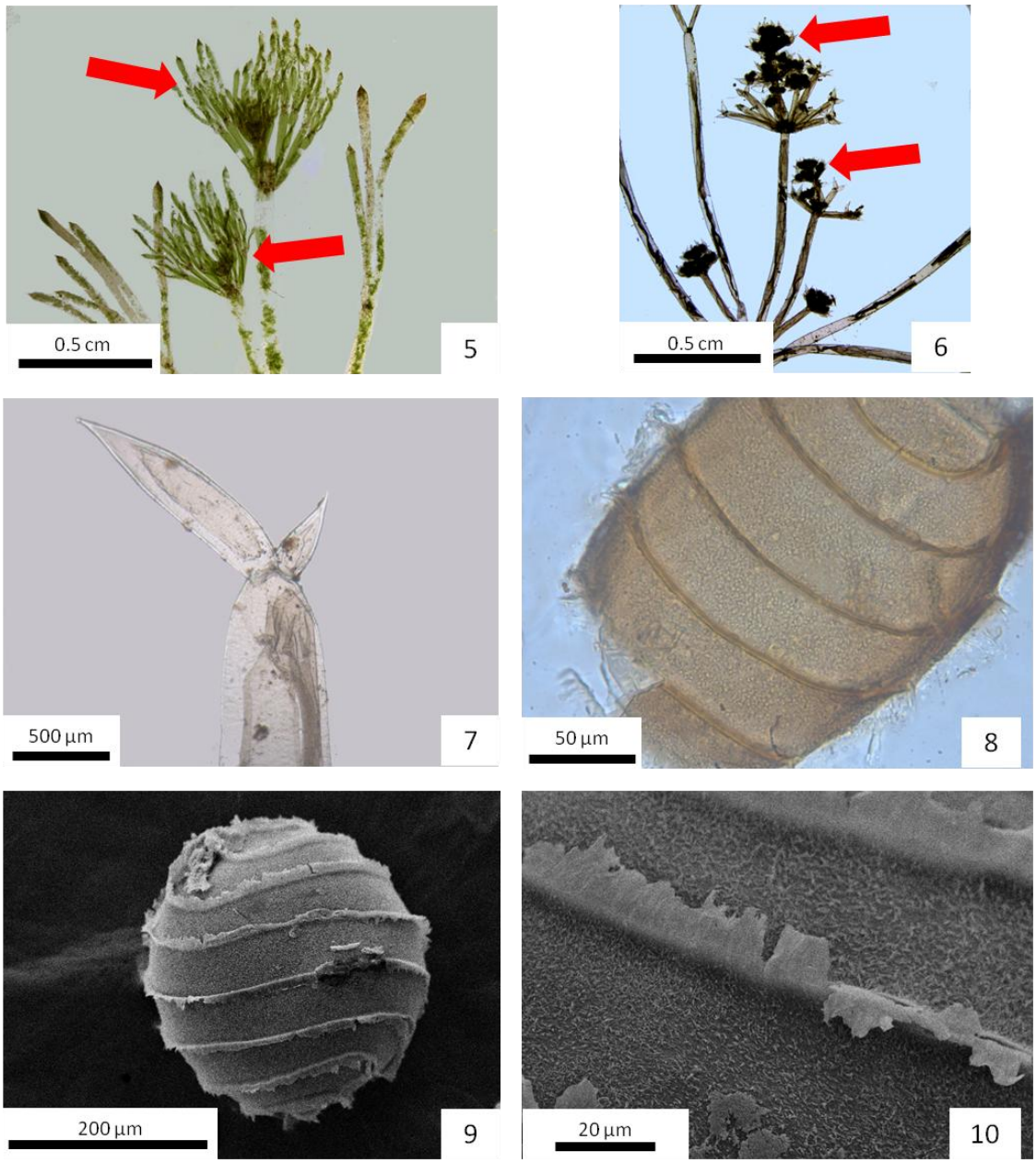
Figs. 5-10.

Plants monoecious, up to 35 cm high, without calcium carbonate deposition. Axes 151-1058 µm in diameter, internodes 0.05-6.7 cm long, 0.14-4.58 (mean 1.25) times as long as the branchlets. Sterile and fertile branchlets similar or different in shape; sterile branchlets (5-)7-9(10) in a whorl, 0.2-4.0 cm long, 1 furcated; rays (5-)7-9(10), 0.05-2.00 cm long, 131-773 µm in diameter; fertile branchlets similar in shape to sterile branchlets, but smaller in length and diameter, and located near apices or forming heads. Dactyls (2)3-4(-6), 1 celled, abbreviated or no, 0.03-1.10 cm long, 55-540 µm in diameter, cylindrical with acuminate apex. Heads, when present, 1-several, terminal or axillary, from loose to compact. Gametangia sejoined or conjoined at fertile branchlets nodes and/or at heads, without mucus. Oogonia 1-3 at a node, 148-496 µm long (excl. nucula), 150-413 µm in wide; convolutions 6-8(9); coronula 24-48 µm high, 42-71 µm wide at base, upper cells usually higher than basal cells; coronular cells convergent, persistent. Oospores brown to dark brown, 211-322 µm long, 181-324 µm wide; striae (5)6-7(8); fossa 28-64 µm across; membrane finely granulate under LM and granulate (only for sample SJRP32572) or scabrous under SEM (all other samples). Antheridia solitary, 126-377 µm in diameter, 8 scutate.

Specimen examined:—BRAZIL. Goiás: BR 070, between the municipalities of Aragarças and Jussara, elev. 339m, 15°51'26.3"S, 51°36'9.5"W, 15 April 2015, coll. *F. R. Borges* (SJRP32572). Mato Grosso: São Nicolau Farm, Municipality of Cotriguaçu, 09°49'27.1"S, 58°15'27.0"W, 25 May 2011, coll. F. R. Borges (SJRP32542). MT10, Municipality of Rosário do Oeste, 14°52'58.5"S, 56°22'24.8"W, 11 May 2014, coll. *F. R. Borges* (SJRP32528). District of Primaverinha, BR163, between the Municipalities of Sorriso and Lucas do Rio Verde, 12°50'43.2"S, 55°49'32.6"W, 11 May 2014, coll. *F. R. Borges* (SJRP32549).Mato Grosso do Sul: Entrance to Recanto Ecológico do Rio da Prata, BR267, Municipality of Jardim, 21°29'21.1"S, 56°26'16.6"W, 15 May 2014, coll. *F. R. Borges* (SJRP32555). Entrance to Recanto Ecológico do Rio da Prata, BR267, Municipality of Jardim, 21°29'21.1"S, 56°26'16.6"W, 15 May 2014, coll. *F. R. Borges* (SJRP32556). Entrance of Araras Farm, BR163, Municipality of Coxim, 18°19'5.9"S, 54°39'56.1"W, 12 May 2014, coll. *F. R. Borges* (SJRP32552). BR163, between the Municipalities of Sonora and Coxim, 17°54'40.5"S, 54°41'24.1"W, 12 May 2014, coll. *F.R. Borges* (SJRP32550). Minas Gerais: BR 369, Municipality of Campos Gerais, elev. 864m, 21°13'39.00"S; 45°38'51.85"W, 30 September 2015, coll. *F.R. Borges* (SJRP32573). São Paulo: Municipality of Neves Paulista, elev. 530m, 20°50'54.6"S, 49°34'13.7"W, 4 September 2014, colls. *F. R. Borges* and *O. Necchi Jr.* (SJRP32565). Municipality of Potirendaba, elev. 443m, 21°00'40.1"S,49°24'40.7"W, 3 September 2014, colls. *F. R. Borges* and *O. Necchi Jr.* (SJRP32529). Municipality of Neves Paulista, elev. 463m, 20°56'48.0"S; Long: 49°41'09.6"W, 4 September 2014, colls. *F. R. Borges* and *O. Necchi Jr.* (SJRP32564). Borá stream, José Domingues Neto Avenue, Municipality of Cedral, elev. 480m, 20°57'03.2"S, 49°20'58.8"W, 3 September 2014, colls. *F. R. Borges* and *O. Necchi Jr.* (SJRP32531). Talhadinho Stream, District of Talhados, Municipality of São José do Rio Preto, elev. 502m, 20°43'45.5"S, 49°17'51.3"W, 4 September 2014, colls. *F. R. Borges* and *O. Necchi Jr.* (SJRP32533). Talhadinho Stream, District of Talhados, Municipality of São José do Rio Preto, elev. 502m, 20°43'45.5"S, 49°17'51.3"W, 4 September 2014, colls. *F. R. Borges* and *O. Necchi Jr.* (SJRP32560). Municipality of José Bonifácio, elev. 440m, 21°00'44.1"S, 49°41'21.6"W, 4 September 2014, colls. *F. R. Borges* and *O. Necchi Jr.* (SJRP32561).

Municipality of Potirendaba, elev. 446m, 21°01'48.9''S,49°23'07.5''W, 3 September 2014, colls. *F. R. Borges* and *O. Necchi Jr.* (SJRP31520). Municipality of José Bonifácio, elev. 440m, 21°00'44.1''S, 49°41'21.6''W, 4 September 2014, colls. *F. R. Borges* and *O. Necchi Jr.* (SJRP32562). Municipality of Ruilândia, elev.445m, 20°55'40.4''S; 49°32'30.9''W, 4 September 2014, colls. *F. R. Borges* and *O. Necchi Jr.* (SJRP31537). Municipality of Potirendaba, elev. 475m, 21°00'04.4''S, 49°25'05.8''W, 3 September 2014, colls. *F. R. Borges* and *O. Necchi Jr.* (SJRP32534). Municipality of Potirendaba, elev. 446m, 21°01'48.9''S, 49°23'07,5''W, 3 September 2014, colls. *F. R. Borges* and *O. Necchi Jr.* (SJRP31519). Municipality of José Bonifácio, elev. 458m, 20°59'08.0''S, 49°40'48.8''W, 4 September 2014, colls. *F. R. Borges* and *O. Necchi Jr.* (SJRP32563).

DNA sequences: *rbc*L- MG010425, MG029152, MG020112, MG010430, MG010428, MG010429, MG020113, MG010426, MG020122, MG010427, MG029156, MG020114, MG029153, MG020118, MG020116, MG020115, MG020117, MG020119, MG029155, MG020121, MG029154, MG020120; ITS2- MG029129, MG029130, MG029134, MG029135, MG029136, MG029133, MG029131, MG029132, MG029137.



FIGURES 5-10. *Nitella acuminata*. 5. Upper whorls with loosely compacted heads. 6. Compacted heads. 7. Dactyls 1-celled, abbreviated with acuminate apex. 8. Oospore membrane with finely granulate aspect under LM. 9. Overview of an oospore under SEM. 10. Oospore membrane with scabrous aspect under SEM.

Nitella axillaris A. Braun (1858: 356)

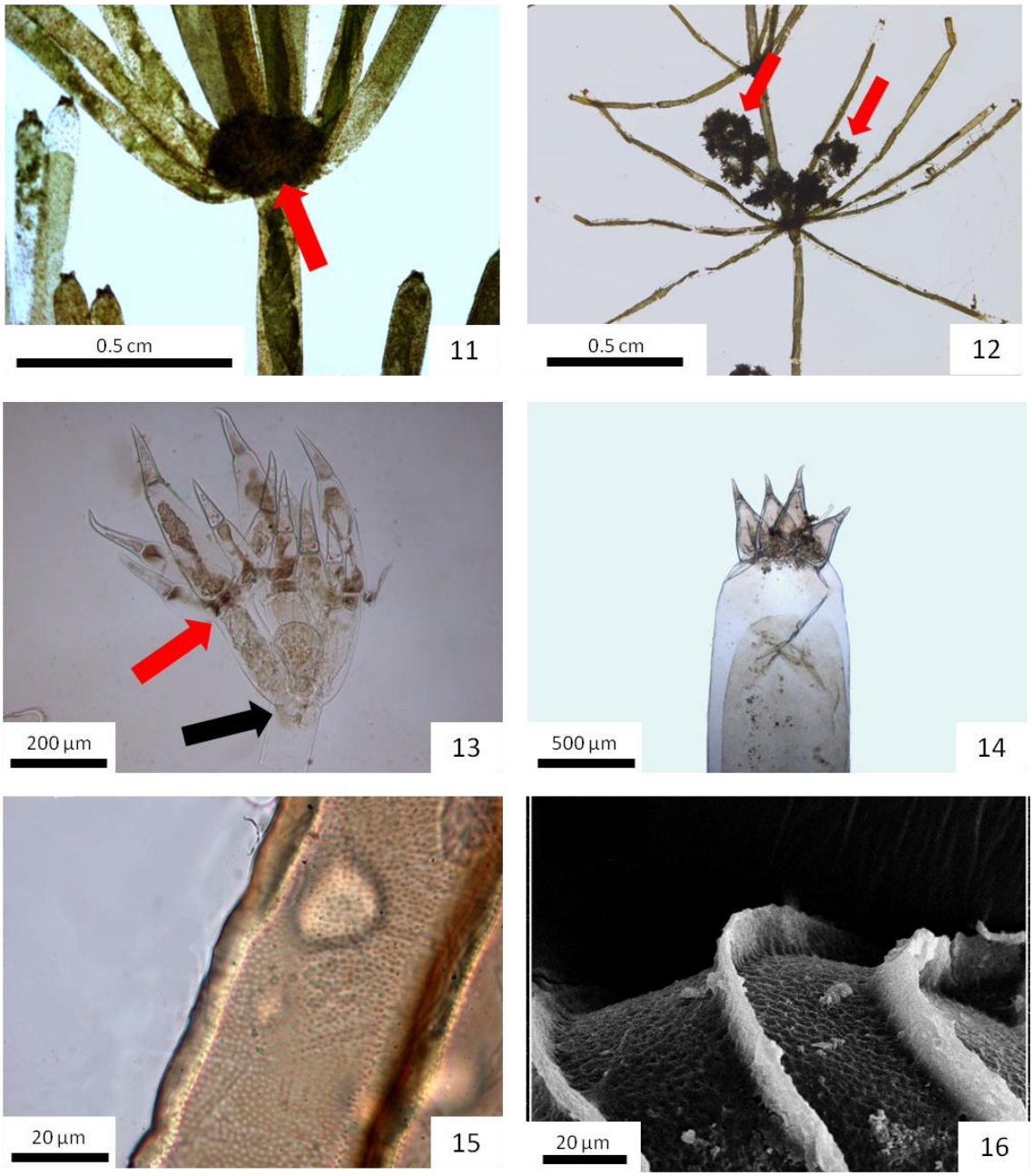
Synonyms: *Nitella translucens* var. *axillaris* (A. Braun) R. D. Wood 1962: 22; *Nitella axilliformis* K. Imahori 1951: 215-216, plate 3; *Nitella translucens* f. *axilliformis* (Imahori) R. D. Wood & Imahori 1965: 689.

Figs. 11-16.

Plants monoecious, up to 26.6 cm high, without calcium carbonate deposition. Axes 299-1182 µm in diameter, internodes 0.7-4.1 cm long, 0.5-3.5 (mean 1.1) times as long as the branchlets. Branchlets sterile and fertile different; steriles (5-)6-8 in a whorl, 0.7-3.7 cm long, 1(2) furcated but appearing simple with tiny terminal coronula; rays (5-) 6-8, constituting the entire branchlet lenght, 283-929 µm in diameter. Dactyls (2)3-4(-6), 2(3)-celled, abbreviated, 0.02-0.04 cm long, 81-279 µm in diameter, penultimate cell tapering gradually towards the base of end cell; end cell conical, acute or acuminate, often deciduous. Fertile branchlets compacted in axillary heads (rarely terminal), 1-2 furcate. Heads numerous, axillary; sessile or stipitate, densely compact or rarely loose, to 0.4cm. Gametangia sejoined or conjoined restricted to branchlet nodes of the heads (rarely at branchlet nodes out of heads). Oogonia 1-2 at a node, 191-411 µm long (excl. nucula), 140-381 µm in wide; convolutions (5)6-7; coronula 26-43 µm high, 46-71 µm wide at base, upper cells usually higher than basal cells; coronular cells convergent, persistent. Oospores brown to dark brown, 236-278 µm long, 224-264 µm wide; striae (5-)6(-7); fossa 39-59 µm across; membrane reticulate under LM and beaded imperfect reticulate to reticulate under SEM. Antheridia solitary, 104-204 µm in diameter, 8 scutate.

Specimen examined:—BRAZIL. Mato Grosso do Sul: Entrance to the Recanto da Barra Farm, BR262, near KM484, between the municipalities of Terenos and Anastácio, 20°30'6.1"S, 55°45'34.7"W, 13 May 2014, coll. *F. R. Borges* (SJRP32553). São Paulo: Pond of Ninféias, State Park, Municipality of São Paulo, 23°38'19.40"S, 46°37'19.18"W, 12 September 2014, coll. *D. F. Peralta* (SJRP32566).

DNA sequences: *rbc*L- MG004810, MG004809; ITS1- MG029146, MG004809 ITS2- MG029146, MG004809.



FIGURES 11-16. *Nitella axillaris*. 11. Whorl with a small, axillary, sessile, compacted head, at a node of the thallus. 12. Whorl with a long, axillary, stipitate and compacted head. 13. 2-furcated fertile branchlet of a head. The black and red arrows indicate, respectively, the primary and the secondary nodes. 14. Dactyls 2-celled, abbreviated, forming a crown. Penultimate cell tapering gradually towards the base of end cell. End cell conical, acute or acuminate. 15. Oospore membrane with reticulate aspect under LM. 16. Oospore membrane with beaded imperfect reticulate aspect under SEM.

Nitella elegans B. P. Pal (1932: 73)

Synonyms: *Nitella pseudoflabellata* f. *elegans* (B.P.Pal) R.D.Wood 1962

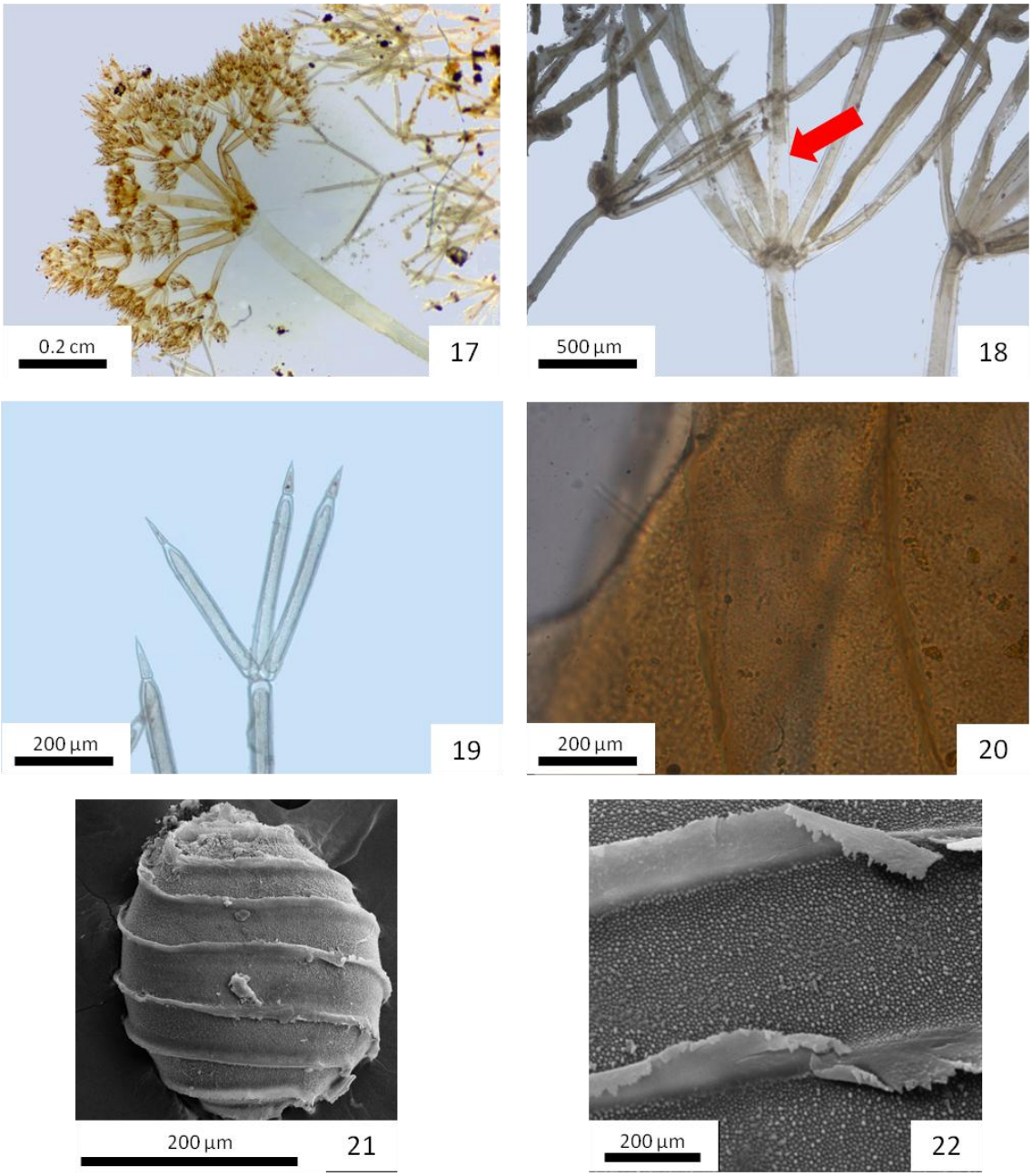
Figs: 17-22.

Plants monoecious, up to 29 cm high, without calcium carbonate deposition, but with or without mucous. Axes 57-385 µm in diameter, internodes 0.21-2.3 cm long, 0.6-4.5 (mean 1.75) times as long as the branchlets. Branchlets sterile and fertile similar or the sterile larger and somewhat less furcate; sterile (5-)7-8(-9) in a whorl, 0.3-2.3 cm long, 3-4(5) furcated; primaries rays (5-)7-8(-9), 0.14-1.10 cm long, 55-240 µm in diameter; secondaries rays (4-)6-8(-10), 0.06-0.60 cm long, 33-152 µm in diameter, with or without central ray; tertiaries rays (3-)5-7, 0.02-0.37 cm long, 28-127 µm in diameter, whit or without central ray; quaternaries (2-)4-6(-8), 0.04-0.24 cm long, 28-109 µm in diameter; quinaries rare. Dactyls (1)2-4(5), 2(-4) celled, commonly abbreviated, 0.01-0.25 cm long, 21-117 µm in diameter, penultimate cell cylindrical tapering distally to base of end cell; end cell conical, acuminate, mucronate. Heads lacking but upper whorls often smaller and somewhat compacted. Gametangia solitary or conjoined at 2^{nd} -3^{rd} (-4^{th}) branchlets nodes with or without mucus. Oogonia 1 at a node, 141-508 µm long (excl. nucula), 109-397 µm in wide; convolutions (5)6-7(8); coronula 27-41 µm high, 43-63 µm wide at base, upper cells normally higher than basal cells; coronular cells convergent, persistent. Oospores light brown to black, 244-301 µm long, 229-268 µm wide; striae 6-7; fossa 40-71 µm across; membrane granulate under LM and finely granulate under SEM. Antheridia solitary, 99-176 µm in diameter, 8 scutate.

Specimen examined:—BRAZIL. Goiás: BR 070, between the Municipalities of Aragarças and Jussara, elev. 339m, 15°51'26.3"S, 51°36'9.5"W, 15 April 2015, coll. *F. R. Borges* (SJRP32571).Mato Grosso: District of Primaverinha, BR163, between the Municipalities of Sorriso and Lucas do Rio Verde, 12°50'43.2"S, 55°49'32.6"W, 11 May 2014, coll. *F. R. Borges* (SJRP32548). São Paulo: Pond of Ninféias, State Park, Municipality of São Paulo, 23°38'19.40"S, 46°37'19.18"W, 12 September 2014, coll. *D. F. Peralta* (SJRP32567).

DNA sequences: *rbc*L- MG004801, MG004800, MG004799; ITS1- MG029150, MG029148; ITS2- MG029150, MG029148, MG029149.

Nitella elegans is here reported for the first time in Brazil.



FIGURES 17-22. *Nitella elegans*. 17. Upper whorls small and somewhat compacted. 18. Primary node of a branchlet. The red arrow indicates a central secondary ray. 19. Dactyls 2-celled. Penultimate cell cylindrical tapering distally towards the base of end cell. End cell conical, acuminate to mucronate. 20. Oospore membrane with granulate aspect under LM. 21. Overview of an oospore under SEM. 22. Oospore membrane with finely granulate aspect under SEM.

Nitella flagellifera J. Groves & G. O. Allen (1927: 337)

Synonyms: *Nitella mucronata* subsp. *flagellifera* (J. Groves & G. O. Allen) R. D. Wood 1962: 18; *Nitella furcata* subsp. *flagellifera* (J. Groves & G. O. Allen) R. D. Wood 1962.

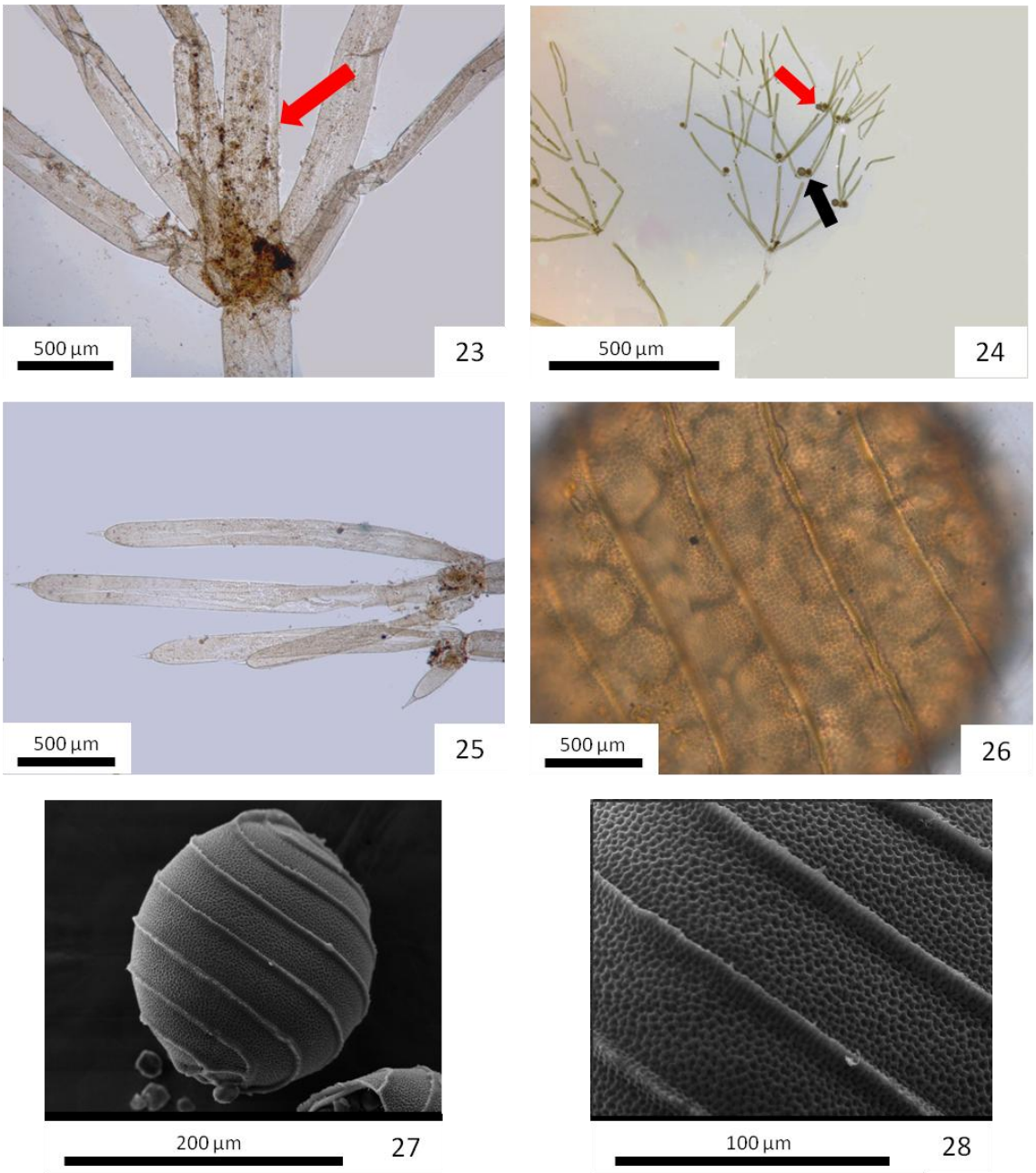
Figs: 23-28.

Plants monoecious, up to 18.5 cm high, without calcium carbonate deposition. Axes 129-581µm in diameter, internodes 0.06-3.9 cm long, 0.12-5.2 (mean 1.0) times as long as the branchlets. Branchlets (4)5-6(7) in a whorl, 0,25-2,3cm long, 2-3(4) furcated; primaries rays (4)5-6(7), 0.08-1.5cm long, 113-453 µm in diameter; secondaries rays 3-6, 0.04-0.6cm long, 75-328 µm in diameter, with or without central ray; tertiaries rays 2-4(5), 0.03-0.4cm long, 57-240 µm in diameter, with or without central ray; quaternaries rays 3, 0.08-0.25cm long, 80-129 µm in diameter. Dactyls 2-3(4), 2(3) celled, elongated or less often abbreviated, 0.01-0.72 cm long, 42-226 µm in diameter, penultimate cell rounded at distal end; end cell conical, acuminate or mucronate. Heads lacking, but axillary and terminal compacted branchlets are frequent (1 per axial node, small or medium size, inverted cone-shaped). Gametangia sejoined or conjoined at 1st (rare), 2nd and 3rd nodes; without mucus. Oogonia 1(2) at a node, 166-510 µm long (excl. nucula), 167-443 µm in wide; convolutions (5)6-7(8); coronula 26-47 µm high, 52-90 µm wide at base, upper cells normally highter than basals, coronular cells convergent ,persistent. Oospores light brown, 253-348 µm long, 226-303 µm wide; striae (5)6-7; fossa 38-71 µm across; membrane reticulate under LM and beaded imperfect reticulate to reticulate under SEM. Antheridia solitary, 88-305 µm in diameter, 8 scutate.

Specimen examined:—BRAZIL. Goiás: District of Placa, Municipality of Pirenópolis, elev. 708m, 15°42'20.7"S, 49°03'40.7"W, 5 September 2009, coll. *O. Necchi Jr.* (SJRP32545). BR 070, between the municipalities of Aragarças and Jussara, elev. 315m, 15°55'4.0"S, 52°00'6.8"W, 15 April 2015, coll. *F. R. Borges* (SJRP32569). Mato Grosso do Sul: BR163, between the Municipalities of Sonora and Coxim, 17°54'40.5"S, 54°41'24.1"W, 12 May 2014, coll. *F.R. Borges* (SJRP32551). Minas Gerais: MG167, between the municipalities of Três Pontas and Varginha, elev. 777m, 21°27'38.8"S, 45°30'55.8"W, 1 October

2015, coll. *F.R. Borges* (SJRP32574).São Paulo: Municipality Potirendaba, elev. 446m, 21°01'48.9''S, 49°23'07.5''W, 3 September 2014, colls. *F.R. Borges & O. Necchi Jr.* (SJRP31518). Municipality of Potirendaba, elev. 443m, 21°00'40.1''S, 49°24'40.7''W, 3 September 2014, colls. *F.R. Borges & O. Necchi Jr.* (SJRP32530). Borá stream, José Domingues Neto Avenue, Municipality of Cedral, elev. 480m, 20°57'03.2''S, 49°20'58.8''W, 13 August 2002, coll. unknown (SJRP32546). Borá stream, José Domingues Neto Avenue, Municipality of Cedral, elev. 480m, 20°57'03.2''S, 49°20'58.8''W, 13 August 2002, coll. unknown (SJRP32547).

DNA sequences: *rbc*L- MG004804, MG004807, MG004805, MG010431, MG004808, MG004803, MG004802, MG004806; ITS1- MG029145, MG029142, MG029144, MG029143; ITS2- MG029145, MG029142, MG029144, MG029143.



FIGURES 23-28. *Nitella flagellifera*. 23. Primary node of a branchlet. The red arrow indicates a central secondary ray. 24. Fertile branchlet with gametangia at second (black arrow) and third (red arrow) nodes. 25. Dactyls 2-celled, elongated. Penultimate cell rounded at distal end. End cell conical and mucronate. 26. Oospore membrane with reticulate aspect under LM. 27. Overview of an oospore under SEM. 28. Oospore membrane with beaded imperfect reticulate to reticulate aspect under SEM.

Nitella microcarpa A. Braun (1958: 357)

Synonyms: *Nitella furcata* (Roxburg ex Bruzelius) C. Agardh emend. R. D. Wood subsp*. mucronata* (A. Braun) R. D. Wood var. *mucronata* f. *mucronata* R. D. Wood & Imahori 1965.

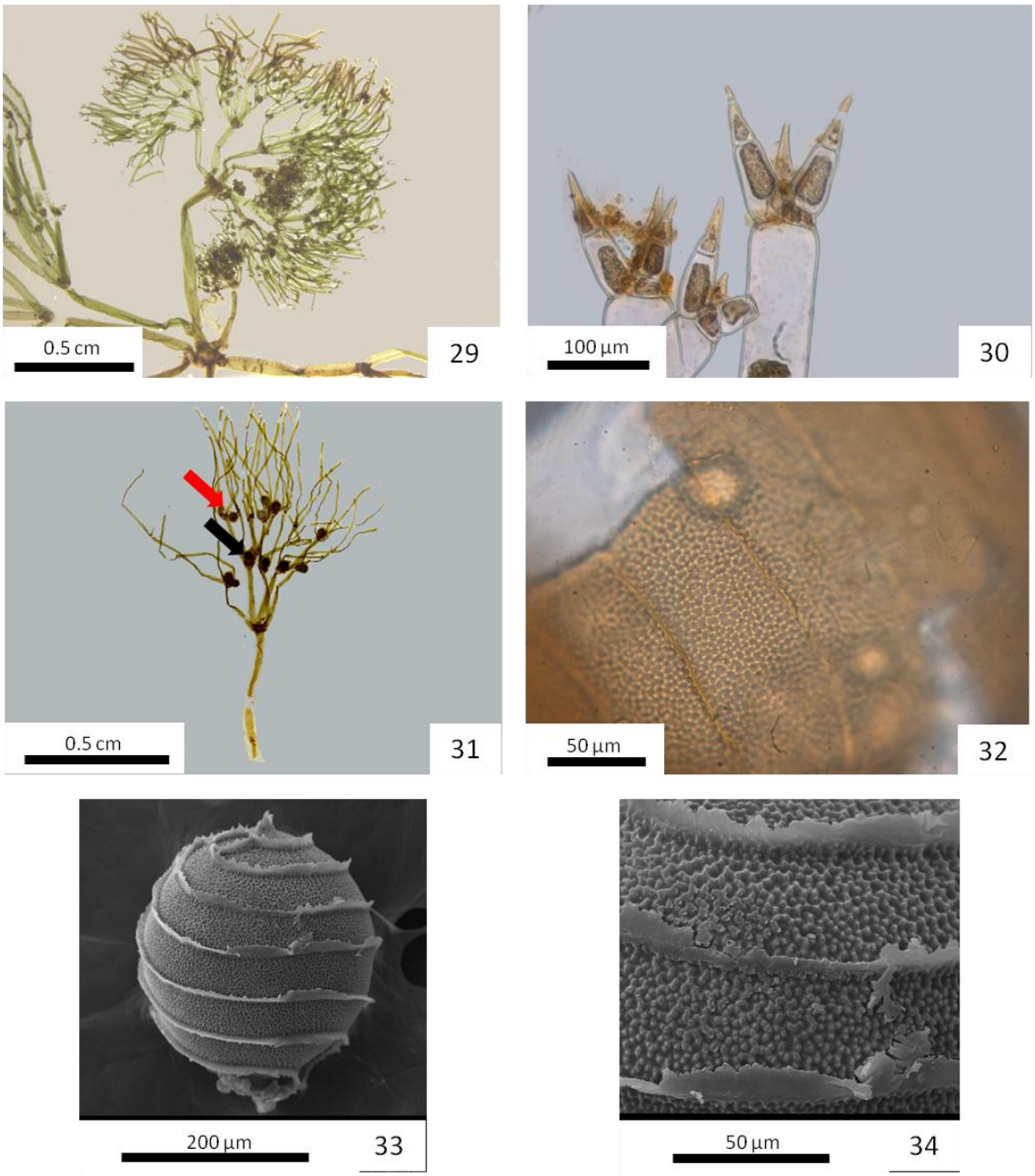
Fig.: 29-34

Plants monoecious, up to 24 cm high, without calcium carbonate deposition. Axes 113-1035 µm in diameter, internodes 0.06-5.3 cm long, 0.16-3.2 (mean 0.8) times as long as the branchlets. Branchlets (4)5-6(-8) in a whorl, 0.24-5.00 cm long, (2)3-4(5) furcated; primaries rays (4)5-6(-8), 0.11-2.00 cm long, 190-795 µm in diameter; secondaries rays (2-)4-6, 0.07-1.2cm long, 76-601 µm in diameter, with or without central ray; tertiaries rays (2)3-4(-6), 0.05-1.55cm long, 52-483 µm in diameter, whit or without central ray; quaternaries 2-4, 0.08-0.8 cm long, 42-425 µm in diameter; quinaries rare. Dactyls (1)2-3(4), 2(3) celled, usually abbreviated, 0.007-0.25 cm long, 25-230 µm in diameter, penultimate cell rounded at distal end; end cell conical, acuminate, mucronate. Heads lacking. Gametangia sejoined or conjoined at all branchlets nodes, without mucus. Oogonia 1-4 at a node, 142-557 µm long (excl. nucula), 128-467 µm in wide; convolutions (4)5-7(8); coronula 30-93 µm high, 33-107 µm wide at base, upper cells normally higher than basal cells; coronular cells convergent, persistent. Oospores light to dark brown, 171-351 µm long, 160-306 µm wide; striae 5-7; fossa 35-80 µm across; membrane reticulate with papillae at the junctions under LM and beaded imperfect reticulate under SEM. Antheridia solitary, 112-244 µm in diameter, 8 scutate.

Specimen examined:—BRAZIL. Goiás: BR 070, between the Municipalities of Aragarças and Jussara, elev.307m, 15°53'38.4"S; 51°39'56.9"W, 15 April 2015, coll. *F. R. Borges* (SJRP32570). Mato Grosso: Entrance to São Nicolau Farm, Municipality of Cotriguaçu, 09°51'25.1"S, 58°15'11.0"W, 25 May 2011, coll. *F. R. Borges* (SJRP32541). São Nicolau Farm, Municipality of Cotriguaçu, 09°49'21.5"S 58°17'11.2"W, 25 May 2011, coll. *F. R. Borges* (SJRP32543). Lagoon located in Parque Florestal de Sinop, Municipality of Sinop, 11°50'02.83"S, 55°30'00.98"W, 2 June 2013, colls. *F. R. Borges* and *O. Necchi Jr.* (SJRP32544). BR 070, between the municipalities of Primavera do Leste

and Barra do Garças, elev. 199m, 15°44'41.8"S, 53°47'8.6"W, 14 April 2015, coll. *F. R. Borges* (SJRP32568).Mato Grosso do Sul: Entrance to the Recanto da Barra Farm, BR262, near KM484, between the municipalities of Terenos and Anastácio, 20°30'6.1"S, 55°45'34.7"W, 13 May 2014, coll. *F. R. Borges* (SJRP32554). São Paulo: Piedade Stream, Municipality of São José do Rio Preto, elev. 507m, 20°52'30"S, 49°22'35.7", 3 September 2014, colls. *F. R. Borges* and *O. Necchi Jr.* (SJRP32532).

DNA sequences: *rbc*L- MG010419, MG010418, MG010421, MG010420, MG010423, MG010422, MG010424; ITS1- MG029141, MG029140, MG029139, MG029138; ITS2- MG029141, MG029151, MG029140, MG029139, MG029138.



FIGURES 29-34. *Nitella microcarpa*. 29. Upper whorls somewhat compacted. 30. Dactyls 2-celled, abbreviated, forming a crown. Penultimate cell rounded at distal end. End cell conical and acuminate. 31. Fertile branchlet with gametangia at second and third nodes. 32. Oospore membrane with reticulate aspect under LM. 33. Overview of an oospore under SEM. 34. Oospore membrane with beaded imperfect reticulate aspect under SEM.

General Remarks

Nitella acuminata has a worldwide distribution, but there are few recent reports, usually in traditional taxonomic studies (e.g. Casanova 2009) and rarely using DNA sequences to show the phylogenetic relationship of this species within the genus (e.g. Pérez *et al.* 2014). Sakayama *et al*. (2004a) concluded that the only sample analyzed under SEM had the oospore wall with a scabrous pattern. Our samples also had a scabrous pattern, except one presenting a granulated ornamentation. This species has been recorded in floristic surveys from Brazil, as well as two other closely related species: *N. subglomerata* and *N. gollmeriana* (e.g. Vieira *et al*. 2002, Picelli-Vicentim *et al*. 2004, Meurer & Bueno 2012, Bueno *et al*. 2011, 2016). Wood & Imahori (1965) treated *N. gollmeriana* as a variety and *N. subglomerata* as a form of *N. acuminata*. *Nitella acuminata* var. *gollmeriana* (*sensu* Wood) is described as having dactyls abreviated, barely visible to naked eyes and dense fertile heads (axillary, rarely terminal), whereas *N. acuminata* f. *subglomerata* (*sensu* Wood) as presenting long dactyls, differing from one another for the lack of heads in *N. acuminata* f. *acuminata* (*sensu* Wood) or if present formed by the compression of the superior whorls. The three taxa, according Wood & Imahori (1965), have oospore wall finely granulate under LM. Tindall (1966) proposed, based on morphological data and geographical distribution of the species studied, that specimens described as *N. gollmeriana*, *N. subglomerata* (and other species, such as *N. stellaris*, *N. lindheimeri*, *N. mexicana*, and possibly *N. subspicata*) represent a rather narrow intergraded series which should be included in a single subgroup of the *N. acuminata* complex. Our samples agreed for all morphological characteristics with *N. acuminata*, *N. gollmeriana* and *N. subglomerata*. Thus, based on the high identity values among the *rbc*L and ITS2 sequences in a large clade with high support (Fig. 2 and 4), we suggest that *N. acuminata*, *N. subglomerata* and *N. gollmeriana* are synonymized, all treated as *N. acuminata*. Studies on the ultrastructure of the oospore wall for this species, although relevant, were not as promising as for other species, since some reports (e.g. John & Moore 1987, Leitch *et al*. 1990, Casanova 2009) have described different patterns of ornamentation, similarly to our results.

Wood & Imahori (1965) lowered *N. axillaris* and *N. axilliformis* to the status of forms of *N. translucens*, based on the presence and development of axillary heads and size of oospores. Sakayama *et al*. (2002, 2004a, 2004b, 2008) raised those species to species level again making clear their separation from *N. translucens* based on molecular data (sequences of *rbc*L and *atp*B) and ornamentation of the oospore wall. They also treated *N. axillaris* and *N. axilliformis* as distinct species, with subtles differences in the oospore wall: the former species presenting sharply developed fused ridges forming a reticulate pattern, while the latter having obtuse fused ridges. They also report distinct size for oospores (335-340 µm for *N. axillaris* and 275-285 µm for *N. axilliformis*). Our trees did not show the separation of *N. axillaris* and *N. axilliformis* and grouped our samples with those from other regions of the world for both species. Thus, in view of a wide and overlapping variation in the diagnostic characters of these two species, as well as the high identity values between the analyzed sequences, we propose that *N. axilliformis* becomes a synonym of *N. axillaris*.

The clade named *N. flagellifera* was formed only by a species of the same name and possesses as sister species *N. oligospira*, both having oospore ornamentation reticulate under SEM. Wood & Imahori (1965) reduced *N. flagellifera* to a subspecies and *N. oligospira* as a form of *N. furcata*. *Nitella flagellifera* was separated from the other subspecies by the presence of a secondary central ray (a characteristic that also distinguishes *N. flagellifera* from *N. oligospira*). Sakayama *et al*. (2002, 2004a, 2004b, 2005, 2008) based on molecular data have shown that *N. oligospira* is distant from *N. furcata* and has as the sister clade composed by *N. axillaris* and *N axilliformis* (in this study called clade *Nitella axillaris*). Our data corroborate the findings of Sakayama *et al.* (2002, 2004, 2004 b, 2005, 2008) and also show the proximity between *N. flagellifera* and *N. oligospira*, both distantly positioned of *N. furcata*. They also showed including *N. oligospira* and *N. flagellifera* under *N. furcata* was artificial. There are several records of *N. flagellifera* for Brazil (Vieira *et al*. 2002, Picelli-Vicentim *et al*. 2004, Bueno *et al*. 2011, 2016). According to Wood & Imahori (1965), the lack of gametangia in the first node of the branchlet is a typical feature of *N. flagellifera*. However, samples SJRP32547 and SJRP32530 had gametangia in the first node, what had also been reported in previous studies

based on Brazilian material (Picelli-Vicentim 2004, Bueno *et al.*, 2011). Our sequences were the first for the species included in Genbank.

The clade named *N. furcata* involves other species that were downgraded to infra-specific taxonomic categories of *N. furcata* by Wood & Imahori (1965): *N. tumulosa*, *N. japonica*, *N. inversa* and our samples of *N. microcarpa*. Using several molecular markers to elaborate highly congruent phylogenetic trees, Sakayama *et al.* (2002, 2004a, 2004b, 2005, 2008) found separate clades with high support for *N. furcata* (along with *N. inversa*), *N. japonica* and *N. tumulosa*, enabling to raise these taxa to species level again. Our trees were similar to those of Sakayama *et al.* (2002, 2004a, 2004b, 2005, 2008), but did not support the separation of all five species mentioned above. We found high values of identity among our sequences and those of other regions, even between samples coming from almost opposite sides of the globe (e.g. Japan and Malaysia). Sakayama *et al*. (2008) points out that all species of this clade present papillate or beaded imperfect reticulate oospore wall under SEM, as we have observed for *N. microcarpa*. In addition, Sakayama *et al*. (2002, 2004a, 2004b) found that *N. furcata* and *N. inversa* had identical sequences for ITS-5.8S rRNA, *atp*B and *rbc*L. Our sequences were the first for the species included in Genbank.

The clade *Nitella pseudoflabellata* was formed by *N. pseudoflabellata* (including a representative of var. *comptonii*), *N. megaspora* and our samples of *N. elegans*. All these species belong to the section *Gioallenia* which, according to Wood & Imahori (1965), is characterized by small to medium sized plants; dactyls with end cells nearly as wide as the apex of penultimate cell and rarely mucronate; presence of absence of heads and/or mucus. Sakayama *et al*. (2005, 2006 and 2008) demonstrated that section *Gioallenia* is polyphyletic. *Nitella elegans* was downgraded by Wood & Imahori (1965) to the form status of *N. pseudoflabellata* and differed morphologically from the other taxa by the presence of secondary central rays, heads with mucus and small oogonia (300-350 µm). Sakayama *et al.* (2002, 2004a, 2004b, 2008) based on molecular data, showed the separation among *N. pseudoflabellata*, *N. megaspora* and *N. pseudoflabellata* var. *comptonii*. Sakayama *et al.* (2006) proposed to elevate the latter variety to species. Our sequences evidenced a separate clade for *N.*

elegans (except for the ITS1 marker), but showed a high identity with the others of the clade. *Nitella elegans* had oospore wall with finely grained ornamentation, as well as the other members of the clade, except *N. pseudoflabellata* var. *comptonii*, with a fibrous type with irregulary arranged granules (Sakayama *et al*. 2006). Our sequences were the first for *N. elegans* to be included in Genbank.

Our data on oospore wall ornamentation and DNA sequences for three molecular markers were mostly consistent with those presented by Sakayama *et al.* (2002, 2004a, 2004b, 2008) and we agree that the use of ornamentation of oospore wall may be extremely useful for the construction of a natural system at section level. In terms of species concept, Wood & Imahori (1965) argued that many species represented a continuum of morphological characters and suggested that 'genetically isolated' populations contributed to the complex pattern of morphological variation. However, such variation does not delineate true species (Karol 2004). Our data showed that even among geographically distant populations of some *Nitella* species, the degree of identity among DNA sequences was high and they exhibited wide variation in morphological characters. Schneider (2016) barcoded 91 *Chara* samples belonging to 14 different taxa using three DNA markers and showed that eight European taxa within the *C. baltica-intermedia*-complex had identical sequences, and only samples from South America differed in one bp from all other samples in the cluster. Some questions need to be answered to refine the taxonomic studies of *Nitella*: what is the weight of molecular data for the species definition? To what extent are morphological features (often having wide inter and intra-population variation, such as gametangia or dactyl size and shape) diagnostic to species delimitation? There is considerable overlap in morphological characteristics used to discriminate species such that uncertainties occur in charophyte species delineation (Boegle *et al.*, 2007, 2010a, 2010b, Schneider *et al* 2016). In addition, different flora treatments differ in their description of one and the same species. Schneider *et al.* (2016) pointed out the lack of knowledge about the relative variation within and among taxa, i.e. whether some morphologically homogeneous taxa are more genetically variable than others, or whether some genetically homogeneous taxa have greater morphological variation than others. They also argued that most of the investigations for *Chara* (and we

extend this to *Nitella*) is restricted to a small number of species and/or a restricted geographical area, increasing the risk of underestimation of genetic and morphological variation. Further studies combining distinct evidences (molecular, ultrastructural and morphological) involving a representative number of samples of distinct regions of the world could properly answer these questions aiming to achieve a more reliable and natural system for *Nitella* taxonomy.

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Table 1. List of taxa with GenBank accession numbers used for tree construction.

Marker	Species	GenBank accession	Strain designation and/or collection information	Reference
*rbc*L	*Nitella gracillima*	AB110874	S053, Japan	Sakayama *et al*. 2003
	N. pseudoflabellata var. *comptonii*	AB236669	S091, Japan	Sakayama *et al*. 2006
	N. pseudoflabellata	AB076065	S032, Japan	Sakayama *et al*. 2002
	N. megaspora	KJ395935	NIES-1628, Unknown	Pérez *et al*. 2014
	N. hyalina	AY823703	KGK0059b, Unknown	Unpublished
	N. furcata	AB076058	S003, Japan	Sakayama *et al*. 2002
	N. inversa	AY804256	Taiwan	Chou *et al*. 2007
	N. tumulosa	AB110869	S060, Malaysia	Sakayama *et al*. 2003
	N. japonica	AB169967	S077, Japan	Sakayama *et al*. 2004
	N. axillaris	AB076070	S005, Unknown	Sakayama *et al*. 2002
	N. translucens	L13482	Unknown	Manhart 1994
	N. axilliformis	KJ395933	NIES-1609, Unknown	Pérez *et al*. 2014
	N. oligospira	AB191732	S095, New Caledonia	Sakayama *et al*. 2005
	N. acuminata	KJ395932	Unknown	Pérez *et al*. 2014
	N. acuminata	AB110866	S057, Malaysia	Sakayama *et al*. 2003
	N. acuminata	DQ076301	Taiwan	Unpublished
	Tolypella intricata	TIU27532	Netherlands	McCourt *et al.*1996

ITS1 e ITS2	*N. pseudoflabellata* var. *comptonii*	AB236678	S091, Japan	Sakayama *et al*. 2006
	N. pseudoflabellata	AB169940	S031, Japan	Sakayama *et al*. 2004
	N. megaspora	AB169944	S073, Japan	Sakayama *et al*. 2004
	N. oligospira	AB191724	S095, New Caledonia	Sakayama *et al*. 2005
	N. axillaris	KR080219	NY00739255, USA	Unpublished
	N. axillaris	AB169949	S005, Unknown	Sakayama *et al*. 2004
	N. axilliformis	AB169950	S056, Japan	Sakayama *et al*. 2004
	N. tumulosa	AB169934	S060, Malaysia	Sakayama *et al*. 2004
	N. japonica	AB169932	S090, Japan	Sakayama *et al*. 2004
	N. furcata	AB169926	S003, Japan	Sakayama *et al*. 2004
	N. gracilima	AB169947	S053, Japan	Sakayama *et al*. 2004
	N. clavata	KR080221	NY:02137576, USA	Unpublished
	N. hyalina	KR080224	NY:02282228, Montenegro	Unpublished
	Tolypella stipitata	KR080243	NY:01475183, USA	Unpublished

CONCLUSÃO GERAL

A presente tese apresentou duas hipóteses gerais. A primeira previa que o número de espécies de *Chara* e *Nitella* descritas para as regiões Sudeste e Centro-Oeste encontra-se equivocado. Por um lado, esse número estaria super-estimado devido à grande quantidade de espécies definidas com base em critérios mal definidos; e por outro sub-estimado pela provável ocorrência de espécies distintas geneticamente, tanto crípticas como diferenciadas por novos critérios diagnósticos. As evidências moleculares associadas aos dados morfológicos tradicionais e ultra-estruturais comprovaram que, ao menos na primeira parte, esta hipótese foi confirmada. Wood & Imahori (1965) propuseram o rebaixamento de várias espécies a níveis taxonômicos infra-específicos, ampliando os limites dos caracteres que delimitavam as espécies, ou seja, utilizando-se do conceito que denominaram de macro-espécie. Além das comprovações moleculares, segundo McCourt *et al.* (2016), o sistema de Wood e Imahori não vem sendo amplamente utilizado porque trabalhos experimentais (McCracken *et al*. 1966, Grant & Proctor 1972, Proctor 1975) mostraram que ele não reflete as diferenças em nível específico em termos de isolamento reprodutivo. Após consulta ao Algae Base (Guiry & Guiry 2017), nos deparamos com vários táxons propostos como níveis infra-específicos por Wood e Imahori e que vêm sendo re-elevados a nível de espécie. Entretanto, a simples elevação ou rebaixamento dos níveis taxonômicos não contribui de forma efetiva para o avanço da taxonomia de Characeae, pois acreditamos que os dados moleculares podem definir melhor as circunscrições das espécies, e complementarmente caracteres morfológicos podem ser selecionados para a diagnose das mesmas. Assim evitaríamos a adoção de um número enorme de espécies e níveis infra-específicos que em muitos casos não são mais do que variações morfológicas (usualmente refletindo respostas ambientais ou variações populacionais) de um mesmo grupo genético. Na presente tese pudemos apontar prós e contras no sistema proposto por Wood e Imahori (1965), comprovando a necessidade de novos estudos, sobretudo através de dados moleculares, antes de adotar ou descartar totalmente o conceito de macroespécie:

1- Como ponto falho no sistema de Wood e Imahori destacamos a proposta de incluir *C. foliolosa*, *C. haitensis*, *C. hydropitys* e *C. rusbyana* como variedades e formas da espécie *Chara zeylanica.* Os três marcadores (*rbc*L, ITS2 e *mat*K) forneceram subsídios para tratarmos cada um destes táxons como uma espécie distinta. Situação semelhante ocorreu para *Nitella flagellifera*, que foi tratada como uma forma de *Nitella furcata*, mas nossos dados evidenciaram tratar-se de uma espécie distinta e até filogeneticamente distante de *N. furcata*, apesar das semelhanças nas características morfológicas.

2- Como ponto positivo, pudemos confirmar o agrupamento de *N. acuminata*, *N. gollmeriana* e *N. subglomerata* como categorias infra-específicas de *Nitella acuminata*. Os marcadores *rbc*L, ITS1 e ITS2 foram eficientes em mostrar que, apesar das diferenças morfológicas, estes três táxons apresentaram-se geneticamente muito semelhantes. Discordamos, entretanto, em adotar categorias infra-específicas e propusemos a inclusão de *N. gollmeriana* e *N. subglomerata* dentro de *N. acuminata*.

Não encontramos nenhuma nova espécie discernível com base em dados genéticos, bem como não conseguimos definir nenhum novo caráter com grande potencial para diagnose das espécies. Nessa linha, o único caso que merece destaque, foram nossos dados moleculares e morfológicos para *C. guairensis*, que mostraram tratar-se de espécie endêmica do Brasil e totalmente distinta das demais espécies comparáveis, tanto para o Brasil quanto para o resto do mundo

A segunda hipótese enunciava que algumas espécies de *Chara* e *Nitella* ocorrentes no Brasil apresentariam, devido ao isolamento geográfico em período relativamente remoto, sequências de DNA bastante divergentes daquelas de outras regiões do mundo (especialmente da América do Norte, Europa, Ásia e Austrália). Esta hipótese foi refutada ao compararmos as sequências de nossas amostras com aquelas provenientes de outras regiões, inclusive coletadas em países bastante distantes, como por exemplo Malásia e Japão, entre outros. As sequências de *rbc*L apresentaram valores de identidade de 98,4% a 100%, as de ITS1 de 90% a 100% e as de ITS2 de

94,4% a 100%, tanto de *Chara* quanto de *Nitella*. As sequências geradas para o marcador *mat*K foram as primeiras a serem incluídas no Genbank para as espécies amostradas neste trabalho, de forma que não há base para comparação com aquelas de outros países. Vale aqui destacar que estes valores foram para clados que, algumas vezes, incluíram mais de uma espécie, como no caso de *Nitella furcata*, que englobou 5 diferentes espécies para o marcador *rbc*L.

Acreditamos que os dados moleculares minimizam a subjetividade na interpretação dos dados, o que é comum em sistemas taxonômicos baseados apenas na morfologia clássica, em que o autor dá maior peso a um caráter em relação a outros. Esta é uma das grandes críticas ao sistema de Wood & Imahori (1965). Com o avanço nos estudos envolvendo maior número de sequências de diversas espécies, provavelmente teremos maior subsídio para determinarmos quais características morfológicas associadas às unidades genéticas melhor se aplicam para separar as espécies.

Quanto à ornamentação da parede do oósporo, a sua aplicabilidade tem grande potencial, uma vez que tem se mostrado como um caráter morfológico bastante conservado em níveis taxonômicos supra-específicos (sub-gêneros e secções), não sendo, porém, tão efetivo para separação de espécies. Isso foi demonstrado por Sakayama *et al*. (2002, 2004a, 2004b, 2008) e corroborado em nosso trabalho.

Por fim, esperamos que esta tese estimule outros pesquisadores brasileiros a realizarem novos trabalhos empregando ferramentas moleculares e ultra-estruturais. O campo a ser explorado no país ainda é vasto, visto que, são reportadas 23 espécies de *Chara* e 27 de *Nitella* para o Brasil (Bueno *et al.* 2015). Esperamos que o uso destas ferramentas, associadas à análise crítica da taxonomia clássica, possam contribuir de forma efetiva para aprimorar o sistema de classificação de Characeae em nível global.

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