UNIVERSIDADE FEDERAL DE SÃO CARLOS

CENTRO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM ECOLOGIA E RECURSOS NATURAIS

Biodiversidade de Selenastraceae (Sphaeropleales, Chlorophyceae): características morfológicas e sequenciamento dos marcadores moleculares 18S rDNA, *rbcL* e ITS como base taxonômica tradicional.

Thaís Garcia da Silva

Tese apresentada ao Programa de Pós-Graduação em Ecologia e Recursos Naturais da Universidade Federal de São Carlos, como parte dos requisitos para obtenção do título de DOUTOR EM CIÊNCIAS, área de concentração: ECOLOGIA E RECURSOS NATURAIS.

Orientador: Prof. Dr. Armando Augusto Henriques Vieira

São Carlos – SP
2016

Ficha catalográfica elaborada pelo DePT da Biblioteca Comunitária UFSCar
Processamento Técnico
com os dados fornecidos pelo(a) autor(a)

Silva, Thaís Garcia da
S586b Biodiversidade de Selenastraceae (Sphaeropleales, Chlorophyceae): características morfológicas e sequenciamento dos marcadores moleculares 18S rDNA, rbcL e ITS como base taxonômica tradicional / Thaís Garcia da Silva. -- São Carlos : UFSCar, 2016.
148 p.

Tese (Doutorado) -- Universidade Federal de São Carlos, 2016.

1. Taxonomia. 2. Sistemática molecular. 3. Selenastraceae. 4. Biodiversidade. 5. Filogenia. I. Título.

UNIVERSIDADE FEDERAL DE SÃO CARLOS

Centro de Ciências Biológicas e da Saúde
Programa de Pós-Graduação em Ecologia e Recursos Naturais

Folha de Aprovação

Assinaturas dos membros da comissão examinadora que avaliou e aprovou a Defesa de Tese de Doutorado da candidata Thaís Garcia da Silva, realizada em 04/02/2016:

Prof. Dr. Armando Augusto Henriques Vieira
UFSCar

Profa. Dra. Odete Rocha
UFSCar

Profa. Dra. Inessa Lacativa Bagatini
UFSCar

Profa. Dra. Andréa Tucci
IB

Prof. Dr. Orlando Necchi Júnior
UNESP

“Longe se vai sonhando demais

Mas onde se chega assim?

Vou descobrir o que me faz sentir

Eu, caçador de mim.”

Milton Nascimento

Agradecimentos

Primeiramente, ao Dr. Armando Augusto Henriques Vieira por ter me aceitado como estagiária e aluna de doutorado. Agradeço imensamente por todo apoio e dedicação nesses 6 anos de trabalho no Laboratório de Ficologia, me mostrando novas possibilidades de aprendizado e estimulando o meu caminhar na pesquisa e conhecimento na ficologia. Sinto-me imensamente honrada por ter trilhado um caminho tão importante de minha vida sob sua tutoria e me espelho no seu exemplo de verdadeiro amor e dedicação à profissão.

À minha co-orientadora Dra. Célia Leite Sant'Anna, Núcleo de pesquisa em Ficologia do Instituto de Botânica, pelo grande estímulo que me deu para que eu prosseguisse na taxonomia e que não desanimasse em momentos decisivos desta jornada, dando valor ao que eu acreditava ser importante neste estudo.

À Dra. Christina Bock, Departamento de Biodiversidade da Universidade Duisburg-Essen, por ter me aceitado como sua aluna, pelo grande conhecimento sobre biologia molecular que humildemente me transmitiu em minha estadia na Alemanha e por ter confiado que eu seria capaz de enfrentar esse desafio "selenastrágico".

À Dra. Sabina Wodniok pelo auxilio nas análises de microscopia eletrônica.

Ao Dr. Jens Boenigk por ter permitido à Christina me receber como aluna no Departamento de Biodiversidade e pelo auxílio burocrático envolvido.

À CAPES pela concessão da bolsa de estudos nos 12 primeiros meses de doutorado.

À Fundação de Amparo à Pesquisa do Estado de São Paulo, FAPESP, pelas concessões das bolsas de estudos no Brasil (Processo n° 2012/19520-1) e, no exterior por meio do programa "Bolsa Estágio de Pesquisa no Exterior" (Processo n° 2013/17457-3).

À Universidade Federal de São Carlos, ao Instituto de Botânica de São Paulo e a Universidade Duisburg-Essen por fornecer a infraestrutura necessária à realização deste

trabalho e ao Programa de Pós-Graduação em Ecologia e Recursos Naturais, pela oportunidade de aprimoramento científico.

Ao Programa de Pós-graduação em Biodiversidade Vegetal e Meio Ambientedo Instituto de Botânica de São Paulo, pela oportunidade de aprimoramento científico por conta das disciplinas cursadas.

Ao Luizinho pelo apoio durante as coletas do Projeto Biota e pelo exemplo profissional que nos dá.

Aos colegas e amigos do Laboratório de Ficologia pelos momentos compartilhados: Naiara Carolina, Cilene, Inessa, Fabrício, Luiz, Letícia, Alessandra, Helena, Guilherme, Ingritt, Chico, Lucas, Érica, Moira (e, possivelmente, mais alguns que devo ter me esquecido...rsrsrs). Meu muito obrigada por tudo o que foi vivido.

À Inessa por clarear meus pensamentos e não me deixar ser tão tendenciosa na taxonomia, no auxílio com a biologia molecular, na redação de artigos e crises existenciais no final deste processo.

À Zezé por ter me ensinado o básico da taxonomia para as análises das minhas amostras quali e quantitativas, ainda na minha iniciação científica. Por um acaso do destino você me sugeriu procurar o Armando para tentar uma pós e aqui cheguei.

Aos meus amigos muito queridos da UDE e que tornaram minha estadia muito agradável: Julia, Lars, Vesna, Yesim, Edward, Elif, Saskia, Philpp, Sabina, Nikoletta, Fernando, Saeed, Farnoush, Sarah, Susy, Beate e Christina.

À Andrea pela companhia agradabilíssima em todas as vezes que fui ao Instituto de Botânica, pelas conversas, hospedagem, por me encorajar na pesquisa e na vida.

Agradeço especialmente ao Luiz pelo apoio cotidiano no laboratório, pelas conversas durante o trabalho e, principalmente, por ter dividido a "nossa" salinha de microscopia por longos dias.

Aos funcionários do PPGERN: João, Roseli e, especialmente Beth (que eu encontrava com frequência ao retornar para casa) pela ajuda com a burocracia envolvida neste doutorado.

Aos meus amigos por todos os momentos durante minha caminhada. Não citarei nomes porque fatalmente esquecer-me-ei de alguém.

Ao Neto, pelos importantes jogos de forca que praticávamos durante as aulas entediantes e por ter feito esses momentos mais leves.

À Priscilla por ter estado ao meu lado por algum tempo neste processo.

Muitos foram importantes pra que eu chegasse aqui mas, agradeço sobretudo à: minha mãe, vó e irmã por estarem ao meu lado e me ensinarem coisas que nem consigo por no papel de tamanha grandeza.

À minha família toda por ter torcido por mim, pelo carinho e pelo porto seguro que só uma família, mesmo que torta, pode propiciar.

Aos meus mestres da vida inteira, que de alguma forma me ajudaram a construir o que sou e olhar para o que quero ser. Por cada momento de ensinamento, obrigada!

Resumo

A filogenia da família Selenastraceae foi investigada por microscopia ótica, análises moleculares dos marcadores 18S rDNA, *rbcL*, ITS1-5,8S-ITS2 e ITS-2. Várias características morfológicas tradicionalmente utilizadas para identificação de gêneros e espécies foram investigadas. Todas as cepas de Selenastraceae estudadas têm pirenóides nus dentro do cloroplasto, exceto o gênero *Chlorolobion*, que apresentou pirenóide amilóide. As análises moleculares mostraram que nenhum critério morfológico isolado considerado até agora é significativo para a sistemática do Selenastraceae, mas o uso de um conjunto de características morfológicas pode ser adequado para identificar espécies dos gêneros *Ankistrodesmus*, *Chlorolobion*, *Kirchneriella*, *Raphidocelis* e *Tetranephris*. As análises filogenéticas moleculares mostraram que os gêneros *Monoraphidium*, *Kirchneriella* e *Selenastrum* são polifiléticos e não distinguíveis como gêneros. O morfotipo de *Selenastrum* revelou três linhagens moleculares diferentes, levando à descrição de dois novos gêneros, *Curvastrum* gen. nov. e *Messastrum* gen. nov. Além disso, as análises filogenéticas revelaram quatro linhagens moleculares atribuídas ao morfotipo de *Kirchneriella*, levando à descrição de cinco espécies novas: Gênero 1 sp. nov. 1, Gênero 2 sp. nov. 1, Gênero 2 sp. nov. 2, *Raphidocelis* sp. nov. e *Tetranephris* sp. nov.

Abstract

The phylogeny of the family Selenastraceae was investigated by light microscopy, molecular analysis of 18S rDNA, *rbcL*, ITS1-5.8S-ITS2 and ITS-2 markers. Several morphological features traditionally used for identification of genera and species were investigated. All Selenastraceae strains studied presented naked pyrenoids within the chloroplast, except for *Chlorolobion*, which presented starched pyrenoid. Molecular analysis showed that no isolated morphological criteria considered so far is significant for the systematic of Selenastraceae, but a set of characteristics may be appropriate to identify species of genera *Ankistrodesmus*, *Chlorolobion*, *Kirchneriella*, *Raphidocelis* and *Tetranephris*. Phylogenetic analyses showed that genera *Monoraphidium*, *Kirchneriella* and *Selenastrum* are polyphyletic and not distinguishable as separate genera. The *Selenastrum* morphotype revealed three different molecular lineages, leading to the description of two new genera, *Curvastrum* gen. nov and *Messastrum* gen. nov. In addition, molecular phylogenetic analysis revealed four lineages assigned to *Kirchneriella* morphotype, leading to the description of five new species: Genus 1 sp. nov. 1, Genus 2 sp. nov. 1, Genus 2 sp. nov. 2, *Raphidocelis* sp. nov. and *Tetranephris* sp. nov.

Lista de siglas e abreviaturas

18S rDNA	18S DNA ribossômico (18S ribosomal RNA)
28S rDNA	28S DNA ribossômico (28S ribosomal RNA)
5.8S rDNA	5.8S DNA ribossômico (5.8S ribossonal RNA)
BP	Probabilidade Bayesiana (Bayesian probability)
CB	Christina Bock
CBC	Mudança de bases compensatórias (Compensatory base changes)
CCMA	Coleção de culturas de Microalgas de Água
Comas	Augusto Abilio Comas González
DNA	Ácido desoxirribonucleico (desoxyribonucleic acid)
gen. nov.	Gênero novo
ITS	Espaçador interno transcrito (Internal transcribed spacer)
ITS1	Espaçador interno transcrito situado entre os genes 18S rDNA and 5.8S rDNA
ITS2	Espaçador interno transcrito localizado entre os genes 5.8S rDNA e 28S rDNA nas algas
KF	Alena Lukešová Culture Collection
KR	Lothar Krienitz
LM	Microscopia ótica (Light microspy)
MCMC	Monte Carlo via Cadeias de Markov (Markov Chain Monte Carlo)
MFE	Energia mínima livre (Minimum free energy)
ML	Máxima verossimilhança (Maximum likelihood)
MP	Máxima parsimônia (Maximum parsimony)
NCBI	National Center for Biotechnology Information
NGS	Sequenciamento de nova geração (Next generation sequencing)

NJ	Agrupamento de vizinhos (Neighbor-joining)
PCR	Reação em cadeia da polimerase (Polimerase Chain Reaction)
PP	Probabilidade posterior (posterior probability)
rbcL	Subunidade grande da RUBISCO (RUBISCO large subunit)
rRNA	Ácido ribonucléico ribossômico (ribosomal rubonucleic acid)
SAG	Sammlung von Algenkulturen der Universität Göttingen
SEM	Microscopia eletrônica de varredura (Scanning Electron microscopy)
sp. nov.	Espécie nova
SSU rRNA	Subunidade menor do ribossomo (ribossome small subunit)
TEM	Microscopia eletrônica de transmissão (Transmission Electron Microscopy)
UTEX	The Culture Collection of Algae at the University of Texas at Austin.
WDCM	World Data Center for Microorganisms

Lista de figuras

Figuras suplementares

Lista de tabelas

Apresentação da tese

A tese foi elaborada para conter os itens: (1) Introdução geral; (2) Hipóteses e Objetivos; (3) Capítulos (com resultados e discussão); (4) Discussão geral; e (5) Conclusões.

Cada capítulo será apresentado no formato de artigo científico: com resumo, introdução, material e métodos, resultados, discussão, referências bibliográficas e material suplementar. Este formato foi escolhido para facilitar a publicação dos resultados obtidos. A introdução geral está em português, bem como a discussão geral e a conclusão.

O primeiro capítulo encontra-se formatado para a publicação na revista Fottea, à qual foi submetido e aceito. Neste capítulo, apresentamos os resultados de uma análise filogenetica utilizando os marcadores *rbcL* e 18S rDNA.

No segundo capítulo da tese abordamos o complexo *Kirchneriella-Raphidocelis-Pseudokirchneriella*, os menores organismos de Selenastraceae, em um estudo filogenético baseado em 18S rDNA e na analise multigene dos marcadores ITS1-5.8S-ITS2, formatado nos moldes da Journal of Phycology, possível periódico para submissão.

Ao final, uma breve discussão geral e as conclusões foram elaboradas baseadas nos capítulos apresentados.

Sumário

Introdução Geral

Sistemática de Chlorophyta

O termo Chlorophyta refere-se, tradicionalmente, ao grupo de algas verdes, que se caracteriza por cloroplastos com membrana dupla, amido como polissacarídeo de reserva, tilacóides empilhados e presença de clorofila a e b (Friedl 1997, Chapman *et al*. 1998). Contudo, alguns gêneros reconhecidamente pertencentes à Chlorophyta, perderam seus pigmentos em processos secundários (Pringsheim 1963). Uma enorme diversidade morfológica está inclusa em Chlorophyta, compreendendo desde organismos unicelulares cocóides ou flagelados, coloniais, filamentosas ramificadas ou não, membranosas e cenocíticas (van den Hoek *et al* 1988).

A divisão Chlorophyta abrange organismos viventes em ambientes de água doce ou marinha, sendo um dos principais produtores primários em ambientes aquáticos (Bock 2010). O hábito dessas algas pode ser epifítico, planctônico e algumas espécies vivem em comunidades edáficas.

O primeiro sistema natural de classificação de algas criado remete há aproximadamente 200 anos e considerou o nível de organização do estado vegetativo como principal característica morfológica para a separação dos grandes grupos (Blackman 1900, Blackman & Tansley 1902, Pascher 1918). Desde então, a sistemática de algas verdes passou por grandes mudanças, sendo aperfeiçoada ao longo dos anos, resultando em uma classificação baseada em ciclos de vida, dados ultra-estruturais, forma e organização das células vegetativas, mitose, composição da parede celular e arquiteturas de células flageladas (Christensen 1962, van den Hoek & Jahns 1978, Ettl & Komárek 1982, Mattox & Stewart 1984, van den Hoek *et al.* 1988, 1995). A partir da combinação de diferentes critérios morfológicos, esses sistemas de classificação

delinearam classes, famílias, gêneros e espécies (Kornmann 1973, Ettl 1981, Komárek & Fott 1983, Mattox & Stewart, 1984).

O advento das análises moleculares aprofundou a visão do sistema natural de classificação de algas verdes (Melkonian & Surek 1995, Friedl 1997, Lewis & McCourt 2004), trazendo o consenso de que as algas verdes evoluiram em duas grandes linhagens, chamadas de Clado Charophyta e Clado Chlorophyceae sensu Lewis & McCourt (2004).

O Clado Charophyta (ou Streptophyta sensu Bremer 1985) compreende as plantas terrestres e um número de algas verdes, grupos como Mesostigmatophyceae, Chlorokybophyceae, Klebsormidiophyceae, Zygnemophyceae, Coleochaetophyceae e Charophyceae.

O Clado Chlorophyceae (ou Chlorophyta) compreende a maioria das algas que eram tradicionalmente referidas como algas verdes. Este clado contém os três grupos monofiléticos de algas verdes (Chlorophyceae, Trebouxiophyceae e Ulvophyceae) e o clado parafilético traz Prasinophyceae, que contém, ao menos, seis diferentes clados (Lewis & McCourt 2004) na parte basal de Chlorophyta (Fawley *et al*. 2000, Lewis & McCourt 2004, Marin *et al.* 2010).

A origem polifilética de várias famílias e gêneros definidos morfologicamente foi revelada ao combinar dados moleculares e morfológicos. Um estudo com o gene SSU rRNA mostrou que a morfologia "*Chlorella*-like" evoluiu independentemente dentro de Chlorellaceae e Trebouxiophyceae (Huss *et al*. 1999). *Chlorella vulgaris* Beijerinck, representada pela autêntica cepa SAG 211-11b, estabeleceu uma linhagem dentro de Chlorellaceae (Trebouxiophyceae), ficando, por conseguinte, o nome genérico *Chlorella* Beijerinck válido apenas para os membros deste grupo (Huss *et al*. 1999, Krienitz *et al.* 2004). Consequentemente, vários novos gêneros foram admitidos para as

algas "*Chlorella*-like" (*Marinichlorella* Aslam *et al.* 2007, *Kalinella* Neustupa *et al.* 2009) e espécies conhecidas (como *Chlorella saccharophila, C. ellipsoidea*) geraram novas combinações para gêneros diferentes (por exemplo, *Chloroidium* Nadson) (Aslam *et al*. 2007, Neustupa *et al*. 2009, Darienko *et al*. 2010).

Assim, admite-se que os caracteres morfológicos são particularmente sujeitos à convergência ou evolução paralela, podendo apresentar plasticidade fenotípica. Formas celulares simples podem subestimar a diversidade genética, como demonstrado no caso de *Chlorella*, onde o formato "bola verde" foi observado em várias linhagens filogenéticas independentes, correspondendo a gêneros e espécies diferentes, definidos por biologia molecular (Huss *et al.* 1999, Aslam *et al*. 2007, Neustupa *et al*. 2009).

Diferentes espécies do gênero *Scenedesmus*, quando submetidos a crescimento sob a influência do zooplâncton *Daphnia*, apresentaram um aumento significativo do tamanho da colônia (Trainor 1998). Ademais, indivíduos isolados de *Scenedesmus* podem exibir características morfológicas que abrangem grupos e conduzirem a sobrestimação da riqueza de espécies (Trainor 1998).

Resta ainda a incerteza de como distinguir espécies sabendo-se da existência de variabilidade genética extremamente elevada versus a quantidade limitada de caracteres morfológicos, levando a expressão de morfologia celular simples, acrescido do fator ambiental que pode ocasionar plasticidade fenotípica dependendo das suas variações.

Taxonomia e filogenia de Selenastraceae (Blackman & Tansley)

A família Selenastraceae é composta de algas verdes cocóides, com células com aspecto fusiforme a cilíndrico, solitárias ou coloniais, cujo principal critério para sua definição é a típica citocinese para a liberação de autósporos. Entretanto, seus gêneros principais nem sempre foram classificados em Selenastraceae.

Blackman e Tansley estabeleceram em 1902 o conteúdo da família Selenastraceae, tendo esta sofrido alterações no decorrer do tempo. Por incluir os gêneros *Selenastrum* Reinsch e *Scenedesmus* Meyen, Scenedesmaceae Bohlin 1904 foi usada por algum tempo como sinônimo de Selenastraceae (Silva 1980). Todavia, West e Fritsch (1927) separaram claramente as duas famílias em Scenedesmaceae, sendo *Scenedesmus* o gênero-tipo, e Selenastraceae, com *Selenastrum* como gênero-tipo.

A partir do modo de reprodução, Brunnthaler (1915) citou os gêneros *Ankistrodesmus* Corda ex Korshikov e *Selenastrum*, com reprodução exclusiva por autosporia, quando dividiu a antiga ordem Protococcales em duas séries denominadas Autosporinae, com reprodução por autosporia, e Zoosporinae, com reprodução por zoósporos.

Korshikov (1953) estabeleceu a família Ankistrodesmaceae como sinônimo de Selenastraceae, incluindo nove gêneros, como *Chlorolobion* Korsikov, *Ankistrodesmus*, *Nephroclamys* (G.S.West) Korshikov e *Kirchneriella* Schmidle. Mais adiante, Bourrelly (1972) inseriu os gêneros *Ankistrodesmus*, *Monoraphidium* Komárková-Legnerová, *Podohedriella* (Duringer) Hindák, *Quadrigula* Printz, *Selenastrum*, *Chlorella*, *Raphidium* Schroeder e *Kirchneriella*, na família Oocystaceae.

Posteriormente, os gêneros *Ankistrodesmus*, *Monoraphidium*, *Podohedriella* e *Quadrigula* e mais 12 gêneros foram colocados na família Chlorellaceae, sub-família Ankistrodesmoideae, por Komárek & Fott (1983), voltando-se a admitir amplamente o

sistema de classificação de Brunnthaler (1915) (Comas, 1996, Hindák, 1984, 1988, 1990).

A divergência entre os autores no que diz respeito à classificação na antiga ordem Chloroccocales, da qual Selenastraceae fez parte, é responsável pelos vários sistemas de classificação propostos até hoje (Sant'Anna 1984). Dependendo dos julgamentos dos autores, os gêneros são colocados no "complexo" de famílias Selenastraceae/Chlorellaceae/Ankistrodesmaceae/Oocystaceae e, frequentemente, nem todas as obras específicas reconhecem todas as famílias deste "complexo".

Na tentativa de estabelecer critérios morfológicos aplicáveis para Selenastraceae, Marvan *et al.* (1984) estudaram a morfologia de 18 gêneros, já classificados em Selenastraceae pelo menos alguma vez, por avaliações numéricas da morfologia e características ontogenéticas (formato celular ou das colônias, o arranjo dos autósporos dentro da célula mãe, a presença/ausência de mucilagem ou de incrustações na parede celular, e a presença, número e tipo de pirenóides) foram usados para a definição morfométrica e qualitativa dos gêneros. Como conclusão, os gêneros diferenciam-se uns dos outros por apenas um caráter acima citado, não apresentando definições precisas dentro do grupo.

O emprego direto das características morfológicas em espécimes coletados no campo é também problemático pela subjetividade dos caracteres empregados, sendo, a variedade morfológica, comumente revelada somente por cultivos das espécies, por exemplo, a presença ou ausência de pirenóides é um caractere utilizado em nível de gênero e espécie, mas em muitos organismos este só é visível em microscopia eletrônica ou, em alguns casos, sua ocorrência/ausência na mesma espécie depende das condições de crescimento. Claramente, os estudos sobre a família Selenastraceae (e o mesmo deve acontecer para outras famílias intimamente relacionadas) mostraram que linhagens

morfologicamente semelhantes podem ser bem diferentes em termos moleculares e que cepas distintas morfologicamente podem ser muito semelhantes em termos do gene 18S rDNA. Ressalta-se que a maioria das espécies de Selenastraceae são descritas como cosmopolitas e nas mais diversas regiões climáticas: dos trópicos até próximo dos ciclos polares, o que significa que a prospecção em ambientes tropicais poderá originar resultados muito diferentes daqueles obtidos em regiões temperadas.

A ocorrência de diversidade críptica e classificações errôneas em nível de gênero, baseadas às vezes em apenas um caractere diacrítico de difícil definição em microscopia óptica, são recorrentes em Selenastraceae (Fawley *et al.* 2005). Isso ocorre principalmente no "complexo" de famílias Ankistrodesmaceae/Selenastraceae/Chlorellaceae/Oocystaceae, grupo reconhecido por ter taxonomia problemática, principalmente em espécies pertencentes aos gêneros *Ankistrodesmus*, *Monoraphidium*, *Selenastrum* e do complexo *Kirchneriella-Pseudokirchneriella-Raphidocelis*.

Com base na variabilidade encontrada na natureza, pode-se especular que devam existir centenas de taxons em Selenastraceae (Fawley *et al.* 2005), tendo em vista que trabalhos anteriores estudaram principalmente espécies isoladas de ambientes temperados do hemisfério norte. O conhecimento atual da diversidade específica e da ecologia de Selenastraceae é, ainda, muito pouco entendido mundialmente, embora esses organismos sejam considerados cosmopolitas e muito frequentes em amostras dos maios diversos corpos de água continentais (Krienitz *et al.* 2011).

Cepas similares à *Selenastrum capricornutum* foram analisadas por filogenia do 18S rDNA e morfologia, revelando que os conceitos morfológicos atribuídos a *Kirchneriella-Pseudokirchneriella-Raphidocelis* são altamente questionáveis, indicando

que a maioria dos táxons podem pertencer a outros clados ou gêneros (Krienitz *et al.* 2011).

Chapman *et al.* (1998) dividiram a classe Chlorophyceae em dois clados: o primeiro inclui as ordens tradicionais (Chlorococcales, Volvocales e Chlorosarcinales) e o segundo clado inclui a ordem monofilética Sphaeropleales, o gênero *Bracteacoccus* e todas as clorofíceas que possuem autosporia. Uma associação próxima ocorre entre as clorofíceas autospóricas, *Scenedesmus* e *Ankistrodesmus* por exemplo, e zoospóricas, como *Sphaeroplea* Agardh e *Neochloris* Starr, com aparato flagelar diretamente oposto, pois há similaridade na estrutura celular e formas de crescimento cenobiais (Chapman *et al.*1998).

De acordo com Wolf *et al.* (2002) deve ser feita uma emenda para incluir diversas clorofíceas autospóricas que, presumivelmente, perderam a habilidade de reprodução por zoosporia e se encontram nas famílias Scenedesmaceae e Selenastraceae.

Estudos recentes (Krienitz *et al.* 2001, Fawley *et al.* 2005), realizados a partir da análise morfológica e filogenia por 18S rDNA, têm mostrado que algumas espécies dos gêneros *Ankistrodesmus*, *Monoraphidium*, *Quadrigula*, e *Podohedriella* estão bem definidos na família Selenastracae. Para Fawley *et al*. (2005), a utilização do 18S rDNA, por ser um gene muito conservado, provavelmente revelou apenas parte da diversidade real, o que sugere que o uso de marcadores moleculares que sofreram maior pressão evolutiva possam ser melhores marcadores filogenéticos. Entretanto, ambos os trabalhos acima citados não encontraram semelhanças quanto às espécies estudadas, o que mostra que a resolução para os gêneros e as espécies de Selenastraceae está ainda muito longe de ser alcançada.

Em uma tentativa para solucionar as dificuldades quanto à morfologia e filogenia de Selenastraceae, o mais apropriado seria o uso de múltiplos genes mais

variáveis do que o 18S rDNA e o aumento da riqueza de espécies estudada. Desta forma, a criação de uma base taxonômica robusta obtida com taxonomia tradicional, dados ecológicos, relações filogenéticas, dados quimiotaxonômicos e observações em cultivos seria um excelente ponto de partida.

Conceitos de espécie em algas verdes

O conceito biológico de espécie aceito amplamente pela comunidade científica é o proposto por Mayr (1948), onde a compatibilidade sexual é critério para a delimitação de espécie, não sendo aplicável a grupos que possuem reprodução assexuada ou cuja reprodução é desconhecida.

A abordagem filogenética com marcadores genéticos é aplicável nesses casos, desde que seja escolhida a região gênica e um marcador molecular apropriado (Bock 2010). Com base na filogenia, o uso de uma região conservada demais distinguirá menos espécies e caracteres morfológicos podem conflitar com a posição das espécies na árvore filogenética. Por outro lado, se uma região altamente variável é escolhida, a quantidade de espécies pode ser superestimada (Hoef-Emden 2007, Rindi *et al.* 2009).

O espaçador transcrito interno 2 (do inglês Internal Transcriber Spacer 2 - ITS2) faz parte do operon rRNA, localizado entre o 5.8S e 28S. As moléculas de rRNA funcionais são obtidas em todo um operon rRNA, que é transcrito como um único rRNA precursor, seguido de processos complexos de excisão de ambas as regiões ITS.

Mudança de bases compensatórias (do inglês Compensatory base changes - CBC) nas estruturas secundárias do ITS2 correlaciona-se com o conceito biológico de espécie (Mayr 1948). CBC ocorrem em uma região pareada de um transcrito primário de RNA quando ambos os nucleotídeos de um sítio sofrem mutação, mantendo o

pareamento (por exemplo, G-C sofre mutações para A-U). A comparação de posições homólogas entre organismos diferentes, em busca de nucleotídeos não conservados, mas que sofreram co-evolução, pode ser revelada pela estabilidade e funcionalidade da estrutura secundária do RNA. A ocorrência de CBC em regiões conservadas do ITS2 coincide com a incompatibilidade sexual entre duas espécies (Coleman & Mai 1997, Mai & Coleman 1997, Coleman 2000, 2003, 2009, Amato *et al*. 2007). A presença de CBCs ou hemi-CBCs (apenas alterações unilaterais de bases) também é usada frequentemente para a delimitação de espécies em grupos cuja morfologia é de difícil resolução ou quando só se conhece a reprodução assexuada (Krienitz *et al*. 2004, Hoef-Emden, 2007).

Tem-se comprovado que o ITS2 é um marcador apropriado para o estudo filogenético de pequena escala entre espécies aparentadas, sendo comum o seu uso entre espécies dentro de um mesmo gênero (Coleman 2003, Coleman & Vacquier 2002, Coleman 2007, Young & Coleman, 2004, Schultz *et al*. 2005). As propriedades altamente divergentes e com rápida evolução legitimam o ITS2 para discriminar organismos estreitamente relacionados, que exibem sequências quase idênticas nos genes rRNA (Wolf *et al*. 2013).

A ordem Sphaeropleales apresenta hélices bem conservadas evolutivamente, preservando a estrutura do ITS2 e promovendo alusões para estudos taxonômicos mais amplos. Uma ramificação incomum da hélice 1 do ITS2 dentro dos gêneros *Hydrodictyon* (Hydrodyctiaceae), *Desmodesmus* e *Scenedesmus* (Scenedesmaceae) foi descrita por van Hannen *et al.* (2002), sendo estes gêneros intimamente relacionados com Selenastraceae.

Além dos estudos conduzidos a partir das regiões nucleares (18S, ITS, 28S), muitos marcadores tem sido utilizados para a delimitação de espécies utilizando o gene

rbcL (ribulose-1,5-bisfosfato, ou RuBisCO), cujos dados são desconhecidos para Selenastraceae e famílias relacionadas. Há décadas o gênero *Ulva* (Ulvophyceae, Ulvaceae) vem sendo amplamente estudado, utilizando também o *rbcL* para resolver problemas taxonômicos (Hayden & Waaland 2002, Hayden *et al.* 2003, Hayden & Waaland 2004, Loughnane *et al*. 2008). Estabelecido entre os grupos de plantas como DNA Barcode, o *rbcL* é considerado um marcador promissor (Hollingsworth *et al*. 2009) por causa de seu uso em estudos taxonômicos e filogenéticos em macroalgas verdes marinhas (Saunders & Kucera 2010). Inferência filogenética em algas verdes com base em *rbcL* está sendo amplamente utilizada principalmente por causa de suas variações e resolução em níveis mais baixos do que o 18S rDNA (Fucivoká *et al*. 2011).

Referências bibliográficas

Amato, A., W. H. Kooistra, J. H. L. Ghiron, D. G. Mann, T. Pröschold & M. Montresor, (2007). Reproductive isolation among sympatric cryptic species in marine diatoms. Protist 158(2):193-207.

Aslam, Z., W. Shin, M. K. Kim, W. T. Im & S. T. Lee, (2007). *Marinichlorella kaistiae* gen. et sp. nov.(Trebouxiophyceae, Chlorophyta) based on polyphasic taxonomy1. Journal of Phycology 43(3):576-584.

Blackman, F. & A. Tansley, (1902). A Revision of the Classification of the Green Algae (Continued) page 72). New Phytologist 1(4):89-96.

Blackman, F. F., (1900). The primitive algae and the flagellata. An account of modern work bearing on the evolution of the algae. Annals of Botany:647-688.

Bock, C., (2011). Genetic diversity and polyphyletic origin of the *Dictyosphaerium* morphotype. Freie Universität Berlin, Germany.

Bourrelly, P., (1972): Les Algues d'eau douce. Initiation à la Systématique: Tome I. Les Algues vertes. Société Nouvelle des Éditions Boubée.

Brunnthaler, J., (1915). Protococcales in A. Pascher's die Susswasserflora Deutschlands, Osterrichs und der Schweiz, Hefts, Chlorophyceae 2. Vena.

Chapman, R. L., M. A. Buchheim, C. F. Delwiche, T. Friedl, V. A. Huss, K. G. Karol, L. A. Lewis, J. Manhart, R. M. McCourt & J. L. Olsen, (1998). Molecular systematics of the green algae Molecular systematics of plants II. Springer, 508-540.

Christensen, T. (1962). Algae. In: Bocher, T. W., Lange, W. & Sorensen, T. (Eds): Botanik, 2, Systematisk botanik. Munksgard, Copenhagen, Denmark, pp. 1-178.

Coleman, A. W., (2000). The significance of a coincidence between evolutionary landmarks found in mating affinity and a DNA sequence. Protist 151(1):1-9.

Coleman, A. W., (2003). ITS2 is a double-edged tool for eukaryote evolutionary comparisons. TRENDS in Genetics 19(7):370-375.

Coleman, A. W., (2007). Pan-eukaryote ITS2 homologies revealed by RNA secondary structure. Nucleic Acids Research 35(10):3322-3329.

Coleman, A. W., (2009). Is there a molecular key to the level of "biological species" in eukaryotes? A DNA guide. Molecular Phylogenetics and Evolution 50(1):197-203.

Coleman, A. W. & V. D. Vacquier, (2002). Exploring the phylogenetic utility of ITS sequences for animals: a test case for abalone (*Haliotis*). Journal of molecular evolution 54(2):246-257.

Comas, A., (1996). Las Chlorococcales dulciacuícolas de Cuba. Bibliotheca Phycologica 99. J. Cramer, Berlin, 192.

Darienko, T., L. Gustavs, O. Mudimu, C. R. Menendez, R. Schumann, U. Karsten, T. Friedl & T. Pröschold, (2010). *Chloroidium*, a common terrestrial coccoid green alga previously assigned to *Chlorella* (Trebouxiophyceae, Chlorophyta). European Journal of Phycology 45(1):79-95.

Ettl, H., (1981). Die neue Klasse Chlamydophyceae, eine natürliche Gruppe der Grünalgen (Chlorophyta)/The New Class Chlamydophyceae, a Natural Group of the Green Algae (Chlorophyta). Plant Systematics and Evolution:107-126.

Ettl, H. & J. Komárek, (1982). Was versteht man unter dem Begriff coccale Grünalgen?(Systematische Bemerkungen zu den Grünalgen II). Algological Studies/Archiv für Hydrobiologie, Supplement Volumes:345-374.

Fawley, M. W., M. L. Dean, S. K. Dimmer & K. P. Fawley, (2006). Evaluating the morphospecies concept in the Selenastraceae (Chlorophyceae, Chlorophyta) Journal of Phycology 42(1):142-154.

Fawley, M. W., Y. Yun & M. Qin, (2000). Phylogenetic analyses of 18S rDNA sequences reveal a new coccoid lineage of the Prasinophyceae (Chlorophyta). Journal of Phycology 36(2):387-393.

Friedl, T., (1997): The evolution of the green algae. Plant systems Evolution [Supplement] 11:87-101.

Fritsch, F., (1927). A treatise on the British freshwater algae in which are included all the pigmented protophyta hitherto found in Bri.

Fučíková, K., J. C. Rada, A. Lukešová & L. A. Lewis, (2011). Cryptic diversity within the genus *Pseudomuriella* Hanagata (Chlorophyta, Chlorophyceae, Sphaeropleales) assessed using four barcode markers. Nova Hedwigia 93(1-2):29-46.

Hayden, H. S., J. Blomster, C. A. Maggs, P. C. Silva, M. J. Stanhope & J. R. Waaland, (2003). Linnaeus was right all along: *Ulva* and *Enteromorpha* are not distinct genera. European Journal of Phycology 38(3):277-294.

Hayden, H. S. & J. R. Waaland, (2004). A molecular systematic study of *Ulva* (Ulvaceae, Ulvales) from the northeast Pacific. Phycologia 43(4):364-382.

Hindák, F. (ed) (1984). Studies on the chlorococcal algae (Chlorophyceae) III, Veda, Bratislava.

Hindák, F. (ed) (1988). Studies on the chlorococcal algae (Chlorophyceae) IV., Veda, Bratislava.

Hindák, F. (ed) (1990). Studies on the chlorococcal algae (Chlorophyceae). V, Veda, Bratislava.

Hoef-Emden, K., (2007). Revision of the genus *Cryptomonas* (Cryptophyceae) II: incongruences between the classical morphospecies concept and molecular phylogeny in smaller pyrenoid-less cells. Phycologia 46(4):402-428.

Hollingsworth, P. M., Forrest L.L., Spouge J.L., Hajibabaei M., Ratnasingham S., Van Der Bank M., C. R. S. Chase M.W., Erickson D.L., Fazekas A. J., Graham S.W., James K.E., Kim K. J., Kress W.J., Schneider H., Van Alphenstahl J., Barrett S. C.H., Van Den Berg C., Bogarin D., Burgess K.S., Cameron K. M., Carine M., Chacon J., Clark A., Clarkson J.J., Conrad F., Devey D. S., Ford C.S., Hedderson T.A.J., Hollingsworth M. L., Husband B.C., Kelly L.J., Kesanakurti P.R., Kim J.S., Kim Y. D., Lahaye R., Lee H.L., Long D.G., Madrinan S., Maurin O., Meusnier I., Newmaster S.G., Park C.W., Percy D.M., Petersen G., Richardson J. E., Salazar G. A., Savolainen V., Seberg O., Wilkinson M.J., Yi D.K. & Little D.P., (2009). A DNA barcode for land plants. Proceedings of the National Academy of Sciences USA 106:12794-12797.

Huss, V. A., C. Frank, E. C. Hartmann, M. Hirmer, A. Kloboucek, B. M. Seidel, P. Wenzeler & E. Kessler, (1999). Biochemical taxonomy and molecular phylogeny of the genus *Chlorella* sensu lato (Chlorophyta). Journal of Phycology 35(3):587-598.

Komárek, J. & B. Fott, (1983). Das Phytoplankton des Sübwassers. Systematik und Biologie. 7. Teil, 1. Hälfte. Chlorophyceae (Grünalgen) Ordnung: Chroococcales. E. Schweizerbart'sche Verlagsbuchhandlung (Nägele u. Obemiller), Stuttgart. 1043p.

Kornmann, P., (1973). Codiolophyceae, a new class of Chlorophyta. Helgoländer Wissenschaftliche Meeresuntersuchungen 25(1):1-13.

Korshikov, O., (1953). Viznachnik prisnovodnih vodorostey Ukrainskoy RSR. V Protococcineae Naukova dumka, Kiıv (in Ukrainian).

Krienitz, L., E. H. Hegewald, D. Hepperle, V. A. Huss, T. Rohr & M. Wolf, (2004). Phylogenetic relationship of *Chlorella* and *Parachlorella* gen. nov.(Chlorophyta, Trebouxiophyceae). Phycologia 43(5):529-542.

Krienitz, L., I. Ustinova, T. Friedl & V. A. Huss, (2001). Traditional generic concepts versus 18S rRNA gene phylogeny in the green algal family Selenastraceae (Chlorophyceae, Chlorophyta). Journal of Phycology 37(5):852-865.

Lewis, L. A. & R. M. McCourt, (2004). Green algae and the origin of land plants. American Journal of Botany 91(10):1535-1556.

Loughnane, C. J., L. M. McIvor, F. Rindi, D. B. Stengel & M. D. Guiry, (2008). Morphology, *rbcL* phylogeny and distribution of distromatic *Ulva* (Ulvophyceae, Chlorophyta) in Ireland and southern Britain. Phycologia 47(4):416-429.

Mai, J. C. & A. W. Coleman, (1997). The internal transcribed spacer 2 exhibits a common secondary structure in green algae and flowering plants. Journal of Molecular Evolution 44(3):258-271.

Marin, B. & M. Melkonian, (2010). Molecular phylogeny and classification of the Mamiellophyceae class. nov. (Chlorophyta) based on sequence comparisons of the nuclear-and plastid-encoded rRNA operons. Protist 161(2):304-336.

Marvan, P., J. Komárek & A. Comas, (1984). Weighting and scaling of features in numerical evaluation of coccal green algae (genera of the Selenastraceae). Algological Studies/Archiv für Hydrobiologie, Supplement Volumes:363-399.

Mattox, K., (1984). Classification of the green algae: a concept based on comparative cytology. Systematics of the green algae:29-72.

Mayr, E., (1947). The bearing of the new systematics on genetical problems; the nature of species. Advances in genetics 3(2):205-237.

Melkonian, M., (1989). Systematics and evolution of the algae Progress in botany. Springer, 214-245.

Neustupa, J., Y. Němcová, M. Eliáš & P. Škaloud, (2009). *Kalinella bambusicola* gen. et sp. nov.(Trebouxiophyceae, Chlorophyta), a novel coccoid Chlorella-like subaerial alga from Southeast Asia. Phycological Research 57(3):159-169.

Pascher, A., (1918). Von einer allen Algenreihen gemeinsamen Entwicklungsregel.

Pringsheim, E., (1963). Chlorophyllarme Algen I *Chlamydomonas pallens* nov. spec. Archives of Microbiology 45(2):136-144.

Rindi, F., D. W. Lam & J. M. López-Bautista, (2009). Phylogenetic relationships and species circumscription in *Trentepohlia* and *Printzina* (Trentepohliales, Chlorophyta). Molecular phylogenetics and evolution 52(2):329-339.

Sant'Anna, C. L., (1984). Chlorococcales (Chlorophyceaea) do Estado de São Paulo, Brasil. Bibliotheca Phycologica 67. J. Cramer, Berlin, 348.

Saunders, G. W. & H. Kucera, (2010). An evaluation of *rbcL,* tufA, UPA, LSU and ITS as DNA barcode markers for the marine green macroalgae. Cryptogamie Algologie 31(4):487-528.

Schultz, J., S. Maisel, D. Gerlach, T. Müller & M. Wolf, (2005). A common core of secondary structure of the internal transcribed spacer 2 (ITS2) throughout the Eukaryota. Rna 11(4):361-364.

Trainor, F., (1998). Biological aspects of *Scenedesmus*: phenotypic plasticity. Schweizerbart'sche Verlagsbuchhandlung, Stuttgart.

van den Hoek, C. & H. M. Jahns, (1978): Algen: Einführung in die Phykologie. Thieme.

van den Hoek, C., W. Stam & J. Olsen, (1988). The emergence of a new chlorophytan system, and Dr. Kornmann's contribution thereto. Helgoländer Meeresuntersuchungen 42(3-4):339-383.

van den Hoek, C., D. Mann & H. M. Jahns, (1995): Algae: an introduction to phycology. Cambridge university press.

van Hannen, E., P. FinkGodhe & M. Lurling, (2002). A revised secondary structure model for the internal transcribed spacer 2 of the green algae *Scenedesmus* and *Desmodesmus* and its implication for the phylogeny of these algae. European Journal of Phycology 37(2):203-208.

Wolf, M., M. Buchheim, E. Hegewald, L. Krienitz & D. Hepperle, (2002). Phylogenetic position of the Sphaeropleaceae (Chlorophyta). Plant Systematics and Evolution 230(3-4):161-171.

Wolf, M., S. Chen, J. Song, M. Ankenbrand & T. Müller, (2013). Compensatory base changes in ITS2 secondary structures correlate with the biological species

concept despite intragenomic variability in ITS2 sequences–a proof of concept. PloS one 8(6):e66726.

Young, I. & A. W. Coleman, (2004). The advantages of the ITS2 region of the nuclear rDNA cistron for analysis of phylogenetic relationships of insects: a *Drosophila* example. Molecular phylogenetics and evolution 30(1):236-242.

Hipóteses

A) O morfotipo *Selenastrum* tem origem polifilética.

As análises moleculares disponíveis na literatura sugerem a origem polifilética de alguns gêneros de Selenastraceae, como *Selenastrum*, gênero tipo da família, *Ankistrodesmus*, *Monoraphidium* e *Kirchneriella*.

B) Uma grande diversidade genética está escondida entre espécies morfológicas.

Espécies frequentes em corpos d'água, como as pertencentes aos gêneros *Ankistrodesmus, Monoraphidium* e *Kirchneriella e Selenastrum,* tem distribuição cosmopolita e ocupam diferentes habitats, segundo a literatura. Alguns autores acreditam que a diversidade genética de microalgas verdes é, por vezes, muito maior do que sua morfologia simples sugere. Estes complexos de espécies genéticas de diferentes gêneros, supostamente, teriam evoluído através de convergência morfológica e localizam-se em diferentes posições filogenéticas.

C) Os marcadores moleculares utilizados revelariam a diversidade genética de Selenastraceae.

As relações entre os gêneros *Kirchneriella* e *Monoraphidium* e as espécies pertencentes a estes, podem revelar a diversidade de organismos dentro desta família, apontando a problemática encontrada para a identificação morfológica do grupo.

Objetivos

Utilizando técnicas de taxonomia tradicional e biologia molecular para estudar a família Selenastraceae, pretendeu-se:

1) Identificar gêneros e espécies pertencentes à família Selenastraceae, grupo reconhecidamente problemático quanto à identificação, principalmente dos gêneros *Ankistrodesmus*, *Monoraphidium*, *Selenastrum* e do complexo *Kirchneriella-Pseudokirchneriella-Kirchneria-Raphidocelis*.

2) Avaliar os genes (18S rDNA, 5.8S e *rbcL*) e o espaçadores intergênicos (ITS1 e ITS2) como potenciais marcadores taxonômicos e moleculares para Selenastraceae, tentando elucidar a variação encontrada entre as espécies estudadas.

3) Detectar as relações filogenéticas dentro da família Selenastraceae por meio da comparação de morfotipos de cepas de diferentes espécies e regiões geográficas.

Capítulo 1:

Selenastraceae (Sphaeropleales, Chlorophyceae): rbcL, 18S rDNA and ITS-2 secondary structure enlightens traditional taxonomy, with description of two new genera, *Messastrum* gen. nov. and *Curvastrum* gen. nov.

1.1. ABSTRACT

The phylogeny of the family Selenastraceae was investigated by light microscopy, 18S rDNA, *rbcL* and ITS-2 analyses. Various morphological features traditionally used for species and genera identification were investigated. All selenastracean strains studied have naked pyrenoids within the chloroplast, except the genus *Chlorolobion*, which presented starch envelope. The molecular analyses showed that no morphological criterion considered so far is significant for the systematics of the Selenastraceae, but a set of features may be suitable to identify the genera *Ankistrodesmus* and *Chlorolobion*. Phylogenetic analyses showed the genera *Monoraphidium*, *Kirchneriella* and *Selenastrum* were not monophyletic and not distinguishable as separate genera, what led to the description of two new genera, *Curvastrum gen. nov* and *Messastrum gen. nov*.

KEYWORDS: *Ankistrodesmus*, *Chlorolobion*, *Kirchneriella*, molecular systematics, morphology, phylogeny, *Selenastrum*.

1.2. INTRODUCTION

Selenastraceae BLACKMAN & TANSLEY 1903 (Chlorophyceae, Sphaeropleales) is a green algae family common in freshwater bodies all over the world, presenting a high morphological diversity (KRIENITZ et al. 2001). This family includes the most common members of phytoplankton in nearly all types of inland waters. They can produce mass developments in lakes, ponds, pools and rivers (MESSYASZ 2003; TAS & GONULOL 2007). Although they commonly occur in freshwater habitats, some species tolerate moderate saline habitats as well, i.e. some taxa are reported from brackish and low saline areas of the Baltic Sea (PANKOW 1990). The Selenastraceae are valuable indicator organisms for ecosystem health, and several species of this family are regularly used as indicator species, e.g. in the frame of the European Water Framework Directive (MISCHKE & KUSBER 2009). The morphology of this group comprises a variety of shapes: from coccoid to elongated, cylindrical to fusiform, sickle-shaped to spirally curved, with sharp or rounded ends, where cell arrangements varies from the solitary to colonial forms (KOMÁREK & FOTT 1983; KORSHIKOV 1987; KOMÁRKOVÁ-LEGNEROVÁ 1969; COMAS 1996; HINDÁK, 1977; HINDÁK 1980; HINDÁK 1984; HINDÁK 1988; HINDÁK 1990; SANT'ANNA 1984). Their reproduction is exclusively by autospore formation, in which the cytokinesis of the mother cell protoplasm gives rise to 2-4-8 young cells (KOMÁREK & FOTT 1983). The combination of cell size, shape, solitary or colonial lifestyle, the releasing process of the autospores and special habitat preferences, are considered to be species-specific (HINDÁK 1977, KOMÁREK & FOTT 1983). Based on these criteria, up to 100 species were described in various genera and included in this family (HINDÁK 1977; HINDÁK 1984; KOMÁREK & FOTT 1983; FAWLEY et al. 2006; KRIENITZ et al. 2011).

Since its description in 1903, the family Selenastraceae has passed by many taxonomic changes, being recognized as: Scenedesmaceae BOHLIN 1904, Selenastraceae WEST & FRITSCH 1927, Ankistrodesmacaeae KORSHIKOV 1953, Oocystaceae BOURRELLY 1972, Chlorellaceae – Ankistrodesmoidea KOMAREK & FOTT 1983. However, first studies in the SSU of the commonly observed genera in this family, e.g. *Ankistrodesmus*, *Selenastrum*, *Monoraphidium*, *Quadrigula*, *Podohedriella* and *Kirchneriella*, show that they form a monophyletic group within the Chlorophyceae (FAWLEY et al. 2006; KRIENITZ et al. 2011; KRIENITZ et al. 2001), apart from other members of Scenedesmaceae (FAWLEY et al. 2006; KRIENITZ et al. 2001), Oocystaceae [which is now placed within the Class Trebouxiophyceae (FRIEDL 1995)] and Chlorellaceae (FRIEDL 1995; KRIENITZ et al. 2001). Since the onset of molecular phylogeny, several genera were excluded from the family due to their molecular traits, e.g., *Closteriopsis* was transferred to the Chlorellaceae and *Hyloraphidium* is in fact a fungus (LUO et al. 2010; USTINOVA et al. 2001).

Despite the monophyly of the family, the genera still need revision, since morphological features are usually not in accordance with molecular data (KRIENITZ et al. 2001; KRIENITZ et al. 2011). For example, defined genera cluster polyphyletic on different clades within the Selenastraceae, e.g. *Selenastrum bibraianum* (type species of *Selenastrum*), and *Selenastrum gracile* belong to different phylogenetic lineages based on 18S rDNA phylogeny (FAWLEY et al. 2006; KRIENITZ et al. 2001) but no taxonomic changes were made in the genus, since the authors suggested further studies with the family to ensure these findings.

Due to the difficulties in their identification and taxonomy, the current knowledge of species diversity and ecology of Selenastraceae is still poorly understood worldwide (FAWLEY et al. 2006; KRIENITZ et al. 2001). In addition, previous studies

were focused on temperate northern hemisphere isolates, whereas the molecular diversity of tropical Selenastraceae remains unknown.

Phylogenetic inference in green algae is mainly based on 18S rDNA gene sequences (BOOTON et al. 1998; BUCHHEIM et al. 2001; FAWLEY et al. 2006; HEGEWALD & HANAGATA 2000; KRIENITZ et al. 2011; KRIENITZ et al. 2003; KRIENITZ et al. 2001; LEWIS 1997). Nevertheless, several studies have shown that the 18S rDNA is in some cases too conserved to distinguish between closely related genera and species (Luo et al. 2010). Different studies take a second marker into account as well, to gain a higher resolution (RINDI et al. 2011). The gene *rbcL* is being widely used mainly because of its higher variations and better resolution than the 18S rDNA at lower taxonomic levels (FUČÍKOVÁ et al. 2011) and is also used as a DNA barcode in marine green macroalgae (SAUNDERS & KUCERA). The ITS-2 has proven to be a suitable marker for small scale phylogenies and it is commonly applied among species within the same genus (BOCK et al. 2011b; COLEMAN 2003; COLEMAN 2007; COLEMAN & VACQUIER 2002; SCHULTZ et al. 2005; YOUNG & COLEMAN 2004) or for the resolution of closely related genera (LUO et al. 2010; LUO et al. 2011b). The highly divergent properties and the rapid evolution legitimate ITS-2 to discriminate closely related organisms, which exhibit nearly identical sequences in rRNA genes (WOLF et al. 2013).

In response to the difficulty in accurately identifying Selenastraceae species worldwide, this study aimed to clarify the taxonomic status of some members of this algae family using morphological traits, and 18S rRNA and *rbcL* gene sequences, and contribute to the knowledge about their diversity. The present study is the first attempt to evaluate Selenastraceae combining morphology, gene sequences of 18S rRNA and *rbcL*, and ITS-2 secondary structure.

1.3. MATERIAL AND METHODS

Algal cultures and microscopy.

Forty five Selenastraceae strains were investigated (Table 1). The algal cultures were obtained from Freshwater Microalgae Culture Collection from Universidade Federal de São Carlos (CCMA – UFSCar, WDCM 835) and from an author personal collection (CB strains). All the strains were grown in WC medium (GUILLARD & LORENZEN 1972) and maintained at of 23 ± 1 ºC, under photoperiod 12/12 hours light/dark, and luminous intensity of ~200 µmol/m2/s.

The whole life cycle of cultured strains were examined using an Axioplan 2 Imaging Zeiss or Nikon Eclipse E600 light microscope with differential interference contrast. Micrographs were taken using an AxioCam with software AxioVision 4.6 (Carl Zeiss Group, Oberkochen, Germany) and a Nikon digital camera DS-Fi1 with Nikon software NIS-Elements D (Nikon Corporation, Tokyo, Japan). The algal strains were identified according to the published keys (KORSHIKOV 1987; KOMÁREK & FOTT 1983; KOMÁRKOVÁ-LEGNEROVÁ 1969; COMAS 1996; HINDÁK 1977; HINDÁK 1980; HINDÁK 1984; HINDÁK 1988; HINDÁK 1990; SANT'ANNA 1984).

Before Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) cells were fixed in 2.5%, glutaraldehyde in culture medium (HEGEWALD et al. 1994) for 24 hours at -5ºC, and dehydrated as follows. The cells were washed three times with culture medium and dehydration series was taken in a graded acetone series: 20, 35, 50, 70, 90% (twice) for 15 minutes each step, and 100% kept overnight. Samples were washed three times with culture medium and postfixed with 1% osmium tetroxide for 2 hours.

For SEM, a Critical Point Dryer (BAL-TEC 030, Germany) was used at 80-90 bars and 30-34 ºC .The samples were placed on a gold-palladium-coater (High Resolution Ion Beam Coater Model 681, Germany) and then 2 depositions of palladium were made (±1 kÅ). SEM images were taken with ESEM Quanta 400 FEG (FEI, The Netherlands).

TEM was performed according to KRIENITZ et al. (2011) with infiltration in epon. Thin sections were prepared on a Reichert UltraCut S (Reichert Inc., Depew, NY, USA), with no poststain and examined in a Hitachi S-4000 Scanning Electron Microscope (IMCES-Imaging Center Essen).

DNA extraction, PCR and sequencing.

For DNA extraction, the algae cultures were grown in the conditions described above for microscopy analyses. The cell suspension was centrifuged at 16.000 xg for 10 minutes and the pellet stored at -80°C until the next step. The cells were disrupted using glass beads (150-212 µm, Sigma-Aldrich), vortex briefly, and extracted using Invisorb® Spin Plant Mini (STRATEC Biomedical AG; Germany) or My-Budget DNA Mini Kit® (Bio-Budget Technologies GmbH; Krefeld; Germany).

PCR amplification was carried out using established target-specific primers and some primers proposed in this study. For 18S rDNA, the primers used were 18SF1 (KATANA et al. 2001), 1F, 300F, 528F, 690F, 920F, 1055F, 1400F, 920R, 1200R, 1520R (HUSS et al. 1999), 18SR1 (5’ – TGATCCTTCTGCAGGTTCACCTA – 3’) modified from KATANA et al. (2001). Four new primers were designed for the *rbc*L: *rbc*L 320 mod (5’ – TATTYGAASAAGGTTCWGTWAC – 3’, modified from RINDI (2008), Selenastraceae *rbcL* F (5’ – CGYTACAAAGGDCGTTGYT – 3’), *rbcL* Orb modified 5’ – CTGGNGCRTTACCCCAAGG – 3’, modified from PAZOUTOVA

unpublished), and Selenastraceae *rbcL* R 5' – RTTACCCCAWGGGTGHCCTA – 3'). These proposed primers were used in association with the following published primers, *rbcL*1, *rbcL*1181, 1421 (NOZAKI et al. 1995), *rbcL* 320 (RINDI et al. 2008), *rbcL* RH1 (MANHART 1994), *rbcL* 1385 (MCCOURT et al. 2000), and *rbcL* ORB (PAZOUTOVA unpublished). For ITS-2 the primers used were 1420F (ROGERS et al. 2006), NS7m and LR1850 (AN et al. 1999) and ITS055R (MARIN et al. 1998). For more details about primers, see Table S3 (supplementary material). The PCR amplifications for *rbcL* gene were performed using the following reaction conditions: 95°C for 5 min followed by 25 cycles, each including 1 min at 95° C, 1 min at 52° C, and 2 min at 72° C with Taq DNA Polymerase QIAGEN® or DreamTaq DNA Polymerase Thermo Scientific®. 18S rDNA PCR amplifications were conducted according to KATANA et al. (2001) and KRIENITZ et al. (2011). ITS-2 PCR amplifications were performed according to BOCK et al. (2011). Each PCR product was electrophoresed in a 1% agarose gel, stained with ethidium bromide.

Purification of the PCR products was conducted using the polyethylene glycol protocol (PEG) according to ROSENTHAL et al. (1993). The PCR products were sequenced by Macrogen Inc. (ABI 3130-Genetic-Analyzer, Applied Biosystems GmbH, Darmstadt, Germany) with the same primers used for amplification. Part of the genomic DNA is stored at the Phycology Lab – UFSCar and Department of Biodiversity – University Duisburg-Essen.

Twenty three new 18S rDNA sequences, 34 new *rbcL* sequences and 8 ITS-2 new sequences were amplified and obtained on this study, totalizing 65 new entries in GenBank (National Center for Biotechnology Information [NCBI], http://www.ncbi.nlm.nih.gov/). The whole dataset used for phylogenetic analyses with

accession numbers are reported in Table 1, including 29 reference sequences acquired from GenBank.

Phylogenetic analyses

Sequences were manually aligned using Align - Manual Sequence Alignment Editor (HEPPERLE 2004). For the phylogenetic analyses, two different datasets were prepared. The *rbcL* analyses contained a dataset of 40 sequences with 767 base positions. For the 18S rDNA analyses, 46 sequences with 1511 base positions were acquired. The two genes (18S rDNA and *rbcL*) were analyzed separately, both in maximum likelihood (ML) on Treefinder (JOBB 2008), distance (neighbor joining; NJ) and maximum parsimony (MP) using PAUP* (portable version 4.0b10) (SWOFFORD 2002). For ML and Bayesian analyses, the evolutive model for both genes (18S rDNA and *rbcL*) (GTR[Optimum, Empirical]: G[optimum]:5) was applied as suggested by MrModeltest (NYLANDER 2004), with tree sampling every 100 generations. The confidence of the tree topology was tested by calculating 1000 bootstrap values for NJ, MP, ML criteria. For all datasets, Bayesian analyses were performed using MrBayes version 3.1. (HUELSENBECK & RONQUIST 2001). Two runs with four chains of Markov chain Monte Carlo (MCMC) iterations were performed (3 million generations for *rbcL* and 8 million generations for 18S rDNA). The stationary distribution was assumed when the average standard deviations of split frequencies between two runs were lower than 0.01 and Tracer V1.4 (RAMBAUT AND DRUMMOND 2007) was used to check the stationary phase and to identify an appropriate burn-in value. The first 25% of the calculated trees were discarded as burn–in. 50% majority–rule consensus trees were calculated for posterior probabilities (PP). The trees were edited using TreeGraph 2 (STÖVER & MÜLLER 2010). Previous publications indicated that *Bracteacoccus*

(KRIENITZ et al. 2001) and *Pediastrum* (FUČÍKOVÁ et al. 2014) members were suitable as an outgroup for the phylogeny of Selenastraceae.

ITS-2 secondary structure prediction.

The ITS-2 model for *Scenedesmus*, proposed by van Hannen, (VAN HANNEN et al. 2002), was used as a template and adapted by hand. The secondary structure was obtained using the RNAfold Webserver (GRUBER et al. 2008) where the minimum free energy (MFE) of the secondary structure of single sequences and the equilibrium base-pairing probabilities were predicted. The RNA secondary structures were visualized with Pseudoviewer 3 (BYUN & HAN 2009).

1.4. RESULTS

Genera and species descriptions.

Messastrum gen. nov. T. S. GARCIA.

Green, planktonic microalgae. Narrow, fusiform to semilunate cells, ends gradually pointed, arcuate. Colonies with 2-4-8 or multi irregularly arranged cells, mostly with the convex side towards the center of the colony. One parietal chloroplast, containing a pyrenoid, no starch cover observed.

Asexual reproduction by autosporulation (2-4-8 autospores per sporangium), sexual reproduction not known. An unseparated fragment of the mother cell membrane remains and is covered by a thin mucous layer. Cells single or on 2-4-8 or multi celled colony formation. Cells often single celled in culture. A difuse thin layer of mucilage is often concentrated as a ring on the middle of the cells on both colonies and free individuals.

Genus differs from other genera in the Selenastraceae based on differences in 18S rDNA and *rbcL* gene sequences.

Typus generis: *Messastrum gracile comb. nov.*

Etymology: From the Latim *mess* (= mess) and *astrum* (= star).

Messastrum gracile comb. nov. (REINSCH) T. S., GARCIA.

Synonym: *Ankistrodesmus gracilis* (REINSCH) KORSHIKOV 1953, *Selenastrum westii* G.M.SMITH 1920, *Dactylococcopsis pannonicus* HORTOBÁGYI 1943.

Basyonym: *Selenastrum gracile* REINSCH 1866: 65, pl. IV: Fig. III.

Cells narrow, fusiform to semilunate, ends gradually pointed, arcuate. Planktonic, solitary or 2-4-8 or multi celled colonies with irregularly arranged cells, mostly with the convex sides towards the center of the colonies. Reproduction by autospore formation, where the sporangium gives rise to 2-4-8 young cells, with parallel or zigzag orientation. Pyrenoid without starch cover, observed just under TEM. One parietal chloroplast. Cell wall covered by a diffuse thin layer of mucilage on both colonies and free individuals. A diffuse thin layer of mucilage is often concentrated as a ring on the middle of the cells on both colonies and free individuals. Cells 19-55 x 1-6 µm, distance between the opposite cell ends 6-34 µm.

Holotype: *Selenastrum gracile* REINSCH 1866: 65, pl. IV: Fig. III.

Epitype (designated here): A formaldehyde fixed sample of strain CCMA-UFSCar 622 is deposited at the Botanical Institute at São Paulo, Brazil, under the designation SP 469319.

Isotype: Material of the authentic strain CCMA-UFSCar 622 (Fig. 1.1), maintained at the Culture Collection of Freshwater Microalgae, Federal University of São Carlos, São Carlos, Brazil.

Etymology: The species epithet is based on a morphological feature, “thin, slender”, kept on this nomenclatural change.

Notes: Epitype - isolated from a pond in Conchas, in the country side of the state of São Paulo, in August 2013. More strains were collected inside the state of São Paulo (CCMA-UFSCar 5 and CCMA-UFSCar 470 (For GPS see Table 1).

Curvastrum gen. nov. T. S., GARCIA.

Green, planktonic microalgae. Narrow, fusiform to semilunate cells, ends gradually pointed, arcuate. Colonies with 4 irregularly arranged cells. One parietal chloroplast, cointaining a pyrenoid without starch cover, observed just under TEM.

Asexual reproduction by autosporulation (four autospores per sporangium), sexual reproduction not known. Cells single or on 4-celled colony formation. Cells often single celled in culture. Cell wall covered by a difuse thin layer of mucilage on both colonies and free individuals. Genus morphologic similar to *Messastrum*, distinguishing for the numbers of cells on colony and 18S rDNA, ITS-2 rDNA and *rbcL* sequences

Typus generis: *Curvastrum pantanale* sp. nov.

Etymology: From the Latim *curvus* (= curved) and *astrum* , (= star).

Curvastrum pantanale sp. nov. T. S., GARCIA.

Cells narrow, fusiform to semilunate, ends gradually pointed, arcuate. Planktonic, solitary or 4-celled colonies with 4 irregularly arranged cells, mostly with the convex sides towards the center of the colonies. Reproduction by autospore formation, where the sporangium gives rise to 4 young cells, with zigzag orientation. Pyrenoid without starch cover. One parietal chloroplast. Cell wall covered by a difuse thin layer of

mucilage on both colonies and free individuals. Cells 8-21 x 1,9-3,5 µm, distance between the opposite cell ends: 4-14 µm.

Holotype: A formaldehydefixed sample of this strain is deposited at the Botanical Institute at São Paulo, Brazil, under the designation SP 469320.

Iconotype: our figure number 1.3-1.4.

Isotype: Material of the authentic strain CCMA-UFSCar 350, maintained at the Culture Collection of Freshwater Microalgae, Federal University of São Carlos, São Carlos, Brazil.

Type locality: Isolated from a water fountain used as a supply for animals on Pantanal (Table 1), a Brazilian wetland, in October 2011. GPS 19°17'59.0"S 55°47'45.0"W.

Etymology: The species epithet was derived from the place of origin of the first isolate of this genus, "Pantanal", which means "great Swamp".

Notes: Also collected inside the state of São Paulo (CCMA-UFSCar 608).

Selenastrum bibraianum (REINSCH) KORSHIKOV 1953

Synonym: *Ankistrodesmus bibraianus* (REINSCH) KORSHIKOV 1953

Basyonym: *Selenastrum bibraianum* REINSCH 1866: 65, pl. IV: Fig. II.

Cells narrow, fusiform to semilunate, ends gradually pointed, arcuate. Planktonic, solitary or 2-4-8 or multi celled colonies with regularly arranged cells, mostly with the convex sides towards the center of the colonies, by their convex side. Reproduction by autospore formation, where the sporangium gives rise to 2-4-8 young cells, with parallel or zigzag orientation. One parietal chloroplast. Cell wall covered by a strong layer of mucilage on colonies. A diffuse thin layer of mucilage is often concentrated as a ring on the middle of the cells on colonies and free individuals. Cells 16-40 x 2-8 µm, distance between the opposite cell ends 2-20 µm.

Holotype: *Selenastrum bibraianum* REINSCH 1866: 65, pl. IV: Fig. II.

Epitype (designated here): A formaldehyde fixed sample of strain CCMA-UFSCar 47 is deposited at the Botanical Institute at São Paulo, Brazil, under the designation SP 469321.

Isotype: Material of the authentic strain CCMA-UFSCar 47 , maintained at the Culture Collection of Freshwater Microalgae, Federal University of São Carlos, São Carlos, Brazil.

Etymology: no reference.

Notes: Epitype - isolated from a reservoir in São Carlos (Represa do Monjolinho), in the country side of the state of São Paulo, in November 2008. More strains were collected inside the state of São Paulo (CCMA-UFSCar 125 and CCMA-UFSCar 630), two strains were isolated from Germany (CB 2009/39 and CB 2009/43) and one from Sweden (CB 2012/47) (Table 1).

Ankistrodesmus arcuatus KORSHIKOV 1953

Synonym: *Monoraphidium arcuatum* (KORSHIKOV) HINDÁK 1970: 24, Figs 19, 10, *Ankistrodesmus pseudomirabilis* KORSHIKOV 1953: 297, Fig. 258 a-f, *Ankistrodesmus sabrinensis* BELCHER & SWALE 1962: 131, Fig. 1:H.

Basyonym: *Ankistrodesmus arcuatus* KORSHIKOV 1953: 296, Fig. 257a, b.

Emend diagnosis: Cell solitary or in colonies, fusiform, narrow or acute at the ends, arched or curved. Four celled colony where cells are arranged by one end attached in mucilage. Presence of mucilage. One parietal chloroplast. Reproduction by autospore formation, where the sporangium gives rise to 4-8 young cells, with parallel orientation. Mother cell wall ruptures along the whole length to autospore liberation. Pyrenoid without starch cover.

Dimensions: 26-60x0,8-4,4 µm, distance between the cell ends: 30 µm.

Holotype: *Ankistrodesmus arcuatus* KORSHIKOV 1953: 296, Fig. 257a, b.

Epitype (designated here): A formaldehyde fixed sample of strain CCMA-UFSCar 24 is deposited at the Botanical Institute at São Paulo, Brazil, under the designation SP 469322.

Isotype: Material of the authentic strain CCMA-UFSCar 24 (Fig. 1.5), maintained at the Culture Collection of Freshwater Microalgae, Federal University of São Carlos, São Carlos, Brazil.

Etymology: The species epithet is based on a morphological feature, "arched", kept on this nomenclatural change.

Note: According to the original diagnosis (KORSHIKOV 1953), the author adds a suggestion that the cells may belong to decomposed colonies of *Messastrum gracile*, differing only by the absence of mucus. Komárková-Legnerová (Komárková-Legnerová 1969) considered a possible colony formation and mucilage presence but did not make a nomenclatural recombination. Epitype: isolated from Broa Reservoir, Itirapina, in the country side of the state of São Paulo, in 1979 (Table 1).

Microscopy

This section is focused on morphological traits that conducted to species differentiation. Cells were examined for their cell shape and dimensions, autospore arrangement, pyrenoid presence or absence and colony formation, respecting the diacritical features of each species (for identification keys, see Material and methods). For a summary see Table 2.

Ankistrodesmus CORDA strains had fusiform and isopolar cells, with diameter of 0,5-5 and width of 15-105 µm. It was observed both solitary cells and colony formation

in culture. Except for *Ankistrodesmus sigmoides* (RABENHORST) BRÜHL & BISWAS (CB 2009/9), which autospores were not observed, all the autospores were arranged in parallel. Mother cell wall remnants were cone-shaped. *Ankistrodesmus spiralis* (TURNER) LEMMERMANN (CB2012/29) had colonies with central twisted bundles, proper from this species. Mucilage was observed on the colonies but not on solitary cells.

Ankistrodesmus stipitatus (CHODAT) KOMÁRKOVÁ-LEGNEROVÁ (CCMA-UFSCar 277 and CCMA-UFSCar 278) were arranged with the cells frequently in parallel in free floating fascicular colonies. Some cells were attached to the walls of the glass tubes, producing basal mucilage on their ends. Autospores seemed to be attached to each other by the middle of the cell even before the mother cell rupture. *Ankistrodesmus fusiformis* CORDA (CB 2012/6CCMA-UFSCar 423, CCMA-UFSCar 611 and CCMA-UFSCar 593) presented cruciform or stellate colonies that were joined by a central mucilaginous area. *Ankistrodesmus fasciculatus* (LUNDBERG) KOMÁRKOVÁ-LEGNEROVÁ (CB 2012/3) presented 2-4 celled colonies connected to each other by their convex side, revealing a fasciculate shape, surrounded by a mucus layer. *Ankistrodesmus sigmoides* had sigmoid colony formation. *Ankistrodesmus arcuatus* (KORSHIKOV) HINDÁK (CCMA-UFSCar 24) arcuated cells were arranged frequently in parallel in free floating fascicular colonies.

Chlorolobion braunii (NÄGELI) KOMÁREK (CCMA-UFSCar 137, CCMA-UFSCar 455,CCMA-UFSCar 462 and CCMA-UFSCar 476) had fusiform, heteropolar to isopolar cells, showing some asymmetry. Many cells were attached to the walls of the glass tubes, producing basal mucilage on their ends, as in *A. stipitatus*. The autospore formation is peculiar and very discernible in this species.

Raphidocelis microscopica (NYGAARD) MARVAN, KOMÁREK & COMAS (CB 2009/6, CB 2009/18 and CB 2012/39) formed colonies with mucilage and very small cells irregularly distributed on the colony.

All *Kirchneriella* SCHMIDLE species were similar in cell shape and in the colony morphology, varying on cell size and on the next exposed features. *Kirchneriella pseudoaperta* KOMÁREK (CCMA-UFSCar 346) presented visible pyrenoid under LM. Mucilage was exhibited when in colony, where mainly 4-celled colonies were observed. Some cells kept connected to each other before leaving the mother cell. *Kirchneriella obesa* (WEST) WEST & WEST (CCMA-UFSCar 345) presented mucilage when in colony and solitary cells were bigger in size than the colony living ones. *Kirchneriella aperta* TEILING (CCMA-UFSCar 482) exhibited mucilage when in colony but not when in solitary cells. *Kirchneriella lunaris* (KIRCHNER) MÖBIUS (CCMA-UFSCar 443) had acute cell apex, with solitary cells bigger than the colonies one, always presenting pyrenoids. *Kirchneriella contorta* var. *elegans* (PLAYFAIR) KOMÁREK (CCMA-UFSCar 447) had circular, crescent-shaped to sigmoid cells with rounded cell apex. *Kirchneriella irregularis* (SMITH) KORSHIKOV (CCMA-UFSCar 348) cells were fusiform to strongly twisted, very small in size and without mucilage.

Genus *Monoraphidium* KOMÁRKOVÁ-LEGNEROVÁ always presented solitary and pyrenoid less cells. *Monoraphidium komarkovae* NYGAARD (CCMA-UFSCar 353 and CCMA-UFSCar 632) had very long and thin cells, without mucilage. *Monoraphidium contortum* (THURET) KOMÁRKOVÁ-LEGNEROVÁ (CCMA-UFSCar 306, CCMA-UFSCar 329, CCMA-UFSCar 349 and CB 2009/10) presented strongly twisted cells, with basal mucilage joining young cells after autospores liberation, like in *A. stipitatus*. *Monoraphidium pseudobraunii* (BELCHER & SWALE) HEYNIG (CCMA-UFSCar 325) showed slightly heteropolarity, with no mucilage and 2 autospores per sporangium.

Monoraphidium indicum HINDÁK (CCMA-UFSCar 549) cells were c-shaped, with two autospores per sporangium and the mother cell wall was constantly found in culture.

Morphologically alike, genera *Selenastrum* (REINSCH) KORSHIKOV, *Curvastrum* T. S., GARCIA and *Messastrum* T. S., GARCIA have similar cell shape, 2-4-8 autospores per sporangium, mother cells ruptures in the meridian part with 4-8 autospores per sporangium. The three genera exhibited a great mucus layer both in colony and in solitary cells.

Selenastrum bibraianum (CCMA-UFSCar 47, CCMA-UFSCar 125, CCMA-UFSCar 630, CB 2009/39, CB 2009/41 and CB 2012/47) and *Messastrum gracile* (CCMA-UFSCar 5, CCMA-UFSCar 470, CCMA-UFSCar 622, CB 2009/3 and CB 2009/35) frequently showed big colonies with an arcuated chain of cells, regularly arranged in colony in the first and irregularly on the second species. *Curvastrum pantanale* T. S., GARCIA (CCMA-UFSCar 350 and CCMA-UFSCar 608) exhibited colonies with no more than 4 cells, irregularly arranged in colony (Fig.1.8a).

TEM micrographs showed similarities among the Selenastraceae strains studied, CCMA-UFSCar 24, CCMA-UFSCar 349 and CCMA-UFSCar 350. A large parietal and cup-shaped chloroplast is a typical feature between the observed strains (as seen on Fig. 1.9, d; Fig.1.10 b and e). The parietal chloroplast is filled with a large number of thylakoids situated in parallel to the cell wall (Fig.1.9, c-e; Fig.1.10, b, d, e). In addition, some polyphosphate vacuoles (Fig.1.9, a, c; Fig.1.10, b, d, e) can be found on cells on stationary phase and often some lipid drops (Fig.1.9, a, b; Fig.1.10, d).The nucleus can be observed at the opposite direction of the pyrenoid (Fig.1.9, d, e; Fig.1.10, a, b, e) and, in between them, there is a dictyosome (Fig. 1.10c). Mitochondria (Fig.1.9, c, e) can be observed at the inner surface of the chloroplast.

In our study, except for *Chlorolobion braunii* (Nägeli) Komárek, all Selenastracean taxa studied have naked pyrenoids (without starch envelopes), both observed by LM or TEM.

Phylogenetic analyses

Representatives of Selenastraceae are included in our *rbcL* (Fig. 1.6) and 18S rRNA gene sequence analyses (Fig. 1.7). Due to the low support of some internal branches in both phylogenies, the relationships among some lineages are not clearly resolved. However, some well-supported clades can be identified in both trees: (i) *Selenastrum gracile*, (ii) *Selenastrum bibraianum*, (iii) *Raphidocelis*, (iv) *Curvastrum pantanale nov. gen. et. sp.*, (v) *Kirchneriella*, (vi) *Chlorolobion* (including *Podohedriella falcata* on the 18S rDNA tree), (vii) *Ankistrodesmus*, (viii) *Monoraphidium*. For *Tetranephris*, *Quadrigula*, *Rhombocystis*, *Nephrochlamys*, one clade for each genera on the 18S rDNA tree was obtained, with no *rbcL* sequences for them. The major clades resolved in our trees correspond well to the main genera traditionally included in Selenastraceae, that are, *Ankistrodesmus* and the distinguishable species of genus *Selenastrum gracile* and *Selenastrum bibraianum* (Reinsch) Korshikov. Due to the different number of strains inside 18S rDNA and *rbcL* trees, different results were obtained for some genera such as, *Monoraphidium*, *Raphidocelis* and *Kirchneriella*.

rbcL phylogenetic analyses (Fig.1.6) shows *Selenastrum gracile* (syn. A*nkistrodesmus gracilis*) strains are closely related to *Ankistrodesmus* (*Ankistrodesmus* clade). 18S rDNA phylogeny (Fig.1.7) reveals *Ankistrodesmus* distributed in two clades: *Ankistrodesmus nannoselene* Skuja (KF673373) (*Kirchneriella* I) and *Ankistrodesmus fusiformis* (type species, *Ankistrodesmus* I). *Selenastrum gracile* strains (*Selenastrum gracilis* clade) (syn. *Ankistrodesmus gracilis*) and *Selenastrum*

bibraianum (syn. *Ankistrodesmus bibraianum*) was placed on two different clades on both phylogenetic analyses.

Therefore some strains are only represented in one of the interfered phylogenies, they are in general congruent (see Figs. 1.6-1.7). The *rbcL* proved to be a difficult gene to amplify in Selenastraceae.

Strains with the Selenastrum-like morphology (*M. gracile*, *C. pantalale* and *S. bibraianum*; details see section morphology) form three separate clades in both analyses with weak statistical support, except for the *Curvastrum*-clade within the *rbcL* phylogeny which has high statistical support in all analyses (1.0 for BI; 98 BS for ML; 100 BS for NJ and MP).

Morphologically similar to *Selenastrum* and *Messastrum*, the fusiform to semilunate cells of *Curvastrum* are organized in colonies with no more than 4 irregularly arranged cells (see results). The irregularly arranged cells in *Curvastrum pantanale* (CCMA-UFSCar 350) form its colony (Fig. 1.3, 1.8a), where cells are grouped in a loose fascicle arrangement (Fig.1.8b) and present parallel arrangement of autospores (Fig.1.8c). The likeness to *Messastrum* was distinguished by molecular data (18S rDNA and *rbcL* sequences). ITS-2 Secondary structure analyses demonstrated that strains of the type species *Curvastrum pantanale* (CCMA-UFSCar 350) when compared to another strain belonging to the same species (CCMA-UFSCar 608) differs from each other in 5 base changes, demonstrating intraspecific variations, with 2 CBC on helix II and 1 on helix III (for ITS-2 secondary structure see Supplementary Fig. 1.A-D). The phylogenetic inference of *rbcL* (Fig. 1.6) allocates *Curvastrum* near *Ankistrodesmus* clade. ITS-2 Secondary structure analyses showed that strains of the type species *S. bibraianum* differ from *M. gracile* type species in 34 base changes and 3 base insertions, where helix I has 1 base insertion and 6 CBC, helix II has 4 CBC, helix III

has 8 CBC and helix IV has 2 base insertions and 4 CBC (for ITS-2 secondary structure see Supplementary Fig. 1.A-D).

Species with *Ankistrodesmus* designation form two clades based on *rbcL* (CCMA-UFSCar 423; CB 2012/3; CB2012/6; CCMA-UFSCar 593; CCMA-UFSCar 611; CB2012/29; CCMA-UFSCar 278; EF113406) and one clade on 18S phylogeny (CCMA-UFSCar 277; X97352; CB2009/9; CCMA-UFSCar 278; X56100). For the strain CCMA-UFSCar 24, based on differences in *rbcL* gene sequences and on the colony formation observed, we decided to consider this species as belonging to genus *Ankistrodesmus*.

Species of the genus *Monoraphidium* are distributed in different clades. Although using different strains, *Raphidocelis* was located near *Monoraphidium* clade I for both *rbcL* and 18S rDNA analyses (Figs. 1.6 and 1.7). One clade constituted by *M. contortum* (strains CCMA-UFSCar 349 and CB 2009/10) closely related to *Raphidocelis microscopica*. A well supported clade for *Monoraphidium contortum* (2 strains), *Monoraphidium indicum* and *Monoraphidium pseudobraunii* was presented on *rbcL* phylogeny. Also, *M. komarkovae*, a needle-shaped representative, constitutes an independent lineage on both phylogenies.

Kirchneriella species, including the type species *Kirchneriella lunaris* (CCMA-UFSCar 443), are distributed on some branches on both phylogenetic analyses, always close to *Monoraphidium* species.

Chlorolobion braunii (*Chlorolobion* clade) forms a well-supported clade based on *rbcL* and the clade that includes sequences designated as *Podohedriella falcata* (X91263) and *Monoraphidium neglectum* (AJ300526) based on 18SrRNA gene interference with strong bootstrap support.

The species *Quadrigula closterioides* (BOHLIN) PRINTZ (Y17924), *Tetranephris brasiliensis* LEITE & BICUDO (HM565929; HM565927), *Rhombocystis complanata* KOMÁREK (HM483518) and *Nephrochlamys subsolitaria* (WEST) KORSHIKOV (HM560960) were distributed at one branch each, on the 18S rDNA tree (Fig.1.7).

1.5. DISCUSSION

This study has presented a large dataset of Selenastraceae species, including many strains of subtropical environments, enlightening the phylogeny of this family. The polyphyly of some genera as *Monoraphidium*, *Kirchneriella* and *Selenastrum* have been confirmed, as already suggested by KRIENITZ et al. (2011; 2001) and respectively two new genera could be described.

The better taxonomical placement of genera and species within the Selenastraceae using molecular data enabled pointing enhanced morphological features to distinguish the members of this complex group for traditional taxonomy, as further discussed.

Morphological criteria with high taxonomic value in traditional systematics of Selenastraceae.

Pyrenoids. The presence or absence of pyrenoids is an important problem for the taxonomy of the studied genera, since using conventional light microscopy (LM), naked pyrenoids are almost impossible to detect, whereas starch covered pyrenoids are easily visualized. For that reason, most Selenastraceae were considered to be pyrenoid-less (KOMÁREK & FOTT 1983; KORSHIKOV 1953) until the study of ELORANTA (1979) that reported the presence of pyrenoids in *Monoraphidium griffithii* (BERKELEY) KOMÁRKOVÁ-LEGNEROVÁ, using TEM images. Later studies detected pyrenoids in all

taxa studied, presenting a matrix naked or covered by starch grains (HEGEWALD et al. 1994; KRIENITZ et al. 2011; KRIENITZ et al. 1985; KRIENITZ et al. 2001). Thus, TEM is necessary for the observation of details on the pyrenoid structure (KRIENITZ et al. 2001). Furthermore, presence or absence of starch-covered pyrenoids may be not a valuable character to the Selenastraceae taxonomy when not in controlled conditions (KRIENITZ & KLEIN 1988; MIYASHI et al. 1986).

In our study, except for *Chlorolobion braunii* (NÄGELI) KOMÁREK, all Selenastracean taxa studied have ellipsoidal or spherical naked pyrenoids (without starch envelopes), both investigated by LM or TEM, but they can also be spherical to irregular in Selenastraceae (see also KRIENITZ, 2011).

Regarding to the pyrenoid differences between genera, the thylakoid membranes permeated the pyrenoid matrix in *Curvastrum pantanale* and *Ankistrodesmus arcuatus* (Fig. 1.9-1.10), but this character demonstrated to be highly variable in the genus *Ankistrodesmus and Monoraphidium.* Similar variation in pyrenoid structures were observed by KRIENITZ *et. al* (2001).

Colony formation. Traditionally used to delimit genera, colony formation is a questionable criterion. Despite the presence of colonies, many solitary cells and intermediates in the formation of colonies can be found, both in fast growing cultures (KRIENITZ et al. 2001) and in environmental samples. Many *Ankistrodesmus* (as an example, see KOMAREK & FOTT, 1983, page 689, plate 193-1), members of *Kirchneriella* and *Chlorolobion* detaches from the colonies when the reproduction begins, due to the lack of mucilage and the protoplasm differentiation process. Similarly, colonies of *Ankistrodesmus* frequently disintegrate into solitary cells and can hardly be differentiated from *Monoraphidium* species. *Ankistrodesmus arcuatus* was

described as solitary cells, and for this reason, later transferred to genus *Monoraphidium* (Syn. *Monoraphidium arcuatum*). Observations on strain CCMA-UFSCar 24 on culture conditions demonstrated that this species is able to form colonies and should be realocated to genus *Ankistrodesmus*, data corroborated by *rbcL* phylogeny.

Arrangement of autospore. A parallel arrangement of autospores was observed for the genera *Ankistrodesmus, Chlorolobion, Messastrum*, *Selenastrum* and *Curvastrum. Monoraphidium* and *Kirchneriella* presented both parallel and serial arrangement of autospores. For *Raphidocelis* species studied, *Raphidocelis microscopica*, the literature does not describe this feature, but it was observed serial arrangement of autospore.

While studying the ontogenesis of genera *Ankistrodesmus* and *Monoraphidium*, KOMÁRKOVÁ-LEGNEROVÁ (1969) observed that the position of autospores was found to be an invariable and stable qualitative characteristic both in nature and in culture.

Cell shape and size. Due to the high variability of these organisms on environmental samples and in culture (FAWLEY et al. 2006; KOMÁRKOVÁ-LEGNEROVÁ 1969), criteria as cell shape and size are problematic. For some species, as *Selenastrum bibraianum* for example, cell shape vary during life cycle, detaching from the colony, and changing from semilunate to fusiform, with an increase in protoplasm content. Cell size is also a doubtful characteristic. The cell dimension described for *A. fusiformis*, for example, is big enough to accommodate *A. fasciculatus* inside the same description (KOMÁREK & FOTT 1983).

The diacritic features of Selenastraceae have been extensively discussed. According to the numerical investigation of MARVAN et al. (1984) several genera inside Selenastraceae differ from each other only by one of the above mentioned characters.

As example, *Selenastrum* differs from *Ankistrodesmus* by the curvature of the cells (KOMÁREK & COMAS GONZÁLEZ 1982), the presence of cell wall incrustations in *Raphidocelis* differs it from *Kirchneriella* (HINDÁK 1977), and *Monoraphidium* differs from *Chlorolobion* by the absence of starched pyrenoid (HEYNIG & KRIENITZ 1982; KOMÁREK 1979). Indeed, these examples could be observed on *rbcL* inference (Fig.1.6).

Morphological features associated with 18S rDNA in Selenastraceae showed that a morphotype can represent different phylotypes, suggesting that the diversity of the family have been considerably underestimated (FAWLEY et al. 2006).

Overall a combination of colony formation, presence/absence of starched pyrenoid and cell shape and size were valuable for the taxonomy of the selenastracean strains.

Remarks on genera.

Selenastrum, Messastrum and C*urvastrum*. The phylogenetic inference of *rbcL* and 18S rDNA (see Figs 1.6, 1.7) showed that *Selenastrum bibraianum* (syn. *Ankistrodesmus bibraianus*), *Selenastrum gracile* (syn. *Ankistrodesmus gracilis*) and *Curvastrum pantanale* represent unrelated lineages. Previous works had presented similar data for *Selenastrum* and *Messastrum* (FAWLEY *et al*. 2006; KRIENITZ *et al*. 2001; KRIENITZ *et al*. 2011), but no taxonomical change was made based on molecular data. The differences between the ITS-2 secondary structure, 18S rDNA and *rbcL* of the type species *S. bibraianum*, *M. gracile* and *C. pantanale* (for ITS-2 secondary structure see supplemental material) were enough to suggest the new genera. *Selenastrum* is distinct from *Messastrum* as indicated by phylogenetic interference and by eight CBCs in the conserved region of Helix III of the ITS2, which has been considered as reliable

criterion for differentiation at species (COLEMAN 2009) and higher taxonomic levels (COLEMAN 2007; 2010).

Curvastrum pantanale sp. nov. was a new genus, proposed by the 18S rDNA and *rbcL* inference and only one diacritical morphological character (number of cells in colony).

Ankistrodesmus. All *Ankistrodesmus*-like strains studied were fusiform, isopolar, arcuate or needle-shaped, with parallel arrangement of autospores. Both phylogenies (Figs 1.6, 1.7) closely related all *Ankistrodesmus* strains. It was possible to recognize the genus using both phylogenies (Figs 1.6, 1.7), but not species within it, since the strains share very similar morphological traits and present a low number of variable positions in the sequences of *rbcL* and 18S rDNA.

The strain *Ankistrodesmus nannoselene* (SAG 202.6), a halfmoon-shaped or croissant-shaped solitary representative, seem to be related to *Kirchneriella* species and is not clearly positioned in Selenastraceae (BOCK et al. 2011a; KRIENITZ & BOCK 2012).

Molecular data revealed that *Monoraphidium arcuatus* (syn. *Ankistrodesmus arcuatus*), here represented by strain CCMA-UFSCar 24, should be recognized as belonging to genus *Ankistrodesmus* (Fig.1.6), since it shows a typical colony formation when attached to a mucilage aggregate (Fig 1.5 - arrowhead).

Monoraphidium. On our *rbcL* inference, *Monoraphidium contortum* showed the same morphotype for different phylotypes, feature also found by FAWLEY et al. (2006) for *Monoraphidium.*

The morphology diversity inside *Monoraphidium* has a huge range of variations, including fusiform to straight cells, spiral to thin crescent-shaped organisms. The absence of typus generis *Monoraphidium griffthii* on this study did not allow the identification of real *Monoraphidium* clade. Further studies including more

representatives of *Monoraphidium* are necessary to elucidate inter and intraspecific relations and designate new genera. The diacritic characters settled for the other genera in Selenastraceae could not distinguish all *Monoraphidium* species.

Two strains of *Monoraphidium convolutum*, one *Raphidocelis subcapitata* (KORSHIKOV) NYGAARD, KOMÁREK, KRISTIANSEN & SKULBERG and *Monoraphidium dybowskii* (WOLOSZYNSKA) HINDÁK & KOMÁRKOVA-LEGNEROVÁ were closely related and these relationships was supported statistically. Recent study (KRIENITZ et al. 2011) placed *M. convolutum* and *Monoraphidium dybowskii* as insertae sedis in Selenastraceae, emphasizing that future studies should be done.

As well as for the genus *Monoraphidium*, other genera as *Quadrigula* and *Nephroclamys* need further studies based on larger taxon sampling.

Raphidocelis and *Kirchneriella.* On our *rbcL* inference, *Raphidocelis microscopica* is closely related to *M. contortum* (strains CCMA-UFSCar 349 and CB 2009/10). This species presents conical, isopolar and capricornutum shape differing from *M. contortum* by cell shape, autospore arrangement, and colony formation.

Further investigations for the genus *Kirchneriella* is needed due to the lack of molecular data. Only two sequences for the same strain (*K. aperta* - SAG 2004) were available on NCBI. As observed on both phylogenies, *Kirchneriella* species present diverse phylotypes inside one morphotype, always closely related to *Monoraphidium*-like members, suggesting that the different morphotypes applied for both genera hides similar genotypes. Similar findings were obtained for *Dictyosphaerium*, which morphotypes formed distinct lineages inside *Chlorella* and *Parachlorella*, presenting independent evolution and confirming the polyphyletic origin of the *Dictyosphaerium* morphotype within the Chlorellaceae (LUO et al. 2010).

Chorolobion. On the 18S rDNA tree, *Chlorolobion braunii* clustered in one well supported branch, with *Podohedriella falcata* and *Monoraphidium neglectum*. We could observe that the strains SAG 202-2, SAG 48.87 analyzed by KRIENITZ *et al*. (2001), and our strains CCMA-UFSCar 137, 462, 477 and 476 refer to three different morphological species. The small difference between these three taxa (up to 20 variable sequence positions) on 18S rDNA was not enough to separate the different morphotypes. Based on 18S rDNA, the distinction among *Podohedriella*, *Monoraphidium*, and *Chlorolobion* remains unclear. However, for *rbcL* inference, the four *Chlorolobion braunii* formed a well-supported clade, with no sequences for *P. falcata* and *M. neglectum*. Thus, since there is few molecular data and the morphology is too similar, not enforcing a nomenclatural change, we maintained our strains as belonging to genus *Chlorolobion*. The numbers of variable sequence positions on 18S rDNA among these six taxa were 0-2

The main morphological differences are that *Chlorolobion braunii* own starch sheathed pyrenoids (KOMÁREK), is fusiform, with serial arrangement of autospores; *Podohedriella falcata* is needle-shaped, representing one of the few periphytic Selenastraceae (DÜRINGER 1958; HINDÁK 1988), and *M. neglectum* have naked pyrenoid serially arranged (KRIENITZ et al. 2001).

Thus, based on morphology and *rbcL* analysis, we maintained our strains as belonging to genus *Chlorolobion*.

General view

Selenastraceae, at this moment, is composed of eleven genera. Except for *Ourococcus* GROBÉTY representatives of all the other ten genera were represented in our

phylogenetic study as well as in other previous studies (FUČÍKOVÁ et al. 2014; KRIENITZ & BOCK 2012).

A joined analysis of *rbcL* and 18S rRNA gene data sets is used in some studies of algal phylogeny (RINDI et al. 2007), since 18S rRNA gene is too conserved to elucidate the phylogenetic trends at genus and species levels. Accordingly, due to the lower variation of the 18S rRNA sequences observed in this study, a better resolution of this group was achieved by using a more variable marker, the *rbcL*. This gene had higher sequence divergence in Selenastraceae, and, therefore, was more useful than the 18S rDNA gene for phylogenetic inference at the genus and species levels. In most algal groups this is commonly the case (GURGEL & FREDERICQ 2004; HAYDEN & WAALAND 2002; HEPPERLE et al. 1998; HOHAM et al. 2002; MÜLLER et al. 2002).

Using both genes, the substantial number of variable sequence positions among the studied taxa (0-76 for 18S rRNA and 0-86 for *rbcL*) revealed the relationship patterns within Selenastraceae.

An usual problem in the systematics of Selenastraceae was the absence or a low bootstrap support for the basis of the trees and a good support only of smaller groups of species or genera (FAWLEY *et al*. 2006; KRIENITZ *et al*. 2001). However, our clusters are in agreement with previous studies in Selenastraceae (FAWLEY *et al*. 2006; KRIENITZ *et al*. 2011; KRIENITZ *et al*. 2001), supporting the probability of the phylogenetic information on the branches, despite some low bootstrap values.

Since MAYR's (1942) species concept cannot be applied to coccoid green algae, limnologists adopted the morphological species concept using morphology based diacritical characteristics as a way to deal with this limitation (KRIENITZ & BOCK 2012).

In addition to the phenotype, discussions about species concepts should consider intraspecific genetic variation (HILT 2006). The description of well-established species,

with known phenotypic population, should be completed by molecular, genetic, physiological, and biochemical features of all those cultured strains that follow to the original type description (WOOD & LEATHAM 1992), leading to the resolution of taxonomic position of morphological variants from the type.

Taxonomical value of diacritic features were discussed based on morphology or ultrastructure (KRIENITZ et al. 2001) suggesting that the concept of "small genera" (KOMÁREK & FOTT 1983) including only a few species for the Selenastraceae is not appropriated, since the existence of many transitional forms, and many diacritic features are considered not reliable (FAWLEY et al. 2006). Since it was detected real phylogenetic lineages in this family conceiving 'small' genera was considered uncertain for Selenastraceae (KRIENITZ et al. 2001). However, some genera presented a set of diacritic characteristics. For its wide variability, the establishment of mucilage as diacritic feature is of limited taxonomic value (KRIENITZ et al. 2012). Our findings suggest that the small genera concept could be applied to *Monoraphidium*, *Kirchneriella* and also for *Selenastrum* and *Messastrum*.

Although establishing smaller genera, differentiated from other genera by only a few diacritic characteristics and containing only a few numbers of species, is a trend (LUO et al. 2010). On the last decades some studies have divided previous morphological well established genus in many smaller ones, as proposed for *Scenedesmus* (AN et al. 1999; KRIENITZ et al. 2003) and *Pediastrum* (BUCHHEIM et al. 2005). *Scenedesmus* Meyen has been differentiated in four genera: *Scenedesmus*, *Acutodesmus* (HEGEWALD) TSARENKO, *Desmodesmus* CHODAT, and *Neodesmus* HINDÁK (AN et al. 1999; KRIENITZ et al. 2003).

Studies on Chlorellaceae (LUO et al. 2010) differentiated six genera within the *Chlorella* clade of Chlorellaceae, mostly by molecular criteria (SSU and ITS-1 and -2)

and revealed that several morphological criteria (mucilage and connecting strands, colonial versus solitary life form and bristle formation) are phenotypic characteristics, representing adaptive responses to environmental factors such as grazing pressure, endosymbiosis or edaphic life strategies.

If in one hand some species may present phenotypic plasticity, on the other hand, analogous morphotypes could hide high genotypic diversity. Crescent-shaped Selenastraceae isolates of belonging to the same morphospecies were found by FAWLEY et al. (2006), with differences regarding SSU rRNA (KRIENITZ et al. 2011) for the same morphotypes studied. High levels of variability among isolates concerned to a particular taxon could actually represent numerous taxa (KOMÁRKOVÁ-LEGNEROVÁ 1969; NYGAARD & KOMAREK 1986).

Despite the possible phenotypical plasticity, the molecular analyses associated with morphological review in different growth phases of the phytoplankton strains, showed that some morphological criteria are important for the systematics of Selenastraceae on genus and species level. Cell shape and size, colony formation, and arrangement of autospores in the mother cell wall demonstrated to be useful for genera separation, but should be applied carefully inside species. The observation of a substantial number of colonies and solitary organisms (10-50 individuals) and observation of the whole life cycle, with special emphasis to autospores liberation and the initial colony formation, should be done in order to identify a taxon.

Unfortunately, some species description were published with missing information on the original publication, regarding morphological information (including plates) not covering all the necessary information to identify many taxa (for morphological references see material and methods). Although 18S rDNA and *rbcL*

were important, morphological characteristics were observed and considered individually (morphology or genetic data) for taxonomical decisions.

However, *Monoraphidium* and *Kirchneriella* seem to be polyphyletic genera and therefore these traditional traits could not be employed, otherwise the identification would not be reliable.

Evidently, there might be a huge number of lineages within the Selenastraceae that are not covered by our study. Unfortunately, no cultures are available for hundreds of taxa designated in this cosmopolitan family and some strains are difficult to obtain all the target genes.

Thus, more efforts and new studies with isolated and cultivable strains are necessary. The many sequences from taxonomic and phylogenetic studies submitted to databases, are fundamental to be used as references for future practical applications and scientific studies (JI et al. 2013), including metabarcoding approaches. The increasingly community studies using high-throughput sequencing still lacks references for phytoplankton species (EILER et al. 2012; PAWLOWSKI et al. 2012).

The coccoid green algae are important in ecosystem studies but little information is available about its geographic distributions, even with the improvement of the taxonomy (NORTON et al. 1996; PADISÁK et al. 2015). It is estimated that exists around 14900 freshwater phytoplankton species (BOURRELLY 1990). Species richness in freshwater lakes has been claimed to be significantly higher in temperate lakes than in tropical ones (LEWIS JR 1978), but this result could be also a consequence of the poorly exploration and understanding about green microalgae diversity and phylogeny in the tropics.

1.6. ACKNOWLEDGEMENTS

The authors would like to thank Fundação de Amparo à Pesquisa do Estado de São Paulo (Processes: 2011/50054-4, 2012/19520-1, 2013/17457-3 and 2013/18083-0) for financial and scientific support and scholarships granted and the staff at the IMCES imaging facility for their help and assistance with imaging and analysis.

1.7. REFERENCES

AN, S.; FRIEDL T. & HEGEWALD E. (1999): Phylogenetic Relationships of *Scenedesmus* and *Scenedesmus*-like Coccoid Green Algae as Inferred from ITS-2 rDNA Sequence Comparisons. - Plant Biology 1(4):418-428.

BOCK, C.; KRIENITZ L. & PRÖSCHOLD T. (2011a): Taxonomic reassessment of the genus *Chlorella* (Trebouxiophyceae) using molecular signatures (barcodes), including description of seven new species. - Fottea 11(2):293-312.

BOCK, C.; PRÖSCHOLD T. & KRIENITZ L. (2011b): Updating the genus *Dictyosphaerium* and description of *Mucidosphaerium* Gen. Nov. (Trebouxiophyceae) based on morphological and molecular data1. - Journal of Phycology 47(3):638-652.

BOOTON, G. C.; FLOYD, G. L. & FUERST P. A. (1998): Origins and affinities of the filamentous green algal orders Chaetophorales and Oedogoniales based on 18S rRNA gene sequences. - Journal of Phycology 34(2):312-318.

BOURRELLY, P. (1990): Les Algues d'eau douce. Initation a la Systématique, I: Les Algues Vertes. - 572pp. N Boubée & Cie, Paris.

BUCHHEIM, M.; BUCHHEIM, J.; CARLSON, T.; BRABAND, A.; HEPPERLE, D.; KRIENITZ, L.; WOLF M. & HEGEWALD E. (2005): Phylogeny of the Hydrodictyaceae (Chlorophyceae): inferences from rDNA data[1]. - Journal of Phycology 41(5):1039-1054.

BUCHHEIM, M. A.; MICHALOPULOS, E. A. & BUCHHEIM J. A. (2001): Phylogeny of the Chlorophyceae with special reference to the Sphaeropleales: a study of 18S and 26S rDNA data. - Journal of Phycology 37(5):819-835.

BYUN, Y. & HAN K. (2009): PseudoViewer3: generating planar drawings of large-scale RNA structures with pseudoknots. - Bioinformatics 25(11):1435-1437.

COLEMAN, A. W. (2003): ITS2 is a double-edged tool for eukaryote evolutionary comparisons. - Trends in Genetics 19(7):370-375.

COLEMAN, A. W. (2007): Pan-eukaryote ITS2 homologies revealed by RNA secondary structure. - Nucleic Acids Research 35(10):3322-3329.

COLEMAN, A. W. (2009): Is there a molecular key to the level of "biological species" in eukaryotes? A DNA guide. - Molecular Phylogenetics and Evolution 50(1):197-203.

COLEMAN, A. W. & VACQUIER V. D. (2002): Exploring the phylogenetic utility of ITS sequences for animals: a test case for abalone (Haliotis). - Journal of molecular evolution 54(2):246-257.

COMAS, A. (1996): Las Chlorococcales dulciacuícolas de Cuba. Bibliotheca Phycologica 99. – 192pp., J. Cramer, Berlin.

DÜRINGER, I. (1958): Über die Verteilung epiphytischer Algen auf den Blättern wasserbewohnender Angiospermen sowie systematisch-entwicklungsgeschichtliche Bemerkungen über einige grüne Algen. - Plant Systematics and Evolution 105(1):1-43.

EILER, A.; DRAKARE, S.; BERTILSSON, S.; PERNTHALER, J.; PEURA, S.; ROFNER, C.; SIMEK, K.; YANG, Y.; ZNACHOR, P. AND LINDSTRÖM E. S. (2013): Unveiling distribution patterns of freshwater phytoplankton by a next generation sequencing based approach. - PLoS ONE 8:e53516.

ELORANTA, V. (1979): The compound internal pyrenoid in cultured cells of the green alga *Monoraphidium griffithii* (Berkel.) Komar.-Legner. - Protoplasma 99(3):229-235.

FAWLEY, M. W.; DEAN, M. L.; DIMMER, S. K. & FAWLEY K. P. (2006): Evaluating the morphospecies concept in the Selenastraceae (Chlorophyceae, Chlorophyta). - Journal of Phycology 42(1):142-154.

FRIEDL, T. (1995): Inferring taxonomic positions and testing genus level assignments in coccoid green lichen algae: a phylogenetic analysis of 18s ribosomal rna sequences from *Dictyochloropsis reticulata* and from members of the genus *Myrmecia* (Chlorophyta, Trebouxiophyceae Cl. Nov.) [1]. - Journal of Phycology 31(4):632-639.

FUČÍKOVÁ, K; LEWIS, P. O.; GONZÁLEZ-HALPHEN D. & LEWIS L. A. (2014): Gene arrangement convergence, diverse intron content, and genetic code modifications in mitochondrial genomes of Sphaeropleales (Chlorophyta). - Genome biology and evolution 6(8):2170-2180.

FUČÍKOVÁ, K.; RADA, J. C.; LUKEŠOVÁ, A. & LEWIS L. A. (2011): Cryptic diversity within the genus *Pseudomuriella* Hanagata (Chlorophyta, Chlorophyceae, Sphaeropleales) assessed using four barcode markers. - Nova Hedwigia 93(1-2):29-46.

GRUBER, A. R.; LORENZ, R.; BERNHART, S. H.; NEUBÖCK, R.; & HOFACKER I. L. (2008): The vienna RNA websuite. - Nucleic acids research 36(suppl 2):W70-W74.

GUILLARD, R. R. & LORENZEN C. J. (1972): Yellow-green algae with chlorophyllide C1, 2. - Journal of Phycology 8(1):10-14..

GURGEL, C. F. D. & FREDERICQ S. (2004): Systematics of the Gracilariaceae (Gracilariales, Rhodophyta): a critical assessment based on *rbcL* sequence analyses[1]. - Journal of Phycology 40(1):138-159.

HAYDEN, H. S. & WAALAND J. R. (2002): Phylogenetic systematics of the Ulvaceae (Ulvales, Ulvophyceae) using chloroplast and nuclear dna sequences. - Journal of Phycology 38(6):1200-1212.

HEGEWALD, E. & HANAGATA N. (2000): Phylogenetic studies on Scenedesmaceae (Chlorophyta). - Archiv für Hydrobiologie Supplementband, Algological studies 136:29-49.

HEGEWALD, E.; L. KRIENITZ & SCHNEPF E. (1994): Studies on *Scenedesmus costato-granulatus* Skuja. - Nova Hedwigia 59(1):97-128.

HEPPERLE, D. (2004): Align Ver. 07/04©. Multisequence alignment-editor and preparation/manipulation of phylogenetic datasets. Win32-Version. Distributed by the author via: http://www. sequentix. de.

HEPPERLE, D.; NOZAKI, H.; HOHENBERGER, S.; HUSS, V.; MORITA, E. & KRIENITZ, L. (1998): Phylogenetic position of the Phacotaceae within the Chlamydophyceae as revealed by analysis of 18S rDNA and *rbcL* sequences. - Journal of Molecular Evolution 47(4):420-430.

HEYNIG, H. & KRIENITZ L. (1982): *Monoraphidium negledum* n. sp. sowie einige Bemerkungen zu den Gattungen *Monoraphidium*, *Chlorolobion* und *Keratococcus* (Chlorococcales). - Archiv für Protistenkunde 125(1):335-344.

HILT, S. (2006): Allelopathic inhibition of epiphytes by submerged macrophytes. - Aquatic Botany 85(3):252-256 doi:DOI 10.1016/j.aquabot.2006.05.004.

HINDÁK, F. (1977): Studies on the chlorococcal algae (Chlorophyceae). Biol. Práce 23: 1–292.

HINDÁK, F. (1980): Studies on the chlorococcal algae (Chlorophyceae) - II. Biol. Práce 26: 1–296.

HINDÁK, F. (1984): Studies on the chlorococcal algae (Chlorophyceae) – III. Biol. Práce 30: 1–263.

HINDÁK, F. (1988): Studies on the chlorococcal algae (Chlorophyceae). – IV. Biol. Práce 34: 1–320.

HINDÁK, F. (1990): Studies on the chlorococcal algae (Chlorophyceae). – V. Biol. Práce 36: 1–227.

HOHAM, R. W.; BONOME, T. A.; MARTIN, C. W. & LEEBENS-MACK, J. H. (2002): A combined 18s rDNA and *rbcL* phylogenetic analysis of *Chloromonas* and *Chlamydomonas* (Chlorophyceae, Volvocales) emphasizing snow and other cold-temperature habitats. - Journal of phycology 38(5):1051-1064.

HUELSENBECK, J. P. & RONQUIST, F. (2001): MRBAYES: Bayesian inference of phylogenetic trees. - Bioinformatics 17(8):754-755.

HUSS, V. A.; FRANK, C.; HARTMANN, E. C.; HIRMER, M.; KLOBOUCEK, A.; SEIDEL, B. M.; WENZELER, P. & KESSLER, E. (1999): Biochemical taxonomy and molecular phylogeny of the genus *Chlorella* sensu lato (Chlorophyta). - Journal of Phycology 35(3):587-598.

JI, Y.; ASHTON, L.; PEDLEY, S. M.; EDWARDS, D. P.; TANG, Y.; NAKAMURA, A.; KITCHING, R.; DOLMAN, P. M.; WOODCOCK, P. & EDWARDS, F. A. (2013): Reliable, verifiable and efficient monitoring of biodiversity via metabarcoding. - Ecology letters 16(10):1245-1257.

JOBB, G. (2008): TREEFINDER version of October 2008. Munich, Germany Distributed by the author at www treefinder de.

KATANA, A.; KWIATOWSKI, J.; SPALIK, K.; ZAKRYŚ, B.; SZALACHA, E. & SZYMAŃSKA, H. (2001): Phylogenetic position of *Koliella* (Chlorophyta) as inferred from nuclear and chloroplast small subunit rDNA. - Journal of Phycology 37(3):443-451.

KOMÁREK, J. (1979): Änderungen in der Taxonomie der Chlorokokkalalgen. - Algological Studies/Archiv für Hydrobiologie, Supplement Volumes:239-263.

KOMÁREK, J. & COMAS GONZÁLEZ, A. (1982): Taxonomical definition of the genera and several species of *Ankistrodesmus* and *Selenastrum* (Chlorococcales). - Algological Studies/Archiv für Hydrobiologie, Supplement Volumes:259-277.

Komárek, J. & Fott, B. (1983): Chlorophyceae (Grünalgen) Ordnung: Chlorococcales. – In: Huber–Pestalozzi, G. (ed.): Das Phytoplankton des Süßwassers 7. Teil, 1. Hälfte. – 1044 pp., Schweizerbart'sche Verlagsbuchhandlung (Nägele u. Obermiller), Stuttgart.

KOMÁRKOVÁ-LEGNEROVÁ, J. (1969): The systematics and ontogenesis of the genera *Ankistrodesmus* Corda and *Monoraphidium* gen. nov. In: FOTT, B. (ed) Studies in Phycology. 75-122.Academia – Praha.

KORSHIKOV, O. A. (1953): Viznachnik prisnovodnihk vodorostey Ukrainsykoi RSR [Vyp] V. Pidklas Protokokovi (Protococcineae). Bakuol'ni (Vacuolales) ta Protokokovi (Protococcales). 1-439. Akad. NAUK URSR – Kyjv.

KORSHIKOV, O. (1987): The freshwater algae of the Ukrainian SSR – V. Sub-class Protococcineae: Vacuolales and Protococcales (translated by J. WG. Lund & W. Tylka). Koeltz Scientific Books, Köenigstein 4:12: 1-412.

KRIENITZ, L. & BOCK, C. (2012): Present state of the systematics of planktonic coccoid green algae of inland waters. - Hydrobiologia 698 (1):295-326.

KRIENITZ, L.; BOCK, C.; KOTUT, K. & PRÖSCHOLD, T. (2012): Genotypic diversity of *Dictyosphaerium*–morphospecies (Chlorellaceae, Trebouxiophyceae) in African inland waters, including the description of four new genera. - Fottea 12:231-253.

KRIENITZ, L.; BOCK, C.; NOZAKI, H. & WOLF, M. (2011): SSU rRNA gene phylogeny of morphospecies affiliated to the bioassay alga "*Selenastrum capricornutum*" recovered the polyphyletic origin of crescent-shaped Chlorophyta. - Journal of Phycology 47(4):880-893.

KRIENITZ, L.; HEGEWALD, E.; HEPPERLE, D. & WOLF, M. (2003): The systematics of coccoid green algae: morphology versus 18S rRNA gene phylogeny. - Biológia 58:437-446.

KRIENITZ, L. & KLEIN, G. (1988): Morphologie und Ultrastruktur einiger Arten der Gattung *Monoraphidium* (Chlorellales) III. *Monoraphidium terrestre* Bristol nov. comb. - Algological Studies/Archiv für Hydrobiologie, Supplement Volumes:447-463.

KRIENITZ, L.; KLEIN, G. & BÖHM, H. (1985): Zur morphologie und ultrastruktur von *Selenastrum gracile* Reinsch 1867 (Chlorellales). - Archiv für Protistenkunde 130(1):79-92.

KRIENITZ, L.; USTINOVA, I.; FRIEDL, T. & HUSS, V. A. (2001): Traditional generic concepts versus 18S rRNA gene phylogeny in the green algal family Selenastraceae (Chlorophyceae, Chlorophyta). - Journal of Phycology 37(5):852-865.

LEWIS JR, W. M. (1978): A compositional, phytogeographical and elementary structural analysis of the phytoplankton in a tropical lake: Lake Lanao, Philippines. - The Journal of Ecology:213-226.

LEWIS, L. A. (1997): Diversity and phylogenetic placement of *Bracteacoccus* Tereg (Chlorophyceae, Chlorophyta) based on 18s ribosomal RNA gene sequence data. - Journal of Phycology 33(2):279-285.

LUO, W.; PRÖSCHOLD, T.; BOCK C. & KRIENITZ, L. (2010): Generic concept in *Chlorella*-related coccoid green algae (Chlorophyta, Trebouxiophyceae). - Plant Biology 12(3):545-553.

MANHART, J. R., (1994): Phylogenetic analysis of green plant *rbcL* sequences. - Molecular phylogenetics and evolution 3(2):114-127.

MARIN, B.; KLINGBERG, M. & MELKONIAN, M. (1998): Phylogenetic relationships among the Cryptophyta: analyses of nuclear-encoded SSU rRNA sequences support the monophyly of extant plastid-containing lineages. - Protist 149(3):265-276.

MARVAN, P.; KOMÁREK, J. & COMAS, A. (1984): Weighting and scaling of features in numerical evaluation of coccal green algae (genera of the Selenastraceae). - Algological Studies/Archiv für Hydrobiologie, Supplement Volumes:363-399.

MAYR, E. (1942): Systematics and the origin of species, from the viewpoint of a zoologist. Columbia University Press. 1-334.

MCCOURT, R. M.; KAROL, K. G.; BELL, J.; HELM-BYCHOWSKI, K. M.; GRAJEWSKA, A.; WOJCIECHOWSKI M. F. & HOSHAW, R. W. (2000): Phylogeny of the conjugating green algae (Zygnemophyceae) based on *rbcL* sequences. - Journal of Phycology 36(4):747-758.

MESSYASZ, B. (2003): Spatial distribution of chlorococcalean genera in the phytoseston of the Wena and Nielba rivers. - Oceanological and Hydrobiological studies(2):33-44.

MISCHKE, U. & KUSBER, W. (2009): Die harmonisierte Taxaliste des Phytoplanktons für Seen und Flüsse in Deutschland. Excel-Datei-Liste mit ausführlichen Anmerkungen. Stand 2009/05/20. Available in: http://www.igb-berlin.de/datenbanken.html.

MIYASHI, T.; KAMATA M. & MUKAI, T. (1986): Oxygenation and [3+ 2]-cycloaddition of methylenecyclopropanes through electron donor-acceptor complexes with tetracyanoethylene by photoexcitation and in the dark. - Journal of the American Chemical Society 108(10):2755-2757.

MÜLLER, K. M.; SHERWOOD, A. R.; PUESCHEL, C. M.; GUTELL R. R. & SHEATH, R. G. (2002): A proposal for a new red algal order, the Thoreales. - Journal of Phycology 38(4):807-820.

NORTON, T. A.; MELKONIAN M. & ANDERSEN, R. A. (1996): Algal biodiversity. - Phycologia 35(4):308-326.

NOZAKI, H.; ITOH, M.; SANO, R.; UCHIDA, H.; WATANABE M. M. & KUROIWA, T. (1995): Phylogenetic relationships within the colonial Volvocales (Chlorophyta) inferred from *rbcL* gene sequence data. - Journal of Phycology 31(6):970-979.

NYGAARD, G.; KOMAREK, J. & SKILBERG, O. (1986): Taxonomic designations of the bioassay alga, NIVA-CHL I (*Selenastrum capricornutum*) and some related strains. - Opera Botanica 90:1-46.

NYLANDER, J. (2004): MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University 2.

PADISÁK, J.; VASAS G. & BORICS, G. (2015): Phycogeography of freshwater phytoplankton: traditional knowledge and new molecular tools. - Hydrobiologia:1-25.

PANKOW, H. (1990): Ostsee-algenflora. Gustav Fischer. Jena. 1-648.

PAWLOWSKI, J.; AUDIC, S.; ADL, S.; BASS, D.; BELBAHRI, L.; BERNEY, C.; BOWSER, S. S.; CEPICKA, I.; DECELLE J. & DUNTHORN, M. (2012): CBOL protist working group: barcoding eukaryotic richness beyond the animal, plant, and fungal kingdoms. - PLoS biology 10(11):e1001419.

RAMBAUT, A. & DRUMMOND, A. (2007): Tracer (http://beast. bio. ed. ac. uk/Tracer). Version.

RINDI, F.; GUIRY M. D. & LÓPEZ-BAUTISTA, J. M. (2008): Distribution, Morphology, And Phylogeny Of *Klebsormidium* (Klebsormidiales, Charophyceae) In Urban Environments In Europe[1]. - Journal of Phycology 44(6):1529-1540.

RINDI, F.; MCIVOR, L.; SHERWOOD, A. R.; FRIEDL, T.; GUIRY M. D. & SHEATH, R. G. (2007): Molecular phylogeny of the green algal order Prasiolales (Trebouxiophyceae, Chlorophyta)[1]. - Journal of Phycology 43(4):811-822.

RINDI, F.; MIKHAILYUK, T. I.; SLUIMAN, H. J.; FRIEDL T. & LÓPEZ-BAUTISTA, J. M. (2011): Phylogenetic relationships in *Interfilum* and *Klebsormidium* (Klebsormidiophyceae, Streptophyta). - Molecular phylogenetics and evolution 58(2):218-231.

ROGERS, J. E.; LEBLOND, J. D. & MONCREIFF, C. A. (2006): Phylogenetic relationship of *Alexandrium monilatum* (Dinophyceae) to other *Alexandrium* species based on 18S ribosomal RNA gene sequences. - Harmful Algae 5.3 275-280.

ROSENTHAL, A.; COUTELLE O. & CRAXTON, M. (1993): Large-scale production of DNA sequencing templates by microtitre format PCR. - Nucleic Acids Research 21(1):173-174.

SANT'ANNA, C. L. (1984): Chlorococcales (Chlorophyceaea) do Estado de São Paulo, Brasil. Bibliotheca Phycologica 67. J. Cramer, Berlin. 1-348.

SAUNDERS, G. W. & H. KUCERA. (2010): An evaluation of *rbcL*, tufA, UPA, LSU and ITS as DNA barcode markers for the marine green macroalgae. - Cryptogamie Algologie 31(4):487-528.

SCHULTZ, J.; MAISEL, S.; GERLACH, D.; MÜLLER, T. & WOLF, M. (2005): A common core of secondary structure of the internal transcribed spacer 2 (ITS2) throughout the Eukaryota. - RNA 11(4):361-364.

STÖVER, B. C. & MÜLLER, K. F. (2010): TreeGraph 2: combining and visualizing evidence from different phylogenetic analyses. - BMC bioinformatics 11(1):7.

Swofford, D.L. (2002): Phylogenetic analysis using parsimony (* and other methods), version 4.0b 10. – Sinauer Associates, Sunderland, Massachusetts.

TAS, B. & GONULOL, A. (2007). An ecologic and taxonomic study on phytoplankton of a shallow lake, Turkey. - Journal of Environmental Biology 28(2):439.

USTINOVA, I.; KRIENITZ L. & HUSS, V. A. (2001): *Closteriopsis acicularis* (GM Smith) Belcher & Swale is a fusiform alga closely related to *Chlorella kessleri* Fott & Nováková (Chlorophyta, Trebouxiophyceae). - European Journal of Phycology 36(04):341-351.

VAN HANNEN, E.; FINKGODHE P. & LURLING, M. (2002): A revised secondary structure model for the internal transcribed spacer 2 of the green algae *Scenedesmus* and *Desmodesmus* and its implication for the phylogeny of these algae. - European Journal of Phycology 37(2):203-208.

WOLF, M.; CHEN, S.; SONG, J.; ANKENBRAND M. & MÜLLER, T. (2013): Compensatory base changes in ITS2 secondary structures correlate with the biological species concept despite intragenomic variability in ITS2 sequences–a proof of concept. - PloS one 8(6):e66726.

WOOD, A. M. & LEATHAM, T. (1992): The species concept in phytoplankton ecology. - Journal of Phycology 28(6):723-729.

YOUNG, I. & COLEMAN, A. W. (2004): The advantages of the ITS2 region of the nuclear rDNA cistron for analysis of phylogenetic relationships of insects: a *Drosophila* example. - Molecular phylogenetics and evolution 30(1):236-242.

Table 1.1. List of studied strains with origin information and GenBank accession numbers for 18S rDNA and *rbcL* genes and ITS-2 secondary structure. Sequences in bold letter acquired from GenBank. CCMA - UFSCar, Coleção de culturas de Microalgas de Água Doce – Universidade Federal de São Carlos ; SAG, Sammlung von Algenkulturen der Universität Göttingen, Germany; UTEX, The Culture Collection of Algae at the University of Texas at Austin. For own isolates or culture collections the initials were given: CB, Christina Bock; KR, Lothar Krienitz; Comas, Augusto Abilio Comas González. KF, Alena Lukešová. The strain AN7-8 belongs to Fawley *et al*, 2004.

		GenBank Accession Numbers			
Taxon	**Strain**	**18S**	***rbcL***	**ITS-2**	**Origin**
Selenastraceae					
Ankistrodesmus arcuatus	CCMA-UFSCar 24		KT355740		Brazil - 22°12'20.6"S 47°52'37.6"W
Ankistrodesmus fusiformis	CCMA-UFSCar 423		KT833565		Brazil - 22°09'57.8"S 48°17'25.0"W
Ankistrodesmus bibraianus	SAG 278-1	**Y16938**			UK - 52°12'08.4"N 0°07'42.0"E
Ankistrodesmus fasciculatus	CB 2012/3		KT355769		Sweden - 56°40'10.4"N 14°10'03.4"E
Ankistrodesmus fusiformis	CB2012/6		KT833576		Sweden - 59°20'00.0"N 12°13'00.0"E
Ankistrodesmus fusiformis	CCMA-UFSCar 593		KT355761		Brazil - 20°25'05.6"S 47°25'41.0"W
Ankistrodesmus fusiformis	CCMA-UFSCar 611		KT833570		Brazil - 23°36'29.1"S 47°13'59.4"W

Table 1.1. cont.

Ankistrodesmus fusiformis	KR 1988/9	**X97352**		Germany - 53°12'31.6"N 13°00'25.5"E
Ankistrodesmus gracilis	KR 1981/231	**HM565930**		Germany - 51°52'55.7"N 12°00'11.6"E
Ankistrodesmus gracilis	SAG 278-2	**Y16937**		UK - 51°57'54.3"N 1°09'39.9"E
Ankistrodesmus nannoselene	SAG 202-6	**KF673373**		Sweden - 59°57'46.0"N 17°07'43.9"E
Ankistrodesmus sigmoides	CB2009/9	KT833600		Kenya - 53°10'06.3"N 13°07'50.9"E
Ankistrodesmus spiralis	CB2012/29		KT833573	Sweden - 55°32'46.4"N 13°15'05.3"E
Ankistrodesmus stipitatus	CCMA-UFSCar 277	KT833580		Brazil - 20°32'01.0"S 46°31'32.9"W
Ankistrodesmus stipitatus	CCMA-UFSCar 278	KT833581	KT355749	Brazil - 20°32'01.0"S 46°31'32.9"W
Ankistrodesmus stipitatus	SAG 202-5	**X56100**	**EF113406**	Czech Republic - 50°04'23.2"N14°26'09.9"E
Chlorolobion braunii	CCMA-UFSCar 137	KT833579	KT355742	Brazil - 21°36'28.5"S 47°46'13.1"W
Chlorolobion braunii	CCMA-UFSCar 455	KT833587	KT355756	Brazil - 22°05'05.3"S 47°44'47.0"W
Chlorolobion braunii	CCMA-UFSCar 462	KT833588	KT355763	Brazil - 22°12'59.2"S 47°37'29.6"W
Chlorolobion braunii	CCMA-UFSCar 476	KT833591	KT355760	Brazil - 21°53'46.9"S 47°20'03.3"W

Table 1.1. cont.

Curvastrum pantanale	CCMA-UFSCar 350	KT833584	KT355754	KU180822	Brazil - 19°17'59.0"S 55°47'45.0"W
Curvastrum pantanale	CCMA-UFSCar 608		KT833569	KU180824	Brazil - 23°15'25.7"S 47°18'18.6"W
Kirchneriella aperta	CCMA-UFSCar 482	KT833592			Brazil - 22°33'09.2"S 48°57'47.5"W
Kirchneriella aperta	SAG 2004	**AJ271859**	**KC145514**		Germany - 53°08'51.7"N 13°01'41.9"E
Kirchneriella contorta var. *elegans*	CCMA-UFSCar 447	KT833586			Brazil - 22°19'01.0"S 48°03'05.4"W
Kirchneriella irregularis	CCMA-UFSCar 348	KT833583	KT355752		Brazil - 19°17'59.0"S 55°47'45.0"W
Kirchneriella lunaris	CCMA-UFSCar 443		KT833566		Brazil - 22°19'01.0"S 48°03'05.4"W
Kirchneriella obesa	CCMA-UFSCar 345		KT833564		Brazil - 19°17'59.0"S 55°47'45.0"W
Kirchneriella pseudoaperta	CCMA-UFSCar 346	KT833582			Brazil - 19°17'59.0"S 55°47'45.0"W
Messastrum gracile	CB 2009/3	KT833596			Germany - 53°00'50.8"N 13°10'00.5"E
Messastrum gracile	CB 2009/35	KT861784			Germany - 52°31'32.1"N13°17'15.5"E
Messastrum gracile	CCMA-UFSCar 470	KT833589	KT355759	KU180823	Brazil - 21°48'38.0"S 47°16'26.0"W
Messastrum gracile	CCMA-UFSCar 5	KT833577	KT355739	KU180819	Brazil - 22°12'20.6"S 47°52'37.6"W
Messastrum gracile	CCMA-UFSCar 622	KT833593	KT355762	KU180825	Brazil - 23°02'28.8"S 48°03'15.9"W

Table 1.1. cont.

Monoraphidium braunii	KR 1986/28	**AJ300527**		Germany - 53°08'37.9"N 13°01'07.3"E
Monoraphidium contortum	CB2009/10		KT833571	Kenya - 53°10'06.3"N 13°07'50.9"E
Monoraphidium contortum	CCMA-UFSCar 306		KT355750	Brazil - 23°44'04.3"S 46°45'44.4"W
Monoraphidium contortum	CCMA-UFSCar 329		KT833563	Brazil - 8°35'17.9"S 63°37'12.3"W
Monoraphidium contortum	CCMA-UFSCar 349		KT355753	Brazil - 19°17'59.0"S 55°47'45.0"W
Monoraphidium convolutum	AS7-3	**AY846377**		USA - 47º17’26.8”N 98º50’07.0”W
Monoraphidium convolutum	KR 1981/262	**HM565926**		Germany - 51°49'37.8"N 11°59'41.7"E
Monoraphidium dybowskii	SAG 202-7e	**Y16939**		France - 43°06'26.3"N 0°05'10.4"W
Monoraphidium indicum	CCMA-UFSCar 549		KT833568	Brazil - 22°04'56.2"S 48°29'20.0"W
Monoraphidium komarkovae	CCMA-UFSCar 353	KT833585	KT355755	Brazil - 19°20'29.0"S 55°43'40.0"W
Monoraphidium komarkovae	CCMA-UFSCar 632	KT833595	KT355763	Brazil - 24°0'19.44"S 48°20'33.41"W
Monoraphidium neglectum	SAG 48.87	**AJ300526**		Germany - 51°44'43.9"N 11°58'48.6"E
Monoraphidium pseudobraunii	CCMA-UFSCar 325		KT355751	Brazil - 8°35'17.9"S 63°37'12.3"W
Monoraphidium terrestre	SAG 49.87	**Y17817**		Germany - 51°47'22.9"N 12°03'53.6"E

Table 1.1. cont.

Nephrochlamys subsolitaria	SAG 243-2a	**HM560960**			UK - 54°55'25.5"N 2°18'02.7"W
Podohedriella falcata	SAG 202-2	**X91263**			Switzerland - 47°10'37.2"N 8°12'35.0"E
Quadrigula closterioides	SAG 12.94	**Y17924**			USA - 47°11'43.2"N 95°10'00.9"W
Raphidocelis microscopica	CB 2009/6		KT355768		Sweden - 0°21'20.1"S 36°03'20.5"E
Raphidocelis microscopica	CB2012/39		KT833574		Sweden - 58°25'11.5"N 14°30'30.6"E
Raphidocelis microscopica	CB 2009/18		KT355764		Kenya - 0°25'49.0"S 36°13'54.6"E
Raphidocelis subcapitata	KR 1991/19	**HM483520**			Germany - 53°08'49.3"N 13°01'53.9"E
Rhombocystis complanata	KR 1998/2	**HM483518**			Dominica - 15°23'29.9"N 61°15'19.6"W
Selenastrum bibraianum	CB2009/41	**HM483514**			Germany - 51°00'47.8"N 9°52'58.2"E
Selenastrum bibraianum	CB 2009/43	KT833599			Germany - 52°27'10.4"N 13°18'25.3"E
Selenastrum bibraianum	CB 2012/47		KT833575	KU180826	Sweden - 55°52'01.4"N 13°33'21.5"E
Selenastrum bibraianum	CB2009/39	KT833597			Sweden - 55°52'01.4"N 13°33'21.5"E
Selenastrum bibraianum	CCMA-UFSCar 125	KT833578	KT355741	KU180821	Brazil - 21°36'28.5"S 47°46'13.1"W

Table 1.1. cont.

Selenastrum bibraianum	CCMA-UFSCar 47		KT833561	KU180820	Brazil - 21°59'08.5"S 47°52'50.6"W
Selenastrum bibraianum	CCMA-UFSCar 630	KT833594			Brazil - 23°49'17.5"S 48°36'02.9"W
Tetranephris brasiliensis	KR 1989/26	**HM565927**			Germany - 53°32'48.5"N 13°15'10.6"E
Tetranephris brasiliensis	Comas 1991/6	**HM565929**			Cuba - 22°17'05.4"N 80°33'32.2"W
Bracteococcaceae					
Bracteacoccus aerius	KF26		**JQ259861**		Sweden - 68°21'03.5"N 18°49'45.2"E
Bracteacoccus bullatus	KF22		**JQ259877**		Czech Republic – 50°04'26.8"N 14°27'02.0"E
Bracteacoccus cohaerens	SAG 2369	**HQ246325**	**HQ246363**		South Africa – 30°07'11.2"S 17°35'26.8"E
Bracteacoccus grandis	UTEX 1246		**GQ985396**		USA - 30°39'27.5"N 97°36'19.1"W
Hydrodictyaceae					
Pediastrum duplex	SAG 28.83	**AY780662**			Germany - 49°17'21.8"N 7°50'59.5"E

Table 1.2. Morphological characteristics of algal strains used in this study. For species marked with asterisk, see taxonomic references on Material and Methods. ND: Not described.

Taxon	**Cell shape (CSh)**	**Cell size (CSz) (µm)**	**Autospore Arrangement (AA)**	**Pyrenoid structure**	**Colony formation**	**Diacritical feature**
Ankistrodesmus arcuatus	Crescent-shaped, circular or arcuate, isopolar	26-60 x 0,8-4,4	Parallel	Naked	Yes	CSz and CSh
Ankistrodesmus fasciculatus	Fusiform, isopolar	15-32 x 1,4-5	Parallel	Naked	Yes	Colony morfology, CSz size and CSh
Ankistrodesmus fusiformis	Fusiform, isopolar	19,2-57 x 1-5,8	Parallel	Naked	Yes	Colony morfology, CSz and CSh
**Ankistrodesmus nannoselene*	Crescent or halfmoon-shaped, isopolar	2,5-5,7 x 0,5-1,8	Parallel	Naked	No	Insertae sedis inside Selenastraceae
Ankistrodesmus sigmoides	Fusiform, isopolar	28 x 2-3	N	Naked	Yes	Colony morfology, CSz and CSh
Ankistrodesmus spiralis	Fusiform, isopolar	36-68 x 1-4,3	Parallel	Naked	Yes	Colony morfology, CSz and CSh
Ankistrodesmus stipitatus	Fusiform, isopolar	43-105 x 1,4-4,6	Parallel	Naked	Yes	Colony morfology, CSz and CSh
Chlorolobion braunii	Fusiform, isopolar to heteropolar	13-52 x 2,5-8	Parallel	Amyloid	No	Colony morfology, CSz and CSh
Curvastrum pantanale	Narrow, fusiform to semilunate, isopolar	7-21 x 1,95-3,49	Zigzag	Naked	Yes	Genus monoespecific
Kirchneriella aperta	Crescent or halfmoon-shaped, isopolar	6-12 x 12	Parallel	Naked	Yes	CSz and CSh
Kirchneriella contorta var. *elegans*	Crescentor halfmoon-shaped, isopolar	6-8 x 2	Parallel	Naked	Yes	CSz and CSh
Kirchneriella irregularis	Fusiform, regularly semicircular	6-21 x 3-6	Parallel	Naked	Yes	CSz and CSh
Kirchneriella lunaris	Crescent-shaped or slightly circular, isopolar	4-15 x 1,2-2,8	Parallel	Naked	Yes	CSz and CSh
Kirchneriella obesa	Circular, isopolar	6-16 x 2-9,5	Parallel	Naked	Yes	CSz and CSh

Table 2. 1 cont.

Kirchneriella pseudoaperta	Crescent or halfmoon-shaped, isopolar	1,6-9,6 x 2,4 x 4,2	Zigzag	Naked	Yes	CSz and CSh, AA
Messastrum gracile	Narrow, fusiform to semilunate, isopolar	19-55 x 1-6	Zigzag	Naked	Yes	CSz and CSh
Monoraphidium braunii	Fusiform, isopolar to heteropolar	13-52 x 2,5-8	Parallel	Amyloid	No	Colony morfology, CSz and CSh
Monoraphidium contortum	Fusiform, crescent-shape, circular, twisted, isopolar	7-40 x 1-5,2	Parallel	Naked	No	CSz and CSh
**Monoraphidium convolutum*	Fusiforme, semicircular, isopolar	5-16 x 1-6	Parallel	Naked	No	CSz and CSh
**Monoraphidium dybowskii*	Cylindrical,fusiform, isopolar	4-15,5 x 1,2-8	Parallel	Naked	No	CSz and CSh
Monoraphidium indicum	Fusiform, circular, isopolar	120-260 x 3-5	Parallel	Naked	No	CSz and CSh
Monoraphidium komarkovae	Needle-shaped, isopolar	25-182 x 1,4-3,5	Parallel	Naked	No	CSz and CSh
**Monoraphidium neglectum*	Fusiform	15-37 x 2-6,5	Serial	Naked	No	CSz and CSh
Monoraphidium pseudobraunii	Fusiform to sigmoid, isopolar or heteropolar	8-25 x 1-2,5	Parallel	Naked	No	CSz and CSh
**Monoraphidium terrestre*	Fusiform, cylindrical	14-30 x 2-8	Serial	Naked	No	CSz and CSh
**Nephrochlamys subsolitaria*	Semilunate, isopolar	3,5-9,6-12	Serial	Naked	Yes	Proximity of cell ends
**Podohedriella falcata*	Needle-shaped, heteropolar	35-50 x 1,5-2,5	Serial	Naked	No	CSz and CSh
**Quadrigula closterioides*	Cylindrical with rounded ends, isopolar	12-30 x 1-4	Parallel	Naked	Yes	Cell end and CSz
Raphidocelis microscopica	Conical, capitata, isopolar	3-5 x 1-2	Serial	Naked	Yes	CSz and CSh
**Raphidocelis subcapitata*	Cylindrical, circular or sigmoid, isopolar	7-23 x 1,2-3	Serial	Naked	Yes	CSz and CSh
**Rhombocystis complanata*	Rhomboidal, isopolar to heteropolar	9,5-14,5x 2,4-3,6	Parallel	Naked	Occasionally	CSz and CSh
Selenastrum bibraianum	Narrow, fusiform to semilunate, isopolar	16-40 x 2,5-4,5	Zigzag	Naked	Yes	CSz and CSh
**Tetranephris brasiliensis*	Bean-shaped	6-7,6 x 4-5,8	Serial	Naked	Yes	CSz and CSh

Table 1.S3. 18s rDNA, *rbcL* and ITS primers used for amplification and sequencing of Selenastraceae.

Primer Name	**Sequence 5'-3'**	**Direction**	**Target gene**	**Reference**
18S F1	AATCTGGTTGATCCTGCCAGT	FW	18S	Katana et al.2001
1 F	CTGGTTGATCCTGCCAG	FW	18S	Huss et al.1999
300 F	AGGGTTCGATTCCGGAG	FW	18S	Huss et al.1999
528 F	CGGTAATTCCAGCTCC	FW	18S	Huss et al.1999
690 F	YAGAGGTGAAATTCT	FW	18S	Huss et al.1999
920F	GAAACTTAAAKGAATTG	FW	18S	Huss et al.1999
1055 F	GGTGGTGCATGGCCG	FW	18S	Huss et al.1999
1400F	TGYACACACCGCCCGTC	FW	18S	Huss et al.1999
920 R	ATTCCTTTRAGTTTC	RV	18S	Huss et al.1999
1200 R	GGGCATCACAGACCTG	RV	18S	Huss et al.1999
1520 R	CYGCAGGTTCACCTAC	RV	18S	Huss et al.1999
18SR1	TGATCCTTCTGCAGGTTCACCTA	RV	18S	Katana et al.2001 (modified)
rbcL 1	ATGGTTCCACAAACAGAAAC	FW	*rbcL*	Nozaki et al. 1995

Table 1.S3. cont.

rbcL 320	TATTCGAAGAAGGTTCAGTAAC	FW	*rbcL*	Rindi et al. 2008
rbcL RH1	ATGTCACCACAAACAGAAACTAAAGC	FW	*rbcL*	Manhart, J.R. 1994
rbcL 320 mod	TATTYGAASAAGGTTCWGTWAC	FW	*rbcL*	Proposed on this study
Selenastraceae *rbcL* F	CGYTACAAAGGDCGTTGYT	FW	*rbcL*	Proposed on this study
rbcL 1181	AAGATTTCAACTAAAGCTGGCA	RV	*rbcL*	Nozaki et al. 1995
rbcL 1385	GGAAAGAAATTAAATTTGAATT	RV	*rbcL*	McCourt et al. 2000
rbcL 1421	TTGTCAATAGTATCAAATTC	RV	*rbcL*	Nozaki et al. 1995
rbcL ORB	CTGGAGCATTACCCCAAGG	RV	*rbcL*	Pazoutova unpublished
rbcL Orb modified	CTGGNGCRTTACCCCAAGG	RV	*rbcL*	Proposed on this study
Selenastraceae *rbcL* R	RTTACCCCAWGGGTGHCCTA	RV	*rbcL*	Proposed on this study
1420F	CAGGTCTGTGATGCCC	FW	ITS	Rogers et al. 2006
NS7m	GGCAATAACAGGTCTGT	FW	ITS	An et al. 1999
ITS055R	CTCCTTGGTCCGTGTTTCAAGACGGG	RV	ITS	Marin et al. 1998
LR1850	CCTCACGGTACTTGTTC	RV	ITS	An et al. 1999

Figure 1.1-1.5. 1.1. *Messastrum gracile*. Original picture of strain CCMA-UFSCar 622 showing a frontal view of colony; 1.2. *Selenastrum bibraianum*. Original picture of strain CCMA-UFSCar 125 showing a frontal view of colony. 1.3-1.4. *Curvastrum pantanale*. (1.3) Original picture of strain CCMA-UFSCar 350, showing free cells and colony; (1.4) Original picture of strain CCMA-UFSCar 350, showing cells in autospore liberation. presenting a cell wall remnant (arrowhead) and protoplasm cleavage (star). 1.5. *Ankistrodesmus arcuatus.* Original picture of strain CCMA-UFSCar 24, showing free cells and colony. Note autospore formation (star) and mucilaginous lump (arrowhead). Scale bar 10 μm.

Figure 1.6: Maximum–likelihood (ML) phylogenetic tree inferred from *rbcL* gene sequences of some members of Selenastraceae. Support values correspond to Bayesian PP (Posterior Probability), ML BP (Bootstrap), MP (Maximum Parsimony) BP, NJ (Neighbor-Joining) BP. Hyphens correspond to values <50% for BP and <0.95 for PP. Scale represents the expected number of substitutions per site. Strain numbers used as mentioned in Table 1.

Figure 1.7: Maximum–likelihood (ML) phylogenetic tree inferred from 18S rDNA gene sequences of some members of Selenastraceae. Support values correspond to Bayesian PP (Posterior Probability), ML BP (Bootstrap), MP (Maximum Parsimony) BP, NJ (Neighbor-Joining)BP. Hyphens correspond to values <50% for BP and <0.95 for PP. Scale represents the expected number of substitutions per site. Strain numbers used as mentioned in Table 1.

Figure 1.8: Scanning electron micrographs of *Curvastrum pantanale* (CCMA-UFSCar 350) in culture. Scale bar, 10 µm. (a) typical colony formation, (b) young cells detaching from each other, (c) young cells, note the autospores position.

Figure 1.9: Transmission electron micrographs of *Curvastrum pantanale* (CCMA-UFSCar 350) in culture. Scale bar, 1 µm. Key to labeling: CW = cell wall, C = chloroplast, D = dictyosome, ER = endoplasmic reticulum, L = lipid drop, M = mitochondrion, N = nucleus, P = pyrenoid, PV = polyphosphate vacuole, S = starch grain. a) longitudinal section; cell presenting lipid drops, polyphosphate vacuoles (arrowhead), chloroplast penetrated with starch grains, and a central pyrenoid. b) detail of figure a, where an endoplasmic reticulum (arrowhead) can be observed. c) longitudinal section; cell presenting polyphosphate vacuoles on both cell apexes, mitochondria, chloroplast filled with starch grains and a central pyrenoid. d) cross section; chloroplast containing starch grains and a pyrenoid situated at the left, a central nucleus can be observed. e) longitudinal section; mature cell containing a central nucleus, mitochondrion and pyrenoid (upper part). All the cell content is surrounded by a cell wall.

Figure 1.10: Transmission electron micrographs of two Selenastraceae in culture. Scale bar, 1 µm. a - c) *Ankistrodesmus arcuatus* (CCMA – UFSCar 24). a) longitudinal section; cell with a nucleus, dictyosome and a chloroplast. b) cross section; a cup-shaped chloroplast penetrated by a pyrenoid (star). On the opposite direction of the pyrenoid, the nucleus is situated. An arrowhead indicates a polyphosphate vacuole. c) detail of figure a, where a dictyosome can be observed. d - e) *Monoraphidium contortum* (CCMA-UFSCar 349), d) longitudinal section; cells on different life cycle

phase. The upper 3 cells are young indidivuals presenting starch grains (arrowhead), polyphosphate vacuoles (arrowhead), chloroplast and nucleus. The lower cell is a mature individual containing many starch grains on the chloroplast, some polyphosphate vacuoles (arrowhead), nucleus and big lipid drops. e) cross section; dense chloroplast with starch grains. Two big polyphosphate vacuoles (arrowhead) and a nucleus can be observed. All the cell content is surrounded by a cell wall.

Supplementary figures

1.A) ITS-2 model for the type strain of *Selenastrum bibraianum* (CCMA-UFSCar 125). In black boxes are the different bases compared to strain *Messastrum gracile* (CCMA-UFSCar 622).

1.B) ITS-2 model for the type strain of *Selenastrum bibraianum* (CCMA-UFSCar 125). In gray boxes are the different bases compared to strain *Selenastrum bibraianum* (CCMA-UFSCar 47) and black boxes compared to *Selenastrum bibraianum* (CB 2012/47).

1.C) ITS-2 model for the type strain of *Messastrum gracile* (CCMA-UFSCar 622). In gray boxes are the different bases of *M. gracile* (CCMA-UFSCar 470) and black boxes *M. gracile* (CCMA-UFSCar 5).

1.D) ITS-2 model for the type strain of *Curvastrum pantanale* (CCMA-UFSCar 350). In gray boxes are the different bases of *C. pantanale* (CCMA-UFSCar 608).

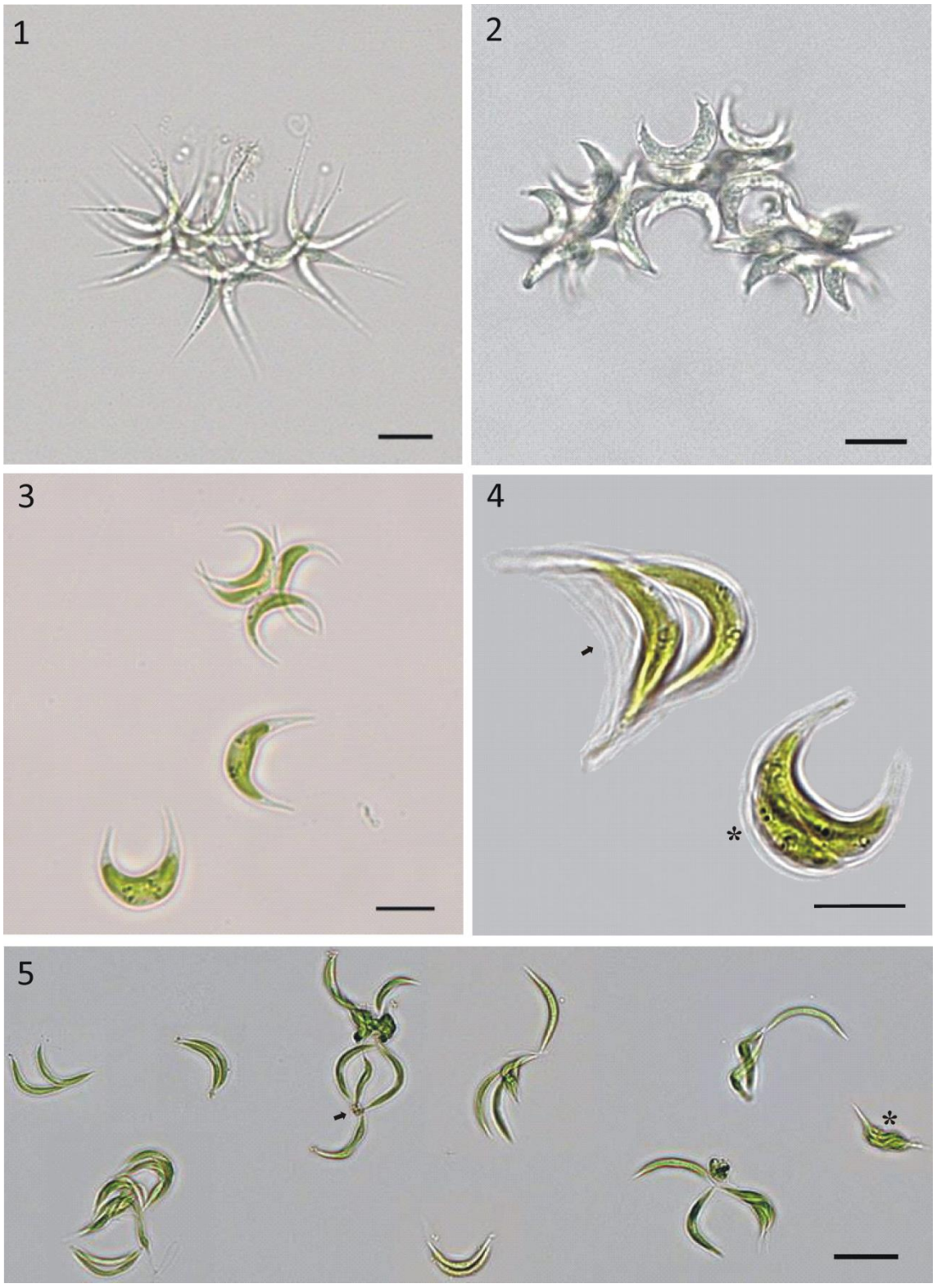


Fig. 1.1-1.5.

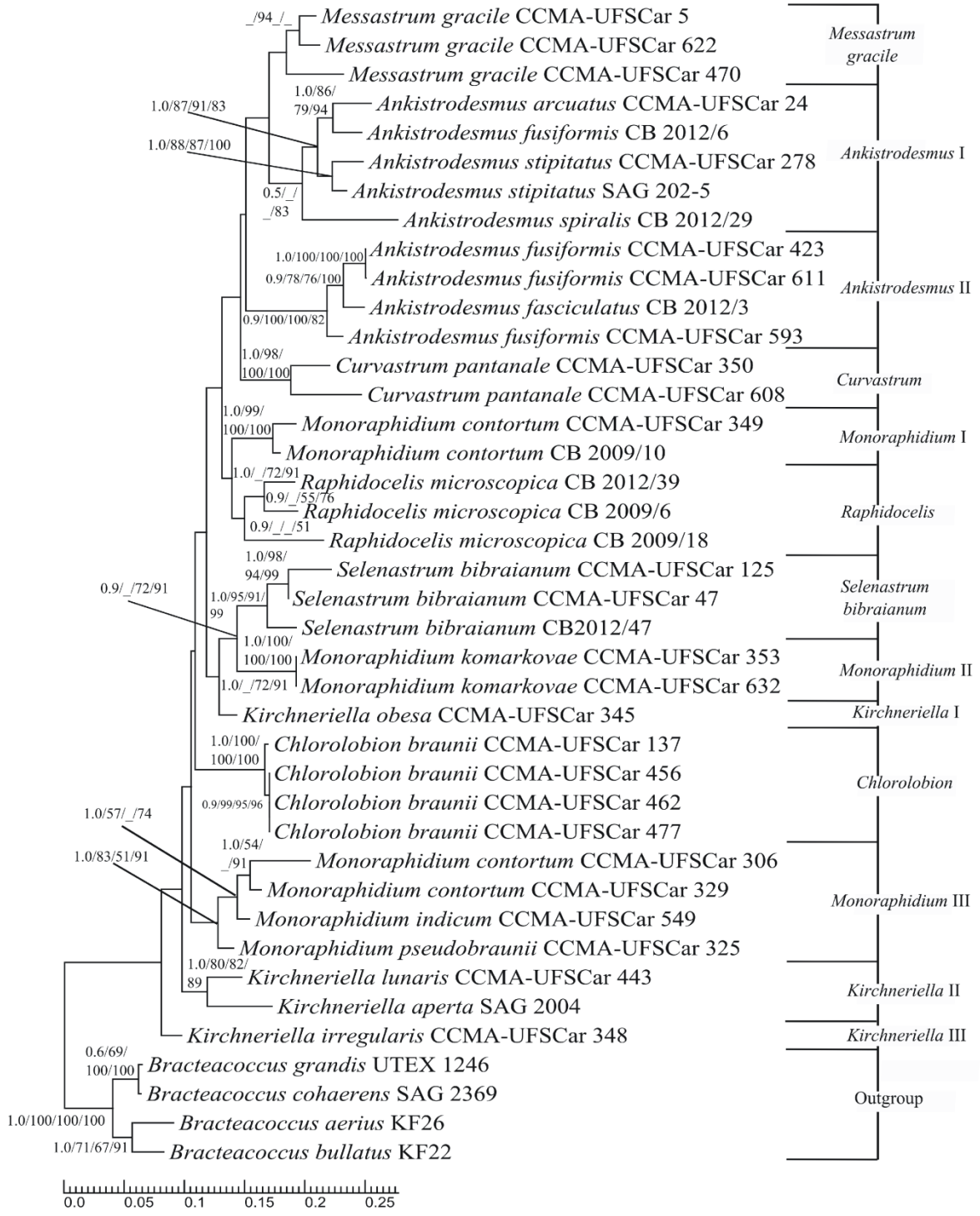

Messastrum gracile CCMA-UFSCar 5
Messastrum gracile CCMA-UFSCar 622
Messastrum gracile CCMA-UFSCar 470
Ankistrodesmus arcuatus CCMA-UFSCar 24
Ankistrodesmus fusiformis CB 2012/6
Ankistrodesmus stipitatus CCMA-UFSCar 278
Ankistrodesmus stipitatus SAG 202-5
Ankistrodesmus spiralis CB 2012/29
Ankistrodesmus fusiformis CCMA-UFSCar 423
Ankistrodesmus fusiformis CCMA-UFSCar 611
Ankistrodesmus fasciculatus CB 2012/3
Ankistrodesmus fusiformis CCMA-UFSCar 593
Curvastrum pantanale CCMA-UFSCar 350
Curvastrum pantanale CCMA-UFSCar 608
Monoraphidium contortum CCMA-UFSCar 349
Monoraphidium contortum CB 2009/10
Raphidocelis microscopica CB 2012/39
Raphidocelis microscopica CB 2009/6
Raphidocelis microscopica CB 2009/18
Selenastrum bibraianum CCMA-UFSCar 125
Selenastrum bibraianum CCMA-UFSCar 47
Selenastrum bibraianum CB2012/47
Monoraphidium komarkovae CCMA-UFSCar 353
Monoraphidium komarkovae CCMA-UFSCar 632
Kirchneriella obesa CCMA-UFSCar 345
Chlorolobion braunii CCMA-UFSCar 137
Chlorolobion braunii CCMA-UFSCar 456
Chlorolobion braunii CCMA-UFSCar 462
Chlorolobion braunii CCMA-UFSCar 477
Monoraphidium contortum CCMA-UFSCar 306
Monoraphidium contortum CCMA-UFSCar 329
Monoraphidium indicum CCMA-UFSCar 549
Monoraphidium pseudobraunii CCMA-UFSCar 325
Kirchneriella lunaris CCMA-UFSCar 443
Kirchneriella aperta SAG 2004
Kirchneriella irregularis CCMA-UFSCar 348
Bracteacoccus grandis UTEX 1246
Bracteacoccus cohaerens SAG 2369
Bracteacoccus aerius KF26
Bracteacoccus bullatus KF22
Messastrum gracile
Ankistrodesmus I
Ankistrodesmus II
Curvastrum
Monoraphidium I
Raphidocelis
Selenastrum bibraianum
Monoraphidium II
Kirchneriella I
Chlorolobion
Monoraphidium III
Kirchneriella II
Kirchneriella III
Outgroup
0.0
0.05
0.1
0.15
0.2
0.25

Figure 1.6.

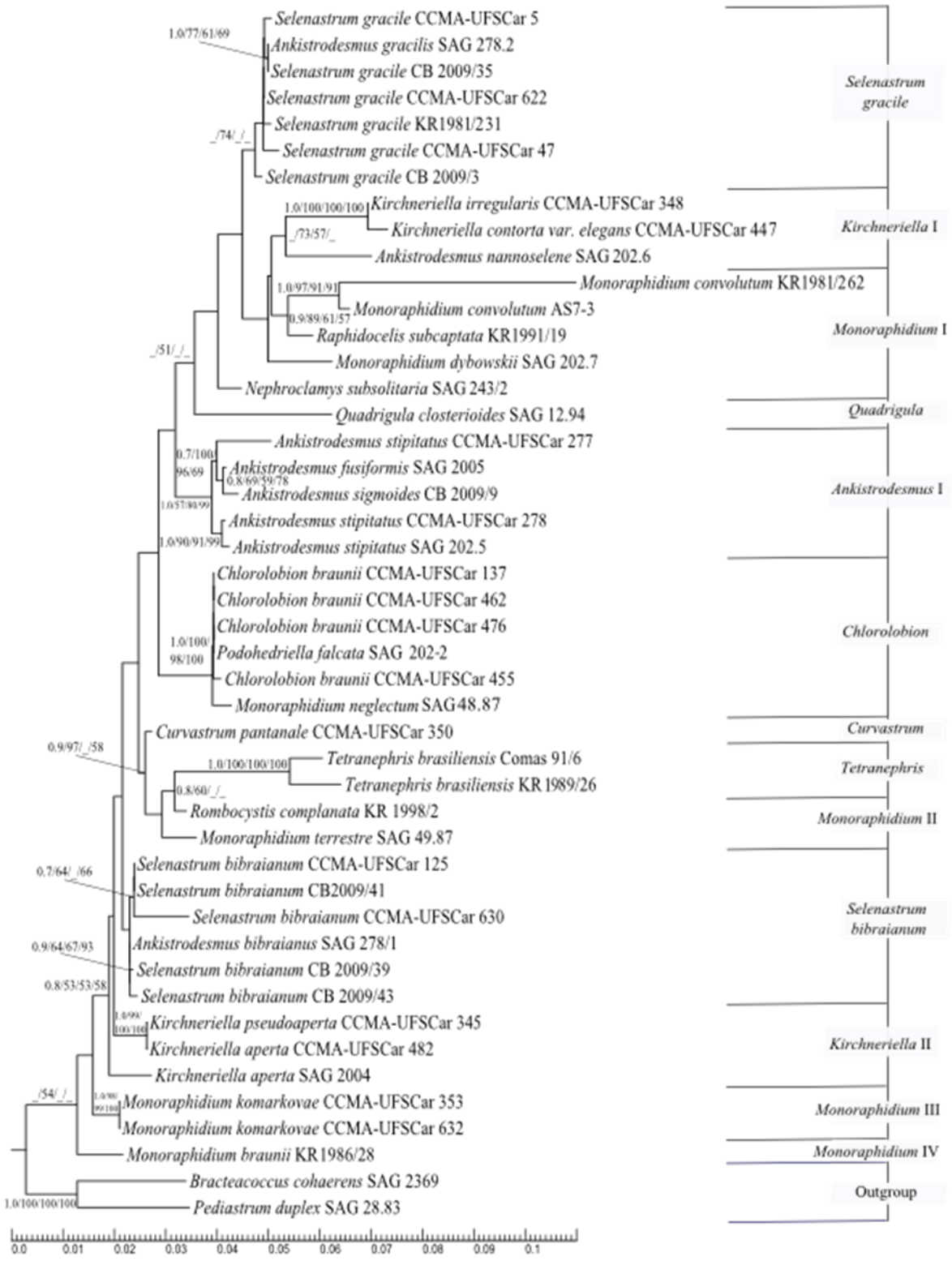

Selenastrum gracile CCMA-UFSCar 5
Ankistrodesmus gracilis SAG 278.2
Selenastrum gracile CB 2009/35
Selenastrum gracile CCMA-UFSCar 622
Selenastrum gracile KR1981/231
Selenastrum gracile CCMA-UFSCar 47
Selenastrum gracile CB 2009/3
Kirchneriella irregularis CCMA-UFSCar 348
Kirchneriella contorta var. elegans CCMA-UFSCar 447
Ankistrodesmus nannoselene SAG 202.6
Monoraphidium convolutum KR1981/262
Monoraphidium convolutum AS7-3
Raphidocelis subcaptata KR1991/19
Monoraphidium dybowskii SAG 202.7
Nephroclamys subsolitaria SAG 243/2
Quadrigula closterioides SAG 12.94
Ankistrodesmus stipitatus CCMA-UFSCar 277
Ankistrodesmus fusiformis SAG 2005
Ankistrodesmus sigmoides CB 2009/9
Ankistrodesmus stipitatus CCMA-UFSCar 278
Ankistrodesmus stipitatus SAG 202.5
Chlorolobion braunii CCMA-UFSCar 137
Chlorolobion braunii CCMA-UFSCar 462
Chlorolobion braunii CCMA-UFSCar 476
Podohedriella falcata SAG 202-2
Chlorolobion braunii CCMA-UFSCar 455
Monoraphidium neglectum SAG48.87
Curvastrum pantanale CCMA-UFSCar 350
Tetranephris brasiliensis Comas 91/6
Tetranephris brasiliensis KR1989/26
Rombocystis complanata KR 1998/2
Monoraphidium terrestre SAG 49.87
Selenastrum bibraianum CCMA-UFSCar 125
Selenastrum bibraianum CB2009/41
Selenastrum bibraianum CCMA-UFSCar 630
Ankistrodesmus bibraianus SAG 278/1
Selenastrum bibraianum CB 2009/39
Selenastrum bibraianum CB 2009/43
Kirchneriella pseudoaperta CCMA-UFSCar 345
Kirchneriella aperta CCMA-UFSCar 482
Kirchneriella aperta SAG 2004
Monoraphidium komarkovae CCMA-UFSCar 353
Monoraphidium komarkovae CCMA-UFSCar 632
Monoraphidium braunii KR1986/28
Bracteacoccus cohaerens SAG 2369
Pediastrum duplex SAG 28.83
0.0
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
0.09
0.1
Selenastrum gracile
Kirchneriella I
Monoraphidium I
Quadrigula
Ankistrodesmus I
Chlorolobion
Curvastrum
Tetranephris
Monoraphidium II
Selenastrum bibraianum
Kirchneriella II
Monoraphidium III
Monoraphidium IV
Outgroup

Figure 1.7.

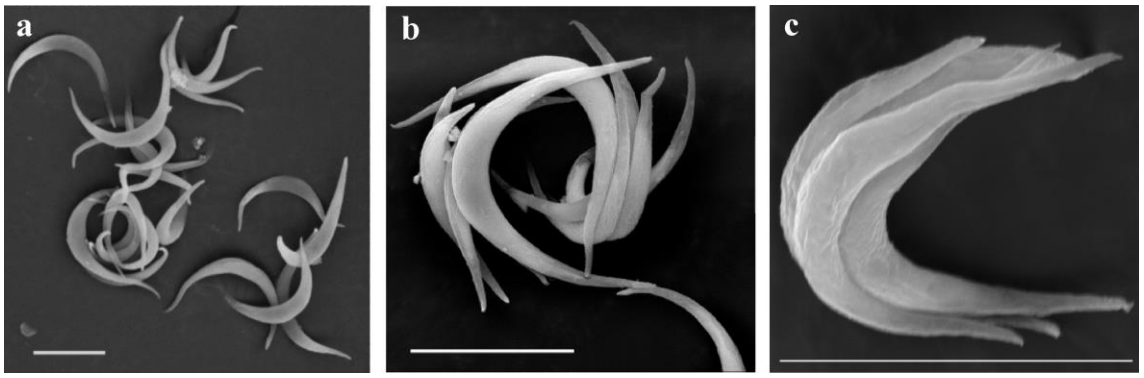


Figure 1.8.

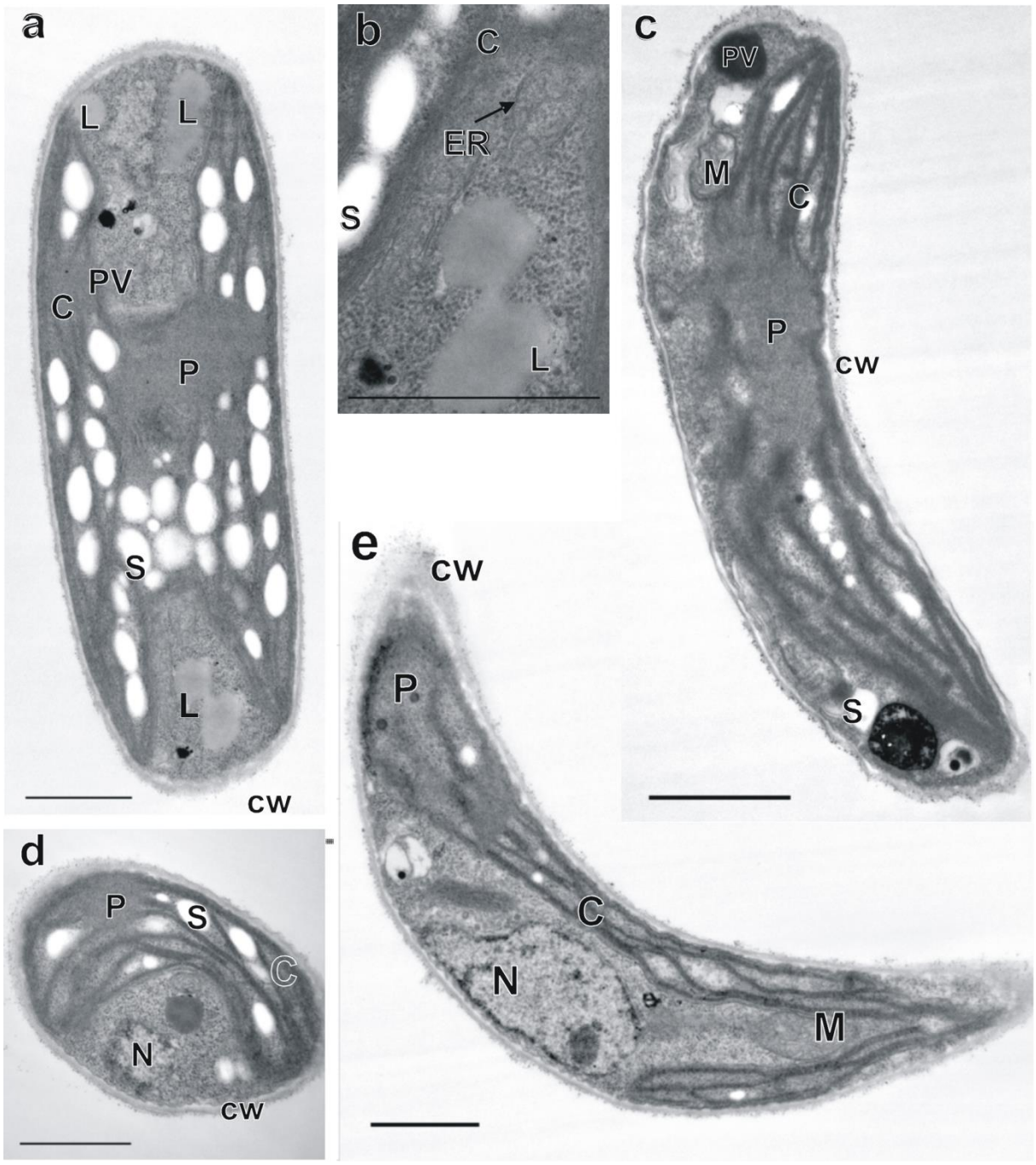


Figure 1.9.

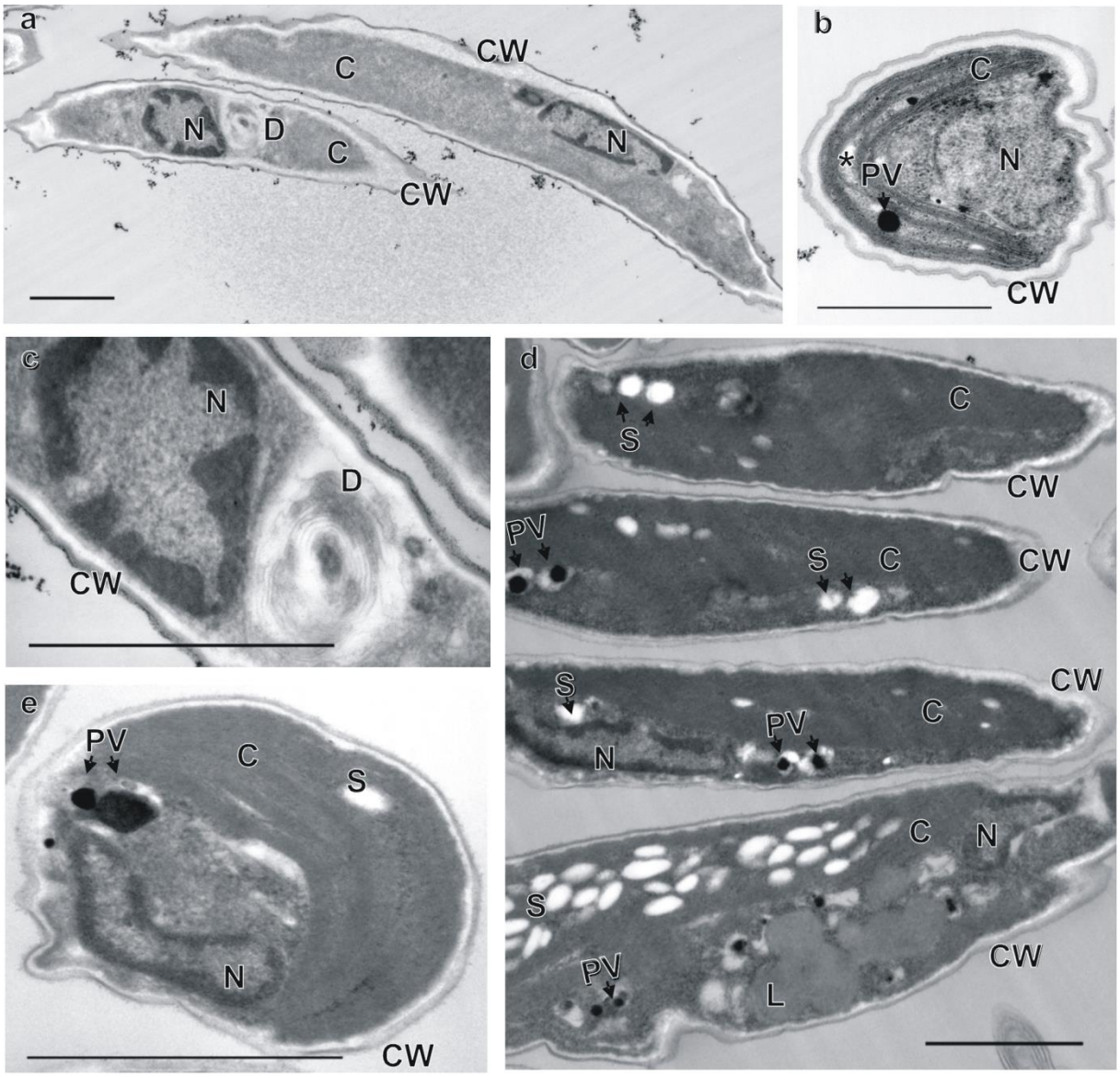
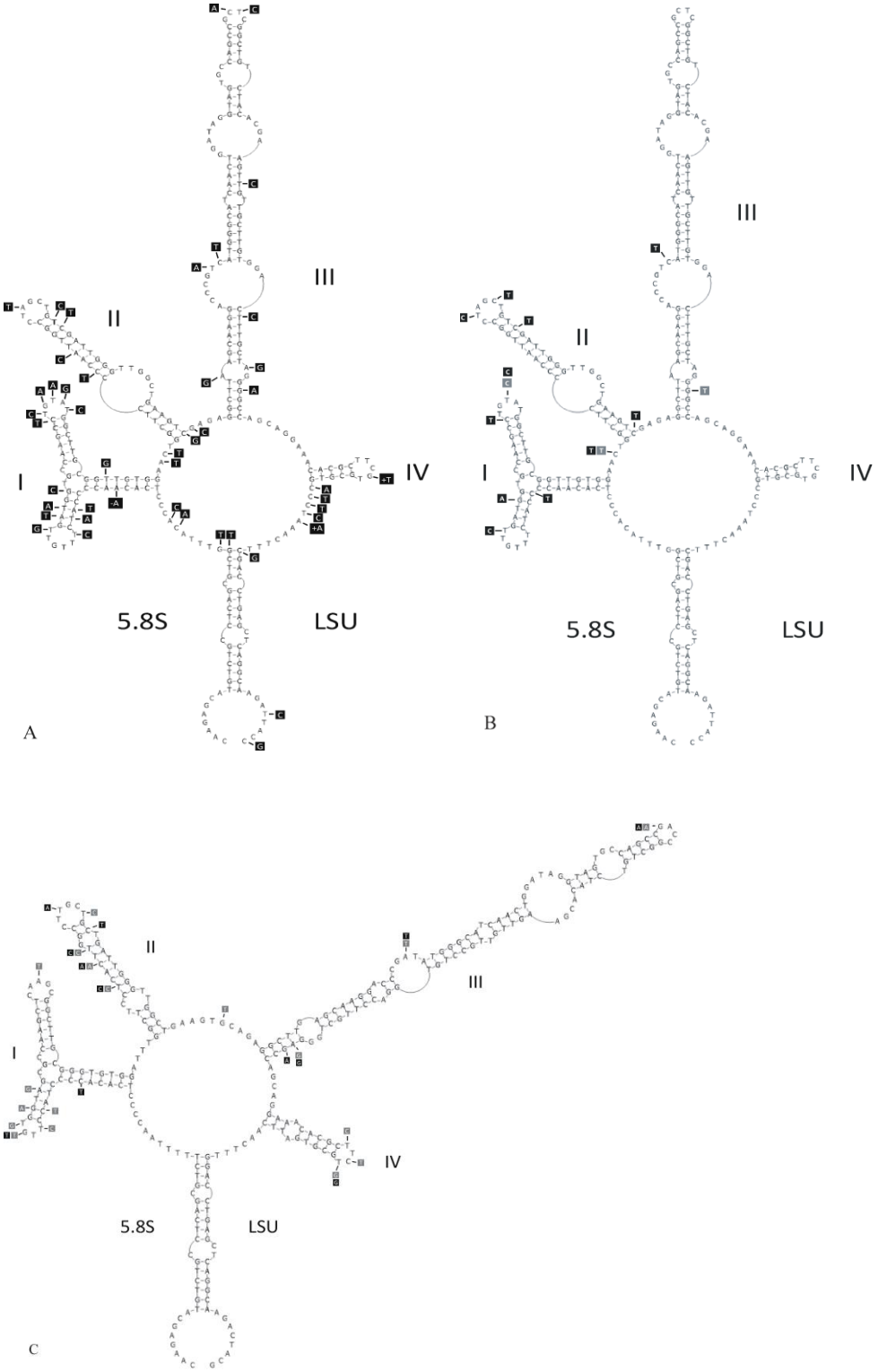
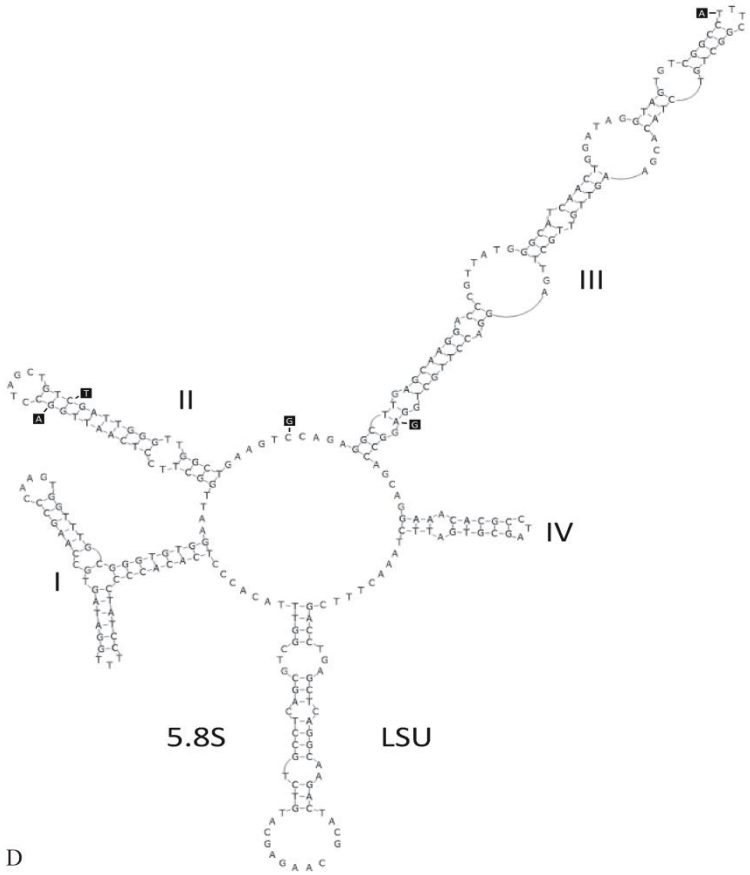

a
C
CW
N
D
C
N
CW
b
C
*
PV
N
CW
c
N
D
CW
d
C
S
CW
PV
S
C
CW
S
CW
PV
C
N
C
N
S
CW
PV
L
e
PV
C
S
N
CW

Figure 1.10.

Supplementary figure

1. A, B, C. D.





D

Capítulo 2:

KIRCHNERIELLA MORPHOTYPE (SELENASTRACEAE, CHLOROPHYCEAE) REVEALS FOUR MOLECULAR LINEAGES, INCLUDING TWO NEW GENERA AND FIVE SPECIES[1].

[1] Paper to be submitted

[2] Corresponding author: thais.garcia.bio@gmail.com.

2.1. ABSTRACT

The phylogeny of the *Kirchneriella* morphotype was investigated by light microscopy, 18S rDNA and ITS1-5.8S-ITS2 analyses. Morphological features traditionally used for species and genera identification were investigated in genera *Kirchneriella*, *Raphidocelis* and *Tetranephris*. Phylogenetic analyses revealed four molecular lineages attributed to *Kirchneriella* morphotype and led to the description of five new species: Genus 1 sp. nov. 1, Genus 2 sp. nov. 1, Genus 2 sp. nov. 2, *Raphidocelis* sp. nov. and *Tetranephris* sp. nov. The molecular analyses showed that a number of morphological features, such as pyrenoid, cell size and shape, may be suitable to identify the genera *Kirchneriella*, *Raphidocelis* and *Tetranephris*.

KEYWORDS: Molecular systematics, morphology, phylogeny, *Kirchneriella*, *Pseudokirchneriella, Raphidocelis, Tetranephris.*

2.2. INTRODUCTION

Kirchneriella Schmidle 1892 (Selenastraceae, Chlorophyceae) is a green algae genus with cosmopolitan distribution in nearly all types of inland waters. The morphology of *Kirchneriella* morphotype contain an assortment of shapes: from coccoid to elongated, cylindrical to fusiform or spirally curved, with sharp or rounded ends, where cell arrangements differs from the solitary to colonial forms (Komárek & Fott 1983; Korshikov 1987; Komárková-Legnerová 1969; Comas 1996; Hindák, 1977; Hindák 1980; Hindák 1984; Hindák 1988; Hindák 1990; Sant'Anna 1984). *Kirchneriella* and relatives show the same reproduction pattern of all members of Selenastraceae: a simple autospore formation, in which the cytokinesis of the mother cell protoplasm gives rise to 2-4-8 young cells (Komárek & Fott 1983).

Since the description of the genus *Kirchneriella*, morphological revaluations regarding presence of pyrenoids and cell wall incrustations (Hindák 1977; 1980; 1984; 1988; 1990; Marvan *et al*. 1984) led to its division in three other morphological defined genera: *Raphidocelis* Hindák (strains with incrustation on cell wall and lacking pyrenoid), *Kirchneria* Hindák (to comprehend the strains with no cell wall incrustations and no pyrenoid) and *Pseudokirchneriella* Hindák (a valid name for *Kirchneria*, since *Kirchneria* was a name already published for a fossile plant). Molecular studies on the SSU of *Kirchneriella* and *Raphidocelis* species placed them as common members of monophyletic group Selenastraceae (Fawley *et al*. 2006; Krienitz *et al.* 2011; Krienitz *et al.* 2001).

The cell size and shape, solitary or colonial organization, releasing process of the autospores and special habitat preferences, considered to be species-specific (Hindák 1977; Komárek & Fott 1983), was used to separate genera inside the family. Those features conducted to the description of up to 81 taxa (including forms and

variations) described in the *Kirchneriella* morphotype (*Kirchneriella*, *Raphidocelis*, *Kirchneria* and *Pseudokirchneriella* complex) (Guiry & Guiry, 2016; Korshikov 1987; Komárek & Fott 1983; Komárková-Legnerová 1969; Comas 1996; Hindák 1977; Hindák 1980; Hindák 1984; Hindák 1988; Hindák 1990; Sant'Anna 1984). As the taxonomic and phylogenetic studies go forward, possibly various genera and species are to be described and included in this complex (Hindák 1977; Hindák 1984; Komárek & Fott 1983; Fawley *et al.* 2006; Krienitz *et al.* 2011; Garcia *et al.* in press).

Thus, a combined molecular approach based on different genetic markers like 18S, *rbcL* or ITS associated with a morphological evaluation and the inclusion of strains from different continents would be a valuable tool for identification and classification not only of Selenastraceae but other green algae as well (Bock *et al.* 2011; Krienitz *et al.* 2012).

Indeed, due to its simple morphology and phenotypic plasticity, including new lineages from different environments and geographically apart may lead to changes in the *Kirchneriella* complex.

In front of the difficulties with species identification and taxonomy, species diversity and ecology of members of Selenastraceae is poorly understood worldwide (Fawley *et al*. 2006; Krienitz *et al.* 2001). Additionally, studies with molecular diversity of tropical Selenastraceae remain largely unknown, since previous studies were focused on temperate northern hemisphere isolates.

Phylogenetic studies in green algae are generally based on 18S rRNA gene sequences (Booton *et al.* 1998; Buchheim *et al.* 2001; Fawley *et al*. 2006; Hegewald & Hanagata 2000; Krienitz *et al.* 2011; Krienitz *et al.* 2003; Krienitz *et al*. 2001; Lewis 1997). However, this gene is in some cases too preserved to distinguish between closely related genera and species (Luo *et al.* 2010). Therefore, some studies have taken into

account the use of a second marker as well, to gain a higher resolution (Rindi *et al.* 2011).

Several studies have employed the ITS for phylogenetic reconstruction in diverse eukaryotes (Bock *et al.* 2011; Coleman 2003; Coleman 2007; Coleman & Vacquier 2002; Schultz *et al.* 2005; Young & Coleman 2004) or for the resolution of closely related genera (Luo *et al.* 2010; Luo *et al.* 2011). The highly divergent properties and the rapid evolution legitimate ITS-2 to discriminate closely related organisms, which exhibit nearly identical sequences in rRNA genes level. Indeed, ITS have been reported to be more informative than gene sequences for those organisms (Coleman 2003) and is increasingly being used as a unique marker between the most frequently utilized DNA region, at least for plant studies (Hershkovitz, *et al.* 1999).

In numerous cases were under consent to employ ITS as barcode region for dinoflagellates (Litaker *et al.* 2007) and fungi (Seifert 2009) since the variation in this DNA region correlates with taxonomic classification in several cases (Coleman, 2009).

Therefore, using a multigene analysis employing conserved genes for a good phylogeny and a second variable marker to obtain higher resolution for lower taxonomic levels is necessary for taxonomically complicated groups as *Kirchneriella* complex. The present study is the first to evaluate *Kirchneriella* morphospecies with 18S rDNA and ITS1, 5.8S rDNA and ITS2 multigene phylogeny, to clarify the phylogenetic relation between genera *Kirchneriella, Raphidocelis and Tetranephris.*

2.3. MATERIAL AND METHODS

Algal cultures and microscopy

The algal cultures were obtained either from Freshwater Microalgae Culture Collection from Universidade Federal de São Carlos (CCMA – UFSCar, WDCM 835) and from an author personal collection (CB strains). KR strains were donated by Professor Lothar Krienitz. Thirty five Selenastraceae strains were investigated (Table 2.1). All the strains were grown at 23 ± 1 ºC under a photoperiod 12/12 hours light/dark and irradiance of ~200 µmol/m^2/s in WC medium (Guillard & Lorenzen).

The morphology of all strains was examined using an Axioplan 2 Imaging Zeiss or Nikon Eclipse E600 light microscope with differential interference contrast. Micrographs were obtained using an AxioCam with software AxioVision 4.6 (Carl Zeiss Group, Oberkochen, Germany) and a Nikon digital camera DS-Fi1 with Nikon software NIS-Elements D (Nikon Corporation, Tokyo, Japan). The species identification were done according to the specialized bibliography: Korshikov 1987a, Komárek & Fott 1983, Komárková-Legnerová 1969, Comas 1996, Hindák 1977, Hindák 1980, Hindák 1984, Hindák 1988, Hindák 1990 and Sant'Anna 1984.

DNA extraction, PCR and sequencing

For DNA extraction, the algal cultures were grown as described for microscopy analyses. The cell cultures were centrifuged at 16.000 xg for 10 minutes and the pellet stored at -80°C until further processing. The cells were mechanically disrupted using glass beads (150-212 µm, Sigma-Aldrich), vortex briefly, and extracted using Invisorb® Spin Plant Mini (STRATEC Biomedical AG; Germany) or My-Budget DNA Mini Kit® (Bio-Budget Technologies GmbH; Krefeld; Germany). PCR amplification, purification and sequencing were carried out as previously reported (Garcia *et al.* in press). Part of the genomic DNA is stored at the Phycology Lab – UFSCar, and Department of Biodiversity – University Duisburg-Essen.

Fifteen new 18S rDNA sequences and 32 ITS-1-5.8S-ITS-2 new sequences were amplified and obtained on this study, totalizing 47 new entries in GenBank (National Center for Biotechnology Information [NCBI], http://www.ncbi.nlm.nih.gov/). The whole dataset used for phylogenetic analyses with accession numbers are reported in Table 1, including 30 references sequences acquired from GenBank and 9 from our previous publication (Garcia *et al*. in press).

Phylogenetic analyses

Sequences were aligned using Align - Manual Sequence Alignment Editor (Hepperle 2004). For the phylogenetic analyses, two different datasets were prepared. For the 18S rDNA analyses, 49 sequences with 1592 base positions were acquired. The concatenated ITS-1-5.8S-ITS2 analyses was conducted with 36 sequences, were ITS-1 had 223 base positions (bp), 5.8S had 159bp and ITS-2 with 213 bp, totalizing 597bp.

Both trees (18S rDNA and ITS-1-5.8S-ITS2) were analyzed separately, both in maximum likelihood (ML) on Treefinder (Jobb 2008), distance (neighbor joining; NJ) and maximum parsimony (MP) using PAUP* (portable version 4.0b10) (Swofford 2002).

For ML and Bayesian analyses, the evolutive model for 18S, ITS-1 and ITS-2 (GTR[Optimum, Empirical]:GI[Optimum]:5) and 5.8S (HKY[{3,1,1,1,1,3},Empirical]:G[Optimum]:5) were applied as suggested Treefinder (Jobb 2008). To assure tree topology, bootstrap analyses (1000) were tested by calculating values for NJ, MP, ML criteria. For both datasets, Bayesian analyses were performed using MrBayes version 3.1. (Huelsenbeck & Ronquist 2001). Two runs with four chains of Markov chain Monte Carlo (MCMC) iterations were performed (1 million generations for ITS-1-5.8S-ITS2and 8 million generations for 18S rDNA). The

stationary distribution was assumed when the average standard deviations of split frequencies between two runs were lower than 0.01 and Tracer V1.4 (Rambaut and Drummond 2007) was used to check the stationary phase and to identify an appropriate burn-in value. The first 25% of the calculated trees were discarded as burn–in. 50% majority–rule consensus trees were calculated for posterior probabilities (PP). The trees were edited using TreeGraph 2 (Stöver & Müller 2010). Previous publications indicated that *Bracteacoccus* (Krienitz *et al.* 2001), *Pediastrum* (Fučíková *et al.* 2014) and Scenedesmaceae (Krienitz *et al.* 2011) members were suitable as an outgroup for the phylogeny of Selenastraceae.

2.4. RESULTS

Taxonomic proposals.

Tetranephris sp. nov. 1 T. S. Garcia & Sant'Anna (Fig 2.1).

Solitary cells or in cross-shaped coenobium with 4-8-16 cells, group of 4 cells always with 2 cells organized in one plane and 2 organized on a perpendicular plane to the others. Cells cylindrical, regularly narrowed towards the ends or gradually obtuse, bean-shaped, slightly arcuate. Cells 3-8 long x 1-4 µm large. Asexual reproduction by autosporulation (4-8 autospores per mother cell, with parallel orientation), sexual reproduction unknown. A diffuse and irregular layer of mucilage is often involving both colonies and free individuals. One parietal chloroplast, no pyrenoid observed.

Holotype: Kenya, Nakuru County, Lake Nakuru National Park. Sample deposited in Herbarium of Institute of Botany, SP, Brazil, under the designation SP 469323.

Type strain: strain CB 2009/6, maintained at the Culture Collection of Freshwater Microalgae, Federal University of São Carlos, São Carlos, Brazil.

Etymology: *to be designated.*

Genus 1gen. nov. T. S. Garcia & Sant'Anna.

Type species: Genus 1 sp nov. 1 T. S., Garcia.

Diagnosis: Green, planktonic microalgae. Colonies with 4-8 or multi-celled, cells with the convex or the concave side outwards the center of the colony. Cells fusiform, regularly narrowed towards the ends or gradually obtuse, regularly curved along its length and slightly bent to one side. Asexual reproduction by autosporulation (4 autospores per mother cell with parallel arrangement), sexual reproduction unknown. Mucilage often involving both colonies and free cells. One parietal chloroplast, containing a pyrenoid, no starch cover observed. Genus differs from other Selenastraceae genera by differences in 18S rDNA and ITS-1-5.8S-ITS2 gene sequences.

Genus 1 sp nov. 1 T. S. Garcia & Sant'Anna (Fig 2.2).

Colonies with 4-8 cells or multi-celled, some with the convex others with the concave side outwards the center of the colony or solitary cells, where two cells are in one plane and two are in a perpendicular plane. Cells fusiform, regularly narrowed towards the ends or gradually obtuse, regularly curved along its length and slightly bent to one side. Cells 6-20 long x 3-7 µm large. Asexual reproduction by autosporulation (4 autospores per mother cell, with parallel orientation), sexual reproduction unknown. A diffuse and irregular layer of mucilage is often involving both colonies and free individuals. One parietal chloroplast, containing a pyrenoid, no starch cover observed.

Holotype: Brazil, São Paulo State, Piracicaba. Sample deposited in Herbarium of Institute of Botany, SP, Brazil, under the designation SP 469324.

Type strain: strain CCMA-UFSCar 230, maintained in the Culture Collection of Freshwater Microalgae, Federal University of São Carlos, São Carlos, Brazil.

Etymology: *to be designated.*

Raphidocelis sp. nov.1 T. S. Garcia & Sant'Anna (Fig. 2.3).

Cells planktonic, solitary or 4-celled to multi-celled colonies with irregularly arranged cells, mostly with the convex sides towards the center of the colonies. Cells narrow, long, cylindrical, arcuate to spirally curved, ends rounded. Cells 15-20 long x 1-2 µm large. Reproduction by autospore formation, where the mother cell gives rise to 2 young cells, with parallel orientation. Cell wall covered by a diffuse layer of mucilage on both colonies and free individuals. One parietal chloroplast. Pyrenoid not observed.

Holotype: Brazil, São Paulo State, Piracicaba. Sample deposited in Herbarium of Institute of Botany, SP, Brazil, under the designation SP 469325.

Type strain: strain CCMA-UFSCar 229, maintained at the Culture Collection of Freshwater Microalgae, Federal University of São Carlos, São Carlos, Brazil.

Etymology: *to be designated.*

Genus 2 gen. nov. T. S. Garcia & Sant'Anna.

Type species: Genus 2 sp nov. 1 T. S., Garcia.

Diagnosis: Cells solitary or in colonies, sometimes formed by mother cell wall before autospore release. Colonies with 4 cells, 2 with the convex side and 2 with the concave side outwards the center of the colony, where two cells are in one plane and two are in a perpendicular plane. Cells cylindrical, lunate, regularly narrowed towards the ends or gradually obtuse, regularly curved along its length. Some single twisted cells present, slightly bent to one side. Asexual reproduction by autosporulation (4 autospores per

mother cell, with parallel orientation), sexual reproduction unknown. Absence of mucilage. One parietal chloroplast, pyrenoid not observed. Genus differs from other Selenastraceae genera by the differences in 18S rDNA and ITS-1-5.8S-ITS2 gene sequences and morphology.

Etymology: *to be designated.*

Genus 2 sp. nov. 1 T. S. Garcia & Sant'Anna (Fig. 2.4).

Cells isolated or in colonies with 4 cells, 2 with the convex side and 2 with the concave side outwards the center of the colony, where two cells are in one plane and two are in a perpendicular plane. Sometimes the colony is surrounded by mother cell wall before autospore release. Cells cylindrical, lunate, regularly narrowed towards the ends or gradually obtuse, regularly curved along its length. Most individuals present twisted cells, slightly bent to one side. Cells 6-15 long x 3-5 µm large. Asexual reproduction by autosporulation (4 autospores per mother cell, with parallel orientation). Absence of mucilage. One parietal chloroplast, pyrenoid not observed.

Holotype: Brazil, Mato Grosso do Sul State, Brazilian Pantanal wetlands. Sample deposited in Herbarium of Institute of Botany, SP, Brazil, under the designation SP 469326.

Type strain: strain CCMA-UFSCar 348, maintained at the Culture Collection of Freshwater Microalgae, Federal University of São Carlos, São Carlos, Brazil.

Etymology: *to be designated.*

Genus 2 sp. nov. 2 T. S. Garcia & Sant'Anna (Fig. 2.5).

Cells isolated or in colonies with 4 cells, 2 with the convex side and 2 with the concave side outwards the center of the colony, where two cells are in one plane and two are in a

perpendicular plane. Cells cylindrical, ends gradually obtuse, bean-shaped, crescent-shaped or slightly arcuate. Cells 7-11 long x 1,2-3 µm large. Asexual reproduction by autosporulation (4-8-multi autospores per mother cell, with parallel orientation), sexual reproduction not known. A diffuse and irregular layer of mucilage is often involving both colonies and free individuals. One parietal chloroplast, no pyrenoid observed.

Holotype: Germany, Saxony-Anhalt State, Zörbig City, Schortewitz Village. Sample deposited in Herbarium of Institute of Botany, SP, Brazil, under the designation SP 469327.

Type strain: strain KR 1979/222, maintained at the Culture Collection of Freshwater Microalgae, Federal University of São Carlos, São Carlos, Brazil.

Etymology: *to be designated.*

Morphological analysis

The morphological investigated parameters were cell shape and dimensions, autospores arrangement, pyrenoid presence/absence and colony formation, respecting the diacritical features of each species. These parameters are shown on Table 2.2.

Kirchneriella species were alike in presence of pyrenoid, cell shape and in the colony morphology. They diverged on cell size and on the following features. *Kirchneriella pseudoaperta* Komárek (CCMA-UFSCar 346 and CCMA-UFSCar 482, Fig. 2.6) presented small but discernable pyrenoid under LM. Mucilage was exhibited when cells were disposed in colony. *Kirchneriella obesa* (West) West &West (CCMA-UFSCar 345 and CB 2012/49, Fig. 2.7) presented mucilage involving the colony, and solitary cells presented bigger size than the cells in colonies. Big colonies typical for *Kirchneriella lunaris* (Kirchner) Möbius (CCMA-UFSCar 87, CCMA-UFSCar 123 and CCMA-UFSCar 443, Fig. 2.8) were observed, exhibiting acute cell apex, with solitary

cells bigger than the colonial one, always presenting pyrenoids and involved in mucilage.

Tetranephris sp. nov. T.S. Garcia & Sant'Anna (CB 2009/6, CB 2009/7 and CB 2009/18) were bean-shaped, slightly arcuate and had narrowed or gradually obtuse apex. Mucilage was observed on free cells and colony formation. The typical cross-shaped colony formation of genus *Tetranephris* Sant'Anna& Bicudo was observed, with 2 cells on a perpendicular plane to the others. Pyrenoid was not observed.

Morphology and molecular data revealed *Raphidocelis* sp. nov. T.S.Garcia & Sant'Anna (CCMA-UFSCar 228 and CCMA-UFSCar 229, Fig. 2.3), a species with long and thin cells, arcuate to spirally curved and rounded apex, without pyrenoid. Colonies were observed with 4 to multi irregularly arranged cells, mostly with the convex sides towards the center of the colonies. It was possible to observe incrustations on cell wall, a typical feature of *Raphidocelis* Hindák species.

Similar to *Kirchneriella* morphotype, two new genera were discovered in our study. Genus 1 gen. nov. sp 1 T.S.Garcia & Sant'Anna (CCMA-UFSCar 230, CCMA-UFSCar 234, CCMA-UFSCar 601 CB 2012/26, Fig. 2.2) presented fusiform cells, regularly narrowed towards the ends or gradually obtuse, usually curved along its length and slightly bent to one side. In this species it was observed colonies with 4-8 or multi-celled, some with the convex others with the concave side outwards the center of the colony or solitary, where two cells are in one plane and two are in a perpendicular plane. Mucilage was frequently involving both colonies and free individuals. Pyrenoid was observed.

Alike to Genus 1 gen. nov. sp.1, Genus 2gen. nov. sp 1 T.S. Garcia & Sant'Anna (CCMA-UFSCar 132, CCMA-UFSCar 348 and CCMA-UFSCar 447, Fig. 2.4) had lunate and regularly curved cells, presenting twisted cells with narrowed or gradually

obtuse cell apex, always small in size and curved along its length. No mucilage was observed both in colony and free living cells. Mother cell wall was often involving young colonies, which were formed by 4 cells, 2 with the convex and 2 with the concave side outwards the center of the colony where two cells are in one plane and two are in a perpendicular plane.

For Genus 2 gen. nov. sp 2 T.S. Garcia & Sant'Anna (CB 2012/21, CB 2012/22 e KR 1979/222, Fig. 2.5) similar characteristics to Genus 2 gen. nov. sp 1 were found. Cells were bean-shaped, crescent-shaped or slightly arcuate, with obtuse apex. Mucilage involved both free living cells and colony. Colony showed the same pattern as Genus 2 gen. nov. sp 1, with 2 cells with the convex and 2 with the concave side outwards the center of the colony disposed in a perpendicular plane to the others.

Thus, the differences regarding both species of Genus 2 gen nov is the cell shape.

Phylogenetic analysis

The ITS1-5.8S-ITS2 (Fig. 2.9) and 18S rDNA (Fig. 2.10) inference were constructed with representative strains of Selenastraceae. Although one of the sequences are missing for few strains, they were considered for the analysis once the inferred phylogenies for both DNA sequences were congruent. The relations among some lineages are resolved although the low support of some external branches in both phylogenies. Some well-supported clades can be identified in both trees: (i) *Messastrum gracile*, (ii) *Selenastrum bibraianum*, (iii) *Raphidocelis*, (iv) *Kirchneriella* (v) *Rhombocystis*, (vi) *Monoraphidium*, (vii) *Ankistrodesmus*, (viii) Genus 1 gen. nov., (iv) Genus 2 gen. nov. and (v) *Tetranephris*. Genera *Quadrigula*, *Nephrochlamys*, *Chlorolobion* and *Podohedriella* were assorted to other ones on the 18S rDNA tree due

to the absence of more similar sequences, with no ITS1-5.8S-ITS2 sequences for them. The main genera traditionally included in Selenastraceae (*Ankistrodesmus*, *Messastrum* and *Selenastrum* are well settled clades in our trees. The different number of strains inside 18S rDNA and ITS1-5.8S-ITS2 trees lead to different results for some genera such as, *Monoraphidium* and *Ankistrodesmus*.

Monoraphidium Komárková-Legnerová species are distributed in different clades on both phylogenies. ITS1-5.8S-ITS2 inference (Fig. 2.9) grouped *Monoraphidium dybowskii* (Woloszynska) Hindák & Komárkova-Legnerová (SAG 202-7e and CB 2009/27) and two strains assigned as *Raphidocelis* sp. (CB 2012/39 and CB 2012/51), designated here as *Monoraphidium dybowskii* clade. *Monoraphidium terrestre* (Bristol) Krienitz & Klein (SAG 49.87) was placed near to *Ankistrodesmus falcatus* (Corda) Ralfs (UTEX 101), forming the *Ankistrodesmus*/*Monoraphidium* clade. A similar result was obtained for 18S rDNA phylogeny where *A. falcatus* and M. *terrestre* were related. The 18S rDNA tree (Fig. 2.10) assigned *Monoraphidium saxatile* Komárková-Legnerová (Mary 9/21 T-5w and NDem 9/21 T-9d) and *Monoraphidium terrestre* (SAG 49.87) as closely related on clade *Monoraphidium*. Also on the 18S rDNA phylogeny, *Monoraphidium* sp. (Itas 9/21 14-6w) and *A. falcatus* (UTEX 101) demonstrated to be related (*Monoraphidium/Ankistrodesmus* clade). *M. dybowskii* strains clustered together on *Monoraphidium dybowskii* clade.

On the 18S rDNA phylogeny, *Monoraphidium neglectum* Heynig & Krienitz (SAG 487.87) demonstrated to be closely related to *Podohedriella falcata* (Düringer) Hindák (SAG 202-2) and *Chlorolobion braunii* (Nägeli) Komárek (CCMA-UFSCar 462) (*Monoraphidium*/*Ankistrodesmus*/*Chlorolobion* clade). *Monoraphidium pusilum* (Printz) Komárková-Legnerová (MDL 1/12-5) and *Monoraphidium contortum* (Thuret) Komárková-Legnerová (AS6-3) clustered together near to *Monoraphidium minutum*

(Nägeli) Komárková-Legnerová (AS3-5) and *Nephroclamys solitaria* (West) Korshikov (SAG 2432a) (*Monoraphidium*/*Nephroclamys* clade). *Monoraphidium convolutum* (Corda) Komárková-Legnerová (AS7-3) and *M. convolutum* (KR 1981/262) clustered with *Monoraphidium* sp. (CCMA-UFSCar 364).

18S rDNA phylogeny (Fig. 2.10) revealed *Ankistrodesmus* Corda distributed in tree clades: *Ankistrodesmus nannoselene* Skuja (SAG 202-6), recognized as *insertae sedis* in Selenastraceae (*Insertae sedis*), *Ankistrodesmus fusiformis* (SAG 2005) and *Ankistrodesmus stipitatus* (Chodat) Komárková-Legnerová (CCMA-UFSCar 277 and SAG 202-5) near *Quadrigula closterioides* (Bohlin) Printz (SAG 12.94) (*Quadrigula*/*Ankistrodesmus* clade). Two clades also had *Ankistrodesmus* as representatives on the ITS1-5.8S-ITS2 inference (Fig. 2.9): *A. falcatus* (A*nkistrodesmus/Monoraphidium* clade) and *A. stipitatus* (A*nkistrodesmus* clade).

For *Messastrum gracile* (Reinsch) T.S. Garcia (CCMA-UFSCar 5, CCMA-UFSCar 622, KR 1981/231 and SAG 278-2) (*Messastrum* clade) a concise and well-supported clade were obtained on both phylogenies. *Selenastrum bibraianum* Reinsch (CCMA-UFSCar 125 and SAG 278-1) (*Selenastrum* clade on 18S rDNA and *Kirchneriella*/*Selenastrum* clade on ITS1-5.8S-ITS2 phylogenies) was closely related to *Kirchneriella*, belonging to the same clade on the ITS1-5.8S-ITS2 tree.

The *Kirchneriella* morphotype strains were focused in this study and were divided into five distinct lineages representing five genera: *Kirchneriella* (10 strains, 4 species), *Raphidocelis* (7 strains, 2 species), *Genus 1* gen. nov. (4 strains, 1 species), *Genus 2 g*en. nov. (6 strains, 2 species) and *Tetranephris* (5 strains,2 species).
All these five genera were highly supported by all bootstraps and Bayesian analyses.

The genus *Kirchneriella* Schmidle (clade *Kirchneriella*, Fig. 2.10 and *Kirchneriella*/*Selenastrum*, Fig. 2.9) contained the ‘true’ crescent to circular shaped

cells, as *K. lunaris* (type species), *Kirchneriella aperta* Teiling, *K. obesa* and *K. pseudoaperta*.

Genus *Tetranephris* (*Tetranephris* clade) had *Tetranephris brasiliense* (18S rDNA phylogeny) and the new species *Tetranephris* sp. nov. T.S.Garcia& Sant'Anna (18S rDNA and ITS1-5.8S-ITS2 phylogenies) as representatives, constituting a cohesive clade.

Members of the genus *Raphidocelis* Hindák (*Raphidocelis* clade), presented *Raphidocelis subcapitata* (Korshikov) Nygaard, Komárek, J.Kristiansen & O.M.Skulberg on both phylogenetic inferences and included *Raphidocelis* sp nov. on the ITS1-5.8S-ITS2 tree (Fig. 2.9).

The species proposed in this study, *Gen. nov.*1 sp1, (*Gen. nov.* 1 clade), *Gen. nov.* 2 sp1 and *Gen. nov.* 2 sp 2(*Gen. nov.* 2 clade) presented good bootstrap values and were positioned inside Selenastraceae, demonstrating closely relation to its representatives.

2.5. DISCUSSION

A large dataset of Selenastraceae species was presented on this study, including strains of subtropical, tropical and temperate environments, improving the phylogeny of this family. Some polyphyletic genera could be observed, as *Monoraphidium*, *Kirchneriella* and *Ankistrodesmus*, also suggested by Krienitz *et al.* (2001; 2011) and respectively two new genera and five species could be described.

The molecular data enabled pointing highlighted morphological features to distinguish the members of this group, reaching better taxonomical placement of genera and species within the Selenastraceae, as further discussed.

Taxonomic and molecular studies on Kirchneriella morphotype

Kirchneriella was briefly described by Schmidle (1893, holotype plate 3, figs. 1-2) as cells more or less pointed at the ends, 3 to 5 µm wide and twice times long, embedded in mucilage, which was continually ignored by the taxonomists. In this publication, Schmidle indicated *Kirchneriella lunata* (Basyonym *Raphidium convolutum* var. *lunare* Kirchner) as the only representative for the genus. Reporting a personal communication with Professor Oskar Kirchner, Professor Schmidle informed that, according to pictures sent to Kirchner, *R. convolutum* var. *lunare* and *K. lunata* belonged to the same taxon. Moreover, Professor Schmidle declared that *Selenastrum obesum* West (Syn. *Kirchneriella obesa* (W.West) Schmidle 1893, *Kirchneriella intermedia* Korsikov 1953) and *Scenedesmus radiatus* (syn. *Scenedesmus bijugatus* var. *radiatus* ? (Reinsch) Hansgirg) pictures should be considered as representatives of the new genus proposed. Probably because of a misunderstanding on Schmidle notes, the scientific community adopted *K. obesa* as type-species for genus *Kircheneriella* (cf. Komarek & Fott 1983; Krienitz 2011; Silva 2013). The correct type-species is *Kirchneriella lunata* Schmidle 1983, according to the Index Nominum Genericorum-Plantarum (Farr & Zijlstra 1996) and Index Nominum Algarum (Silva 1996). Möbius in 1894 unnecessarily corrected the name to *K. lunaris* (Silva *et al.* 1997), referring to an error on epithet reference.

After Schmidle publication, taxonomic studies revealed dozens of species belonging to the newly described genus (Korshikov 1987; Komárek & Fott 1983; Komárková-Legnerová 1969; Comas 1996; Hindák 1977; Hindák 1980; Hindák 1984; Hindák 1988; Hindák 1990).

According to Korshikov (1953) the composition of genus *Kirchneriella* was insufficiently studied and the species descriptions were brief, with little substantial and,

in some cases, possibly inexact, what led to mismatch of the same species in different literatures. In addition, precise designation and species variability are doubtful inside the genus (Korshikov 1953).

Granulated cell walls (or "surface ornamentation", in Hindák 1988) and visible pyrenoids in some *Kirchneriella* species were features that lead Hindák (Hindák1977) propose *Raphidocelis* was as a new genus, suggesting *Raphidocelis sigmoidea* Hindák as type species. *Raphidocelis extensa* (Korshikov) Komárek, a species without incrustations on the cell wall, was also included in *Raphidocelis* (Komárek 1979).

Hindák (1988) established *Kirchneria* as *Kircheneriella*-like cells without pyrenoid and cell wall granulation, restricting *Raphidocelis* to the ones with cell wall incrustations, which Marvan *et al*. (1984) had formerly transferred to *Raphidocelis*, appointing *K. subcapitata* (Korshikov) Hindák (syn. *Raphidocelis subcapitata*) as specie type. On 1990, Hindák introduced *Pseudokirchneriella* as a new name for *Kirchneria*, once he realized that the name had already been used for a fossile plants. Consequently, *Pseudokirchneriella subcapitata* (Hindák) Hindák was the type species designated for the created genus. 18S rDNA phylogeny conducted to the right position of *Raphidocelis subcapitata* (Korshikov) Nygaard, Komárek, Kristiansen& Skulberg. (Syn. *Pseudokirchneriella subcapitata*) elucidating the journey of *Selenastrum capricornutum* Printz (Krienitz et al. 2011a) and showed that *Pseudokirchneriella* was an uneven genus, since it presents pyrenoid under Transmission Electron Microscopy.

The beginning of molecular studies on Selenastraceae evidenced *Raphidocelis* really represents a molecular lineage not related to *Kirchneriella* (Fawley et al. 2006 ; Krienitz et al. 2001; Krienitz et al. 2011).

Morphotypical concepts of the genus *Raphidocelis* sensu Hindák (1977) and Marvan *et al.* (1984) are highly questionable according to Krienitz *et*. al, (2011). The

referred publication considerate that crescent-shaped morphology with only sporadically starch-covered or naked pyrenoids were distributed in 10 different clades, and highlighted that could be expected that the majority of taxa assigned to *Raphidocelis* by Marvan *et al.* (1984) probably belong to other genera, particularly to *Nephrochlamys* and *Tetranephris*.

Facing the limitation of Mayr's species concept (1942) to coccoid green algae, limnologists adopted the morphological species concept, based on morphological diacritical characteristics as a way to deal with this limitation (Krienitz & Bock 2012).

Feature as presence or absence of pyrenoids is an important problem of generic importance concerning the Selenastraceae. Naked pyrenoids are almost impossible to distinguish using conventional LM. On the other hand, starch covered pyrenoids are visualized simply using conventional LM. For that reason, most Selenastraceae were considered to be pyrenoid-less (Korshikov 1953, Komárek and Fott 1983). Eloranta (1979) was the first to report the presence of pyrenoids in a Selenastracean taxa, *Monoraphidium griffithii* (Berkeley) Komárková-Legnerová, by TEM images. Later studies detected this structure in all taxa studied, some presenting naked matrix and some exhibiting starch grains covering the pyrenoid matrix (Krienitz *et al.* 1985, 2001; Krienitz & Scheffler, 1994; Krienitz *et al.* 2011).

The physiological stage of the algae can alter pyrenoid structure, as demonstrated by Eloranta (1979), for *Monoraphidium*. Depending on the culture technique, pyrenoids can develop in a different way within the chloroplast or even disappear, once influenced by the concentration of CO_2 (Miyachi *et al.* 1986; Krienitz and Klein 1988). Consequently, TEM is necessary for the observation in details of the pyrenoid structure (Krienitz *et al.* 2001) and presence or absence may be not a valuable character to the Selenastraceae taxonomy when not in controlled conditions.

Kirchneriella and Genus 1 sp nov 1 had ellipsoidal or spherical naked pyrenoids (without starch envelopes), both investigated by LM (Table 2). All the other taxa did not present such structure under LM observations. After Eloranta (1979) report on naked-pyrenoids in *Monoraphidium*, Marvan *et al.* (1984) accepted that members of *Raphidocelis* could similarly contain such pyrenoids. Pyrenoids were also observed in *R. subcapitata* and *T. brasiliense* (strains Comas 1991/6, KR 1989/26 and CB 2009/7) on ultrastructure observations (Krienitz *et al.*2011).

Colony morphology was considered a species specific attribute, since all studied taxa presented such character. Cross-shaped colonies were observed in *Tetranephris* sp nov 1. Genus 1 gen nov and Genus 2 gen nov. presented 2 cells with the convex side and 2 with the concave side outwards the center of the colony, in two perpendicular planes to each other. *Raphidocelis* sp nov presented irregular arranged cells on colony or with the convex side towards the center of the colony; *Kirchneriella* species presented irregularly arranged cells, mostly with the convex side towards the center of the colony.

When identifying *Kirchneriella*-like species, cell size and shape and cell curvature or cell twist should be considered, since these features were crucial for strains identification. A parallel arrangement of autospores was observed in all studied strains.

A combination of colony formation, presence/absence of starched pyrenoid and cell shape and size demonstrated to be valuable for the taxonomy of the selenastracean strains (Garcia *et al.* in press).

Accordingly, subtle differences regarding cell shape and size, mucilage, cell arrangement in colony were significant to distinct members of the different lineages. Nevertheless, the establishment of mucilage was considered a diacritic feature of limited taxonomic value (Krienitz *et al.* 2012), since its presence is variable.However, the

monomeric composition and glycosidic bonds are being studied as diacritic feature in Selenastraceae and a pattern has been observed inside these taxa (Meccheri *et al*. in development).

Taxonomic value of generic features based on morphology or ultrastructure, usually applied by one single criterion (as pyrenoid presence/absence or granulations on cell wall), was considered not appropriated for Selenastraceae (Krienitz *et al.* 2011). The changing views about the use of diacritic characters in the Selenastraceae and the fluctuations of generic circumscription were reflected on the description of new morphological genera, such *Raphidocelis* (Krienitz *et al.* 2011) and *Pseudokirchneriella*.

18S rDNA phylogeny in Selenastraceae revealed that a morphotype can represent dissimilar phylotypes, suggesting that the diversity of the family have been considerably underestimated when morphologically evaluated (Fawley *et al.* 2006). Based on morphology and molecular data, Selenastraceae is compounded of twelve genera at the moment (Fučíková *et al.* 2014; Krienitz & Bock 2012, Garcia *et al*. in press), wherein *Ourococcus* was the only representatives that was not represented in our phylogenetic inference.

The lower variation of the 18S rRNA sequences observed in Selenastraceae suggested the use of a more variable marker, with higher sequence divergence, as ITS1 and ITS2, to improve the resolution of the group, also employed to phylogenetic studies of other green algal phylogeny (Luo *et al.* 2009; Bock *et al.* 2011; Hegewald *et al.* 2013).

The number of variable sequence positions was improved among the studied taxa by using a higher number of variable markers, (0-68 for 18S rRNA and 0-132 for ITS1-5.8S-ITS2) revealing the relationship arrangements within Selenastraceae.

The absence or a low bootstrap support for the basis of the trees and a good support only of smaller groups of species or genera is a known problem in the systematics of Selenastraceae (Fawley *et al*. 2006; Krienitz *et al*. 2001; Garcia *et al.* in press). Although for some important clades as *Kirchneriella* and *Kichneriella/Selenastrum* the support using both DNA sequences was no good, they were clustered using such different DNA regions, a very conservative and a variable one. Furthermore, the clusters obtained are in agreement with previous studies in Selenastraceae (Fawley *et al*. 2006; Krienitz *et al*. 2011; Krienitz *et al*. 2001; Garcia *et al.* in press), supporting the probability of the phylogenetic information on the branches.

Remarks on genera

Kirchneriella. *Kirchneriella* "true species" are the ones related to *K. lunaris* (type species). Apparently, this genus contains dozens of morphologically related species and varieties (Komarek & Fott 1983). Species identification in taxa with simple morphology is a known issue, since diacritic features can be overlapped, as in *K. lunaris* and *K. dianae*, which cell size and shape are nearly the same, presenting similar colony formation, similar cell shape and presence of mucilage and pyrenoid (Komárek &Fott 1983; Komarek 1983; Hindák 1980; Korsikov 1953; Comas 1996).

Our phylogenetic analyses present 4 species associated to *K. lunaris*: *K. dianae* (SAG 2004), *K. obesa, K. aperta* and *K. pseudoaperta. K. dianae* sequence was acquired from GenBank and it was not possible to evaluate its morphology. All *Kirchneriella* strains that belong to CCMA-UFSCar were identified respecting their type description.

Previous 18S rDNA phylogenetic study with Selenastraceae demonstrated that *S. bibraianum* was closely related to *K. aperta*, justified by their crescent-shaped or

semilunate morphotype and ultrastructure features (Krienitz *et al* 2001). Type species for Selenastraceae, *S. bibraianum* is recognized as a typical morphotype of this family. At the present time, we do not want to assume taxonomical conclusions between *Kirchneriella* and *Selenastrum* relation. Nomenclatural changes can only be reached after more molecular data from these taxa are available.

Raphidocelis. Regarding its importance as a famous experimental organism, *Raphidocelis subcapitata* NIVA-CHL1 was one of the first strain of Selenastraceae subjected to SSU rRNA gene sequence analyses (Booton *et al.* 2004, NCBI access. no. AF169628). NIVA-CHL1, CCMA-UFSCar 48 (NIVA-CHL1, donated by Dr. Skulberg in 1983), KR 1991/19 and MDL 1/12-3 demonstrated to be a molecular lineage and a morphological species well established both for taxonomic and phylogenetic inferences (Krienitz *et al.* 2001; Fawley *et al.* 2006; Krienitz *et al.* 2011). The strains CCMA-UFSCar 228 and CCMA-UFSCar 229, settled in *Raphidocelis* clade, has uniformly granulated cell wall, one of the diacritic features to separate *Raphidocelis* from *Kirchneriella* (Komárek and Fott 1983). *Raphidocelis* sp. nov., a new species proposed in this study, presented similar morphology to *Raphidocelis contorta* var. *elongata* (G.M.Smith) Marvan, Komárek & Comas (Syn. *Kirchneriella elongata* G.M.Smith, *Pseudokirchneriella elongata* (G.M.Smith) F.Hindák, *Kirchneriella contorta* var. *elongata* (G.M.Smith) Komárek 1979). The holotype for *Raphidocelis contorta* var. *elongata* is a drawing (Schmidle 1894: 44, pl. VII: fig. 2) and does not have the type strain in culture collections and any sequence deposited on internet database (as NCBI, for example). Therefore, it is not possible to claim both species as belonging to the same taxon.

According to the 18S rDNA, *M. convolutum* and *Monoraphidium* sp. (also on the ITS1-5.8S-ITS2) are closely related to *Raphidocelis*. Previous study already discussed the similarity between *M. convolutum*, *Raphidocelis van-goori* and *Tetranephris*, suggesting that further *Tetranephris* species continue undiscovered below other crescent-shaped genera (Krienitz *et al.* 2011). We believe that these strains recognized as *M. convolutum* (AS7-3 and KR 1981/262) and *Monoraphidium* sp. (CCMA-UFSCar 364) may belong to a genus not yet described. Further molecular studies with tropical strains are being conducted and are necessary to elucidate *Monoraphidium* and *Raphidocelis* relation, improving Selenastraceae phylogeny.

Genus 1 gen nov. Besides *Kirchneriella*, *Raphidocelis* and *Tetranephris*, Genus 1 gen nov. represents a monoespecific genus in Selenastraceae, described in this study, according to the molecular data. The geographic origin of the studied strains are São Paulo State country side (CCMA-UFSCar 174, 230, 234 and 601) and one was isolated from France (CB 2012/26), where cosmopolitan distribution is suggested by molecular and morphological data, which consider them as an identical species. *Kirchneriella irregularis* var. *spiralis* Korshikov is morphologically similar to Genus 1 gen nov. The holotype for *K. irregularis* var. *spiralis* is a drawing (Korsikov 1953 : 319, fig. 292) and does not have the type strain in culture collections and any sequence deposited on internet database (as NCBI, for example). Therefore, it is not possible to claim both species as belonging to the same taxon.

Tetranephris. Previously assigned to the Scenedesmaceae (Sant'Anna & Bicudo, 1977; Komárek & Fott 1983), a study with 18S rDNA allocated the genus *Tetranephris* as belonging to the Selenastraceae (Krienitz *et al.* 2011). The 18S rDNA similarity to

strains Comas 1991/6 and KR 1989/26, recognized as *T. brasiliense* (Krienitz *et al.* 2011), permitted the proposal of *Tetranephris* sp nov in this study. Regarding cell size and shape *Tetranephris* sp nov (3-8 long x 1-4 µm large) does not correspond to any described species of the genus at the moment (cf. Komarék & Fott 1983), since *T. brasiliense* (6-7,6 long x 4-5,8 large) and *T. europae* (4-8 long x 2-4 large) are bigger in size. The strain CB 2009/7 was formerly studied and was identified as *T. brasiliense*, but investigations under LM revealed the smaller size compared to the others, not in agreement with *T. brasiliense* diagnosis. The geographic origins of the studied strains were Cuba (strain Comas 1991/6), Kenya (CB 2009/6, CB 2009/7, CB 2009/18) and Germany (KR1989/26).

Genus 2 gen nov. According to the molecular data, a new *Kirchneriella*-like genus is proposed in this study. Genus 2 gen nov. is composed of 2 species at the moment, with good bootstrap values for 18S rDNA and ITS1-5.8S-ITS2 phylogenies.

Kirchneriella irregularis var. *irregularis* Korsikov (1953) (Syn. *Kirchneriella lunaris* var. *irregularis* G.M Smith 1920, *Kirchneria irregularis* (G.M.Smith) Hindák 1988, *Kirchneriella irregularis* (G.M.Smith) Korshikov 1953, *Kirchneriella lunaris* var. *dianae* Bohl sensu G.M. Smith 1920) is morphologically similar to Genus 2 sp nov. 1, except for its bigger cell size (6-21 long X 3-6 large, according to the species diagnosis). The holotype for *K. irregularis* var. *irregularis* is a drawing (Korsikov 1953: 319, fig. 291a, b) and does not have the type strain in culture collections and any sequence deposited on internet database (as NCBI, for example). Therefore, it is not possible to relate both species as belonging to the same taxon. The geographic origin of the studied strains from Genus 2 sp nov 1 are from São Paulo State country side

(CCMA-UFSCar 132 and 447) and Pantanal, a Brazilian wetland (CCMA-UFSCar 348).

Also placed at Genus 2 gen nov, Genus 2 sp nov 2 is morphologically and molecular similar to Genus 2 sp nov 1. Regarding morphology, the known species *Kirchneriella pinguis* Hindák resembles Genus 2 sp nov 2, except for its bigger cell size (5-10 long X 2-4 large, according to the species diagnosis). The holotype for *K. pinguis* is a drawing (Hindák 1977: 91, figs 33, 34) and does not have the type strain in culture collections and any sequence deposited on internet database (as NCBI, for example). Therefore, it is not possible to appoint both species as belonging to the same taxon. The geographic origin of the studied strains from Genus 2 sp nov 2 are from Slovakia (CB 2012/21 and CB 2012/22) and Germany (KR 1979/222).

For taxa review on *Kichneriella*, *Raphidocelis*, *Tetranephris* and Genus 1 gen nov and Genus2 gen nov, including latest taxonomic bibliography, see table 2.3.

Selenastraceae: highlights on small-celled genera phylogeny

Morphological and modern molecular phylogenetic approaches in systematics concerning the concept of genera in coccoid green algae have lately motivated controversy (Luo *et al.* 2009). Several genera have recently passed though reformulation after molecular studies. Recently, some morphological well-known genera were divided in many smaller ones as proposed for *Scenedesmus* Meyen (An *et al.* 1999; Krienitz *et al.* 2003) and *Pediastrum* (Buchheim *et al.* 2005) in 18S rDNA phylogenetic approaches.

The idea that some morphological criteria represent phenotypical adaptations to environment conditions and do not effectively replicate phylogenetic relationships was supported by (Krienitz *et al*. 2004; Luo *et al.* 2006).

A molecular study differentiated six genera within the *Chlorella* Beyerinck [Beijerinck]clade of Chlorellaceae (SSU and ITS-1 and -2) and revealed that several morphological criteria used on identification (mucilage and connecting strands, colonial or solitary life form and bristle formation) were phenotypic characteristics and represented adaptive responses to environmental factors such as grazing pressure, endosymbiosis or edaphic life strategies (Luo *et al.* 2010).

The significant variability among isolates concerned to a particular taxon in Selenastraceae could in fact represent numerous taxa (Komárková-Legnerová 1969; Nygaard & Komarek 1986). The concept of small genera, including a small number of species (cf. Komarék & Fott 1983; Krienitz *et al.* 2001; Krienitz *et al.* 2011; Krienitz & Bock 2012) for Selenastraceae has been recently discussed and confirmed. The small genera concept was suggested for genera *Kirchneriella* on our previous phylogenetic inference (Garcia *et al.* in press).

Also, the existence of many transitional forms and a wide morphospecies concept would result in a considerable underestimation of species diversity (Krienitz, 2001; Krienitz, 2012).

It was observed that analogous morphotypes can hide high genotypic diversity inside *Kirchneriella*, *Tetranephris* and *Raphidocelis*. Our data corroborates to previous findings on Selenastracean crescent-shaped isolates, which exhibited the same morphotypes and differences regarding SSU rRNA (Krienitz *et al.* 2011; Fawley *et al.* 2006).

The combination of molecular analyses and morphological review in different growth phases of strains was already exposed as important for the systematics of Selenastraceae on genus and species level (Garcia *et al.* in press). Also, diacritic features as cell shape and colony formation, arrangement of autospores in the mother

cell wall, cell shape and size and cell twist proved to be convenient for genera separation, being applied carefully inside species (Garcia et al in press). However, polyphyletic genera, as *Monoraphidium* and *Ankistrodesmus*, should employ these traditional traits carefully, otherwise there could be misidentification of species.

Although morphological characteristics were observed and considered individually in this study, the 18S rDNA and ITS1-5.8S-ITS2 phylogenies were crucial to designate taxonomical decisions in these small coccoid green algae.

The absence of cultures of the type strains for most of the taxa elected in this cosmopolitan family and the few number of sequences on databases did not propitiated an enhanced coverage of lineages within the Selenastraceae. This scenery linked to the phenotypic plasticity of this group reinforces how necessary are the new studies involving isolated and cultivable strains to elucidate phylogenetic relation on coccoid green algae.

Such phylogenetic studies are crucial to provide reference sequences to practical applications and scientific studies including metabarcoding approaches (Ji *et al.* 2013) and high-throughput sequencing, which lacks references for phytoplankton species (Eiler *et al.* 2012; Pawlowski *et al.* 2012).

Some species descriptions in *Kircheneriella* complex seem to be lost and some were published with missing information on the original publication (including plates with holotype or old "iconotypus"), mainly the material published by the end of 1800's.

The missing material could serve as subsidiary for many taxonomic reviews absolutely necessary for the relevant reassessment of many genera inside Selenastraceae, as *Monoraphidium* and *Ankistrodesmus*.

Additionally, little is known about geographic distributions of the coccoid green algae even though their importance in ecosystem and the advance in the taxonomy of

the group (Norton *et al.* 1996; Padisák *et al.* 2015). Biodiversity in the tropics is considered substantially low compared to temperate lakes (Lewis Jr 1978), suggesting a poor exploration and understanding about green microalgae diversity and phylogeny in these areas.

2.6. ACKNOWLEDGEMENTS

The authors would like to thank Fundação de Amparo à Pesquisa do Estado de São Paulo (Processes: 2011/50054-4, 2012/19520-1, 2013/17457-3 and 2013/18083-0) for financial and scientific support and scholarships granted. A special thanks to Prof. Lothar Krienitz, who donated us his lovely green algae collection.

2.7. REFERENCES

An, S., T. Friedl & E. Hegewald, (1999). Phylogenetic Relationships of *Scenedesmus* and *Scenedesmus*-like Coccoid Green Algae as Inferred from ITS-2 rDNA Sequence Comparisons. *Plant Biology* 1(4):418-428.

Bock, C., 2011. Genetic diversity and polyphyletic origin of the *Dictyosphaerium* morphotype. Ph.D. thesis. Freie Universität Berlin,Berlin, 48 pp.

Bock, C., T. Pröschold & L. Krienitz, 2011. Updating the genus *Dictyosphaerium* and description of *Mucidosphaerium* gen. nov. (Trebouxiophyceae) based on morphological and molecular data. *Journal of Phycology* 47(3):638-652.

Booton, G. C., G. L. Floyd & P. A. Fuerst, 1998. Polyphyly of tetrasporalean green algae inferred from nuclear small-subunit ribosomal DNA. *Journal of Phycology* 34(2):306-311.

Buchheim, M., J. Buchheim, T. Carlson, A. Braband, D. Hepperle, L. Krienitz, M. Wolf & E. Hegewald, 2005. Phylogeny of the Hydrodictyaceae (Chlorophyceae): inferences from rDNA data. *Journal of Phycology* 41(5):1039-1054.

Buchheim, M. A., E. A. Michalopulos & J. A. Buchheim, 2001. Phylogeny of the Chlorophyceae with special reference to the Sphaeropleales: a study of 18S and 26S rDNA data. *Journal of Phycology* 37(5):819-835.

Coleman, A. W., 2003. ITS2 is a double-edged tool for eukaryote evolutionary comparisons. *Trends in Genetics* 19(7):370-375.

Coleman, A. W., 2007. Pan-eukaryote ITS2 homologies revealed by RNA secondary structure. *Nucleic Acids Research* 35(10):3322-3329.

Coleman, A. W., 2009. Is there a molecular key to the level of "biological species" in eukaryotes? A DNA guide. *Molecular Phylogenetics and Evolution* 50(1):197-203.

Coleman, A. W. & V. D. Vacquier, 2002. Exploring the phylogenetic utility of ITS sequences for animals: a test case for abalone (*Haliotis*). *Journal of Molecular Evolution* 54(2):246-257.

Comas, A., 1996. *Las Chlorococcales dulciacuícolas de Cuba*. Bibliotheca Phycologica 99. J. Cramer, Berlin, 192.

Eiler, A., S. Drakare, S. Bertilsson, J. Pernthaler, S. Peura, C. Rofner, K. Simek, Y. Yang, P. Znachor, and E. S. Lindström. 2013. Unveiling distribution patterns of freshwater phytoplankton by a next generation sequencing based approach. *PLoS ONE* 8:e53516.

Eloranta, V., 1979. The compound internal pyrenoid in cultured cells of the green alga *Monoraphidium* griffithii (Berkel.) Komar.-Legner. *Protoplasma* 99(3):229-235.

Farr, E. & G. Zijlstra, 1996. Index Nominum Genericorum (Plantarum).< h ttp. botany si edu/ing/>[accessed 05 January 2016].

Fawley, M. W., M. L. Dean, S. K. Dimmer & K. P. Fawley, 2006. Evaluating the morphospecies concept in the Selenastraceae (Chlorophyceae, Chlorophyta) *Journal of Phycology* 42(1):142-154.

Fucikova, K., P. O. Lewis & L. A. Lewis, 2014. Widespread desert affiliation of Trebouxiophycean algae (Trebouxiophyceae, Chlorophyta) including discovery of three new desert genera. *Phycological Research* 62(4):294-305 doi:10.1111/pre.12062.

Garcia, T. S., Bock C., Sant'Anna C. L., Bagatini I. L., Wodniok S., Vieira A. A. H., 2016. Selenastraceae (Sphaeropleales, Chlorophyceae): *rbcL*, 18S rDNA and ITS-2 secondary structure enlightens traditional taxonomy, with description of two new genera, *Messastrum* gen. nov. and *Curvastrum* gen. nov. *Fottea*, in press.

Guillard, R. R. & C. J. Lorenzen, 1972. Yellow-green algae with chlorophyllide C1, 2. *Journal of Phycology* 8(1):10-14.

Guiry, M.D. & Guiry, G.M. 2016. *AlgaeBase*. World-wide electronic publication, National University of Ireland, Galway. http://www.algaebase.org; searched on 5 January 2016.Hegewald, E., C. Bock & L. Krienitz, 2013. A phylogenetic study on Scenedesmaceae with the description of a new species of *Pectinodesmus* and the new genera *Verrucodesmus* and *Chodatodesmus* (Chlorophyta, Chlorophyceae). *Fottea* 13(2):14.

Hegewald, E. & N. Hanagata, 2000. Phylogenetic studies on Scenedesmaceae (Chlorophyta). *Archiv für Hydrobiologie Supplementband, Algological Studies* 136:29-49.

Hepperle, D., 2004. Align Ver. 07/04©. Multisequence alignment-editor and preparation/manipulation of phylogenetic datasets. Win32-Version. Distributed by the author via: http://www. sequentix. de.

Hershkovitz, M., E. Zimmer & W. Hahn, 1999. Ribosomal DNA sequences and angiosperm systematics. *Molecular systematics and plant evolution* (Systematics Association special volume 57:268-326.

Hindák, F., 1977. *Studies on the Chlorococcal algae (Chlorophyceae).* Veda, Publishing House of the Slovak Academy of Science, Bratislava:186.

Hindák, F. 1980. *Studies on the chlorococcal algae (Chlorophyceae) II*., Veda, Bratislava.

Hindák, F. 1984. *Studies on the chlorococcal algae (Chlorophyceae) III*, Veda, Bratislava.

Hindák, F. 1988. *Studies on the chlorococcal algae (Chlorophyceae) IV*., Veda, Bratislava.

Hindák, F. 1990. *Studies on the chlorococcal algae (Chlorophyceae) V*, Veda, Bratislava.

Huelsenbeck, J. P. & F. Ronquist, 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17(8):754-755.

Ji, Y., L. Ashton, S. M. Pedley, D. P. Edwards, Y. Tang, A. Nakamura, R. Kitching, P. M. Dolman, P. Woodcock & F. A. Edwards, 2013. Reliable, verifiable and efficient monitoring of biodiversity via metabarcoding. *Ecology letters* 16(10):1245-1257.

Jobb, G., 2008. TREEFINDER version of October 2008. Munich, Germany Distributed by the author at www treefinder de.

Komárek, J., 1979. Änderungen in der Taxonomie der Chlorokokkalalgen. *Algological Studies/Archiv für Hydrobiologie,* Supplement Volumes:239-263.

Komárek, J. & B. Fott, 1983. *Das Phytoplankton des Sübwassers. Systematik und Biologie.* 7. Teil, 1. Hälfte. Chlorophyceae (Grünalgen) Ordnung: Chroococcales. E. Schweizerbart'sche Verlagsbuchhandlung (Nägele u. Obemiller), Stuttgart. 1043p.

Komárková-Legnerová, J., 1969. *The systematics and ontogenesis of the genera Ankistrodesmus Corda and Monoraphidium gen. nov.* In Fott, B. (ed) Studies in Phycology. Academia, Praha, 75-122.

Korshikov, O., 1953. *Viznachnik prisnovodnih vodorostey Ukrainskoy RSR. V Protococcineae Naukova dumka,* Kiıv (in Ukrainian).

Korshikov, O. A. 1987. *The Freshwater Algae of the Ukrainian SSR. V. Sub-Class Protococcineae Vacuolales and Protococcales.* (trans: Lund, J. W. G. T., W.). Bishen Singh Mahendra Pal Singh and Koeltz Scientific Book. Translated by Lund, J. W. G. & Tylka, W., Dehra Dun, India.

Krienitz, L. & C. Bock, 2012. Present state of the systematics of planktonic coccoid green algae of inland waters. *Hydrobiologia* 698(1):295-326.

Krienitz, L., C. Bock, K. Kotut & T. Pröschold, 2012. Genotypic diversity of *Dictyosphaerium*–morphospecies (Chlorellaceae, Trebouxiophyceae) in African inland waters, including the description of four new genera. *Fottea* 12:231-253.

Krienitz, L., C. Bock, H. Nozaki & M. Wolf, 2011. SSU rRNA gene phylogeny of morphospecies affiliated to the bioassay alga "*Selenastrum capricornutum*" recovered the polyphyletic origin of crescent-shaped Chlorophyta. *Journal of Phycology* 47(4):880-893.

Krienitz, L., E. H. Hegewald, D. Hepperle, V. A. Huss, T. Rohr & M. Wolf, 2004. Phylogenetic relationship of *Chlorella* and *Parachlorella* gen. nov. (Chlorophyta, Trebouxiophyceae). *Phycologia* 43(5):529-542.

Krienitz, L. & G. Klein, 1988. Morphologie und Ultrastruktur einiger Arten der Gattung *Monoraphidium* (Chlorellales) III. Monoraphidium terrestre Bristol nov. comb. *Algological Studies/Archiv für Hydrobiologie*, Supplement Volumes:447-463.

Krienitz, L., G. Klein & H. Böhm, 1985. Zur morphologie und ultrastruktur von *Selenastrum gracile* Reinsch 1867 (chlorellales). *Archiv für Protistenkunde* 130(1):79-92.

Krienitz, L. & W. Scheffler, 1994. The Selenastraceae of the oligotrophic Lake Stechlin (Brandenburg, Germany). *Biologia Ser A* (Slovakia).

Krienitz, L., I. Ustinova, T. Friedl & V. A. Huss, 2001. Traditional generic concepts versus 18S rRNA gene phylogeny in the green algal family Selenastraceae (Chlorophyceae, Chlorophyta). *Journal of Phycology* 37(5):852-865.

Krienitz, L., M. Wolf, E. Hegewald & D. Hepperle, 2002. Systematics of Coccoid Green Algae: Morphology vs. 18S rRNA Gene Phylogeny. *Journal of Phycology* 38(s1):19-20.

Lewis Jr, W. M., 1978. A compositional, phytogeographical and elementary structural analysis of the phytoplankton in a tropical lake: Lake Lanao, Philippines. The *Journal of Ecology*: 213-226.

Lewis, L. A., 1997. Diversity and phylogenetic placement of *Bracteacoccus* Tereg (Chlorophyceae, Chlorophyta) based on 18s ribosomal RNA gene sequence data. *Journal of Phycology* 33(2):279-285.

Litaker, R. W., M. W. Vandersea, S. R. Kibler, K. S. Reece, N. A. Stokes, F. M. Lutzoni, B. A. Yonish, M. A. West, M. N. Black & P. A. Tester, 2007. Recognizing dinoflagellate species using its rDNA sequences. *Journal of Phycology* 43(2):344-355.

Luo, W., C. Bock, H. Li, J. Padisák & L. Krienitz, 2011. Molecular and microscopic diversity of planktonic eukaryotes in the oligotrophic Lake Stechlin (Germany). *Hydrobiologia* 661(1):133-143.

Luo, W., S. Pflugmacher, T. Pröschold, N. Walz & L. Krienitz, 2006. Genotype versus phenotype variability in *Chlorella* and *Micractinium* (Chlorophyta, Trebouxiophyceae). *Protist* 157(3):315-333.

Luo, W., T. Pröschold, C. Bock & L. Krienitz, 2010. Generic concept in *Chlorella*-related coccoid green algae (Chlorophyta, Trebouxiophyceae). *Plant Biology* 12(3):545-553.

Marvan, P., J. Komárek & A. Comas, 1984. Weighting and scaling of features in numerical evaluation of coccal green algae (genera of the Selenastraceae). *Algological Studies/Archiv für Hydrobiologie*, Supplement Volumes:363-399.

Mayr, E., 1942: *Systematics and the origin of species, from the viewpoint of a zoologist*. Harvard University Press.

Meccheri, F. S., Polissacarídeos extracelulares de microalgas: quimiotaxonomia e prospecção de interações em filmes nanoestruturados. (PhD thesis on development)., Universidade Federal de São Carlos.

Miyachi, S., M. Tsuzuki, I. Maruyama, M. Gantar, S. Miyachi & H. Matsushima, 1986. Effects of CO2 concentration during growth on the intracellular structure of *Chlorella* and *Scenedesmus* (Chlorophyta). *Journal of Phycology* 22(3):313-319.

Norton, T. A., M. Melkonian & R. A. Andersen, 1996. Algal biodiversity. *Phycologia* 35(4):308-326.

Nygaard, G. & Komarek, J. & Skilberg, O.,1986 Taxonomic designations of the bioassay alga, NIVA-CHL I (*Selenastrum capricornutum*) and some related strains. *Opera Botanica* 90:1-46.

Padisák, J., G. Vasas & G. Borics, 2015. Phycogeography of freshwater phytoplankton: traditional knowledge and new molecular tools. *Hydrobiologia*:1-25.

Pawlowski, J., S. Audic, S. Adl, D. Bass, L. Belbahri, C. Berney, S. S. Bowser, I. Cepicka, J. Decelle & M. Dunthorn, 2012. CBOL protist working group: barcoding eukaryotic richness beyond the animal, plant, and fungal kingdoms. *PLoS biology* 10(11):e1001419.

Rambaut, A. & A. J. Drummond, (2007). Tracer v 1. 4.

Rindi, F., T. I. Mikhailyuk, H. J. Sluiman, T. Friedl & J. M. López-Bautista, 2011. Phylogenetic relationships in *Interfilum* and *Klebsormidium* (Klebsormidiophyceae, Streptophyta). *Molecular phylogenetics and evolution* 58(2):218-231.

Sant'Anna, C. L., 1984. Chlorococcales (Chlorophyceae) do Estado de São Paulo, Brasil. Bibliotheca Phycologica 67. J. Cramer, Berlin, 348.

Schultz, J., S. Maisel, D. Gerlach, T. Müller & M. Wolf, 2005. A common core of secondary structure of the internal transcribed spacer 2 (ITS2) throughout the Eukaryota. *RNA* 11(4):361-364.

Schmidle, W. (1893). *Beiträge zur Algenflora des Schwartzwaldes und der Rheinebene*. Bericht der Naturforschenden Gesammelt Freiberg 7: 68-112.

Schultz, J., S. Maisel, D. Gerlach, T. Müller & M. Wolf, (2005). A common core of secondary structure of the internal transcribed spacer 2 (ITS2) throughout the Eukaryota. *RNA* 11 (4):361-364.

Seifert, K. A., 2009. Progress towards DNA barcoding of fungi. *Molecular Ecology Resources* 9(s1):83-89.

Silva, D., C. L. Sant'Anna, A. Tucci & A. Comas, 2013. New planktic species of *Kirchneriella* Schmidle (Chlorophyceae, Selenastraceae) from Brazilian freshwaters. *Brazilian Journal of Botany* 36(2):153-157.

Silva, P.C. (1996-to date). *Index Nominum Algarum, University Herbarium*, University of California, Berkeley http://ucjeps.berkeley.edu/INA.html. Updated continuously.

Stöver, B. C. & K. F. Muller, 2010. TreeGraph 2: combining and visualizing evidence from different phylogenetic analyses. *BMC bioinformatics* 11:7-7.

Young, I. & A. W. Coleman, 2004. The advantages of the ITS2 region of the nuclear rDNA cistron for analysis of phylogenetic relationships of insects: a Drosophila example. *Molecular phylogenetics and evolution* 30(1):236-242.

Table 2.1. List of studied strains with origin information and GenBank accession numbers for 18S rDNA and ITS1-5.8S-ITS-2. Sequences in bold letter acquired from GenBank. ACOI, Coimbra Collection of Algae; CCMA - UFSCar, Coleção de culturas de Microalgas de Água Doce – Universidade Federal de São Carlos ; SAG, Sammlung von Algenkulturen der Universität Göttingen, Germany; UTEX, The Culture Collection of Algae at the University of Texas at Austin; AS, Itas, Mary, NDem and MDL acquired from Fawley *et al*., 2006. For own isolates, the initials of the isolator were given: CB, Christina Bock; KR, Lothar Krienitz; Comas, Augusto Abilio Comas González. Accession number indicated with x will be deposited prior to publication.

Strain	**Taxon**	**GenBank Accession Numbers**		**Origin**
		18S	**ITS1-5.8S-ITS-2**	
Selenastraceae				
SAG 278-1	*Ankistrodesmus bibraianus*	**Y16938**		UK - 52°12'08.4"N 0°07'42.0"E
UTEX 101	*Ankistrodesmus falcatus*	**JN630515**	**KC145459**	USA - 39°10'12.0"N 86°31'43.5"W
SAG 2005	*Ankistrodesmus fusiformis*	**X97352**		Germany - 53°12'46.1"N 13°01'44.8"E
KR1981/231	*Ankistrodesmus gracilis*	**HM565930**		Germany - 51°52'55.7"N 12°00'11.6"E
SAG 278-2	*Ankistrodesmus gracilis*	**Y16937**		Germany - 51°52'55.7"N 12°00'11.6"E
SAG 202-6	*Ankistrodesmus nannoselene*	**HM483519**		Sweden - 59°57'46.0"N 17°07'43.9"E

Table 2.1cont.

CCMA-UFSCar 277	*Ankistrodesmus stipitatus*	KT833580	x	Brazil - 20°32'01.0"S 46°31'32.9"W
SAG 202-5	*Ankistrodesmus stipitatus*	**X56100**		Czech Republic - 50°04'23.2"N14°26'09.9"E
CCMA-UFSCar 462	*Chlorolobion braunii*	KT833588		Brazil - 22°12'59.2"S 47°37'29.6"W
CCMA-UFSCar 601	Genus1 sp1	x	x	Brazil - 20°53'05.8"S 48°14'20.7"W
CCMA-UFSCar 234	Genus1 sp1	x	x	Brazil - 21°53'06.9"S 47°46'53.6"W
CCMA-UFSCar 230	Genus1 sp1	x	x	Brazil - 22°42'45.8"S 47°37'53.5"W
CCMA-UFSCar 174	Genus1 sp1	x		Brazil - 22°42'45.8"S 47°37'53.5"W
CB 2012/26	Genus1 sp1		x	France - 47°31'29.3"N 2°03'31.0"E
CB 2009/6	*Tetranephris* sp nov	x	x	Kenya - 0°21'20.1"S 36°03'20.5"E
CB 2009/7	*Tetranephris* sp nov	x		Kenya - 0°21'20.1"S 36°03'20.5"E
CB 2009/18	*Tetranephris* sp nov	x		Kenya - 0°25'49.0"S 36°13'54.6"E
CCMA-UFSCar 132	Genus 2 sp1	x	x	Brazil - 21°36'28.5"S 47°46'13.1"W
CCMA-UFSCar 447	Genus 2 sp1	x	x	Brazil - 22°19'01.0"S 48°03'05.4"W
CCMA-UFSCar 348	Genus 2 sp1	KT833583	x	Brazil - 19°17'59.0"S 55°47'45.0"W

Table 2.1. cont.

CB 2012/21	Genus 2 sp2		x	Slovakia - 48°51'48.0"N 20°22'48.8"E
CB 2012/22	Genus 2 sp2		x	Slovakia - 48°51'48.0"N 20°22'48.8"E
KR 1979/222	Genus 2 sp2		x	Germany - 51°38'57.7"N 12°01'46.3"E
SAG 2004	*Kirchneriella aperta*	**AJ271859**	**KC145464**	Germany - 53°08'51.7"N 13°01'41.9"E
ACOI 287	*Kirchneriella dianae*	**HM483512**		Portugal - 40°26'29.0"N 8°45'20.5"W
CCMA-UFSCar 123	*Kirchneriella lunaris*	x	x	Brazil - 21°36'28.5"S 47°46'13.1"W
CCMA-UFSCar 87	*Kirchneriella lunaris*	x	x	Brazil - 21°36'28.5"S 47°46'13.1"W
CCMA-UFSCar 443	*Kirchneriella lunaris*	x	x	Brazil - 22°19'01.0"S 48°03'05.4"W
CCMA-UFSCar 345	*Kirchneriella obesa*	x	x	Brazil - 19°17'59.0"S 55°47'45.0"W
CB 2012/49	*Kirchneriella obesa*		x	Sweden - 55°52'01.4"N 13°33'21.5"E
ACOI 3125	*Kirchneriella obesa*	**HM483513**		Portugal - 40°12'07.5"N 8°24'53.9"W
CCMA-UFSCar 346	*Kirchneriella pseudoaperta*	KT833582	x	Brazil - 19°17'59.0"S 55°47'45.0"W
CCMA-UFSCar 482	*Kirchneriella pseudoaperta*	KT833592	x	Brazil - 22°33'09.2"S 48°57'47.5"W
CCMA-UFSCar 5	*Messastrum gracile*	KT833577	x	Brazil - 22°12'20.6"S 47°52'37.6"W

Table 2.1. cont.

CCMA-UFSCar 622	*Messastrum gracile*	KT833593	x	Brazil - 23°02'28.8"S 48°03'15.9"W
Itas 9/21 14-6w	*Monoraphidium* sp.	**AY846379**		USA - 47°12'57.9"N 95°12'23.2"W
CCMA-UFSCar 364	*Monoraphidium* sp.	x	x	Brazil - 19°01'27.0"S 55°55'28.0"W
AS6-3	*Monoraphidium contortum*	**AY846382**		USA - 47º17’26.8”N 98º50’07.0”W
AS7-3	*Monoraphidium convolutum*	**AY846377**		USA - 47º17’26.8”N 98º50’07.0”W
KR 1981/262	*Monoraphidium convolutum*	**HM565926**		Germany - 51°49'37.8"N 11°59'41.7"E
SAG 202-7e	*Monoraphidium dybowskii*	**Y16939**		France - 43°06'26.3"N 0°05'10.4"W
CB 2009/27	*Monoraphidium dybowskii*	**HM483515**	x	Kenya - 0°22'29.9"S 35°55'34.4"E
AS3-5	*Monoraphidium minutum*	**AY846380**		USA - 47º17’26.8”N 98º50’07.0”W
MDL 1/12-5	*Monoraphidium pusillum*	**AY846376**		USA - 46°56'48.0"N 99°42'19.0"W
SAG 48.87	*Monoraphidium neglectum*	**AJ300526**		Germany - 51°44'43.9"N 11°58'48.6"E
Mary 9/21 T-5w	*Monoraphidium saxatile*	**AY846384**		USA - 45°49'39.6"N 95°28'24.6"W
NDem 9/21 T-9d	*Monoraphidium saxatile*	**AY846385**		USA - 47°11'40.6"N 95°09'51.4"W
SAG 49.87	*Monoraphidium terrestre*	**Y17817**	x	Germany - 51°47'22.9"N 12°03'53.6"E

Table 2.1cont.

SAG 243-2a	*Nephrochlamys subsolitaria*	**HM560960**		UK - 54°55'25.5"N 2°18'02.7"W
SAG 202-2	*Podohedriella falcata*	**X91263**		Switzerland - 47°10'37.2"N 8°12'35.0"E
SAG 12.94	*Quadrigula closterioides*	**Y17924**		USA - 47°11'43.2"N 95°10'00.9"W
CB 2012/51	*Raphidocelis* sp.		x	France - 47°31'29.3"N 2°03'31.0"E
CB 2012/39	*Raphidocelis* sp.		x	Sweden - 58°25'11.5"N 14°30'30.6"E
CCMA-UFSCar 228	*Raphidocelis* sp1		x	Brazil - 22°42'45.8"S 47°37'53.5"W
CCMA-UFSCar 229	*Raphidocelis s*p1		x	Brazil - 22°42'45.8"S 47°37'53.5"W
KR 1979/236	*Raphidocelis subcapitata*		x	Germany - 51°45'18.4"N 11°57'38.9"E
KR1991/19	*Raphidocelis subcapitata*	**HM483520**		Germany - 53°08'49.3"N 13°01'53.9"E
NIVA-CHL1				
(CCMA-UFSCar 48)	*Raphidocelis subcapitata*		x	Norway - 59°59'07.3"N 10°59'16.0"E
MDL 1/12-3	*Raphidocelis subcapitata*	**AY846381**		USA - 46°56'48.0"N 99°42'19.0"W
KR 1998/1	*Rhombocystis complanata*		x	Dominica - 15°23'29.9"N 61°15'19.6"W
KR 1998/2	*Rhombocystis complanata*	**HM483518**		Dominica - 15°23'29.9"N 61°15'19.6"W

Table 2.1. cont.

CCMA-UFSCar 125	*Selenastrum bibraianum*	KT833578	x	Brazil - 21°36'28.5"S 47°46'13.1"W
NIVA-CHL1	*Raphidocelis subcapitata*	**AF169628**		Norway - 59°59'07.3"N 10°59'16.0"E
Comas 1991/6	*Tetranephris brasiliense*	**HM565929**		Cuba - 22°17'05.4"N 80°33'32.2"W
KR 1989/26	*Tetranephris brasiliense*	**HM565927**		Germany - 53°30'28.8"N 13°12'48.4"E
Bracteacoccaceae				
SAG 23.69	*Bracteacoccus cohaerens*	**HQ246325**		South Africa – 30°07'11.2"S 17°35'26.8"E
Hidrodyctiaceae				
SAG 28.83	*Pediastrum duplex*	**AY780662**		Germany - 49°17'21.8"N 7°50'59.5"E
Scenedesmaceae				
SAG 276-3a	*Acutodesmus obliquus*	**X56103**		Not found
UTEX 76	*Scenedesmus communis*	**X739941**		USA - 42°22'23.9"N 71°06'29.2"W

Table 2.2. Morphological characteristics of *Kirchneriella*-like strains used in this study. Aa: autospore arrangement; P: pyrenoid; C: colony; M: mucilage.

Taxon	Cell shape	Cell size (long x large µm)	Aa	P	C	M
Genus 1 *sp. nov.* 1	Fusiform regularly curved to bent to one side., with narrowed or obtuse ends.	6-20 x 3-7	Parallel	+	+	+
Genus 2 *sp. nov.* 1	Lunate, curved or twisted cells, slightly bent to one side, with narrowed or obtuse ends.	6-15 x 2,7-5	Parallel	-	+	-
Genus 2 *sp. nov.* 2	Bean-shaped, crescent-shaped or slightly arcuate, ends gradually obtuse.	7-11 x 1,2-3	Parallel	-	+	+
Kirchneriella lunaris	Half-moon or horseshoe-shaped.	4-15 x 1,2-8	Parallel	+	+	+
Kirchneriella obesa	Half-moon or horseshoe-shaped.	6-16 x 2-9,5	Parallel	+	+	+
Kirchneriella pseudoaperta	Half-moon or horseshoe-shaped.	4-8 x 2-4	Parallel	+	+	+
Raphidocelis sp. nov. 1	Narrow and long cell, arcuate to spirally curved with rounded ends.	15-20 x 1-2	Parallel	-	+	+
Tetranephris sp. nov. 1	Bean-shaped to slightly arcuate, narrowed or obtuse ends.	3-8 x 1-4	Parallel	-	+	+

Table 2.3.Revision of taxa included in *Kirchneriella*, *Raphidocelis*, Gen 1 gen nov, Gen 2 gen nov and *Tetranephris*. Names marked with asterisks represents organisms with 18S rDNA sequences available on NCBI and used on previous phylogenetic studies.

Taxon
Kirchneriella
Kirchneriella pseudoaperta Komárek 1983: 126, 176, pl. 21: 50
**Kirchneriella aperta* Teiling 1912: 276, fig. 9
Kirchneriella brasiliana Silva, Sant'Anna, Tucci & Comas2013, fig. 1
Kirchneriella cornuta Korshikov 1953: 319, fig. 293a-d
**Kirchneriella dianae* (Bohlin) Comas Gonzalez 1980: 4
Kirchneriella dianae var. *major* (Korshikov) Comas (= *Kirchneriella lunaris* var. *acuta* Playfair?, cf. Komarek & Fott, 1983)
Kirchneriella hindakiana P.Marvan, J.Komárek & A.Comas 1984: 390
Kirchneriella incurvata J.H.Belcher & Swale 1962: 125, fig. 1: Q-S
Kirchneriella irregularis (G.M.Smith) Korshikov 1953: 319, fig. 291a, b
Kirchneriella irregularis var. *spiralis* Korshikov 1953: 319, fig. 292
Kirchneriella lagerheimii Teiling; Unverified - Missing reference

Table 2.3. cont.

Kirchneriella lunaris (Kirchner) Möbius 1894: 331

Kirchneriella lunaris var. *acuta* Playfair; Unverified - Missing reference

Kirchneriella major C.Bernard 1908: 179, figs 398, 399

**Kirchneriella obesa* (West) West & G.S.West 1894: 16

Kirchneriella obtusa (Korshikov) Komárek 1979: 255

Kirchneriella phaseoliformis Hortobágyi 1952: 237, 241, 243, fig. 14

Kirchneriella pinguis Hindák 1977: 91, figs 33, 34

Raphidocelis

Raphidocelis contorta var. *elongata* (G.M.Smith) P.Marvan, J.Komárek & A.Comas 1984

Raphidocelis contorta (Schmidle) Marvan, Komárek & Comas 1984: 368, fig. 12

Raphidocelis arcuata (G.M.Smith) P.Marvan, J.Komárek &A.Comas 1984: 386

Raphidocelis contorta var. *gracílima* (Bohlin) P.Marvan, J.Komárek & A.Comas 1984: 386

Raphidocelis danubiana (Hindák) Marvan, Komárek & Comas 1984: 386, fig. 9B: 9 a-e

Raphidocelis extensa (Korshikov) Komárek 1979: 257

Table 2.3. cont.

Raphidocelis inclinata Nygaard, J.Komárek, J.Kristiansen&O.M.Skulberg
Raphidocelis inclinata var. *serialis* Nygaard, J.Komárek, J.Kristiansen&O.M.Skulberg
Raphidocelis mayorii (G.S.West) Marvan, Komárek & Comas 1984: 387
Raphidocelis microscopica (Nygaard) Marvan, Komárek & Comas 1984: 387
Raphidocelis mucosa (Korshikov) Komarek 1979: 258
Raphidocelis pseudomucosa Krienitz 1986: 310, figs. 6, 7
Raphidocelis roselata (Hindák) Marvan, Komárek & Comas 1984: 387
Raphidocelis rotunda (Korshikov) Marvan, Komárek & Comas 1984: 387, fig. 9B: 7
Raphidocelis sigmoidea Hindák 1977: 61, pl. 23: figs 13, 24
**Raphidocelis subcapitata* (Korshikov) Nygaard, Komárek, J.Kristiansen&O.M.Skulberg 1987: 31
Raphidocelis turfosa G.Uherkovich 1986
Raphidocelis valida Nygaard, Komárek, J.Kristiansen&O.M.Skulberg 1987: 42, fig. 13
Raphidocelis van-goorii var. *decussata* Nygaard, J.Komárek, J.Kristiansen&O.M.Skulberg1987
Raphidocelis van-goorii Nygaard, Komárek, J.Kristiansen&O.M.Skulberg 1987: 41, pl. 4: figs 12, 16, 32-34

Table 2.3. cont.

Raphidocelis sp nov T. S. Garcia & Sant'Anna
Genus 1gen nov
Genus 1 sp nov 2 T.S.Garcia& Sant'Anna
Genus 2gen nov
Genus 2 sp nov 1 T.S.Garcia & Sant'Anna
Genus 2 sp nov 2 T.S.Garcia & Sant'Anna
Tetranephris
**Tetranephris brasiliense* C.R.Leite & C.E.M.Bicudo 1977: 231-233, figs 1-8
Tetranephris europaea (Hindák) Komárek 1979: 260
Tetranephris sp. nov. T.S.Garcia & Sant'Anna

Fig. 2.1-2.5. Drawings of light microscopical characters. 1. *Tetranephris* sp. nov. (CB 2009/6). Note the cell wall remnant (arrowhead); 2. Gen. nov. 1 sp1 (CCMA-UFSCar 230); 3. *Raphidocelis* sp. nov. (CCMA-UFSCar 229). Note the cell wall remnant (arrowhead); 4. Gen. nov. 2 sp 1. (CCMA-UFSCar 342); 5. Gen. nov. 2 sp 2. (KR 1979/222). Note the cell wall remnant (arrowhead). Scale bar 10 μm.

Fig. 2.6-2.8. Drawings of light microscopical characters. 6. *Kirchneriella pseudoaperta* (CCMA-UFSCar 346). 7. *Kirchneriella obesa* (CCMA-UFSCar 345). 8. *Kirchneriella lunaris* (CCMA-UFSCar 87). Scale bar 10 μm.

Figure 2.9: Maximum–likelihood (ML) phylogenetic tree inferred from ITS1-5.8S-ITS2 gene sequences of some members of Selenastraceae. Support values correspond to Bayesian PP (Posterior Probability), ML BP (Bootstrap), MP (Maximum Parsimony) BP, NJ (Neighbor-Joining)BP. Hyphens correspond to values <50% for BP and <0.95 for PP. Scale represents the expected number of substitutions per site. Strain numbers used as mentioned in Table 2.1.

Figure 2.10: Maximum–likelihood (ML) phylogenetic tree inferred from 18S rDNA gene sequences of some members of Selenastraceae. Support values correspond to Bayesian PP (Posterior Probability), ML BP (Bootstrap), MP (Maximum Parsimony) BP, NJ (Neighbor-Joining)BP. Hyphens correspond to values <50% for BP and <0.95 for PP. Scale represents the expected number of substitutions per site. Strain numbers used as mentioned in Table 2.1.

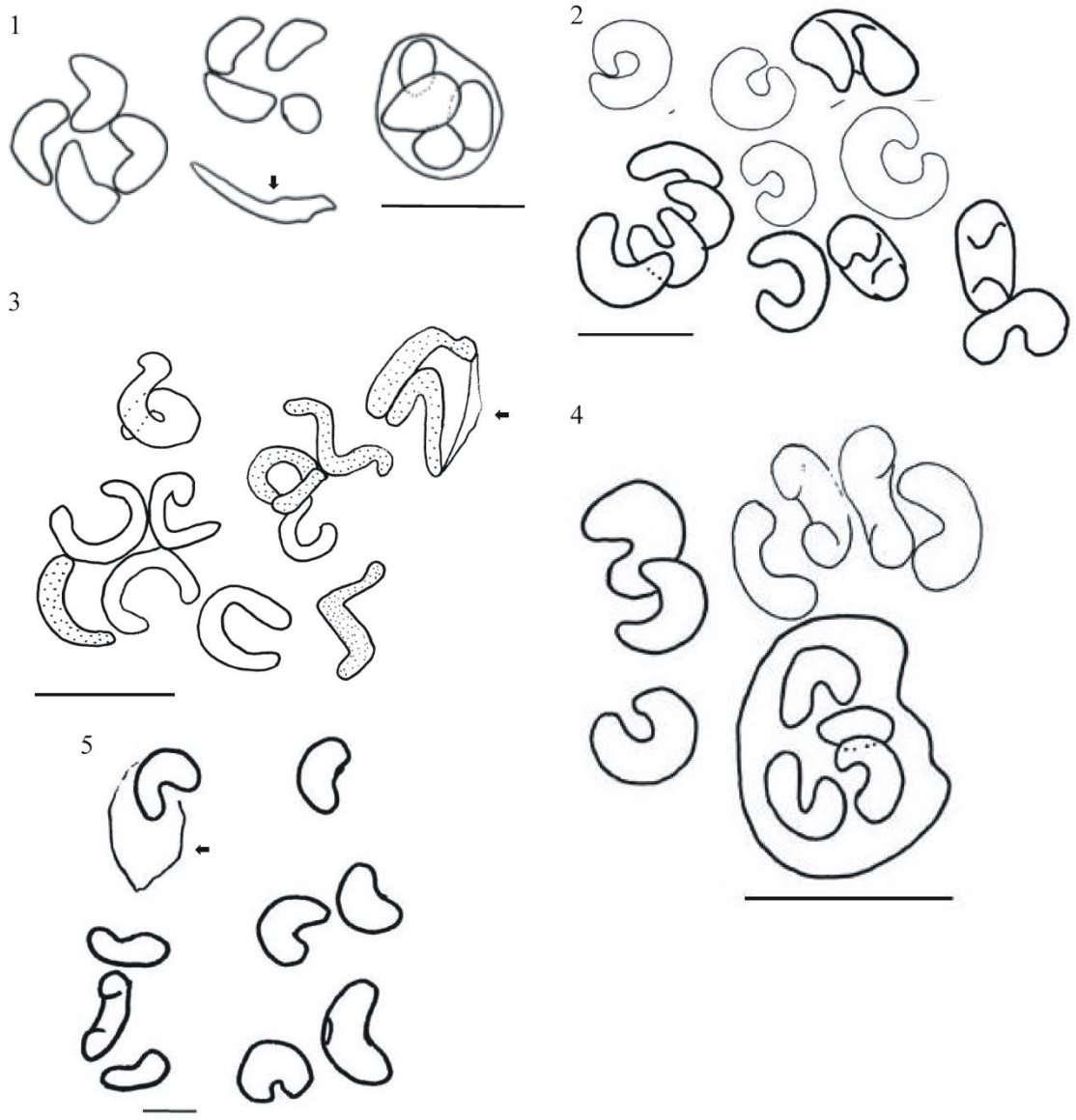
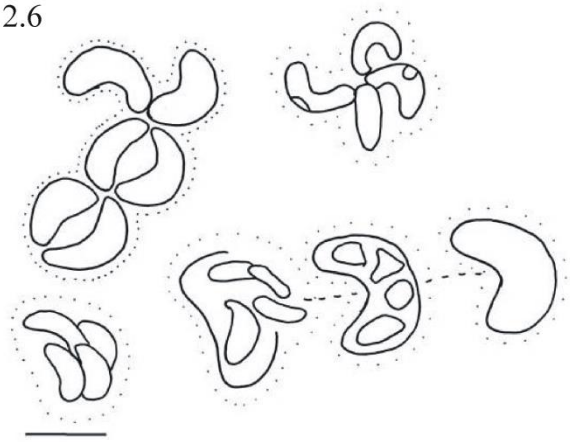
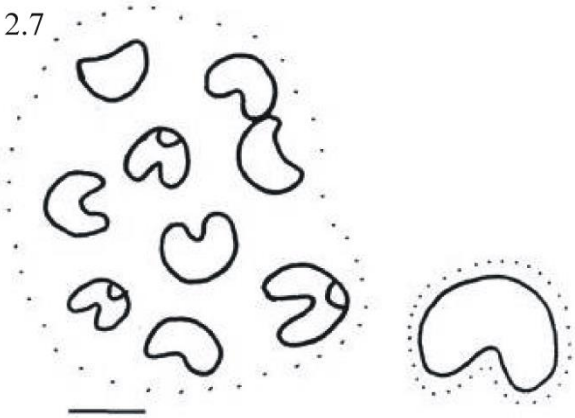


Fig. 2.1-2.5

2.6



2.7



2.8

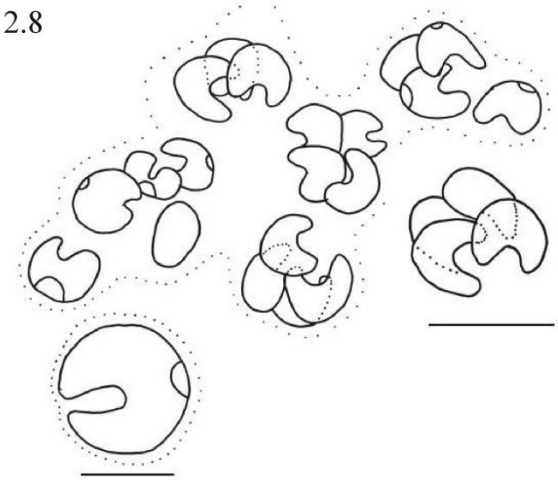


Fig. 2.6-2.8

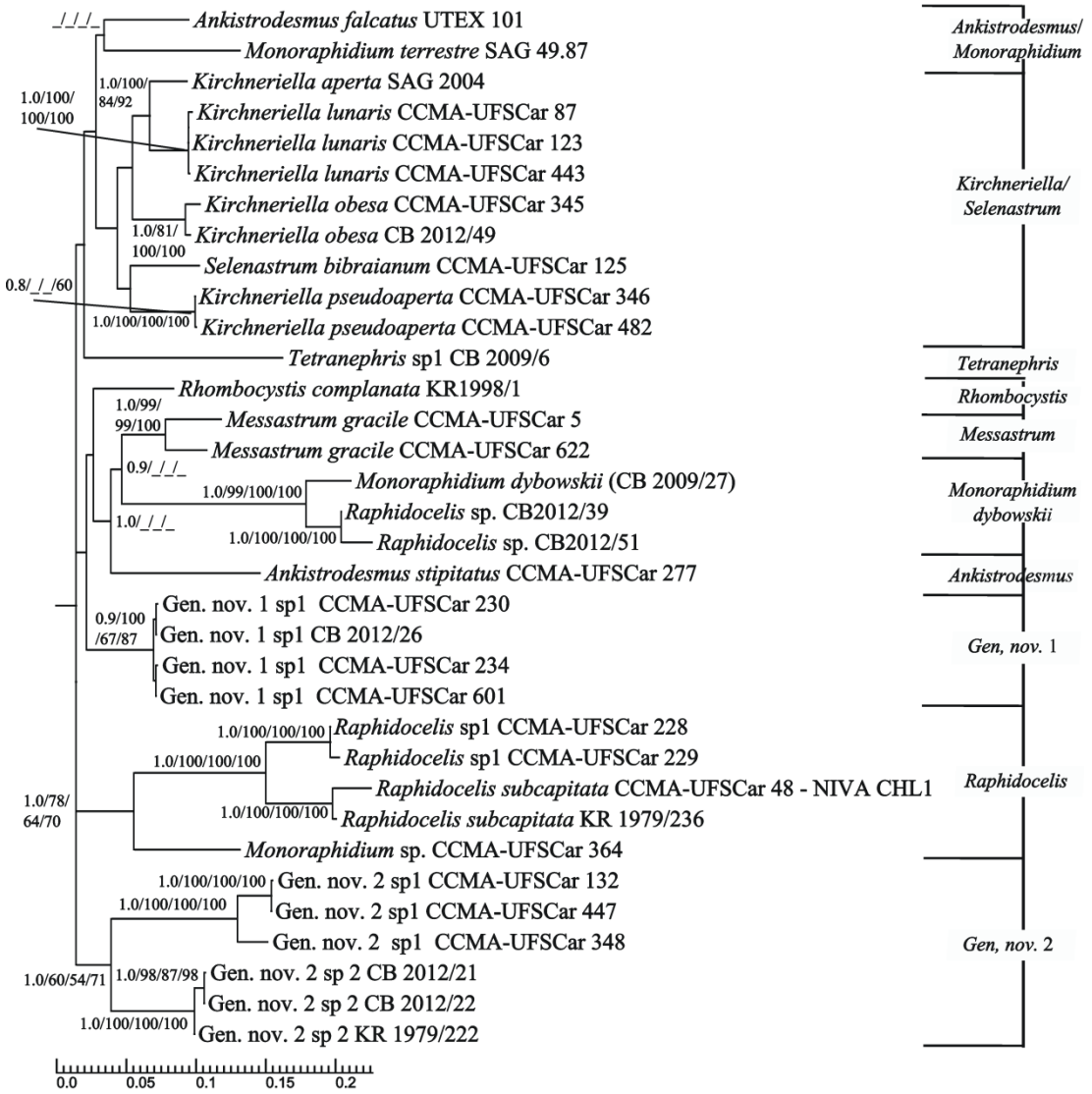

_/_/_
Ankistrodesmus falcatus UTEX 101
Monoraphidium terrestre SAG 49.87
1.0/100/
84/92
Kirchneriella aperta SAG 2004
1.0/100/
100/100
Kirchneriella lunaris CCMA-UFSCar 87
Kirchneriella lunaris CCMA-UFSCar 123
Kirchneriella lunaris CCMA-UFSCar 443
Kirchneriella obesa CCMA-UFSCar 345
1.0/81/
100/100
Kirchneriella obesa CB 2012/49
Selenastrum bibraianum CCMA-UFSCar 125
0.8/_/_/60
Kirchneriella pseudoaperta CCMA-UFSCar 346
1.0/100/100/100
Kirchneriella pseudoaperta CCMA-UFSCar 482
Tetranephris sp1 CB 2009/6
Rhombocystis complanata KR1998/1
1.0/99/
99/100
Messastrum gracile CCMA-UFSCar 5
Messastrum gracile CCMA-UFSCar 622
0.9/_/_
1.0/99/100/100
Monoraphidium dybowskii (CB 2009/27)
Raphidocelis sp. CB2012/39
1.0/_/_
1.0/100/100/100
Raphidocelis sp. CB2012/51
Ankistrodesmus stipitatus CCMA-UFSCar 277
Gen. nov. 1 sp1 CCMA-UFSCar 230
0.9/100
/67/87
Gen. nov. 1 sp1 CB 2012/26
Gen. nov. 1 sp1 CCMA-UFSCar 234
Gen. nov. 1 sp1 CCMA-UFSCar 601
1.0/100/100/100
Raphidocelis sp1 CCMA-UFSCar 228
1.0/100/100/100
Raphidocelis sp1 CCMA-UFSCar 229
Raphidocelis subcapitata CCMA-UFSCar 48 - NIVA CHL1
1.0/78/
64/70
1.0/100/100/100
Raphidocelis subcapitata KR 1979/236
Monoraphidium sp. CCMA-UFSCar 364
1.0/100/100/100
Gen. nov. 2 sp1 CCMA-UFSCar 132
1.0/100/100/100
Gen. nov. 2 sp1 CCMA-UFSCar 447
Gen. nov. 2 sp1 CCMA-UFSCar 348
1.0/60/54/71
1.0/98/87/98
Gen. nov. 2 sp 2 CB 2012/21
Gen. nov. 2 sp 2 CB 2012/22
1.0/100/100/100
Gen. nov. 2 sp 2 KR 1979/222
0.0
0.05
0.1
0.15
0.2
Ankistrodesmus/
Monoraphidium
Kirchneriella/
Selenastrum
Tetranephris
Rhombocystis
Messastrum
Monoraphidium
dybowskii
Ankistrodesmus
Gen, nov. 1
Raphidocelis
Gen, nov. 2

Fig. 2.9

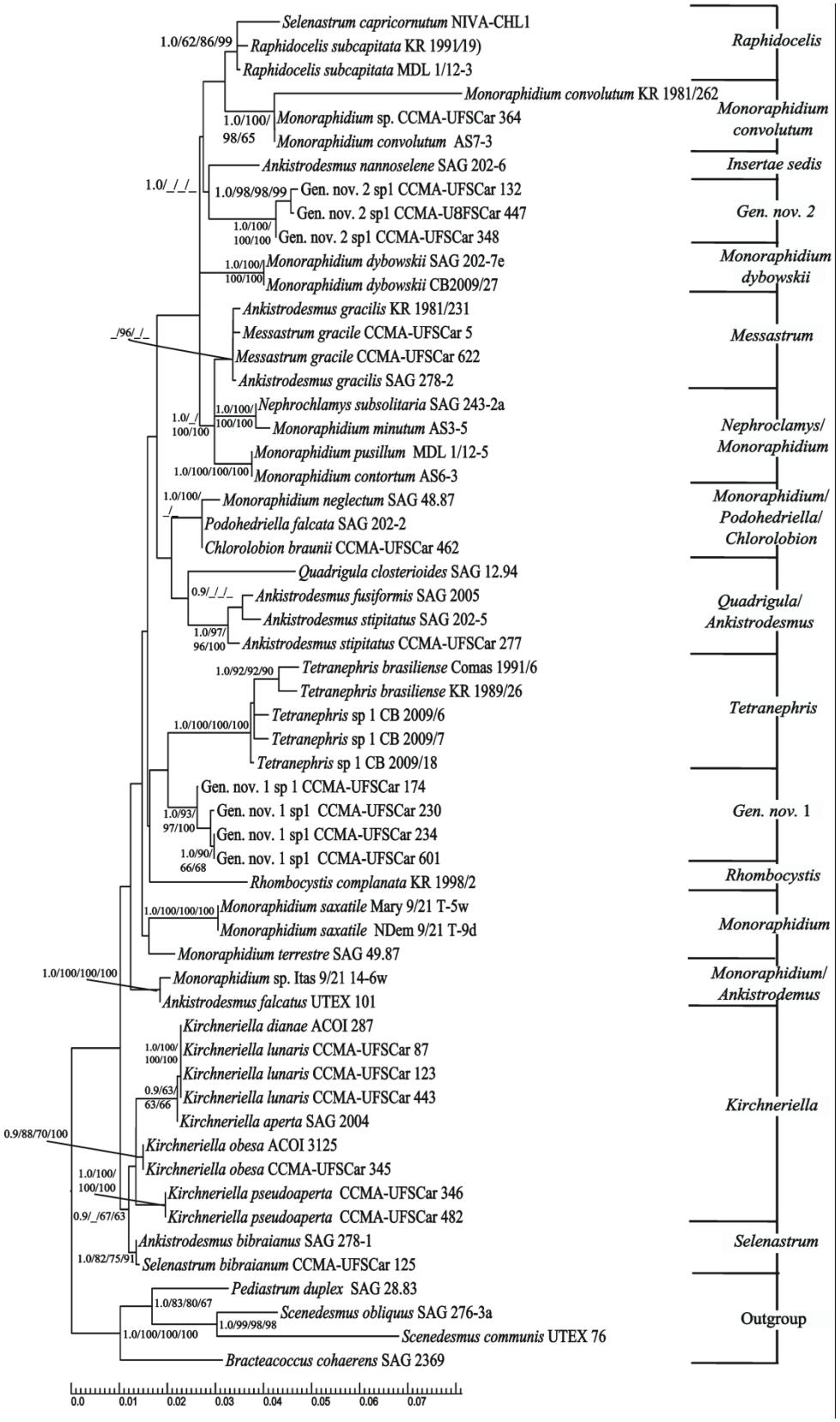

Selenastrum capricornutum NIVA-CHL1
1.0/62/86/99 Raphidocelis subcapitata KR 1991/19)
Raphidocelis subcapitata MDL 1/12-3
Raphidocelis
Monoraphidium convolutum KR 1981/262
1.0/100/ 98/65 Monoraphidium sp. CCMA-UFSCar 364
Monoraphidium convolutum AS7-3
Monoraphidium convolutum
Ankistrodesmus nannoselene SAG 202-6
Insertae sedis
1.0/_/_/_
1.0/98/98/99 Gen. nov. 2 sp1 CCMA-UFSCar 132
Gen. nov. 2 sp1 CCMA-U8FSCar 447
1.0/100/ 100/100 Gen. nov. 2 sp1 CCMA-UFSCar 348
Gen. nov. 2
1.0/100/ 100/100 Monoraphidium dybowskii SAG 202-7e
Monoraphidium dybowskii CB2009/27
Monoraphidium dybowskii
Ankistrodesmus gracilis KR 1981/231
_/96/_/_
Messastrum gracile CCMA-UFSCar 5
Messastrum gracile CCMA-UFSCar 622
Ankistrodesmus gracilis SAG 278-2
Messastrum
1.0/100/ 100/100 Nephrochlamys subsolitaria SAG 243-2a
1.0/_/ 100/100 Monoraphidium minutum AS3-5
Monoraphidium pusillum MDL 1/12-5
1.0/100/100/100 Monoraphidium contortum AS6-3
Nephroclamys/ Monoraphidium
1.0/100/ _/ Monoraphidium neglectum SAG 48.87
Podohedriella falcata SAG 202-2
Chlorolobion braunii CCMA-UFSCar 462
Monoraphidium/ Podohedriella/ Chlorolobion
Quadrigula closterioides SAG 12.94
0.9/_/_/_ Ankistrodesmus fusiformis SAG 2005
Ankistrodesmus stipitatus SAG 202-5
1.0/97/ 96/100 Ankistrodesmus stipitatus CCMA-UFSCar 277
Quadrigula/ Ankistrodesmus
1.0/92/92/90 Tetranephris brasiliense Comas 1991/6
Tetranephris brasiliense KR 1989/26
1.0/100/100/100 Tetranephris sp 1 CB 2009/6
Tetranephris sp 1 CB 2009/7
Tetranephris sp 1 CB 2009/18
Tetranephris
Gen. nov. 1 sp 1 CCMA-UFSCar 174
1.0/93/ 97/100 Gen. nov. 1 sp1 CCMA-UFSCar 230
Gen. nov. 1 sp1 CCMA-UFSCar 234
1.0/90/ 66/68 Gen. nov. 1 sp1 CCMA-UFSCar 601
Gen. nov. 1
Rhombocystis complanata KR 1998/2
Rhombocystis
1.0/100/100/100 Monoraphidium saxatile Mary 9/21 T-5w
Monoraphidium saxatile NDem 9/21 T-9d
Monoraphidium
Monoraphidium terrestre SAG 49.87
1.0/100/100/100 Monoraphidium sp. Itas 9/21 14-6w
Ankistrodesmus falcatus UTEX 101
Monoraphidium/ Ankistrodemus
Kirchneriella dianae ACOI 287
1.0/100/ 100/100 Kirchneriella lunaris CCMA-UFSCar 87
Kirchneriella lunaris CCMA-UFSCar 123
0.9/63/ 63/66 Kirchneriella lunaris CCMA-UFSCar 443
Kirchneriella aperta SAG 2004
0.9/88/70/100 Kirchneriella obesa ACOI 3125
Kirchneriella obesa CCMA-UFSCar 345
1.0/100/ 100/100 Kirchneriella pseudoaperta CCMA-UFSCar 346
0.9/_/67/63 Kirchneriella pseudoaperta CCMA-UFSCar 482
Kirchneriella
Ankistrodesmus bibraianus SAG 278-1
1.0/82/75/91 Selenastrum bibraianum CCMA-UFSCar 125
Selenastrum
Pediastrum duplex SAG 28.83
1.0/83/80/67 Scenedesmus obliquus SAG 276-3a
1.0/99/98/98 Scenedesmus communis UTEX 76
1.0/100/100/100
Outgroup
Bracteacoccus cohaerens SAG 2369
0.0 0.01 0.02 0.03 0.04 0.05 0.06 0.07

Fig. 2.10

Discussão Geral

A partir da grande quantidade de dados moleculares e morfológicos obtidos no presente estudo, tanto de cepas tropicais como de ambientes temperados, pode-se observar que os gêneros *Monoraphidium*, *Kirchneriella* e *Ankistrodesmus* demonstraram ser polifiléticos, também sugerido em trabalhos anteriores (Krienitz *et al.* 2001, Krienitz *et al.* 2011).

A inferência filogenética baseada em 18S rDNA e *rbcL* reafirmam a separação de *Selenastrum bibraianum* e *Selenastrum gracile* (Syn. *Messastrum gracile*) em dois gêneros distintos, levando ao surgimento de *Messastrum* e a descrição de um novo gênero, *Curvastrum*, morfologicamente similar à *Selenastrum*.

O emprego do 18S rDNA e a análise multigene com ITS1-5.8S-ITS2 mostrou que, para a identificação de espécies semelhantes à *Kirchneriella*, tamanho, forma, curvatura ou torção celular são características sutis e relevantes, uma vez que estas características foram cruciais para a identificação das espécies. Ainda, as análises filogenéticas revelaram que o morfotipo associado à *Kirchnriella* ocultava quatro linhagens filogenéticas, levando à descrição de uma espécie nova de *Raphidocelis*, uma espécie nova de *Tetranephris* e dois novos gêneros.

Estudos filogenéticos em Selenastraceae, baseados em 18S rDNA e ITS1-5.8S-ITS2, revelaram que um tipo morfológico pode representar diferentes filotipos, como *Selenastrum* e *Kirchneriella*, sugerindo que a diversidade da família tem sido subestimada consideravelmente quando avaliada morfologicamente (Fawley *et al.* 2006).

O conceito de gêneros em algas verdes cocóides, bastante debatido em trabalhos recentes sobre sistemática de algas, têm gerado discussão (Luo *et al.* 2009) e demonstra ser uma tendência (Luo *et al.* 2010). Vários gêneros têm passado recentemente por reformulação após estudos moleculares. Atualmente, alguns gêneros com morfologia bem conhecidas foram divididos em gêneros muitos menores, como proposto para *Scenedesmus* Meyen (An *et al.* 1999, Krienitz *et al.* 2003) e *Pediastrum* (Buchheim *et al.* 2005), em abordagens filogenéticas com o 18S rDNA.

Embora morfologicamente semelhantes, estudos moleculares evidenciaram que *Raphidocelis* representa uma linhagem molecular não relacionada com *Kirchneriella*, corroborando a separação morfológica destes gêneros (Fawley *et al.* 2006, Krienitz *et al.* 2001, Krienitz *et al.* 2011). Morfotipos semelhantes podem esconder alta diversidade genética dentro dos gêneros *Kirchneriella*, *Tetranephris* e *Raphidocelis*.

Achados semelhantes foram obtidos para *Dictyosphaerium*, onde os morfotipos formaram linhagens distintas dentro de *Chlorella* e *Parachlorella*, apresentando evolução independente e confirmando o polifiletismo do morfotipo *Dictyosphaerium* dentro de *Chlorellaceae* (Luo *et al.* 2010).

Além do fenótipo, discussões sobre conceitos de espécies devem considerar a variação genética (Hilt 2006). A descrição de espécies bem estabelecidas, com população fenotípica conhecida, deve ser investigada de acordo com características moleculares, genéticas, fisiológicas e bioquímicas de todas essas cepas cultivadas que seguem a descrição do tipo de origem (Wood & Leatham 1992), levando à resolução da posição taxonômica das variantes morfológicas do tipo.

O valor taxonômico de caracteres diacríticos utilizados em Selenastraceae foi discutido com base na morfologia e na ultra-estrutura (Krienitz *et al.* 2001)

demonstrando que o conceito de "gêneros pequenos" (Komárek & Fott 1983), incluindo apenas algumas espécies, para a Selenastraceae não era apropriado, uma vez que a existência de muitas formas de transição, e muitas características diacríticas não são confiáveis (Fawley *et al.* 2006). Portanto, a ideia gêneros "pequeno" foi considerada incerta para Selenastraceae (Krienitz *et al.* 2001). Entretanto, em um trabalho posterior Krienitz *et al.* (2011) reempregou esse conceito ao estudar os morfotipos associados à alga teste *Selenastrum capricornutum*, cujo morfotipo está associado a cerca de 10 linhagens evolutivas diferentes.

Nossos resultados sugeriram que o conceito de gêneros pequenos poderia ser aplicado em *Monoraphidium*, *Kirchneriella*, *Selenastrum* e *Messastrum*.

A observação de um número substancial de colônias e organismos solitários (10-50 indivíduos) e observação de todo o ciclo de vida, com especial ênfase para a liberação de autósporos e a formação de colônias, foi proposta para a identificaçao acurada dos taxa compreendidos em Selenastraceae (Garcia *et al.* no prelo)

Algumas descrições originais das espécies foram publicadas faltando informações quanto à morfologia (incluindo holotipos ou iconotipos) não fornecendo todas as informações necessárias para a identificação de muitos taxa.

A combinação das análises moleculares e avaliação morfológica em diferentes fases de crescimento das cepas demonstrou ser importante para a sistemática de Selenastraceae (Garcia *et al.* no prelo).

Somado às informações do ciclo de vida em que estão faltando informações, a ausência de culturas de espécies tipo de centenas de taxa pertencentes a essa família cosmopolita associada à dificuldade de amplificação de alguns marcadores moleculares,

como o *rbcL*, limitam os estudos taxonômicos. Desta forma, faz-se necessário mais esforço para o isolamento de linhagens e posterior estudo taxonomico.

Com o passar dos anos houve uma grande flutuação nas circunscrições dos gêneros e na mudança no ponto de vista dos taxonomistas no que se refere à delimitação morfológica das espécies, refletindo na descrição de novos gêneros morfológicos, tais como *Raphidocelis* (Krienitz *et al.* 2011) e *Pseudokirchneriella*.

Alguns critérios morfológicos podem representar adaptações fenotípicas às condições ambientais e não refletir efetivamente as relações filogenéticas, como a presença e ausencia de pirenóides (Krienitz *et al.* 2004;. Luo *et al.* 2006).

O emprego dos marcadores SSU, ITS-1 e ITS-2 diferenciaram seis gêneros dentro do morfotipo de *Chlorella* Beyerinck [Beijerinck], revelando que vários critérios morfológicos utilizados na identificação (mucilagem e fios de conexão entre as células, forma de vida colonial ou solitária e formação de cerdas) foram considerados características fenotípicas e representaram respostas adaptativas a fatores ambientais, tais como a pressão por grazing, endossimbiose ou estratégias para adaptação ao ambiente terrestre (Luo *et al.* 2010).

O modo de reprodução das algas cocóides não permite o emprego do conceito de espécies de Mayr (1942) limitando os limonologistas à adoção do conceito de espécie morfológica, usando características diacríticas baseadas em morfologia como uma maneira de lidar com essa limitação (Krienitz & Bock 2012).

Estima-se que existam cerca de 14.900 espécies de fitoplâncton de água doce (Bourrelly 1990). Afirma-se que a riqueza de espécies em lagos temperados de água doce é significativamente maior do que nos lagos tropicais (Lewis Jr 1978). Tal

afrmação dá-se como consequência da pouca exploração e conhecimento sobre a diversidade de microalgas verdes e a filogenia de algas nos trópicos.

Além disso, pouco se sabe sobre as distribuições geográficas de algas verdes cocóides apesar de sua importância nos ecossistema de água doce e do avanço na taxonomia do grupo (Norton *et al.* 1996;. Padisák *et al.* 2015).

Estudos filogenéticos têm papel fundamental para fornecer sequências de referência para aplicações práticas de alguns estudos científicos, como abordagens metabarcoding (Ji *et al.* 2013) e seqüenciamentos de nova geração, que carecem de referências para espécies de fitoplâncton (Eiler *et al.* 2012;. Pawlowski *et al.* 2012).

Referências bibliográficas

An, S., T. Friedl & E. Hegewald, (1999). Phylogenetic Relationships of *Scenedesmus* and *Scenedesmus*-like Coccoid Green Algae as Inferred from ITS-2 rDNA Sequence Comparisons. Plant Biology 1(4):418-428.

Bourrelly, P., (1990). Les Algues d'eau douce. Initation a la Systématique, I: Les Algues Vertes. 572pp. N Boubée & Cie Paris.

Buchheim, M., J. Buchheim, T. Carlson, A. Braband, D. Hepperle, L. Krienitz, M. Wolf & E. Hegewald, (2005). Phylogeny of the Hydrodictyaceae (Chlorophyceae): inferences from rDNA data1. Journal of Phycology 41(5):1039-1054.

Eiler, A., S. Drakare, S. Bertilsson, J. Pernthaler, S. Peura, C. Rofner, K. Simek, Y. Yang, P. Znachor, and E. S. Lindström. 2013. Unveiling distribution patterns of freshwater phytoplankton by a next generation sequencing based approach. PLoS ONE 8:e53516.

Fawley, M. W., M. L. Dean, S. K. Dimmer & K. P. Fawley, (2006). Evaluating the morphospecies concept in the Selenastraceae (Chlorophyceae, Chlorophyta) Journal of Phycology 42(1):142-154.

Hilt, S., (2006). Allelopathic inhibition of epiphytes by submerged macrophytes. Aquatic Botany 85(3):252-256 doi:DOI 10.1016/j.aquabot.2006.05.004.

Ji, Y., L. Ashton, S. M. Pedley, D. P. Edwards, Y. Tang, A. Nakamura, R. Kitching, P. M. Dolman, P. Woodcock & F. A. Edwards, (2013). Reliable, verifiable and efficient monitoring of biodiversity via metabarcoding. Ecology letters 16(10):1245-1257.

Komárek, J. & B. Fott, (1983). Das Phytoplankton des Sübwassers. Systematik und Biologie. 7. Teil, 1. Hälfte. Chlorophyceae (Grünalgen) Ordnung: Chroococcales. E. Schweizerbart'sche Verlagsbuchhandlung (Nägele u. Obemiller), Stuttgart. 1043p.

Krienitz, L. & C. Bock, (2012). Present state of the systematics of planktonic coccoid green algae of inland waters. Hydrobiologia 698(1):295-326.

Krienitz, L., C. Bock, H. Nozaki & M. Wolf, (2011). SSU rRNA gene phylogeny of morphospecies affiliated to the bioassay alga "*Selenastrum capricornutum*" recovered the polyphyletic origin of crescent-shaped Chlorophyta. Journal of Phycology 47(4):880-893.

Krienitz, L., E. Hegewald, D. Hepperle & M. Wolf, (2003). Systematics of coccoid green algae: morphology versus 18S rRNA gene phylogeny. Biologia 58:437-446.

Krienitz, L., I. Ustinova, T. Friedl & V. A. Huss, (2001). Traditional generic concepts versus 18S rRNA gene phylogeny in the green algal family Selenastraceae (Chlorophyceae, Chlorophyta). Journal of Phycology 37(5):852-865.

Lewis Jr, W. M., (1978). A compositional, phytogeographical and elementary structural analysis of the phytoplankton in a tropical lake: Lake Lanao, Philippines. The Journal of Ecology:213-226.

Luo, W., T. Pröschold, C. Bock & L. Krienitz, (2010). Generic concept in *Chlorella*-related coccoid green algae (Chlorophyta, Trebouxiophyceae). Plant Biology 12(3):545-553.

Mayr, E., (1942). Systematics and the origin of species, from the viewpoint of a zoologist. Harvard University Press.

Norton, T. A., M. Melkonian & R. A. Andersen, (1996). Algal biodiversity. Phycologia 35(4):308-326.

Padisák, J., G. Vasas & G. Borics, (2015). Phycogeography of freshwater phytoplankton: traditional knowledge and new molecular tools. Hydrobiologia:1-25.

Pawlowski, J., S. Audic, S. Adl, D. Bass, L. Belbahri, C. Berney, S. S. Bowser, I. Cepicka, J. Decelle & M. Dunthorn, (2012). CBOL protist working group: barcoding eukaryotic richness beyond the animal, plant, and fungal kingdoms. PLoS biology 10(11):e1001419.

Wood, A. M. & T. Leatham, (1992). The species concept in phytoplankton ecology. Journal of Phycology 28(6):723-729.

Garcia, T. S., Bock C., Sant'Anna C. L., Bagatini I. L., Wodniok S., Vieira A. A. H., 2016. Selenastraceae (Sphaeropleales, Chlorophyceae): *rbcL*, 18S rDNA and

ITS-2 secondary structure enlightens traditional taxonomy, with description of two new genera, *Messastrum* gen. nov. and *Curvastrum* gen. nov. Fottea, in press.

Krienitz, L., E. H. Hegewald, D. Hepperle, V. A. Huss, T. Rohr & M. Wolf, 2004. Phylogenetic relationship of *Chlorella* and *Parachlorella* gen. nov. (Chlorophyta, Trebouxiophyceae). Phycologia 43(5):529-542.

Luo, W., S. Pflugmacher, T. Pröschold, N. Walz & L. Krienitz, 2006. Genotype versus phenotype variability in *Chlorella* and *Micractinium* (Chlorophyta, Trebouxiophyceae). Protist 157(3):315-333.

Conclusões

1. O morfotipo associado à *Selenastrum* revelou três linhagens evolutivas distintas, conduzindo à separação de *Selenastrum bibraianum* e *Selenastrum gracile* (Syn. *Messastrum gracile*) em dois gêneros, levando ao surgimento de *Messastrum* e à descrição de um novo gênero, *Curvastrum*, morfologicamente similar à *Selenastrum*.

2. Embora morfologicamente semelhantes Raphidocelis representa uma linhagem molecular não relacionada com *Kirchneriella*;

3. O morfotipo associado à *Kirchneriella* revelou quatro linhagens evolutivas distintas, conduzindo a descrição de dois novos gêneros, uma nova espécie de *Raphidocelis* e uma nova espécie de *Tetranephris*;

4. A análise molecular demonstrou que os gêneros *Kirchneriella*, *Selenastrum* e *Monoraphidium* possuem origem polifilética;

5. Embora as características morfologicas tenham sido avaliadas e consideradas importantes para a diferenciação dos taxa, a filogenia baseada em 18S rDNA, *rbcL* e ITS1-5.8S-ITS2 foram cruciais para subsidiar as decisões taxonômicas neste grupo de algas verdes cocóides;

6. As caracteristicas diacríticas utilizadas em Selenastraceae se mostraram relevantes para a separação dos taxa dessa família. Deve-se, entretanto, utilizá-los em conjunto e não individualmente;

7. A combinação das análises moleculares e avaliação morfológica em diferentes fases de crescimento das cepas foi importante para a sistemática de Selenastraceae;

8. O uso de cepas provenientes de ambientes tropicais e temperados, provenientes de três continentes distintos, comprovou o cosmopolitismo de Selenastraceae.