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Raquel Filipa de Almeida Amaral

Diversity, Phylogeny and Pigment Studies of Eustigmatophyceae, a Poorly Known Group of Microalgae

Doctoral thesis in Biosciences, specialization in Microbiology,
under the supervision of Professor Doctor Lília Maria Antunes dos Santos and
co-supervision of Professor Doctor Marek Eliáš and Professor Doctor Sérgio Seixas de Melo,
presented to the Life Sciences Department of the Faculty of Sciences and Technology
of the University of Coimbra.

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DEPARTAMENTO DE CIÊNCIAS DA VIDA
FACULDADE DE CIÊNCIAS E TECNOLOGIA
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Publications

(updated papers publication status, which reflects the time of the defense)

Work resulting from this PhD originated three publications as a first author:

(1) Amaral R., Melo JSS and Santos LMA, 2020. Pigments from Eustigmatophyceae: relevance of the violaxanthin cycle for carotenoid production. *Journal of Applied Phycology*, accepted. – chapter 6

(2) Amaral R, Fawley KP, Němcová Y, Ševčíková T, Lukešová A, Fawley MW, Santos LMA and Eliáš M, 2020. Towards modern classification of eustigmatophytes including the description of Neomonodaceae, fam. nov., and three new genera. *Journal of Phycology* 56: 630-648 – chapter 2

(3) Amaral R, Eliáš M and Santos LMA, 2019. *Characiopsis* Borzì belongs to the Eustigmatophyceae, 2020. *European Journal of Phycology* published online https://doi.org/10.1080/09670262.2020.1808712. – chapter 3

International collaborations during the PhD work also originated the following publications:

(1) Ševčíková T, Yurchenko T, Fawley KP, Amaral R, Strnad H, Santos LMA, Fawley MW, Eliáš M, 2019. Plastid Genomes and Proteins Illuminate the Evolution of Eustigmatophyte Algae and Their Bacterial Endosymbionts. *Genome Biology and Evolution* 11(2): 362-379.

(2) Yurchenko T, Ševčíková T, Přibyl P, El Karkouri K, Klimeš V, Amaral R, Zbránková V, Kim E, Raoult D, Santos LMA, Eliáš M, 2019. A gene transfer event suggests a long-term partnership between eustigmatophyte algae and a novel lineage of endosymbiotic bacteria. *The ISME Journal* 12: 2163–2175.

(3) Eliáš M, Amaral R, Fawley KP, Fawley MW, Němcová Y, Neustupa J, Přibyl P, Santos LMA, and Ševčíková T, 2017. Eustigmatophyta. *In*: Handbook of the Protists. John M. Archibald, Alastair G.B. Simpson, Claudio H. Slamovits, Lynn Margulis, Michael Melkonian, David J. Chapman, John O. Corliss (Eds). Springer International Publishing, Switzerland, pp. 1-39.

4) Assunção MFG, Amaral R, Martins CB, Ferreira JD, Ressurreição S, Santos SD, Varejão JMTB and Santos LMA, 2016. Screening microalgae as potential sources of antioxidants. *Journal of Applied Phycology* 29: 865–877.

Abbreviations

18S rRNA – Small subunit of the ribossomal RNA

ACOI – Coimbra Collection of Algae

ANOVA – Analysis of variance

BCCO – Biology Centre Collection of Organisms

CAUP - Culture Collection of Algae at Charles University

DIC – Differential Interference Contrast

DMF – dimethylformamide

HPLC – DAD – High Performance Liquid Chromatography with Diode array Detector

IUPAC – International Union of Pure and Applied Chemistry

MeOH – methanol

PCR – Polymerase Chain Reaction

*rbc*L – Ribulose bisphosphate carboxylase large chain

TEM – Transmission Electron Microscopy

UTEX – Culture Collection of Algae at the University of Texas, Austin

Resumo

A classe de microalgas Eustigmatophyceae foi estabelecida em 1970 devido à transferência de organismos previamente incluídos na classe Xanthophyceae, cuja estrutura celular de células vegetativas e reprodutoras se apresentava única. Diferenças pigmentares corroboraram esta separação de microalgas predominantemente de água doce e que exibem uma cor verde-amarelada dos seus cloroplastos. Durante cerca de quatro décadas, os estudos morfológicos, pigmentares ou moleculares focaram-se num número muito reduzido de taxa atribuídos à nova classe, o que condicionou o progresso no seu conhecimento. O isolamento seletivo de potenciais novos taxa permitiu que presentemente a Algoteca de Coimbra (ACOI) detenha um elevado número de estirpes de géneros pouco estudados.

Assim, os objetivos deste trabalho foram definidos com foco nos taxa existentes em ACOI e consistiram em (1) aplicar uma abordagem polifásica ao seu estudo, combinando dados morfológicos com dados moleculares, para confirmar a sua posição na classe Eustigmatophyceae e revelar a sua filogenia e (2) determinar o conteúdo pigmentar das estirpes para complementar a abordagem anterior e verificar o perfil típico da classe, focando o conteúdo em carotenoides de potencial interesse biotecnológico.

Para clarificar a taxonomia e filogenia dos taxa pouco estudados, foram selecionadas estirpes ACOI dos géneros *Characiopsis* e *Pseudostaurastrum*. Foram utilizadas técnicas de microscopia ótica e eletrónica para obtenção de dados morfológicos. Para o estudo taxonómico e filogenético sequenciaram-se o gene nuclear 18S rRNA, para a obtenção da filogenia da classe e o gene cloroplastidial *rbc*L gene, para filogenia com maior resolução.

Os estudos citológicos e moleculares revelaram que o género *Characiopsis* pertence à classe Eustigmatophyceae e não à classe Xanthophyceae. As estirpes de *C. ovalis*, *C. minima* and *C.*

aquilonaris posicionam-se numa linhagem nova, a família Neomonodaceae fam. nov. que passa a incluir organismos cujas células não possuem pirenoide, ficando os nomes anteriores como sinónimos de géneros estudados. A nova família contém quatro géneros, um destes, *Pseudellipsoidion*, foi reavaliado e a taxonomia atualizada, e três novos géneros, *Neomonodus* gen. nov., *Characiopsiella* gen. nov. e *Munda* gen. nov.. Ficou provado que outras estirpes, nomeadamente *C. pernana*, *C. acuta*, *C. longipes*, *C. minutissima* e *C. cedercreutzii* são eustigmatofíceas e o género *Characiopsis* foi formalmente transferido da classe Xanthophyceae para a classe Eustigmatophyceae. O género *Pseudostaurastrum*, reconhecido como uma linhagem filogenética profunda do grupo ordinal *Goniochloridales*, com duas estirpes conhecidas, foi ampliado com o presente estudo. A coleção única de estirpes ACOI permitiu a obtenção de árvores filogenéticas mais completas do género e confirmar a sua monofilia por análise do gene *rbc*L.

A determinação do conteúdo pigmentar de 27 estirpes pertencentes a 10 géneros diferentes, por cromatografia líquida de alta performance com detetor de fotodíodos (HPLC-DAD), permitiu confirmar o padrão pigmentar típico das eustigmatofíceas, com clorofila a, ausência de clorofila b, e três pigmentos mais abundantes, por ordem decrescente: violaxantina, vaucheriaxantina e β-caroteno. Foram determinados valores elevados de violaxantina, representando cerca de metade do total de pigmentos em *Monodopsis unipapilla* ACOI 2938. O cultivo em baixa luminosidade é o fator apontado para a obtenção dos valores elevados neste carotenoide. O perfil pigmentar de estirpes de Neomonodaceae e de *Characiopsis* foi obtido pela primeira vez.

As conclusões principais são que o género *Characiopsis* pertence à classe Eustigmatophyceae e na sua forma anterior era polifilético. Algumas espécies posicionam-se no Eustigmataceae group e retêm o nome genérico *Characiopsis*, enquanto outras compõem a nova família Neomonodaceae fam. nov., distribuindo-se pelos géneros *Neomonodus* gen. nov.,

Pseudellipsoidion, *Characiopsiella* gen. nov., *Munda* gen. nov., não tendo sido detetado pirenoide nestes organismos. O género *Pseudostaurastrum* era anteriormente pouco estudado, foi agora ampliado em 19 estirpes, aumentando a diversidade em mais três grupos moleculares, correspondendo a *P. hastantum*, *P. lobulatum* e um terceiro sem atribuição taxonómica. Ficou consolidado o perfil pigmentar das eustigmatofíceas, em todas as estirpes estudadas foi detetada clorofila a, violaxantina, vaucheriaxantina e β-caroteno. A quantificação relativa mostrou que as eustigmatofíceas são ricas em violaxantina, sendo sempre o carotenoide mais abundante nas estirpes estudadas.

Com este estudo, a classe Eustigmatophyceae aumentou significativamente, com a adição de 66 novas estirpes, 55 das quais isoladas e mantidas em ACOI e 10 estirpes de outras coleções. É também agora sabido que o perfil pigmentar das eustigmatofíceas é consistente, tendo ficando triadas as melhores produtoras de pigmentos com potencial uso em biotecnologia.

Palavras-chave:

Eustigmatophyceae, Neomonodaceae, *Characiopsis*, *Pseudostaurastrum*, filogenia, taxonomia, 18S rRNA, *rbc*L, pigmentos, HPLC.

Abstract

The microalgal class Eustigmatophyceae was established in 1970 by the transfer of organisms previously included in the Xanthophyceae, due to their unique vegetative and reproductive cell structure. Differences in pigment content confirmed the separation of mostly freshwater microalgae with yellow-green chloroplasts. During the following four decades, morphological, pigment and molecular studies were focused in the reduced number of taxa which were attributed to the class, which limited progress in its knowledge. The selective isolation of potentially new taxa originated a high number of understudied genera held at Coimbra Collection of Algae (ACOI).

The objectives of this work were defined with a focus on the taxa held at ACOI and consisted in (1) the application of a polyphasic approach combining morphological studies with molecular data for confirming their position in class eustigmatophyte and for revealing their phylogeny and (2) to determine the pigment content of the strains in order to complement the study and to verify the typical eustigmatophyte pigment profile with a focus on the carotenoids with potential biotechnological interest.

In order to clarify the taxonomy and phylogeny of understudied taxa, the ACOI genera *Characiopsis* and *Pseudostaurastrum* were selected. Optical and electron microscopy techniques were used for obtaining morphological data. For taxonomy and phylogeny studies, 18S rRNA gene sequences were obtained for backbone overview of the phylogeny, and *rbc*L gene sequences for allowing the determination of internal phylogeny.

The cytological and molecular studies revealed that the large genus *Characiopsis* belongs to the Eustigmatophyceae rather than the Xanthophyceae. Strains of *C. ovalis*, *C. minima* and *C. aquilonaris* are positioned in the new familial lineage, the Neomonodaceae fam. nov. which

includes organisms devoid of a pyrenoid with previous names rendered synonyms of the newly established taxa. Other strains with *Characiopsis* morphology namely *C. pernana*, *C. acuta*, *C. longipes*, *C. minutissima*, *C. cedercreutzii*, were also proved to be eustigmatophytes and the genus was formally transferred from the Xanthophyceae to the Eustigmatophyceae. The genus *Pseudostaurastrum* was already known to be a deep monophyletic lineage of the ordinal clade *Goniochloridales*, however with only two known strains. This genus was enlarged with the present study. The unique ACOI collection of these sensitive and rare organisms enabled a broader phylogenetic overview of this genus and its monophyly was proven by *rbc*L gene analysis.

The determination of the pigment content of 27 strains belonging to 10 different genera was performed by high performance liquid chromatography with diode array detection (HPLC-DAD). The characteristic eustigmatophyte pattern was confirmed, with the detection of chlorophyll a and no chlorophyll b, and the three major carotenoids namely from the most to the least abundant: violaxanthin, vaucheriaxanthin and β-carotene. The study revealed high amount of violaxanthin, representing around half the total pigments in *Monodopsis unipapilla* ACOI 2938. The low light conditions in cultivation of these strains is the factor pointed out as justifying the high content in this carotenoid. The pigment profile of Neomonodaceae and *Characiopsis* strains was obtained for the first time.

The main conclusions are that the genus *Characiopsis* belongs to the Eustigmatophyceae and it was polyphyletic in its previous form. Some strains are positioned in the Eustigmataceae group and retain the generic name *Characiopsis* while others compose the new family Neomonodaceae fam. nov., and distribute by the genera *Neomonodus* gen. nov., *Pseudellipsoidion*, *Characiopsiella* gen. nov., *Munda* gen. nov., devoid of a pyrenoid. Genus *Pseudostaurastrum* was understudied and it is now enlarged in 19 strains, with 3 new molecular groups, corresponding to *P. hastantum*, *P. lobulatum* and a third taxonomically undetermined

group. The eustigmatophyte pigmentary profile was consolidated, in all studied strains chlorophyll a, violaxanthin, vaucheriaxanthin and β-carotene were detected. The relative quantification showed that the eustigmatophyceae are rich in violaxanthin, the most abundant carotenoid in the studied strains.

With this study, the class Eustigmatophyceae enlarged significantly, with the addition of 66 new strains, 55 of which are ACOI isolates and 10 are cultures from other collections. Is is also now known that the pigmentary profile of the eustigmatophytes is consistent and the strains were screened, and the best producers of biotechnologically interesting strains were listed.

Keywords:

Eustigmatophyceae, Neomonodaceae, *Characiopsis*, *Pseudostaurastrum*, Phylogeny, taxonomy, 18S rRNA, *rbc*L, pigments, HPLC.

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Figures and Tables

1.

General Introduction

The Eustigmatophyceae is a class of nearly ubiquitous yellow-green microalgae, with a peculiar story. The chronology of the class may be divided into three main periods, corresponding to the methodological approaches applied to their study, through times of evolving analytical scientific resources. The study of this class was performed by researchers who pursued the objective of finding new members and informally referring to them affectionately as "eustigs".

1.1. 1970–80s: The rise of Eustigmatophyceae by uncovering misplaced organisms

The very first acknowledgement that microalgal class Xanthophyceae was polyphyletic goes back to 1969, when Whittle and Casselton found that among the xanthophytes they were surveying for pigment analysis, some strains had different pigment contents, strikingly lacking antheraxanthin and having violaxanthin as the major xanthophyll. The authors refer to Hibberd's personal communication that these strains were eligible to be included in the new class Eustigmatophyceae, to be described shortly after (Hibberd and Leedale 1970). The name of the class evokes a conspicuous eyespot in its unique zoospores, described by comparative examination of vegetative cells and zoospores with some xanthophyte strains (Hibberd and Leedale, 1971 and 1972). The eustigmatophytes exhibit i) an extraplastidial eyespot associated with a flagellar swelling present at the basis of the long, mastigoneme-bearing flagellum in the zoospores, ii) chloroplasts devoid of a girdle lamella, iii) lamellate vesicles scattered throughout the cytoplasm, in both vegetative cells and zoospores, and iv) the presence of a reddish globule in vegetative cells (Hibberd and Leedale 1970, 1971).

Still under the taxonomic turbulence environment around these yellow-green organisms, one of the founders of Eustigmatophyceae, GF Leedale, was visiting the lab of Lee and Bold who were inspecting some material isolated from a Texas site, with some organisms having

an attaching stipe. He gave them his taxonomic opinion that they had isolated one possible member of the newly described class and detailed studies gave rise to the new eustigmatophyte stipitate *Pseudocharaciopsis texensis* (Lee and Bold 1973).

Later, DJ Hibberd published a review with the intent of establishing some organization within the class (Hibberd 1981). He gave the basis for accurate identification of eustigmatophyte organisms and established the taxonomy of the class. The taxonomical scheme featured in this monographic review of eustigmatophytes became the most frequently adopted (Table 1.1.). It consisted in division Eustigmatophyta, with a single class Eustigmatophyceae and a single order Eustigmatales comprising four families, the Eustigmataceae, the Pseudocharaciopsidaceae, the Chlorobotryaceae and the Monodopsidaceae (Hibberd 1981). A first mention of an order for the Eustigmatophyceae was the Pseudocharaciopsidales, following the establishment of Lee and Bold's systematic approach for genus *Pseudocharaciopsis* (Lee and Bold 1973) but it was considered as not validly published (Hibberd 1981).

Table 1.1. Taxonomy of the class Eustigmatophyceae, according to Hibberd (1981).

Order	**Family**	**Genus**
Eustigmatales	Eustigmataceae	*Vischeria* *Eustigmatos*
	Pseudocharaciopsidaceae	*Pseudocharaciopsis*
	Chlorobotryaceae	*Chlorobotrys*
	Monodopsidaceae	*Monodopsis* *Nannochloropsis*

Since its establishment, the diversity of the class has enlarged in these decades by i) the transfer of members, mostly from the Xanthophyceae (Lee and Bold 1973, Hibberd 1974, Antia et al. 1975, Hibberd 1981)- in this case, the use of the epithet *Pseudo* prior to the old xanthophyte name was sometimes used, eg. *Pseudocharaciopsis*; ii) by adding newly isolated strains to the growing list of eustigmatophytes (Lubián 1982, Preisig and Wilhelm 1989).

The pigment profile of the Eustigmatophyceae was a relevant segregating characteristic since the establishment of the class. Complementary pigment studies of these organisms were therefore developing in parallel with the morphological studies (Whittle and Casselton 1969, 1975, Antia et al. 1975, Antia and Cheng 1982, Brown 1987, Preisig and Wilhelm 1989).

1.2. 1990–2000: Eustigmatophyte diversity within the ACOI Collection

The ACOI Culture Collection was started in 1972 as an academic collection of the Department of Botany, University of Coimbra, for enabling the immediate provision of microalgal strains for ultrastructural studies (Santos and Santos 2004). It was established by initiative of cytologist professor JF Mesquita with the collaboration of MF Santos, a researcher on algal taxonomy that became responsible for the isolation, maintenance and taxonomic identification of the cultures until her retirement. A first list of ACOI strains was published in 1986 with 167 taxa (Santos and Mesquita 1986) and two additional lists of more isolates were later reported, accounting for 88 (Santos 1988) and 194 (Santos et al. 1993). Most isolations were made from sites located in the center of Portugal.

During the 90's a substantial amount of ultrastructural and morphologically based studies were performed. Following her studies on cytology and ultrastructure of Eustigmatophyceae (Santos 1990, Santos and Leedale 1991, Vicente and Santos 1991, Santos and Leedale 1992, Santos and Leedale 1995, Santos 1996, Santos et al. 1997), LMA Santos' concern was to

determine the diversity of eustigmatophytes. So, she started a field campaign to find and isolate new eustigmatophytes, in collaboration with MF Santos. This effort originated ca. 80 putative eustigmatophytes to the ACOI collection and many isolates from other taxonomic groups, namely euglenophytes (Santos and Santos 2004). Most of the isolated eustigmatophytes kept at ACOI are stipitates with *Characiopsis*-like morphology, others are rare or difficult strains to cultivate and many are not available in any other worldwide collection. ACOI is therefore a treasure trove of microalgae in general (about 4000 strains) and specifically of eustigmatophytes (acoi.ci.uc.pt).

Light and electron microscopy studies were performed in several of these isolates during the 90's and afterwards, in order to confirm their eustigmatophyte nature (Osório et al. 1999, Santos and Santos 2001). However, much of this knowledge remained unpublished, since LMA Santos wanted to combine and complete the obtained morphological data with molecular data, what is now called a polyphasic approach, and such tools were not yet easily available. The use of molecular data for eustigmatophyte studies was starting, with studies confirming the monophyletic nature of the class (Bhattacharya et al. 1992, Andersen et al. 1998) and the 18S gene sequencing of *Nannochloropsis granulata* (Karlson et al. 1996). Other light and electron microscopic studies were published during this period to report new eustigmatophyte species (Schnepf et al. 1995/96, Karlson et al. 1996).

1.3. 2000s: Molecular phylogeny studies and the growing interest on eustigmatophytes for biotechnological purposes

Molecular studies in the Eustigmatophyceae

Although the use of molecular data for determining the positions of heterokont classes of microalgae started in the 1990's with the use of *rbc*L gene analysis (Daugbjerg and Andersen 1997), as previously mentioned, molecular methods were not commonly used for

eustigmatophyte studies during the first decade of the millennium. Only two eustigmatophyte genera were analyzed and their phylogeny clarified. The description of *Pseudellipsoidion edaphicum* was still based on morphology (Neustupa and Němcová 2001) and the unknown diversity of the class was evident (Santos and Santos 2001). First attempts of collaboration for the purpose of molecular data on ACOI strains were done in 1993 and 1995 with the german groups of M Melkonian and T Friedl, respectively. However, these data did not generate published phylogenies. The phylogenetic position of *Pseudotraedriella kamillae* was then determined (Hegewald et al. 2007), followed by another study focused on *Nannochloropsis* phylogeny which generated the first molecular based overview of eustigmatophyte phylogeny (Prior et al. 2009).

With the liberalization of molecular techniques through their growingly generalized use at lower prices, new genera were added to the class at a higher pace during the second decade of the millennium, namely *Trachydiscus* (Přibyl et al. 2012), *Vacuoliviride* (Nakayama et al. 2015), *Microchloropsis* (Fawley et al. 2015) and *Paraeustigmatos* (Fawley et al. 2019). Substantial cultivation efforts were conducted by K Fawley and M Fawley, who provided a phylogenetic characterization of an array of new organisms isolated from U.S.A. freshwater environments (Fawley et al. 2014). This molecular analysis strikingly unveiled a new deeply diverged clade at the ordinal rank, informally called clade *Goniochloridales*.

During the second decade the taxonomy of Eustigmatophyceae was still basically Hibberd´s scheme (Hibberd 1981) with some modifications (Table 1.3.1.). The traditional order Eustigmatales comprised 3 clades at the family level, including two of the original families described by Hibberd, the Eustigmataceae and the Monodopsidaceae, and a third clade informally called the *Pseudellipsoidion* group; the Loboceae previously reported was considered as invalid and abandoned (Fawley et al. 2014). The Eustigmataceae was informally called the Eustigmataceae group because its taxonomic limits were, and still remain, under study. It is

interpreted as a monophyletic family that merges the traditional Hibberd's families Eustigmataceae, Chlorobotrydaceae and Pseudocharaciopsidaceae (Fawley et al. 2014, Eliáš et al. 2017). A third monophyletic clade of Eustigmatales was acknowledged and informally named the *Pseudelliopsoidion* group (Fawley et al. 2014, Eliáš et al. 2017).

Table 1.3.1. Taxonomy of the Eustigmatophyceae in 2014 (Fawley et al. 2014).

Order	**Family**	**Taxa**
Eustigmatales	Eustigmataceae group	*Vischeria* *Eustigmatos* *Chlorobotrys regularis* *Pseudocharaciopsis minuta*
	Monodopsidaceae	*Monodopsis* *Nannochloropsis* *Pseudotëtraedriella kamillae*
	Pseudellipsoidion group	*Pseudellipsoidion edaphicum* *Pseudocharaciopsis ovalis* several undescribed strains from U.S.A.
clade *Goniochloridales*		*Trachydiscus minutus* *Goniochloris sculpta* *Pseudostaurastrum*

Interest on eustigmatophytes as a source of biotechnologically interesting compounds

During the second decade of the millennium, the bioprospecting of microalgae for discovering biotechnologically interesting compounds doubled in number of published papers when compared to the three previous decades. The most studied topic was the determination of

lipidic content and productivity for alternative biofuel applications (Stoyneva-Gärtner et al. 2019b).

The Eustigmatophyceae have gained growing attention from the biotechnological community, with applied research most dedicated to (by order of importance): lipids, medicine and cosmetics, pigments, nutrition (food and feed), environmental applications and vitamins (Stoyneva-Gärtner et al. 2019b). Many compounds found in eustigmatophytes exhibit antioxidant activity (Table 1.3.2.), which is commonly linked with anti–inflammatory and anti–cancer activities, (Lauritano et al. 2016) also detected in eustigmatophytes (Table 1.3.2.).

The most studied compounds are the lipids, either for biofuel or nutritional applications with a focus on the genus *Nannochloropsis* and *Vischeria*. The first studies were in the 1970s but most work was performed already in the 2000s (Table 1.3.2).

During the second decade of the millennium there is a predominance of studies dedicated to evaluating the possible use of eustigmatophytes for medicine and cosmetic uses (Stoyneva-Gärtner et al. 2019b), as well as for nutritional purposes (see also Assunção et al. 2019), again with a predominance for *Nannochloropsis* (and its derived genus *Microchloropsis*) and *Vischeria* (Table 1.3.2.). These two genera have been by far the most studied in all biotechnological fields (Stoyneva-Gärtner et al. 2019b).

A study dedicated to bioprospect the antioxidant activity of extracts from ACOI strains included several different taxa of major taxonomic groups: Cyanophyceae, Haptophyceae, Chrysophyceae, Crytophyceae, Rhodophyceae, Chlorophyta, Xanthophyceae, Euglenophyceae and also Eustigmatophyceae (Assunção et al. 2016). One strain of *Vischeria*, *Goniochloris, Pseudostaurastrum, Dioxys,* two *Chlorobotrys* and seven strains identified as *Characiopsis* were tested. The eustigmatophyte extracts proved to have the highest antioxidant capacity observed in the whole study, with values higher than fresh raspberry determined for *Vischeria helvetica* ACOI 299 and *Munda aquilonaris* ACOI 2424 (Assunção et al. 2016).

The interest on the commercial use of eustigmatophyte pigments started in the 1980s with the discovery of asthaxanthin in *N. oculata* (Antia and Cheng 1982). Asthaxanthin is one of the most lucrative microalgal-derived pigment already in market (Li et al. 2011) so it was a significative finding for the purpose of bioprospecting eustigmatophytes. It was only after 2000 that most studies were performed, mostly in *Vischeria* until recently a comprehensive study of eustigmatophyte pigments was released (Stoyneva-Gärtner et al. 2019a). The most valued pigments pointed out by the biotechnology sector, with potential use in commercial applications are asthaxanthin, β-carotene, violaxanthin, lutein, zeaxanthin, canthaxanthin and chlorophyll a (Table 1.3.2. and references therein). *Nannochloropsis*/*Microchloropsis* have a presence in the aquaculture market (Ferreira et al. 2009) and have been tested to be used as fertilizer (Fui et al. 2018). A less popular yet important application as vitamin sources has been tested in *N. oculata* (Durmaz 2007) and *M. subterraneus* (Spolaore et al. 2006).

Table 1.3.2. Biotechnological interest of compounds produced by the Eustigmatophyceae, based on the comprehensive review of Stoyneva-Gärtner et al. (2019b) and in the biodiesel section, based on Nobre et al. (2012).

Compound	Biotechnological interest	Reference	Present in the Eustigmatophyceae	Reference
Lipids				
fatty acids				
	participate in many metabolic processes, play a role in cycle of cardiac cells, reduce cholesterol, diabetes, and ocular disease, arthritis and cystic fibrosis	Ristić-Medić et al. 2013; Honoré et al. 1994; Simopoulos 1991; Herbaut 2006; Landmark and Alm 2006; Reiffel and McDonald 2006	*Monodus subterraneus*	Mercer et al. 1974
	human diet	Bae and Hur 2011	*Nannochloropsis* and unidentified strains	Patterson et al. 1994
			Nannochloropsis	Bae and Hur 2011; Seto et al. 1984; Sukenik et al. 1989; Rebolloso-Fuentes et al. 2001; Safafar et al. 2016; Neumann et al. 2018
			Vischeria, Ellipsoidion	Gao et al. 2016; Liu and Lin 2005; Cohen 1994; Nianjun and Xuecheng 2001; Iwamoto and Sato 1986; Hu et al. 1997; Vazhappilly and Chen 1998; Volkman et al. 1999; Xu et al. 2001; Khozin-Goldberg et al. 2002; Khozin-Goldberg and Cohen 2006; Iliev et al. 2010; Rezanka et al. 2010; Gigova et al. 2012
			Trachydiscus	Pilatova 2013
			N. limnetica	Krienitz and Wirth 2006

Biofuel				
TAG	alternative fuel sources	Khozin-Goldberg and Boussiba 2011	*Nannochloropsis*	Volkman et al. 1992; Volkman et al. 1993; Moazami et al. 2012; Dinesh et al. 2018; Volkman et al. 1998; Doan et al. 2011
			Vischeria	Volkman et al. 1993
			Vischeria polyphem	Zhang et al. 2013
			Vischeria stellata	Gao et al. 2016
fatty acids	biodiesel	Nobre et al. 2012	*Nannochloropsis*	Nobre et al. 2012
biohydrogen	alternative fuel sources	Nobre et al. 2012	*Nannochloropsis*	Nobre et al. 2012
Total nutrients (biomass)				
	alternative food sources	Neumann et al. 2018; Tibbetts et al. 2015	*Nannochloropsis*	Neumann et al. 2018; Tibbets et al. 2015
			N. granulata	Tibbets et al. 2015
Medicine				
	discovery of an operon encoding for antibiotic or other protective activity	Yurchenko et al. 2018	*Characiopsis acuta*	Yurchenko et al. 2018
Cosmetic				
	lipids and tanning effect of canthaxantin for creams	Koller et al. 2014; Durmaz 2007	*Nannochloropsis*	Koller et al. 2014; Mourelle et al. 2017
	antioxidant and collagen synthesis improvement	Stolz and Obermayer 2005; Letsiou et al. 2017	*N. oculata*, *M. gaditana*	Stolz and Obermayer 2005; Letsiou et al. 2017

	patented ingredient patented Pentapharm	Mourelle et al. 2017; Spolaore et al. 2006; Stolz and Obermayer 2005; Mourelle et al. 2014	*N. oculata*	Mourelle et al. 2017; Spolaore et al. 2006; Stolz and Obermayer 2005; Mourelle et al. 2014
Pigments				
Asthaxanthin AsX	animal feed to impart coloration (salmon, shrimp, crabs, chicken eggs etc.)	Higuera-Ciapara et al. 2006; Ambati et al. 2014; Shah et al. 2016	*N. oculata*	Antia and Cheng 1982
	dietary supplement (antioxidant, anti-inflammatory, anti-cancer, skin and eye care, immune response enhancer)	Ambati et al. 2014; Park et al. 2010; McCall et al. 2018	*Vischeria*	Stoyneva-Gartner et al. 2019a
β-carotene	provitamin A activity stimulates the immune system	Li et al. 2012a; Li et al. 2012b	*Vischeria*	Li et al. 2012a; Li et al. 2012b
	antioxidant, prevents heart disease and anti-cancer, use in food, pharmaceutic and cosmetic industries	Prieto et al. 2011		
Violaxanthin ViX	strong antioxidant, anti-proliferative, anti-inflammartory, anti-cancer – potential use in health care products	Talero et al. 2015; Wang et al. 2018	Eustigmatophyceae	Stoyneva-Gartner et al. 2019a
Lutein	feed, food nutraceutical, pharmaceutical	Stoyneva-Gartner et al. 2019a	*Vischeria*	Stoyneva-Gärtner et al. 2019a
	supplements for reducing macular degeneration, cataract formation, support ocular function, anti-cancer, prevents cardiovascular disease	Miranda et al. 2015; Li et al. 2010; Nolan et al. 2013; Koo et al. 2014; Pinazo-Durán et al. 2014; Wang et al. 2014; Bernstein et al. 2016		
Zeaxanthin	similar to lutein	Li et al. 2010; Bernstein et al. 2016	*Vischeria*	Stoyneva-Gärtner et al. 2019a

Canthaxanthin CaX	antioxidant, protective of hatching eggs in hatcheries	Surai 2007; Surai 2012	Eustigmatophyceae	Stoyneva-Gärtner et al. 2019a
	studied for use in tanning creams due to anti-cancer, anti-dermatosis activity	Sujak et al 2005; Koller et al. 2014; Mourelle et al. 2017		
	studied for use as chemotherapy adjuvant	Eid et al. 2012		
Chlorophylls and chlorophyllins	controversial health benefits include indications as detoxication, antioxidant, boosting immune system, wound healing, weight loss, anti-cancer	Mishra et al. 2012		
	food and beverage coloration			
Aquaculture				
	feed for animals in farming chain	Spolaore et al. 2006	*M. gaditana*	Ferreira et al. 2009
			Nannochloropsis sp.	Bae and Hur 2011
Environmental applications				
	fertilizer	Fui et al. 2018	*N. oculata*	Fui et al. 2018
vitamins				
vitamin E (tocopherol)	antioxidant activity in vivo, prevention of eye and skin pathologies, atherosclerosis, cardiovascular desease and cancer	Durmaz 2007	*N. oculata*	Durmaz 2007
	animal breeding enhancement of survival rate	Durmaz 2007	*Monodus subterraneus*	Spolaore et al. 2006
	fish larvae feed	Liu and Lin 2005		

2.

Towards modern classification of eustigmatophytes, including the description of Neomonodaceae, fam. nov. and three new genera

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2.1. Abstract

The class Eustigmatophyceae includes mostly coccoid, freshwater algae, although some genera are common in terrestrial habitats and two are primarily marine. The formal classification of the class developed decades ago, does not fit the diversity and phylogeny of the group as presently known and is in urgent need of revision. This study concerns a clade informally known as the *Pseudellipsoidion* group of the order Eustigmatales, which was initially known to comprise seven strains with oval to ellipsoidal cells, some bearing a stipe. We examined those strains as well as ten new ones and obtained 18S rDNA and *rbc*L gene sequences. The results from phylogenetic analyses of the sequence data were integrated with morphological data of vegetative and motile cells. Monophyly of the *Pseudellipsoidion* group is supported in both 18S rDNA and *rbc*L trees. The group is formalized as the new family Neomonodaceae comprising, in addition to *Pseudellipsoidion*, three newly erected genera. By establishing *Neomonodus* gen. nov. (with type species *Neomonodus ovalis* comb. nov.) we finally resolve the intricate taxonomic history of a species originally described as *Monodus ovalis* Chodat and later moved to the genera *Characiopsis* and *Pseudocharaciopsis*. *Characiopsiella* gen. nov. (with the type species *Characiopsiella minima* comb. nov.) and *Munda* gen. nov. (with the type species *Munda aquilonaris*) are established to accommodate additional representatives of the polyphyletic genus *Characiopsis*. A morphological feature common to all examined Neomonodaceae is the absence of a pyrenoid in the chloroplasts, which discriminates them from other morphologically similar yet unrelated eustigmatophytes (including other *Characiopsis*-like species).

2.2. Introduction

The Eustigmatophyceae constitute a well-defined clade of ochrophyte (heterokontophyte) algae that is considered a separate class related to Chrysophyceae, Synchromophyceae, and

possibly Pinguiophyceae (Yang et al. 2012, Ševčíková et al. 2015, Eliáš et al. 2017). Eustigmatophytes are coccoid algae, solitary or in loose colonies, reproducing via autosporogenesis or, occasionally in some taxa, by zoospores with unique features (for a review see Eliáš et al. 2017). Eustigmatophytes occur primarily in freshwater and soil, but research on the class has been concentrated on the primarily marine genera *Nannochloropsis* and *Microchloropsis* (Fawley et al. 2015) which have shown potential for biotechnological exploitation (Ma et al. 2016).

The existence of eustigmatophytes as an independent group was realized in the early 1970's upon investigation of the ultrastructure and pigment composition of several algae previously classified as Xanthophyceae (Hibberd and Leedale 1970, 1971). Since then, the class has been growing in diversity, both by recruiting additional traditional xanthophytes (Hibberd 1981, Santos 1990, Schnepf et al. 1996, Santos and Santos 2004, Přibyl et al. 2012) and description of brand new taxa (e.g. Preisig and Wilhelm 1989, Neustupa and Němcová 2001, Hegewald et al. 2007, Nakayama et al. 2015, Fawley et al. 2019). It is likely that this process of reassigning misclassified xanthophycean taxa will continue when other previously described yet poorly documented species are reinvestigated with modern methods, as demonstrated by the recent study of *Tetraëdriella subglobosa*, re-isolated from the original type locality and proved to be a eustigmatophyte by 18S rRNA and *rbc*L gene sequencing (Fawley and Fawley 2017). Hundreds of described xanthophytes have not been studied by transmission electron microscopy (TEM) or molecular approaches (see Ettl 1978), so they represent a particularly attractive target for investigation.

The need to clarify the diversity and phylogeny of the Eustigmatophyceae and to provide proper identifications of strains held in culture collections is now more urgent than ever before, given the rapid growth of interest in eustigmatophytes other than *Nannochloropsis* and *Microchloropsis* that has been stimulated by the fact that all studied Eustigmatophyceae

produce valuable compounds such as lipids (Pal et al. 2013, Gao et al. 2018), carotenoids (Lubián et al. 2000, Li et al. 2012a) and antioxidants (Assunção et al. 2016). The first consolidated classification of eustigmatophytes developed by Hibberd (1981) recognized a single order Eustigmatales divided into four families. The growth of newly recognized or described eustigmatophytes and the advent of molecular phylogenetics quickly challenged Hibberd's scheme. The inadequacy of the existing eustigmatophyte classification has become even more obvious with molecular characterization of new freshwater isolates (Prior et al. 2009, Fawley et al. 2014, Fawley and Fawley 2017) and environmental DNA surveys (Lara et al. 2011, Nikouli et al. 2013, Villanueva et al. 2014), which revealed the existence of substantial undescribed phylogenetic diversity within this group.

Eustigmatophytes are thus now known to encompass two deeply separated principal lineages, one corresponding to the order Eustigmatales (Hibberd 1981) and the other comprised of eustigmatophytes recognized or described only after Hibberd's seminal work (Eliáš et al. 2017). This second putative order not formalized under the International Code of Nomenclature for Algae, Fungi and Plants is presently referred to as the clade *Goniochloridales* and validated under the PhyloCode (Fawley et al. 2014). The same authors also described the existence of three robustly separated clades within the Eustigmatales. One clade corresponds to the Monodopsidaceae *sensu* Hibberd (1981), expanded by the addition of *Pseudotetraëdriella kamillae*, a species described and placed in the family Loboceae by Hegewald et al. (2007). The second clade referred to as the Eustigmataceae group comprises members of the families Eustigmataceae and Chlorobotrydaceae *sensu* Hibberd (1981), the strain *Pseudocharaciopsis minuta* UTEX 2113, an isolate identified as *Characiopsis saccata* plus several unidentified isolates (Fawley et al. 2014).

The present study concerns the third clade of the Eustigmatales, informally named the *Pseudellipsoidion* group by Fawley et al. (2014) according to its representative *Pseudellipsoidion*

edaphicum, an organism described by Neustupa and Němcová (2001) as a eustigmatophyte but not formally classified into any family. Molecular characterization of several unidentified isolates showed one highly supported lineage comprising four unnamed strains positioned together with *P. edaphicum* (Fawley et al. 2014) and a second lineage within the *Pseudellipsoidion* group that included two *Pseudocharaciopsis ovalis* strains. These findings suggested that the genus *Pseudocharaciopsis* as circumscribed by Hibberd (1981) is polyphyletic. The taxonomy of the *Pseudellipsoidion* group is therefore in need of revision. To meet this objective, the present study provides morphological and molecular data (18S rRNA and *rbc*L gene sequences) for seven original *Pseudellipsoidion* group members and ten additional strains, nine previously assigned to the genus *Characiopsis* in the Xanthophyceae. The establishment of a new eustigmatophyte family, the Neomonodaceae, is proposed to include four genera, three of them newly described. In addition, clades at the species level are indicated for future further analysis.

2.3. Materials and Methods

Algal cultures

A total of seventeen strains of microalgae isolated from freshwater, soil, peat bogs and mines were studied (Table 2.1.). Seven strains are Portuguese isolates held at the Coimbra Collection of Algae (ACOI) (acoi.ci.uc.pt) maintained in liquid Desmideacean Medium (Schlösser 1994), pH 6.4 to 6.6, at 20 °C, under 12:12 h photoperiod and under 10 $\mu mol.m^{-2}.s^{-1}$ light intensity provided by cool white fluorescent lamps. Four strains are isolates from Itasca State Park, Minnesota, U.S.A. and one strain was isolated from a small pond in Arkansas, U.S.A; these strains are kept on agar slants of WH+ medium (Fawley et al. 2014). Three strains are soil isolates from the Czech Republic, held at the Culture Collection of Algae at Charles University (CAUP) (botany.natur.cuni.cz/algo/caup.html) in Bold's Basal Medium (BBM) (Bischoff and Bold 1963). Two strains are isolates from inhospitable

environments, namely coal and lignite mines in the Czech Republic and Germany; their cultures are kept at the Institute of Soil Biology in the Biology Centre Collection of Organisms (BCCO) (www.soilalgae.cz) on BBM agar slants, pH 6 to 6.4, at 15°C, under continuous low light, and also cryopreserved under -150 °C.

Light microscopy observations

Morphological evaluation of the cells was performed using a Leica DMRB either by light microscopy analysis or by DIC microscopy using 60x and 100x PLAN APO objectives. Micrographs were acquired with a Leica DFC420 digital camera. A Nikon Ni-U microscope equipped with a 100x Plan Apo objective and DIC was used for investigating the strains from the collection of Karen and Marvin Fawley. Observations and measurements were performed in young and old cultures (5 and 30 days). The presence of zoospores was recorded from one hour to two days after adding fresh culture medium to an old batch culture (more than one month). Drawings were obtained by digital tracing micrographs in Photoshop Elements using a Wacom Bamboo drawing tablet. Cell size was assessed using the digital image analysis software LAS V4.6 or Nikon Elements BR by measuring 5 cells of each strain, 5 and 30 days after sub-culturing.

Transmission Electron Microscopy

For TEM a suspension of cells was fixed for 2 h or 2.5 h with 2% or 2.5% glutaraldehyde in 0.05M phosphate buffer, pH 6.8 and then washed with the same buffer by centrifugation one to three times for 5 min at 2000 rpm. The cell suspension was embedded in 1.5% or 2% agar and post-fixed in 1% osmium tetroxide solution (prepared 1:1 v/v with the same phosphate buffer) for 2 hours in the dark. The fixative was then washed out by centrifugation (2x buffer then 2x deionized water or 3x buffer, 5 min at 2000 rpm). Samples were

dehydrated in an ethanol series (70%, 96% and 100% or 70%, 80%, 95% and 100%), each for 15 min and then embedded in Spurr´s resin with butanol or ethanol (5%, 10%, 25%, 50%, 75%, 95% and 100% or 33%, 50% and 66%) and kept overnight in a desiccator. Resin blocks were then cut with an ultramicrotome (Ultracut E, Reichert-Jung) and ultrathin sections were mounted on copper grids and stained with 1% or 2% uranyl acetate and 0.2% lead citrate. Samples were examined in a JEOL 1011 or a FEI-Tecnai G2 Spirit Bio Twin electron microscope. Direct preparations of zoospores were obtained by fixing a drop of zoospore suspension on a formvar/carbon-coated grid in 2% osmium tetroxide vapor, drying at room temperature and shadowcasting with gold/palladium.

PCR amplification and DNA sequencing

Cells were collected by centrifugation of 2 ml culture or harvested from agarized medium and disrupted using a mixer mill (MM200 Retsch, Haan, Germany) for 5 minutes. Genomic DNA was extracted using Spin Plant Mini Kit (Invisorb®, Invitek). PCR was performed with the MyTaq™ Red DNA Polymerase (Bioline, United Kingdom), under following conditions: denaturation 95°C for 2 minutes followed by 35 cycles of 95°C for 30 seconds, 52°C for 30 seconds, 72°C for 2.5 minutes and final extension at 72°C for 5 minutes. PCR products from amplification of the 18S rRNA and rbcL genes were purified using GenElute™ PCR Clean-Up Kit (SIGMA). Sequencing reactions were performed using BigDye® Terminator v3.1 Cycle Sequencing Kit (ThermoFisher scientific) and analyzed using the 3130xl Genetic Analyzer in the DNA Sequencing Laboratory of the Faculty of Science, Charles University in Prague. Primers used for obtaining full sequences of the 18S rRNA gene included the amplification primers 18S-F and 18S-R and internal sequencing primers according to Katana et al. (2001). Primers used for amplification of *rbc*L were EU-rbcL-F1 (5´- ATGTTTCAATCTGTAGAAGAAAG-3') and the reverse primer EU-*rbc*L-R1

(5´- CCTTGTGTTAATCTCACTCTTC-3'), which were newly designed based on a comparison of complete rbcL genes obtained as parts of fully sequenced eustigmatophyte plastid genome sequences (Ševčíková et al. 2015). They allow for highly efficient amplification of essentially a complete rbcL gene from diverse eustigmatophytes (see also Fawley et al. 2015, Fawley and Fawley 2017, Fawley et al. 2019). For sequencing reactions, the amplification primers were used along with the newly designed sequencing primers (Table 2.1.). Sequencing reads were assembled with SeqAssem (SequentiX, http://www.sequentix.de/software_seqassem.php), and manually edited by visual inspection of sequencing chromatograms. Sequence data from the strains from the collection of Karen and Marvin Fawley (the five "*Pseudellipsoidion* sp." strains) were obtained using the procedures and primers described in Fawley and Fawley (2017). Sequences were trimmed to exclude primer regions and deposited at GenBank (accession numbers provided in Table 2.2.).

Table 2.1. Primers used for the amplification and sequencing of the 18S rRNA and *rbc*L genes of the studied strains. amp – amplification primer, seq – sequencing primer.

Region	Type	Name	5´ sequence 3´	Reference
18S rRNA	amp	F	AACCTGGTTGATCCTGCCAGT	Katana et al. 2001
	amp	R	TGATCCTTCTGCAGGTTCACCTACG	Katana et al. 2001
	amp	Eustig-F1	GACAATAAATAACAATGCCGG	this paper
	amp	Eustig-R1	GTTATAAACTCGTTGAACGCA	Fawley et al. 2014
	seq	402-23F	GCTACCACATCCAAGGAAGGCA	Katana et al. 2001
	seq	416-37R	ATTTGCGCGCCTGCTGCCTTCC	Katana et al. 2001
	seq	895-916F	GTCAGAGGTGAAATTCTTGGAT	Katana et al. 2001
	seq	1308-39R	CTCGTTCGTTAACGGAATTAACC	Katana et al. 2001
	seq	1323-44F	CGAACGAGACCTCAGCCTGCTA	Katana et al. 2001
*rbc*L	amp	EustigrbcLF	GATCCRATTGAAGCTGC	this paper
	amp	DPrbcL7 (R)	AARCAACCTTGTGTAAGTCTC	Jones et al. 2005

	amp	EU-rbcL-F1	ATGTTTCAATCTAGAAGAAAG	this paper
	amp	EU-rbcL-R1	CCTTGTGTTAATCTCACTCTTC	this paper
	seq	EUSrbcL-sF1	AACTCWCAACCWTTCATGCGT	this paper
	seq	EUSrbcL-sR1	AACGCATGAAWGGTTGWGAGT	this paper
	seq	Q301rbcL-sF2	GCTTCTGGTGGTATTCACTGTG	this paper
	seq	Q301rbcL-sR2	CACAGTGAATACCACCAGAAGC	this paper
	seq	DPrbcL7 (R)	AARCAACCTTGTGTAAGTCTC	Jones et al. 2005
	seq	Pseudell-rbcL-SF1	CTTAGGTGCAACTGTAAAACC	this paper
	seq	Pseudell-rbcL-SR1	GGTTTTACAGTTGCACCTAAG	this paper
	seq	Pseudell-rbcL-SF2	GTGAYCCTTTAATGGTTAAAG	this paper

Phylogenetic analyses

The complete dataset for analyses of the 18S rRNA gene sequences included in total 565 sequences and consisted of the 10 newly obtained sequences of the Neomonadaceae family, an exhaustive set of 539 non-redundant eustigmatophyte 18S rDNA sequences gathered from the GenBank database based on extensive blast searches and preliminary analyses (which also led us to exclude some low-quality and/or apparently chimeric sequences), and a selection of 14 sequences from phylogenetically diverse ochrophytes to provide an outgroup. The sequences were aligned with MAFFT 7.429 (Katoh and Frith 2012, Katoh and Standley 2013), using the "Add" option and a preexisting master alignment of ochrophyte 18S rRNA gene sequences manually curated to take into account the conserved secondary structure of 18S rRNA molecules (Eliáš et al. 2017). Redundant sequences were removed in BioEdit version 7.0.5 (Hall 1999) and the resulting final alignment was used in two different analyses. The first utilized a subset of 99 sequences (all Neomonodaceae sequences, 75 additional eustigmatophyte sequences representing all main lineages in the group, and the outgroup sequences). Trimming the alignment with GBlocks 0.91b (Castresana 2000) to remove unreliably aligned positions left 1614 positions in the final

alignment. In the second analysis, the full alignment was trimmed with trimAl v1.4. rev6 using 0.02 similarity threshold (Capella-Gutiérrez et al. 2009; https://www.genome.jp/tools/ete/), leaving 1756 positions for tree inference. For the *rbc*L gene analysis, a selection of 40 eustigmatophyte sequences available from GenBank (retaining only one sequence per described species for non-Neomonodaceae representatives) and the 13 newly obtained or updated sequences were aligned with MAFFT 7.429. The termini of the alignment were trimmed in GeneDoc (Nicholas and Nicholas 1997) to remove positions with a high percentage of missing data, leaving 1347 positions. Trees were inferred using the maximum likelihood (ML) method implemented in RAxML (8.2.12) at the Cyberinfrastructure for Phylogenetic Research (CIPRESS) Portal (http://www.phylo.org/sub_sections/portal) (Miller et al. 2010) using the strategy of Stamatakis et al. (2008) for obtaining the highest likelihood tree. The evolutionary model used was the default GTR+Γ. In the case of the *rbc*L gene, two analyses were done, one considering the whole alignment as one partition and the other considering separate partitions for the three codon positions. Bootstrap analyses were performed with the rapid bootstrapping procedure, with the adequate number of replicates detected by the program itself ("halt bootstrapping automatically" option); the number of bootstrap replicated for each tree is specified in the respective figure legends. Trees were drawn with the aid of the iTOL tool (Letunic and Bork 2016; https://itol.embl.de/).

2.4. Results

Expanded phylogenetic diversity of the family Neomonodaceae (*Pseudellipsoidion* group)

The phylogenetic tree inferred from 18S rRNA gene sequences (Fig. 2.1.) shows the deep separation of eustigmatophyte into two clades, Goniochloridales and Eustigmatales. The latter

is further resolved into three strongly to fully supported subclades, the Monodopsidaceae, the Eustigmataceae group, and the Neomonodaceae (i.e. *Pseudellipsoidion* group), plus a deep lineage represented solely by the recently described *Paraeustigmatos columelliferus* (Fawley et al. 2019). The Neomonodaceae is expanded by ten newly characterized strains. The strain Beav 4/26 T-6w proved to be closely allied with *P. edaphicum* and previously reported unidentified strains Tow 8/18 T-12d, WTwin 8/18 T-5d, Tow 9/21 P-2w and Mary 8/18 T-3d. The clade comprising these six strains, further referred to as the genus *Pseudellipsoidion*, is supported by a bootstrap value of 89% and separated from other lineages in the Neomonodaceae. Another clade, which we later formally describe as the new genus *Neomonodus*, is maximally supported and includes five strains previously identified as *Pseudocharaciopsis ovalis* or *Characiopsis ovalis*, three of them newly characterized here. Specifically, the strains BCCO_30_2917 and BCCO_30_2918 have the same 18S rRNA gene sequence as *P. ovalis* CAUP Q 302, whereas the 18S rRNA gene sequence of the strain *Neomonodus* sp. ACOI 2437 exhibited two and one nucleotide differences from *P. ovalis* CAUP Q 301 and *P. ovalis* CAUP Q 302, respectively.

The remaining six Neomonodaceae strains constitute two separate novel lineages (Fig. 2.1.). One lineage comprises two strains from the ACOI culture collection (ACOI 2426 and ACOI 2423A) identified by us as *Characiopsis minima*. These two strains had identical 18S rRNA gene sequences and are described below as the new genus *Characiopsiella*. The second new lineage included strains identified as *Characiopsis aquilonaris* (ACOI 2424, 2424A, 2424B) and *Characiopsis* sp. (ACOI 2428); it is described below as the new genus *Munda*. The 18S rRNA gene sequences of the strains in this new lineage were also identical. The phylogenetic analysis of the 18S rRNA gene suggested that *Neomonodus* and *Munda* are sister lineages and that *Characiopsiella* is sister to the Neomonodus-Munda clade, but bootstrap support for the latter relationship is weak (61%). The family Neomonodaceae is strongly supported (bootstrap value of 99%) as monophyletic and clearly separated as one of the four main clades in the order Eustigmatales.

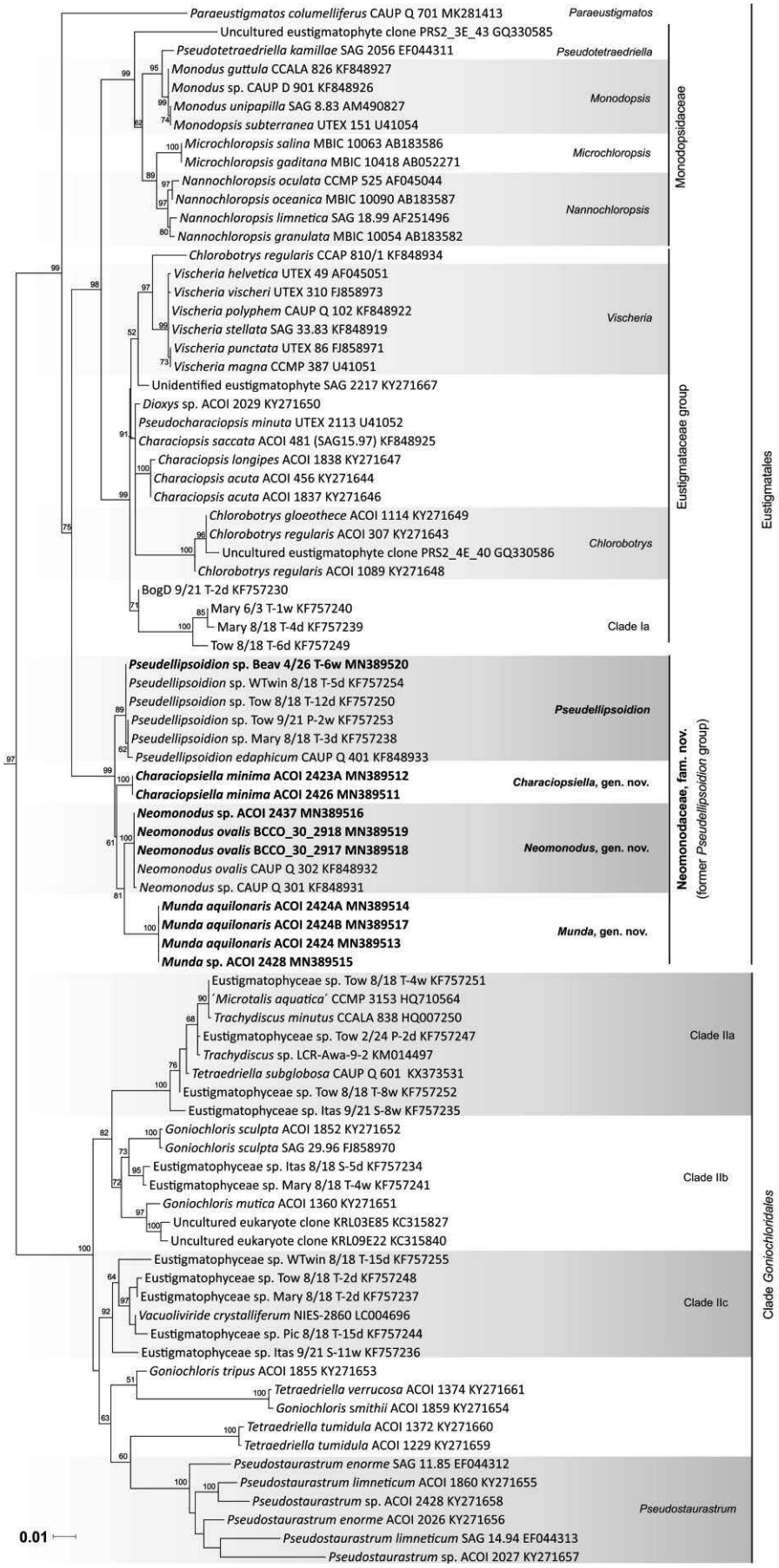
Paraeustigmatos columelliferus CAUP Q 701 MK281413
Paraeustigmatos
Uncultured eustigmatophyte clone PRS2_3E_43 GQ330585
Pseudotetraedriella kamillae SAG 2056 EF044311
Pseudotetraedriella
Monodus guttula CCALA 826 KF848927
Monodus sp. CAUP D 901 KF848926
Monodus unipapilla SAG 8.83 AM490827
Monodopsis subterranea UTEX 151 U41054
Monodopsis
Microchloropsis salina MBIC 10063 AB183586
Microchloropsis gaditana MBIC 10418 AB052271
Microchloropsis
Nannochloropsis oculata CCMP 525 AF045044
Nannochloropsis oceanica MBIC 10090 AB183587
Nannochloropsis limnetica SAG 18.99 AF251496
Nannochloropsis granulata MBIC 10054 AB183582
Nannochloropsis
Monodopsidaceae
Chlorobotrys regularis CCAP 810/1 KF848934
Vischeria helvetica UTEX 49 AF045051
Vischeria vischeri UTEX 310 FJ858973
Vischeria polyphem CAUP Q 102 KF848922
Vischeria stellata SAG 33.83 KF848919
Vischeria punctata UTEX 86 FJ858971
Vischeria magna CCMP 387 U41051
Vischeria
Unidentified eustigmatophyte SAG 2217 KY271667
Dioxys sp. ACOI 2029 KY271650
Pseudocharaciopsis minuta UTEX 2113 U41052
Characiopsis saccata ACOI 481 (SAG15.97) KF848925
Characiopsis longipes ACOI 1838 KY271647
Characiopsis acuta ACOI 456 KY271644
Characiopsis acuta ACOI 1837 KY271646
Chlorobotrys gloeothece ACOI 1114 KY271649
Chlorobotrys regularis ACOI 307 KY271643
Uncultured eustigmatophyte clone PRS2_4E_40 GQ330586
Chlorobotrys regularis ACOI 1089 KY271648
Chlorobotrys
BogD 9/21 T-2d KF757230
Mary 6/3 T-1w KF757240
Mary 8/18 T-4d KF757239
Tow 8/18 T-6d KF757249
Clade Ia
Eustigmataceae group
Pseudellipsoidion sp. Beav 4/26 T-6w MN389520
Pseudellipsoidion sp. WTwin 8/18 T-5d KF757254
Pseudellipsoidion sp. Tow 8/18 T-12d KF757250
Pseudellipsoidion sp. Tow 9/21 P-2w KF757253
Pseudellipsoidion sp. Mary 8/18 T-3d KF757238
Pseudellipsoidion edaphicum CAUP Q 401 KF848933
Pseudellipsoidion
Characiopsiella minima ACOI 2423A MN389512
Characiopsiella minima ACOI 2426 MN389511
Characiopsiella, gen. nov.
Neomonodus sp. ACOI 2437 MN389516
Neomonodus ovalis BCCO_30_2918 MN389519
Neomonodus ovalis BCCO_30_2917 MN389518
Neomonodus ovalis CAUP Q 302 KF848932
Neomonodus sp. CAUP Q 301 KF848931
Neomonodus, gen. nov.
Munda aquilonaris ACOI 2424A MN389514
Munda aquilonaris ACOI 2424B MN389517
Munda aquilonaris ACOI 2424 MN389513
Munda sp. ACOI 2428 MN389515
Munda, gen. nov.
Neomonodaceae, fam. nov.
(former Pseudellipsoidion group)
Eustigmatales
Eustigmatophyceae sp. Tow 8/18 T-4w KF757251
'Microtalis aquatica' CCMP 3153 HQ710564
Trachydiscus minutus CCALA 838 HQ007250
Eustigmatophyceae sp. Tow 2/24 P-2d KF757247
Trachydiscus sp. LCR-Awa-9-2 KM014497
Tetraedriella subglobosa CAUP Q 601 KX373531
Eustigmatophyceae sp. Tow 8/18 T-8w KF757252
Eustigmatophyceae sp. Itas 9/21 S-8w KF757235
Clade IIa
Goniochloris sculpta ACOI 1852 KY271652
Goniochloris sculpta SAG 29.96 FJ858970
Eustigmatophyceae sp. Itas 8/18 S-5d KF757234
Eustigmatophyceae sp. Mary 8/18 T-4w KF757241
Goniochloris mutica ACOI 1360 KY271651
Uncultured eukaryote clone KRL03E85 KC315827
Uncultured eukaryote clone KRL09E22 KC315840
Clade IIb
Eustigmatophyceae sp. WTwin 8/18 T-15d KF757255
Eustigmatophyceae sp. Tow 8/18 T-2d KF757248
Eustigmatophyceae sp. Mary 8/18 T-2d KF757237
Vacuoliviride crystalliferum NIES-2860 LC004696
Eustigmatophyceae sp. Pic 8/18 T-15d KF757244
Eustigmatophyceae sp. Itas 9/21 S-11w KF757236
Clade IIc
Goniochloris tripus ACOI 1855 KY271653
Tetraedriella verrucosa ACOI 1374 KY271661
Goniochloris smithii ACOI 1859 KY271654
Tetraedriella tumidula ACOI 1372 KY271660
Tetraedriella tumidula ACOI 1229 KY271659
Pseudostaurastrum enorme SAG 11.85 EF044312
Pseudostaurastrum limneticum ACOI 1860 KY271655
Pseudostaurastrum sp. ACOI 2428 KY271658
Pseudostaurastrum enorme ACOI 2026 KY271656
Pseudostaurastrum limneticum SAG 14.94 EF044313
Pseudostaurastrum sp. ACOI 2027 KY271657
Pseudostaurastrum
Clade Goniochloridales
0.01

Figure 2.1. Phylogeny of Eustigmatophyceae based on sequences of the 18S rRNA gene. The phylogeny shown was inferred using the maximum likelihood method implemented in RAxML (employing the GTR+Γ substitution model) with bootstrap analysis followed by thorough search for the ML tree. Bootstrap values correspond to the percentage calculated from 300 replicates and are shown when higher than 50. For simplicity, the outgroup (a selection of diverse ochrophyte 18S rRNA sequences) is omitted from the figure. Terminal leaves are labelled with the species/strain name (sometimes different from the name in the respective GenBank record to reflect recent taxonomic changes) and the GenBank accession number of the sequence. New sequences are highlighted in boldface.

We performed a second phylogenetic analysis of eustigmatophyte 18S rRNA gene sequences that also included partial sequences (~500 to ~600 bp) obtained by surveying environmental DNA from an east African freshwater lake (Villanueva et al. 2014) and a tropical coastal lagoon (Alves-de-Souza et al. 2017). The former study reported the existence of five clades comprising sequences from uncultivated eustigmatophytes, denoted Group 1 to Group 5. Our analysis, which benefited from a substantial improvement of the sampling of cultured eustigmatophytes and employing a more sophisticated method of phylogenetic inference, enabled us to more precisely place these five groups within eustigmatophytes (Figs. 2.2. and Figure S1.). Group 1 is confirmed as a cluster within the clade *Goniochloridales*, Group 2 is now revealed to correspond to the basal Eustigmatales lineage typified by *Paraeustigmatos columelliferus*, and Group 4 and Group 5 constitute a larger clade branching off basally in the Eustigmataceae group. Most significantly for our main focus here, the Group 3 of Villanueva et al. (2014) represents a novel, apparently diverse lineage within Neomonodaceae, potentially sister to the genus *Characiopsiella*. The partial sequences from the coastal lagoon (Alves-de-Souza et al. 2017) all fall within the genus *Microchloropsis* (Fig. S1.).

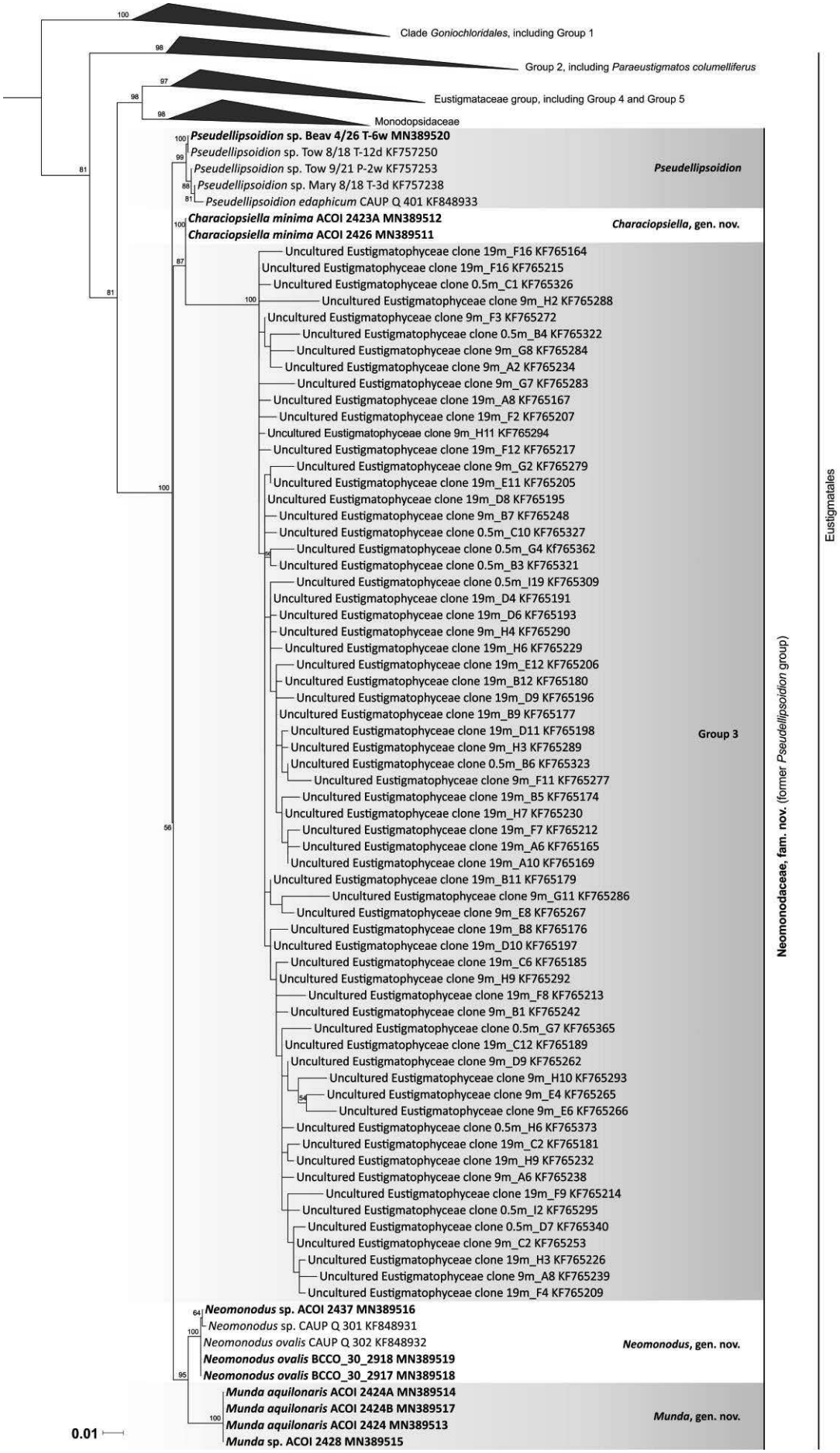
Clade Goniochloridales, including Group 1
Group 2, including Paraeustigmatos columelliferus
Eustigmataceae group, including Group 4 and Group 5
Monodopsidaceae
Pseudellipsoidion sp. Beav 4/26 T-6w MN389520
Pseudellipsoidion sp. Tow 8/18 T-12d KF757250
Pseudellipsoidion sp. Tow 9/21 P-2w KF757253
Pseudellipsoidion sp. Mary 8/18 T-3d KF757238
Pseudellipsoidion edaphicum CAUP Q 401 KF848933
Pseudellipsoidion
Characiopsiella minima ACOI 2423A MN389512
Characiopsiella minima ACOI 2426 MN389511
Characiopsiella, gen. nov.
Uncultured Eustigmatophyceae clone 19m_F16 KF765164
Uncultured Eustigmatophyceae clone 19m_F16 KF765215
Uncultured Eustigmatophyceae clone 0.5m_C1 KF765326
Uncultured Eustigmatophyceae clone 9m_H2 KF765288
Uncultured Eustigmatophyceae clone 9m_F3 KF765272
Uncultured Eustigmatophyceae clone 0.5m_B4 KF765322
Uncultured Eustigmatophyceae clone 9m_G8 KF765284
Uncultured Eustigmatophyceae clone 9m_A2 KF765234
Uncultured Eustigmatophyceae clone 9m_G7 KF765283
Uncultured Eustigmatophyceae clone 19m_A8 KF765167
Uncultured Eustigmatophyceae clone 19m_F2 KF765207
Uncultured Eustigmatophyceae clone 9m_H11 KF765294
Uncultured Eustigmatophyceae clone 19m_F12 KF765217
Uncultured Eustigmatophyceae clone 9m_G2 KF765279
Uncultured Eustigmatophyceae clone 19m_E11 KF765205
Uncultured Eustigmatophyceae clone 19m_D8 KF765195
Uncultured Eustigmatophyceae clone 9m_B7 KF765248
Uncultured Eustigmatophyceae clone 0.5m_C10 KF765327
Uncultured Eustigmatophyceae clone 0.5m_G4 Kf765362
Uncultured Eustigmatophyceae clone 0.5m_B3 KF765321
Uncultured Eustigmatophyceae clone 0.5m_I19 KF765309
Uncultured Eustigmatophyceae clone 19m_D4 KF765191
Uncultured Eustigmatophyceae clone 19m_D6 KF765193
Uncultured Eustigmatophyceae clone 9m_H4 KF765290
Uncultured Eustigmatophyceae clone 19m_H6 KF765229
Uncultured Eustigmatophyceae clone 19m_E12 KF765206
Uncultured Eustigmatophyceae clone 19m_B12 KF765180
Uncultured Eustigmatophyceae clone 19m_D9 KF765196
Uncultured Eustigmatophyceae clone 19m_B9 KF765177
Uncultured Eustigmatophyceae clone 19m_D11 KF765198
Uncultured Eustigmatophyceae clone 9m_H3 KF765289
Uncultured Eustigmatophyceae clone 0.5m_B6 KF765323
Uncultured Eustigmatophyceae clone 9m_F11 KF765277
Uncultured Eustigmatophyceae clone 19m_B5 KF765174
Uncultured Eustigmatophyceae clone 19m_H7 KF765230
Uncultured Eustigmatophyceae clone 19m_F7 KF765212
Uncultured Eustigmatophyceae clone 19m_A6 KF765165
Uncultured Eustigmatophyceae clone 19m_A10 KF765169
Uncultured Eustigmatophyceae clone 19m_B11 KF765179
Uncultured Eustigmatophyceae clone 9m_G11 KF765286
Uncultured Eustigmatophyceae clone 9m_E8 KF765267
Uncultured Eustigmatophyceae clone 19m_B8 KF765176
Uncultured Eustigmatophyceae clone 19m_D10 KF765197
Uncultured Eustigmatophyceae clone 19m_C6 KF765185
Uncultured Eustigmatophyceae clone 9m_H9 KF765292
Uncultured Eustigmatophyceae clone 19m_F8 KF765213
Uncultured Eustigmatophyceae clone 9m_B1 KF765242
Uncultured Eustigmatophyceae clone 0.5m_G7 KF765365
Uncultured Eustigmatophyceae clone 19m_C12 KF765189
Uncultured Eustigmatophyceae clone 9m_D9 KF765262
Uncultured Eustigmatophyceae clone 9m_H10 KF765293
Uncultured Eustigmatophyceae clone 9m_E4 KF765265
Uncultured Eustigmatophyceae clone 9m_E6 KF765266
Uncultured Eustigmatophyceae clone 0.5m_H6 KF765373
Uncultured Eustigmatophyceae clone 19m_C2 KF765181
Uncultured Eustigmatophyceae clone 19m_H9 KF765232
Uncultured Eustigmatophyceae clone 9m_A6 KF765238
Uncultured Eustigmatophyceae clone 19m_F9 KF765214
Uncultured Eustigmatophyceae clone 0.5m_I2 KF765295
Uncultured Eustigmatophyceae clone 0.5m_D7 KF765340
Uncultured Eustigmatophyceae clone 9m_C2 KF765253
Uncultured Eustigmatophyceae clone 19m_H3 KF765226
Uncultured Eustigmatophyceae clone 9m_A8 KF765239
Uncultured Eustigmatophyceae clone 19m_F4 KF765209
Group 3
Neomonodus sp. ACOI 2437 MN389516
Neomonodus sp. CAUP Q 301 KF848931
Neomonodus ovalis CAUP Q 302 KF848932
Neomonodus ovalis BCCO_30_2918 MN389519
Neomonodus ovalis BCCO_30_2917 MN389518
Neomonodus, gen. nov.
Munda aquilonaris ACOI 2424A MN389514
Munda aquilonaris ACOI 2424B MN389517
Munda aquilonaris ACOI 2424 MN389513
Munda sp. ACOI 2428 MN389515
Munda, gen. nov.
0.01
Neomonodaceae, fam. nov. (former Pseudellipsoidion group)
Eustigmatales

Figure 2.2. Phylogeny of Eustigmatophyceae based on sequences of the 18S rRNA gene including partial sequences from environmental DNA surveys. The tree was inferred using the same procedure as the tree shown in Fig. 2.1. For simplicity, the outgroup (a selection of diverse ochrophyte 18S rRNA sequences) is omitted from the figure and the main eustigmatophyte branches are collapsed as triangles, except for the family Neomonodaceae. Bootstrap values were calculated from 354 replicates and are shown when higher than 50. The positions of the five groups of partial sequences from uncultured eustigmatophytes obtained by Villanueva et al. (2014) are indicated. The full version of the tree is provided as Fig. S1.

A phylogenetic analysis of *rbc*L sequences confirmed with maximal support the monophyly of the Neomonodaceae and its placement in the order Eustigmatales. Within the Eustigmatales, the Neomonodaceae was sister to the Eustigmataceae group, although with low bootstrap support (<50%) (Fig. 2.3.). All four clades treated here as separate Neomonodaceae genera were each resolved as monophyletic with maximal support and clearly separated from each other. However, their mutual relationships differed from the inferred tree topology that resulted from analysis of 18S rRNA gene sequence data (Figs. 2.1. and 2.2.). Specifically, *Pseudellipsoidion* and *Characiopsiella* appeared as sister to each other, with Munda and Neomonodus branching off as more basal lineages (Fig. 2.3.). An analysis considering the three codon positions of the *rbc*L gene as separate partitions yielded a tree with the same topology as the with no partitioning employed, with minor differences in bootstrap support values only (Fig. 2.3.). The *rbc*L sequences revealed a degree of genetic diversity within each of the four main clades that was not apparent from the 18S rRNA gene. Thus, within *Munda*, the strain ACOI 2428 differed from the remaining three strains by four nucleotides whereas the 18S rRNA sequences were identical.

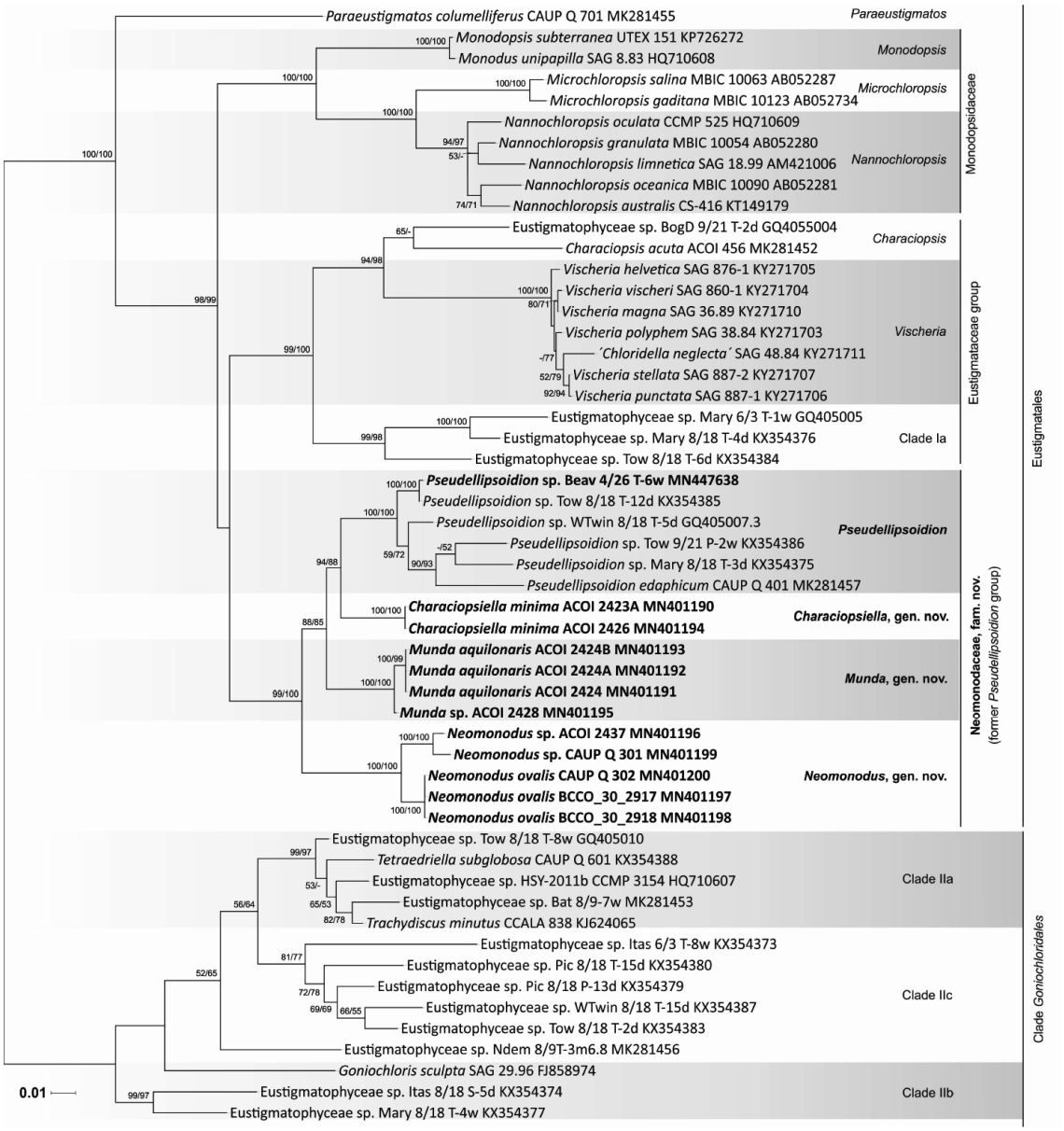

Figure 2.3. Phylogeneny of Eustigmatophyceae based on sequences of the *rbc*L gene. The phylogeny shown was inferred using maximum likelihood method implemented in RAxML (employing the GTR+Γ substitution model) with bootstrap analysis followed by thorough search for the ML tree. The topology of the tree reflects a result obtained without defining separate partitions for different codon positions of the *rbc*L gene. Two sets of bootstrap support values (calculated from 354 replicates) are given, one from the non-partitioned analysis, the other (separated by a slash) an analysis with partitions. Only values higher than 50 are shown. Labels at terminal leaves comprise the strain updated taxonomic name followed by the collection reference number when applicable and the GenBank accession number. New sequences highlighted in boldface. The root of the tree is placed between the order Eustigmatales (including *Paraeustigmatos columelliferus*) and the clade *Goniochloridales*,

following results of phylogenetic analyses of the 18S rRNA gene (see also Fig. 2.1.) and multiple plastid-encoded proteins (Ševčíková et al. 2019).

Morphological and ultrastructural characterization of the Neomonodaceae

The main morphological characters showing variation among different Neomonodaceae representatives are summarized in Table 2.2. Vegetative cells of the Neomonodaceae (Figs. 2.4. to 2.7.) are light green with different oval, ellipsoidal or elongated shapes simultaneously found in the same culture. Cell size is also quite variable, 8-11 × 4-5 μm (without stipe) with much smaller (6 × 3 μm) or larger cells (up to 30 × 10 μm) occasionally observed. Generally, the cells widen and sometimes round up when the cultures age. Many are free-floating cells with the anterior end rounded (Figs. 2.4. C and 2.6. A, C), acute (Figures 2.6. A, 2.7. D) or with a papilla (Fig. 2.4. A, 2.4. B). Sometimes these morphologies are seen in different cells in the same culture. Sessile cells with a marked polarity were also observed (Fig. 2.4. A, 2.6. E, 2.7. B). An attaching stipe and/or a disc was positioned at the posterior end of the cells of some species providing cell polarity. The stipe is always short (usually ≤ 1 μm) and consists of an extension of the cell wall with cell content (Fig. 2.6. B, 2.7. C). Substances from the surrounding medium may adhere to the stipe, causing it to become dark orange-brown. In a morphologically similar yet not directly related eustigmatophyte *Pseudocharaciopsis minuta*, this coloration was shown to be due to the accumulation of metals such as Mn (Wujek 2012). Vegetative cells of *Neomonodus* (Fig. 2.4.), *Characiopsiella* (Fig. 2.6.) and *Munda* (Fig. 2.7.) are mainly populated with stipitate cells, whereas those of *Pseudellipsoidion* (Fig. 2.5.) are exclusively free-floating. Due to their resemblance to the genera *Characiopsis* and *Monodus*, the cells with a stipe have been referred to as *Characiopsis*-like and those without a stipe as *Monodus*-like (Lee and Bold 1973, Neustupa and Němcová 2001).

Neomonodaceae cells display a cell wall in one piece, usually smooth. One to several chloroplasts are present in the cells (Fig. 2.4. A) with a typical eustigmatophycean lamellate structure with a few evenly spaced thylakoids not bounded by a girdle lamella (Fig. 2.7. E). No pyrenoid was observed under the light microscope and its absence was further noted in TEM sections of all genera (Figs. 2.4. E, 2.5. C, 2.6. B and 2.7. C). Special attention was paid to the clarification of the presence of a pyrenoid in *Pseudellipsoidion edaphicum* CAUP Q 401, where no pyrenoid was found (see the emended diagnosis of *Pseudellipsoidion edaphicum* below). One or more nearly spherical nuclei may be found in the cell (Fig. 2.7. C) with a central nucleolus often observed (Fig. 2.7. E). An apparent connection between the chloroplast endoplasmic reticulum and the nuclear envelope was observed in some cells of Neomonodaceae (Fig. 2.7. F, arrowheads), although in some sections the nucleus and the chloroplasts stay quite apart (Fig. 2.7. C).

Although it may be small or undetected in young cells, a very conspicuous orange-reddish globule is usually found in vegetative cells observed under light microscopy (Figs. 2.4. C, 2.5. D and 2.7. A); sometimes more than one. Sections show this red body composed of many adjacent droplets and not bounded by a membrane (Fig. 2.4. G). Oil droplets are frequently observed in old cells of the Neomonodaceae (Figs. 2.4. D and 2.6. A). Lamellate vesicles with refractive properties under light microscopy (Figures 2.4. A and 2.5. A) are scattered throughout the cytoplasm (Figs. 2.4. E, 2.6. B and 2.7. C) and display a finely lamellate structure (Fig. 2.4. F). Other structures and organelles common in eukaryotic cells can be found in the cytoplasm, such as tubular mitochondria (Figs. 2.4. D and 2.7. E) or a small Golgi body lying next to the nucleus (Fig. 2.7. E).

Table 2.2. Morphological characters, collection sites, former and new taxonomic assignment and GenBank accession numbers for 18S rRNA and *rbc*L gene sequences of the Neomonodaceae strains. ACOI - Coimbra Collection of Algae, Portugal; CAUP - Culture Collection of Algae at Charles University in Prague, Czech Republic, Tow, WTwin and Mary strains are kept in the laboratory of K. and M. Fawley, University of the Ozarks, U.S.A; BCCO strains as well as CAUP Q 302 are kept in the Culture Collection of Soil Algae and Cyanobacteria of the Institute of Soil Biology BC CAS, member of BCCO (Biology Centre Collection of Organisms).

Strain	Revised identification	Original identification	Cell shape	Attaching structure	Anterior end	Size (µm)[1]	Strain origin	18S rRNA	*rbc*L
CAUP Q301	*Neomonodus* sp.	*Pseudocharaciopsis ovalis*	Round/ oval/ elongated	Small point	Round/ some acute	9 x 6	Near Třtice, central Bohemia, Czech Republic; peat-bog soil; 1998	KF848931[3]	MN401199
ACOI 2437	*Neomonodus* sp.	*Characiopsis anabaena*	oval/ elongated	Small point	Acute/ some round	11 x 5	Caramulo, Portugal; 2002	MN389516	MN401196
CAUP Q 302 (=BCCO_30_2523)	*Neomonodus ovalis*	*Pseudocharaciopsis ovalis*	Round/ oval/ elongated	Small point	Round/ some acute	8 x 5	Acidophilic oak forest near Netolice, South Bohemia, Czech Republic; forest soil; 1987	KF848932[3]	MN401200
BCCO_30_2917	*Neomonodus ovalis*	---	Round/ oval/ elongated	Short stipe	Acute w. tip/ some wo. tip/ some round	9 x 5	Sokolov, Vintířov, Czech Republic; former coal mine; 1998	MN389518	MN401197
BCCO_30_2918	*Neomonodus ovalis*	---	Round/ oval/ elongated	Short stipe	Acute w. tip/ some rounded	9 x 6	Domsdorf, near Cottbus, Germany; former lignite mine; 1998	MN389519	MN401198
Beav 4/26 T-6w	*Pseudellipsoidion* sp.	---	oval	---	Rounded		Pond near Lake Monticello, Drew County, Arkansas, USA; 2010	MN389520	MN447638
Tow 8/18T-12d	*Pseudellipsoidion* sp.	---	oval	---[2]	Rounded	8,5 x 6	Itasca, State Park, Minnesota, "Tower Pond"; 2001	KF757250[3]	KX354385[4]

WTwin 8/18T-5d	*Pseudellipsoidion* sp.	---	oval	---[2]	Rounded	7,5 x 5	Itasca, State Park, Minnesota, West Twin Lake; 2001	KF757254[3]	GQ405007.3 [5] [6]
Tow 9/21P-2w	*Pseudellipsoidion* sp.	---	oval	---	Rounded	7 x 4,5	Itasca, State Park, Minnesota, "Tower Pond"; 2000	KF757253[3]	KX354386[4]
Mary 8/18 T-3d	*Pseudellipsoidion* sp.	---	oval	---[2]	Rounded	8 x 6,5	Itasca, State Park, Minnesota, Mary Lake; 2001	KF757238[3]	KX354375[4]
CAUP Q401	*Pseudellipsoidion edaphicum*	*Pseudellipsoidion edaphicum*	oval	---	Rounded	10 x 4	Near Třtice, central Bohemia, Czech Republic; peatbog soil; 1998	KF848933[3]	MK281457
ACOI 2426	*Characiopsiella mínima*	*Characiopsis minima*	Round/ oval/ elongated	Short stipe	Rounded	8 x 4	Mondego river (old branch), Coimbra, Portugal; plankton; 2000	MN389511	MN401194
ACOI 2423A	*Characiopsiella mínima*	*Characiopsis* sp.	Round/ oval/ elongated	Short stipe	Rounded/ some acute w./wo. tip	7 x 4	Minas de S. Domingos, Mértola, Portugal; mine; 2000	MN389512	MN401190
ACOI 2428	*Munda* sp.	*Characiopsis* sp.	elongated	Short stipe	Acute w. tip/ some wo. tip/ some round	9.5 x 3	Mondego river (old branch), Casal Novo do Rio, Portugal; plankton; 2000	MN389515	MN401195
ACOI 2424	*Munda aquilonaris*	*Characiopsis aquilonaris*	elongated	Short stipe	Acute w. tip/ some wo. tip/ some round	10 x 4	Lagoa da Chanca, Rabaçal, Portugal; plankton; 2000	MN389513	MN401191
ACOI 2424A	*Munda aquilonaris*	*Characiopsis aquilonaris*	elongated	Short stipe	Acute w. tip/ some wo. tip/ some round	11 x 4	Lagoa da Chanca, Rabaçal, Portugal; plankton; 2000	MN389514	MN401192
ACOI 2424B	*Munda aquilonaris*	*Characiopsis aquilonaris*	elongated	Short stipe	Acute w. tip/ some wo. tip/ some round	9 x 4	Lagoa da Chanca, Rabaçal, Portugal; plankton; 2000	MN389517	MN401193

[1] Average vegetative cell size given as cell length x cell width, excluding extremely large cells (> 50 µm long); [2] Very rarely a short stipe was seen (< 1 µm long); [3] Fawley et al. 2014; [4] Fawley and Fawley 2017; [5] Prior et al. 2009, [6] Shorter sequence reported by Prior et al. (2009) updated here to cover the same region as the newly sequenced *rbc*L genes.

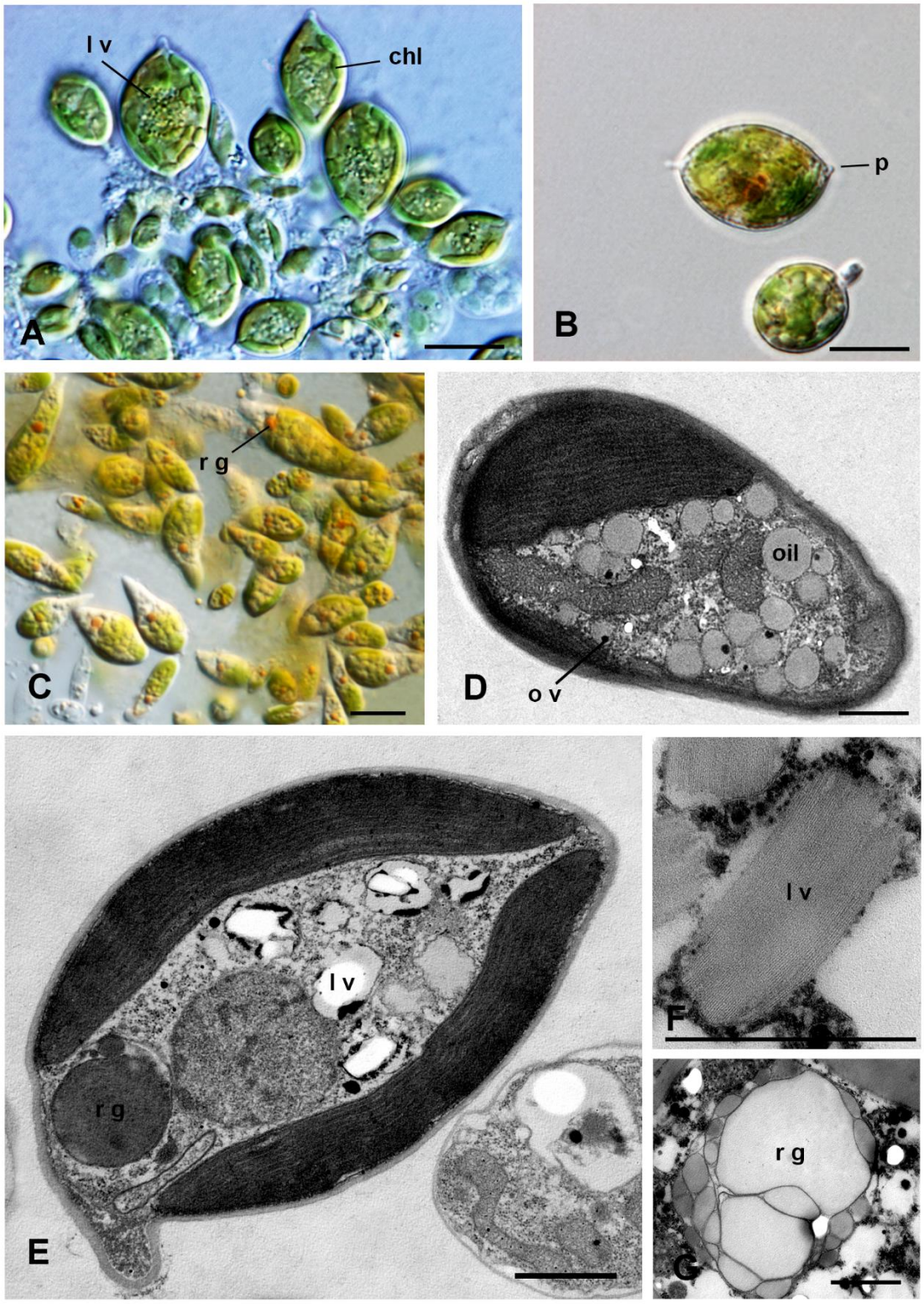

Figure 2.4. Vegetative cells of *Neomonodus ovalis* BCCO_30_2918 (A, B), *Neomonodus* sp. ACOI 2437 (C, D) and *Neomonodus ovalis* CAUP Q 302 (E - G) observed under light and electron microscopy. Apical papilla (p), chloroplast (chl), lamellate vesicles (lv), mitochondrion (m), oil droplets (oil), osmiophilic vesicles (ov), reddish globule (rg). Light micrographs with DIC, bar 10 μm; TEM micrographs, bar 1 μm.

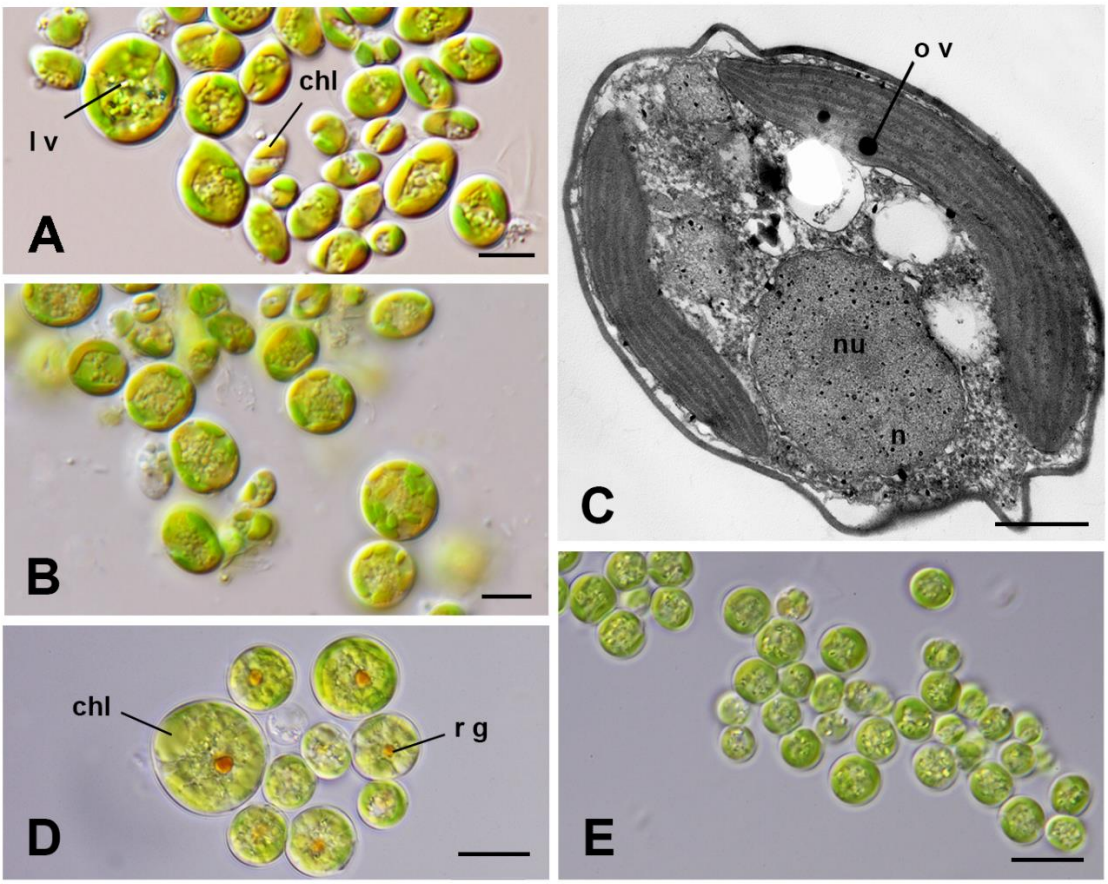

Figure 2.5. Vegetative cells of *Pseudellipsoidion* sp. WTwin 8/18 T-5d (A), *Pseudellipsoidion* sp. Mary 8/18 T-3d (B) and *Pseudellipsoidion edaphicum* CAUP Q 401 (C, D, E), observed under light and electron microscopy. Chloroplast (chl), lamellate vesicles (lv), nucleolus (nu), nucleus (n), osmiophilic vesicles (ov), reddish globule (r g). Light photographs with DIC, bar 10 μm; TEM micrographs, bar 1 μm.

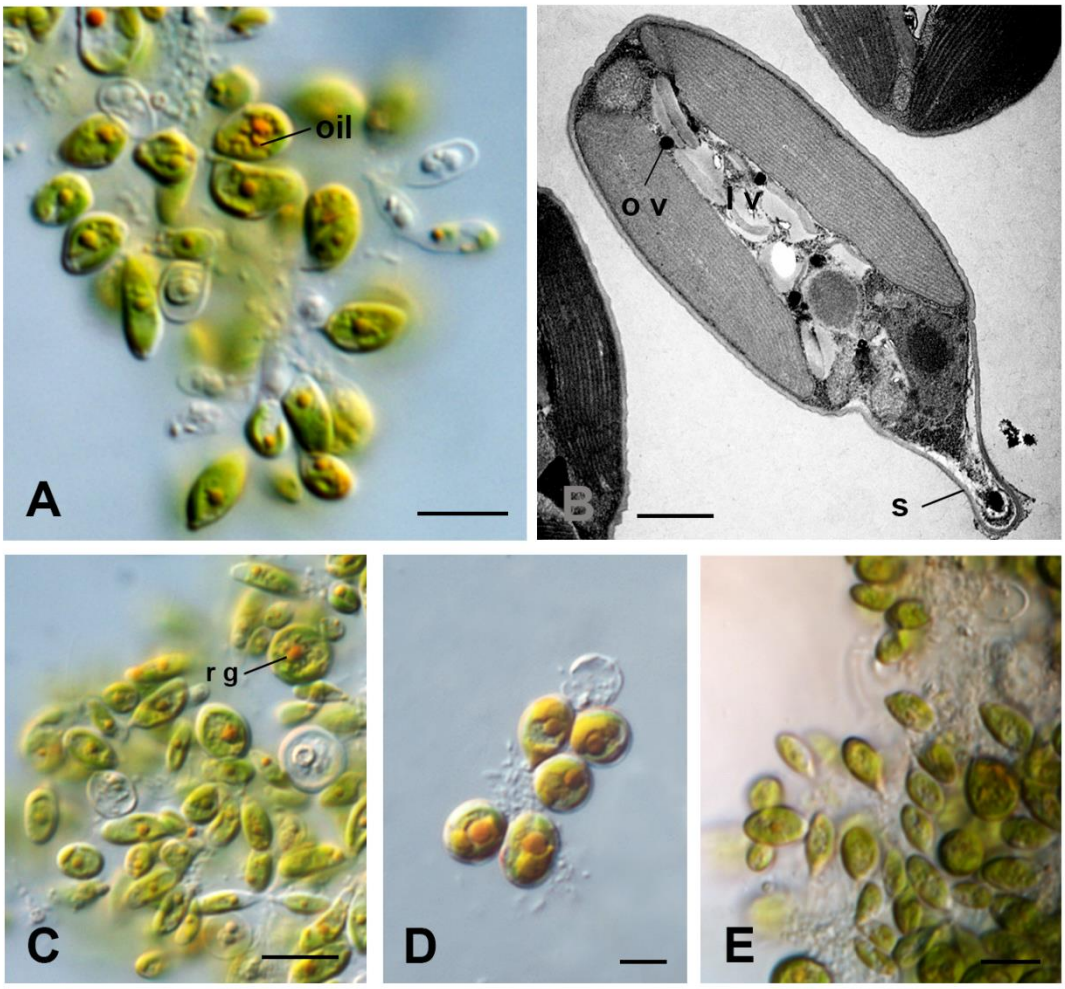

Figure 2.6. Vegetative cells of *Characiopsiella minima* ACOI 2426 (A, B, C) and *Characiopsiella minima* ACOI 2423A (D and E) observed under light and electron microscopy. Lamellate vesicle (lv), oil droplets (oil), osmiophilic vesicles (ov), reddish globule (rg), stipe (s). Light micrographs with DIC, bar 10 μm; TEM micrographs, bar 1 μm.

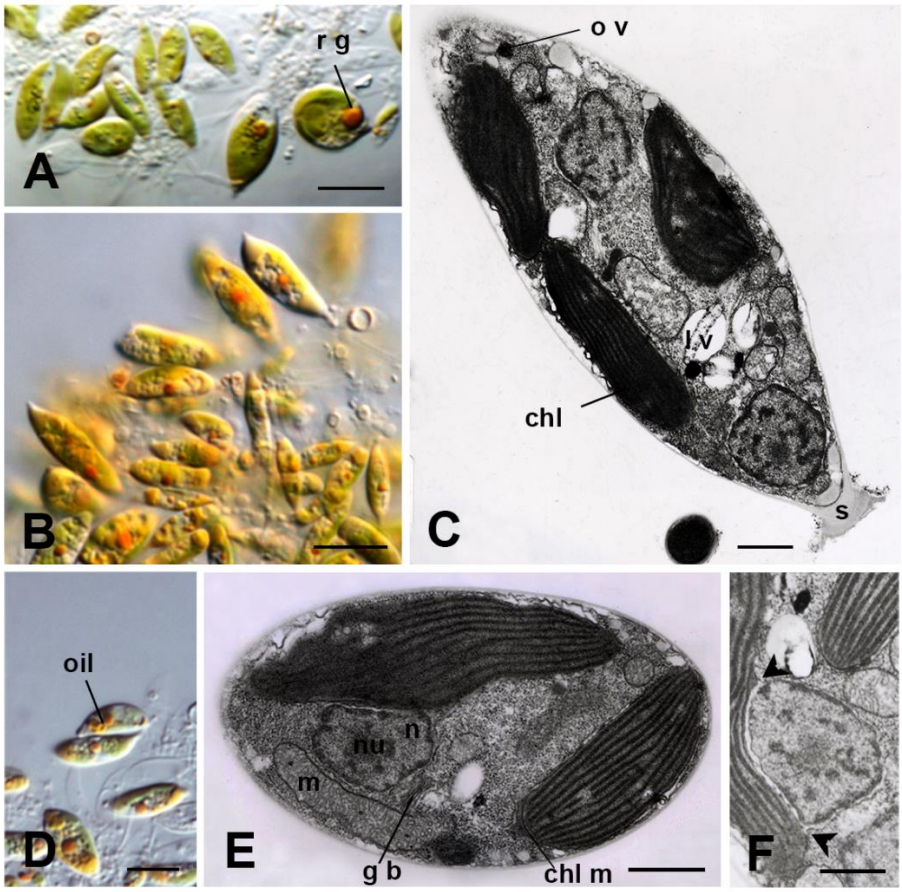

Figure 2.7. Vegetative cells of *Munda aquilonaris* ACOI 2424A (A), ACOI 2424B (B), ACOI 2424 (C, E, F) and *Munda* sp. ACOI 2428 (D), observed under light and electron microscopy. Chloroplast (chl), chloroplast membrane (chl m), Golgi body (gb), lamellate vesicles (lv), mitochondrion (m), nucleolus (nu), nucleus (n), osmiophilic vesicles (ov), reddish globule (rg), stipe (s), connection between the chloroplast endoplasmic reticulum and the nuclear envelope (arrowheads). Light micrographs with DIC, bar 10 µm; TEM micrographs, bar 1 µm.

Regarding reproductive cells, the formation of autospores was observed, followed by their release after mother cell wall disruption (Fig. 2.8. A) and rounded or elongated flask-shaped zoospores were observed in liquid cultures of all Neomonodaceae strains examined (examples shown in Fig. 2.8. B to 2.8. D). Zoospore movement was observed under the light microscope, with a visible long flagellum (Fig. 2.8. B, 2.8. C). Shadowcast preparations in *Pseudellipsoidion* sp. WTwin 8/18 T-5d and in *Munda aquilonaris* ACOI 2424 revealed a second shorter and thinner emergent flagellum (Fig. 2.8. D, 2.8. E). An extra-plastidial eyespot,

associated with a swelling at the base of the anterior, long and mastigoneme-bearing flagellum has been detected in TEM sections of zoospores (Fig. 2.8. F, 2.8. G).

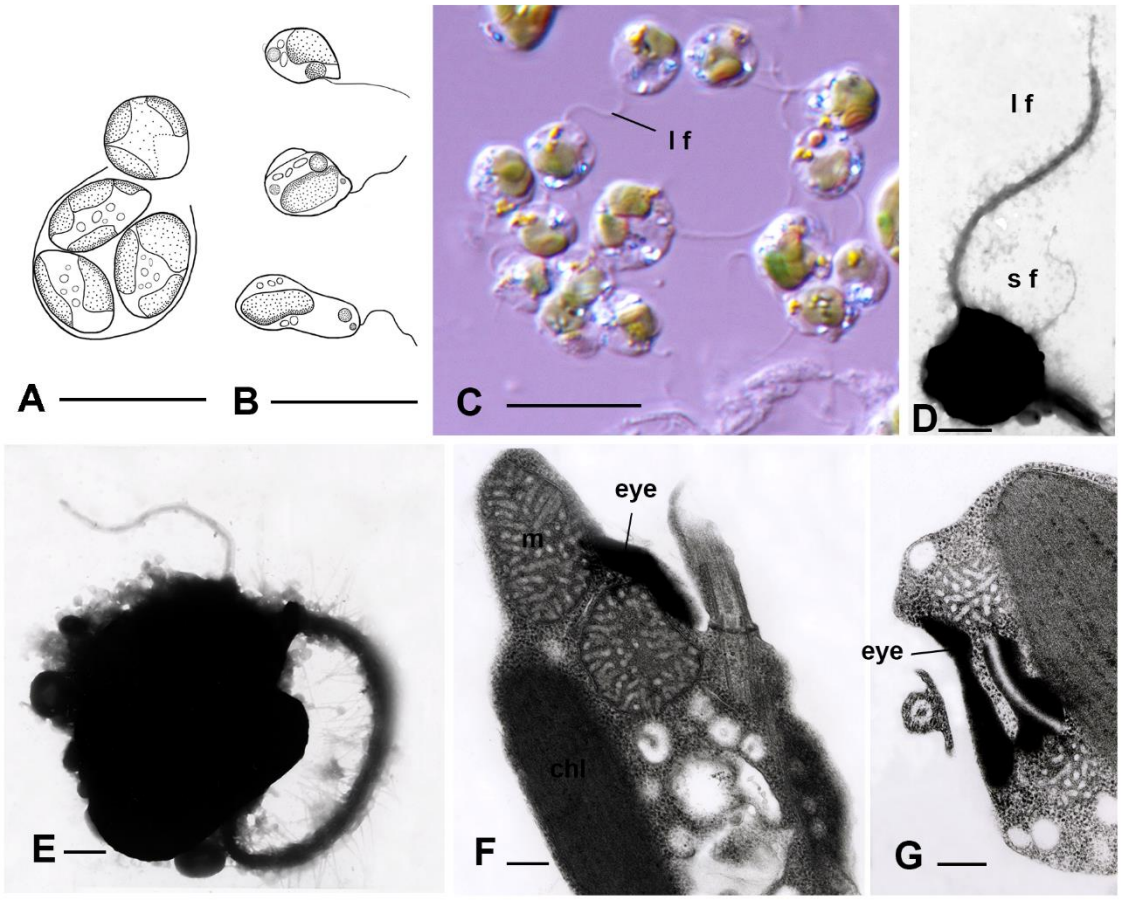

Figure 2.8. Reproductive cells of the Neomonodaceae, observed under light and electron microscopy. Autospore release in *Pseudellipsoidion* sp. Tow 8/18 T-12d (A), biflagellate zoospores of *Pseudellipsoidion* spp. strains Tow 8/18 T-12d, Mary 8/18 T-3d and WTwin 8/18 T-5d (B), *Pseudellipsoidion* sp. WTwin 8/18 T-5d (C), *Pseudellipsoidion* sp. WTwin 8/18 T-5d (D) and *Munda aquilonaris* ACOI 2424 (E - G). Chloroplast (chl), eyespot (eye), long flagellum (lf), mitochondrion (m), short flagellum (sf).

2.5. Discussion

Phylogeny of the Neomonodaceae

The overall structure of the phylogenetic tree inferred from 18S rRNA gene sequences (Fig. 2.1.) agrees well with previous similar analyses (Fawley et al. 2014, Nakayama et al. 2015, Eliáš et al. 2017, Fawley and Fawley 2017, Kryvenda et al. 2018, Fawley et al. 2019). The chief difference compared to previous studies is the expansion of the *Pseudellipsoidion* group – here formalized as the family Neomonodaceae – by ten newly characterized strains. Three of them belong to the lineage here described as the genus *Neomonodus* and one joins a group of five previously characterized strains that we here classify as the genus *Pseudellipsoidion*. The remaining six new strains added much more phylogenetic novelty to the Neomonodaceae by constituting two novel lineages deeply separated from the genera *Pseudellipsoidion* and *Neomonodus*, here established as new genera *Characiopsiella* and *Munda*. The monophyly of Neomonodaceae as a whole and of each of its four genera recognized here is independently supported by an analysis *rbc*L sequences, although the two phylogenetic markers suggest a different branching order among the genera. Future studies, ideally employing genome-scale sequence data (such as complete organellar genomes), will help resolve the internal phylogeny of this group. The degree of genetic diversity within the individual Neomonodaceae genera found using the *rbc*L gene is higher than that apparent from 18S rRNA gene sequence comparisons, in agreement with the known higher evolutionary rate of *rbc*L in comparison to the 18S rRNA gene (e.g. Patwardhan et al. 2014).

The separate status of the Neomonodaceae is also supported by the recent phylogenomic analysis of multiple plastid genes including one representative of the group, *P. edaphicum* CAUP Q 404 (Ševčíková et al. 2019). The latter analysis placed *P. edaphicum* with maximal support as a sister lineage to the family Monodopsidaceae and the Eustigmataceae group combined, in agreement with our 18S rRNA gene phylogenies (Figs. 2.1. and 2.2.) but not

the *rbc*L gene phylogeny, which shows, albeit without support, Neomonodaceae as a sister lineage of the Eustigmataceae group (Fig. 2.3.). Although the branching order of the main Eustigmatales lineages certainly needs to be corroborated by further investigations including multigene phylogenetic analyses of nuclear and mitochondrial sequences, the status of the Neomonodaceae as a family-level lineage separated from all other previously described eustigmatophyte families is firmly established.

Interestingly, inclusion of partial gene sequences from a previous environmental DNA survey (Villanueva et al. 2014) in the analysis of 18S rDNA sequence data revealed that the phylogenetic diversity of the Neomonodaceae is not limited to the four recognized genera. A diverse cluster of environmental DNA sequences is nested within the Neomonodaceae clade as a lineage that seems to correspond to a hitherto unknown separate genus, if not multiple separate genera (Fig. 2.2.). This cluster was previously referred to as the Group 3 (to distinguish it from four additional clusters represented solely by sequences from uncultivated eustigmatophytes; Villanueva et al. 2014) and the authors could not recognize its actual phylogenetic position within Eustigmatales because of the lack of 18S rRNA sequences from characterized members of the Neomonodaceae at that time. The number of different yet related genotypes constituting the Group 3, which all come from a single lake, is surprising. It may reflect a true genetic (and presumably taxonomic) diversity of this novel clade, but the presence of multiple different copies of the 18S rRNA gene in the same genome (i.e. its intragenomic heterogeneity) might also partly account for this apparent diversity (e.g., Alverson and Kolnick 2005). Isolation of the algae representing the Group 3 and their careful investigation is crucial for proper interpretation of the results of the environmental DNA survey.

Interestingly, we could now also illuminate the identity of the Group 2 defined by Villanueva et al. (2014) (see Fig. S1.). From our results, the Group 2 lineage includes the recently described

Paraeustigmatos columelliferus (Fawley et al. 2019), which represents a novel separate lineage sister to all the previously known Eustigmatales including Neomonodaceae (see also the position of *P. columelliferus*, referred to as strain Mont 10/10-1w, in the plastid phylogenomic analysis by Ševčíková et al. 2019). Thus, *P. columelliferus* may be the first encountered representative of a diverse eustigmatophyte clade for which a new formal taxon – perhaps a new family – may be established in the future. Interpretation of the family status of two more clusters of eustigmatophyte environmental DNA sequences, i.e. Groups 4 and 5, will also depend on direct characterization of the organisms behind the sequences and on the eventual formal taxonomic treatment of the phylogenetically adjacent Eustigmataceae group.

Morphology and ultrastructure of the Neomonodaceae

The cytology of all studied members of the Neomonodaceae (Figs. 2.4. to 2.8.) conforms to the diagnostic features used to segregate the Eustigmatophyceae from the Xanthophyceae. The most distinctive features are the presence of a reddish globule (sometimes more than one) in the vegetative cell and the exclusively eustigmatophycean lamellate vesicles, also present in the zoospores. The zoospores have a unique eyespot composition of extraplastidial droplets positioned near the long flagellum (Hibberd and Leedale 1970, 1971, 1972, Hibberd 1980). Although an absence of a connection between the chloroplast endoplasmic reticulum and the nuclear envelope has been considered a general eustigmatophyte characteristic separating them from other ochrophytes (Hibberd and Leedale 1970, 1972), a connection was observed in TEM preparations of the Neomonodaceae member *Munda aquilonaris*. The preservation of the continuity of those membranes has previously been documented for *Monodopsis* and *Nannochloropsis* species (Antia et al. 1975, Lubián 1982, Maruyama et al. 1986, Santos and Leedale 1995). This suggests that a more detailed investigation by employing electron tomography is needed to

rule out that the nucleus-chloroplast connection has simply been overlooked in the majority of eustigmatophytes.

One of the most conspicuous characteristics of eustigmatophytes is an orange-reddish globule usually found in vegetative cells, often more than one in larger or older cells. It is present in all Neomonodaceae, seen in light microscopy (Figs. 2.4. C, 2.5. D, and 2.7. A). It has a typical structure composed of many adjacent droplets not bound by a membrane (Fig. 2.4. G), as previously reported for other members of the eustigmatophyte class (Hibberd and Leedale 1972, Santos and Leedale 1995, Santos 1996, Eliáš et al. 2017). Its lipidic nature was hypothesized by Hibberd (1980), and a possible relation with lipid globules released from the chloroplast in Trachydiscus minutus has been considered (Přibyl et al. 2012). Lipids are the most acknowledged reserve material found in eustigmatophytes and are of biotechnological importance, especially for biofuel and food purposes (Gao et al. 2018). The accumulation of lipid droplets in the cytoplasm often has been reported (Schnepf et al. 1995/96, Přibyl et al. 2012). Lipid droplets were frequently observed in old cells of the Neomonodaceae (Figs. 2.4. D and 2.6. A). Lamellate vesicles have been described as another typical feature of eustigmatophytes (Hibberd and Leedale 1972, Santos and Leedale 1995), so far consistently found in all analyzed eustigmatophytes (Santos 1996), including the Neomonodaceae (Figs. 2.4. A, E, F, 2.5. A, 2.6. B, 2.7. C). The origin, composition and function of these structures remains unclear, but a polysaccharide nature, possibly paramylon-like, has been suggested (Schnepf et al. 1995/96).

Reproduction of eustigmatophytes is usually autosporic and production of zoospores can occur in some genera; however, sexual reproduction has not been reported (Eliáš et al. 2017). In the Neomonodaceae reproduction is achieved by both the formation of autospores and the formation of rounded or elongated flask-shaped zoospores (Fig. 2.8.). The presence of a unique type of extra-plastidial eyespot in zoospores, associated with a swelling at the base of

the anterior, long and mastigoneme-bearing flagellum is one of the most typical features of the Eustigmatophyceae and is the basis of its name (Hibberd and Leedale 1972). This structure has been detected in TEM sections of the studied strains, as expected. Zoosporic eustigmatophytes are characterized by an emerging long mastigoneme-bearing flagellum and a second flagellum that may be reduced to the basal body or emerge from the cell as a second shorter and thinner flagellum (Hibberd 1970, Santos 1996). Two emergent flagella were detected in representatives of the Neomonodaceae, with shadowcast preparations in *Pseudellipsoidion* sp. WTwin 8/18 T-5d and in *Munda aquilonaris* ACOI 2424 revealing a shorter flagellum (Fig. 2.8. D, E). The previous report on *P. edaphicum* CAUP Q 401 indicated only one, long emerging flagellum (Neustupa and Němcová 2001) but the presence of a second smaller flagellum may be interpreted from the published shadowcast photo (Fig. 16 in Neustupa and Němcová 2001); the description of the species has been emended below to reflect this. Zoospores with two flagella were previously reported for *Pseudocharaciopsis ovalis* CAUP Q 301 (Neustupa and Němcová 2001), here classified in the genus *Neomonodus*. Hence, zoospores with two flagella are probably a common characteristic of Neomonodaceae, although this needs to be confirmed for the genus *Characiopsiella*.

No morphological character stands out as potentially synapomorphic for Neomonodaceae, but two traits – the absence of a pyrenoid and zoospores having two flagella – are noteworthy, as their combination may be unique for this family. The consistent absence of a pyrenoid in the Neomonodaceae constitutes a distinctive morphological character separating the members of this family from *Characiopsis*-like eustigmatophytes belonging to the Eustigmataceae group (Amaral et al. 2011, Amaral et al. resubmitted). Note that a pyrenoid was originally reported by light microscopy in some cells of *Pseudellipsoidion edaphicum* CAUP Q 401 (Neustupa and Němcová 2001). However, the strain was re-evaluated in the present study and no pyrenoid was found, so the absence of a pyrenoid is considered a common feature for all studied members of the family.

A polyhedral pyrenoid in the vegetative cells was originally listed as one of the characteristics of the Eustigmatophyceae, with a possible exception noted for *Ellipsoidion acuminatum* CCAP 822/1 (Hibberd and Leedale 1970, 1971) that was subsequently confirmed by the authors (Hibberd and Leedale 1972). The strain was later re-identified as *Monodus ovalis* and reclassified as *Pseudocharaciopsis ovalis* by Hibberd (1981). The author pointed to the fact that the absence of a pyrenoid in *P. ovalis* contrasts with the presence of a spherical stalked pyrenoid in *P. minutum* but considered this difference not substantial enough to place the two species in different genera. The other morphological and ultrastructural characters of both vegetative cells and zoospores of the CCAP 822/1 strain as reported by Hibberd are indeed consistent with his species identification, implying the strain might be *Neomonodus ovalis* or a closely allied species. It is impossible now to verify this identity by molecular data, because the culture CCAP 822/1 maintained in the Culture Collection of Algae and Protozoa now represents a scenedesmacean alga (data not shown, but see also images of the strain provided by the CCAP collection, https://www.ccap.ac.uk/strain_info.php?Strain_No=822/1) and the original alga has most likely been lost.

Additional pyrenoid-less eustigmatophytes, unrelated to Neomonodaceae, are now known (Eliáš et al. 2017, Fawley et al. 2019). It remains to be determined whether the distribution of pyrenoid-less taxa in the eustigmatophyte phylogeny reflects multiple independent origins or multiple independent losses of the pyrenoid. In contrast, biflagellated zoospores are clearly a plesiomorphic character in eustigmatophytes, retained by Neomonodaceae and at least one independent lineage represented by *Pseudocharaciopsis minuta* (=*P. texensis*) in the Eustigmataceae group (Lee and Bold 1973). In addition, biflagellated zoospores were documented from *Botryochloropsis similis* (Preisig and Wilhelm 1989), a colonial eustigmatophyte with a phylogenetic position that remains undetermined because of the lack of molecular data (the culture is no longer available). Since *B. similis* also lacks a pyrenoid, it may in fact belong to the Neomonodaceae. The other eustigmatophytes without a pyrenoid

are known to produce uniflagellate zoospores, as is the case of *Pseudostaurastrum limneticum* and *Trachydiscus minutus* (Schnepf et al. 1995/96, Přibyl et al. 2012) or do not produce zoospores (at least at conditions tested), which is the case of the genera *Nannochloropsis* and *Microchloropsis* (Eliáš et al. 2017) and the recently described *Paraeustigmatos columelliferus* (Fawley et al. 2019).

There are no striking morphologic characters distinguishing the organisms belonging to the four Neomonodaceae genera, so molecular data is crucial for distinguishing the genera in this family. There are nevertheless some differences which may indicate the genus when molecular data is not yet available. *Pseudellipsoidion* stands out of the other three genera because its cells are devoid of a stipe. The stipitate genera *Neomonodus*, *Characiopsiella* and *Munda* present narrow morphological differences, with *Characiopsiella* having on average smaller cells than those found in the cultures of *Neomonodus* and *Munda* although some small cells may also be seen in the latter, so this characteristic must be examined and used carefully. For these reasons the genera here presented are delimited based on molecular clades defined by 18S rRNA and *rbc*L gene phylogenies.

Taxonomic considerations

Three genera of Neomonodaceae are established here to accommodate species that have been placed in the genus *Characiopsis* since their description (*Characiopsiella*, *Munda*) or at least for a transient period of time (*Neomonodus*). They form a weakly supported clade excluding *Pseudellipsoidion* in the 18S rRNA gene tree (Figs. 2.1. and 2.2.), but the *rbc*L tree suggests (with stronger bootstrap support) that they are paraphyletic with respect to *Pseudellipsoidion* (Fig. 2.3.). Regardless of the uncertain branching order within Neomonodaceae, the molecular data justify the description of these three *Characiopsis*-like lineages as separate genera, since in both 18S rRNA and *rbc*L gene trees they are resolved as lineages just as deeply diverged from

each other and from the *Pseudellipsoidion* lineage as are various other pairs of eustigmatophyte taxa classified is separate genera (e.g. *Nannochloropsis* and *Microchloropsis*; Figs. 2.1. and 2.3.).

Another question is whether three new genera are needed for the three *Characiopsis*-like Neomonodaceae lineages or whether any of them could retain its current generic assignment or be placed into another existing genus. An obvious possibility is that one of the lineages equates to the genus *Characiopsis*. The identity of this genus and its actual phylogenetic provenance (Eustigmatophyceae versus Xanthophyceae) have remained unclear, partly because of the uncertainties concerning the type of the genus discussed by Hibberd (1981). However, as we will discuss in detail elsewhere (Amaral et al. resubmitted), there is now little doubt that *Characiopsis* is a eustigmatophyte typified by the species *Characiopsis minuta*, presently referred to as *Pseudocharaciopsis minuta* and belonging to the Eustigmataceae group (Fig. 2.1.). Hence, the name *Characiopsis* is not applicable to any of the lineages of the Neomonodaceae family.

No alternative generic placements have been previously proposed for the species presently known as *Characiopsis aquilonaris* and *Characiopsis minima*, so new genera need to be established for them. Our proposal to establish the third new genus, Neomonodus, requires a more elaborate justification. Chodat (1913) described *Monodus ovalis* as a species of the new genus *Monodus* he erected in the same study. Subsequently, Chodat (in Poulton 1925) transferred the species to the genus *Characiopsis* as *Characiopsis ovalis*, but Hibberd (1981) later moved the species to still another genus, *Pseudocharaciopsis*. Molecular phylogenetic evidence from multiple isolates that morphologically fit the description of *Monodus ovalis* clearly shows it cannot be placed in the genus *Pseudocharaciopsis*, since the 18S rRNA gene sequence from the authentic strain of the type species of the genus (*P. texensis* UTEX 2113, now referred to as *P. minuta* UTEX 2113) places it robustly in the Eustigmataceae group (Fig. 2.1.). The species

also cannot stay remain in the genus *Characiopsis* (see above), so its original placement in the genus *Monodus* must be revisited.

Indeed, as discussed by Silva (1980), *Monodus ovalis* should be regarded as the original type of the genus *Monodopsis* Chodat. However, the transfer of *M. ovalis* to the genus *Characiopsis* by Chodat made the status of other described *Monodus* species uncertain, which motivated Silva to propose conservation of the genus *Monodus* with a different type, *Monodus acuminatus*. This proposal was later approved by the Committee for Algae of the International Association for Plant Taxonomy (Silva 1994). Hence, accepting *Monodus ovalis* as a member of the genus *Monodus* would imply that it is specifically related to *M. acuminatus*, at present usually referred with a changed orthography as *M. acuminata* (see the respective AlgaeBase record at http://www.algaebase.org/search/species/detail/?species_id=62247; Guiry and Guiry 2019). However, the description of *M. acuminata* differs from the morphological characteristics of the members of the "*P. ovalis*" clade in important details, namely in the shape of the cells being always round at one end and sharply acute at the opposite, the presence of a single chloroplast lying only on one side of a cell, and in the absence of an attaching stipe (Ettl 1978). Hence, erecting the new genus *Neomonodus* for *Monodus ovalis* and its allies appears to be the best way to finally settle the taxonomic status of this species.

Because authentic strains of the species of the newly established genera *Neomonodus*, *Characiopsiella* and *Munda* no longer exist, we below designate epitypes for the species to stabilize their definition for the future. Each epitype is derived from an existing culture that could be identified without reasonable doubts to the species level.

New and emended taxonomic diagnoses

Neomonodaceae R. Amaral, K.P. Fawley, Y. Němcová, T. Ševčíková, A. Lukešová, M.W. Fawley, L.M.A. Santos et M. Eliáš, fam. nov.

Unicellular with oval, ellipsoidal or slightly curved elongated cells, sometimes simultaneously present in culture. Free cells without polarity or cells possessing a posterior short attaching stipe and an anterior end rounded, acute or with a papilla. Vegetative cells with one to several chloroplasts, no pyrenoid detected, with a reddish globule and lamellate vesicles. Reproduction by formation of autospores and biflagellate zoospores. Found in ponds, lakes, soil, peat bog soil and metal mine tailings.

TYPE GENUS: *Neomonodus* gen. nov.

REMARKS: The family as delimited here presently includes four genera (together with four formally described species) confirmed as belonging to the family by DNA sequences. *Botryochloropsis similis*, currently classified as a eustigmatophyte *incertae sedis* (Eliáš et al. 2017), may be an additional member of Neomonodaceae based on its morphological characteristics.

Neomonodus R. Amaral, K.P. Fawley, Y. Němcová, T. Ševčíková, A. Lukešová, M.W. Fawley, L.M.A. Santos et M. Eliáš, **gen. nov.**

Very diverse cell morphologies and sizes in culture (8-11 × 4-5 μm). Most cells with a short stipe (0.2-1.5 μm) and anterior end acute or with a papilla. Usually more than two parietal chloroplasts. The genus is distinguished from other genera with a similar morphology on the basis of 18S rRNA and rbc*L sequences.*

TYPE SPECIES: ***Neomonodus ovalis*** (Chodat) R. Amaral, K.P. Fawley, A. Němcová, T. Ševčíková, A. Lukešová, M.W. Fawley, L.M.A. Santos et M. Eliáš **gen. et comb. nov.**

BASIONYM: *Monodus ovalis* Chodat 1913, In Materiaux pour la Flore Cryptogamique Suisse 4 (2). Berne: 182.

HOLOTYPE: fig. 156-159 in Chodat 1913.

HOMOTYPIC SYNONYMS: *Characiopsis ovalis* (Chodat) Chodat ex Poulton 1925, In Étude sur les Hétérokontes, thèse no. 777, Université de Genève. Geneva: 32. *Pseudocharaciopsis ovalis* (Chodat) Hibberd 1981, In Notes on the taxonomy and nomenclature of the algal classes Eustigmatophyceae and Tribophyceae (synonym Xanthophyceae). Botanical Journal of the Linnean Society of London 82: 110.

ETYMOLOGY: the ancient Greek prefix *neo* meaning new plus the original name *Monodus*. The genus name was proposed by the late Prof. Paul C. Silva.

EPITYPE (designated here to support holotype): strain CAUP Q 302 permanently preserved in a metabolically inactive state (cryopreserved in liquid nitrogen), deposited at Coimbra Collection of Algae (ACOI), University of Coimbra.

REMARKS: DNA sequence data revealed the separation of the five *Neomonodus* strains into two internal groups (Fig. 2.2.), although the strains do not exhibit striking morphological differences. One group includes three strains with the morphological characteristics of *Monodus* (*Pseudocharaciopsis*) *ovalis* (CAUP Q 302, BCCO_30_2917, and BCCO_30_2918). The second clade comprises strains CAUP Q 301 and ACOI 2437 previously identified as *Pseudocharaciopsis ovalis* and *Characiopsis anabaenae*, respectively. Most cells of ACOI 2437 resemble *C. ovalis*; however, narrower cells are similar to those of *Characiopsis anabaenae* Pascher 1938. A rigorous comparative morphological study of the *Neomonodus* clade combined with data from multiple genetic markers are required to decide whether all five strains represent one or multiple separate species. For this reason, we cautiously recommend the strains CAUP Q 301 and ACOI 2437 be considered as unidentified *Neomonodus* species.

REFERENCE MOLECULAR DATA (GenBank accession numbers): 18S rRNA gene – KF848932, *rbc*L gene – MN401200.

Pseudellipsoidion (Neustupa et Němcová) Němcová, emend.

Oval to ellipsoidal cells without a stipe. Cells without a pyrenoid. Biflagellate zoospore production observed.

TYPE SPECIES: *Pseudellipsoidion edaphicum* Neustupa et Němcová

Vegetative cell shape globular, oval or ellipsoidal. The cell does not possess a pyrenoid. Production of biflagellate zoospores.

REMARKS: *Pseudellipsoidion* was erected by Neustupa and Němcová (2001) in order to accommodate *P. edaphicum* CAUP Q 401. Present reinvestigation of this strain revealed, contrary to the initial report, the absence of a pyrenoid and zoospores being biflagellate rather than having only one flagellum, necessitating emendation of the original diagnosis. Sequences of the *rbc*L gene show the existence of five substantially different internal lineages within *Pseudellipsoidion*, which may be interpreted at the species level once more morphological and molecular data are available. Additional strains of *Pseudellipsoidion* spp. may also be required to fully assess the species-level taxonomy.

REFERENCE MOLECULAR DATA (GenBank accession numbers): 18S rRNA gene – KF848933, *rbc*L gene – MK281457.

Characiopsiella R. Amaral, K.P. Fawley, A. Němcová, T. Ševčíková, Y. Lukešová, M.W. Fawley, L.M.A. Santos et M. Eliáš, **gen. nov.**

Small cells 5-8 × 3-4 μm, oval or ellipsoidal, with a short attaching stipe, producing zoospores. The genus is distinguished from other genera with a similar morphology on the basis of 18S rRNA and rbc*L sequences.*

TYPE SPECIES: *Characiopsiella minima* (Pascher) R. Amaral, K.P. Fawley, A. Němcová, T. Ševčíková, Y. Lukešová, M.W. Fawley, L.M.A. Santos et M. Eliáš, **gen. et** comb. nov.

BASIONYM: *Characiopsis minima* Pascher 1938, In Heterokonten, Kryptogamen-Flora von Deutschland, Österreich und der Schweiz. (Rabenhorst, L. Eds) Vol. 11, Teil 5, Akademische Verlagsgesellschaft, Leipzig: 731-732.

HOLOTYPE: fig. 582 in Pascher 1938.

EPITYPE (designated here to support holotype): strain ACOI 2426 permanently preserved in a metabolically inactive state (cryopreserved in liquid nitrogen), deposited at Coimbra Collection of Algae (ACOI), University of Coimbra.

ETYMOLOGY: The name is derived from *Characiopsis* and the Latin diminutive suffix *–ella* in reference to the morphological resemblance to *Characiopsis* species and the small size of the cells.

REMARKS: The genus is presently considered monotypic, as the two strains representing it (ACOI 2426 and ACOI 2423A) are morphologically highly similar and exhibit identical 18S rRNA and *rbc*L gene sequences.

REFERENCE MOLECULAR DATA (GenBank accession numbers): 18S rRNA gene – MN389511, *rbc*L gene – MN401194.

Munda R. Amaral, K.P. Fawley, Y. Němcová, T. Ševčíková, A. Lukešová, M.W. Fawley, L.M.A. Santos et M. Eliáš, **gen. nov.**

Cells 9-11 × 3-4 μm, elliptical to cylindrical with a short stipe and fewer than five large parietal chloroplasts. Production of biflagellate zoospores. The genus is distinguished from other genera with a similar morphology on the basis of 18S rRNA and rbc*L sequences.*

TYPE SPECIES: ***Munda aquilonaris*** (Skuja) R. Amaral, K.P. Fawley, Y. Němcová, T. Ševčíková, A. Lukešová, M.W. Fawley, L.M.A. Santos et M. Eliáš, **gen. et comb. nov**.

BASIONYM: *Characiopsis aquilonaris* Skuja 1964, In Grundzüge der Algenflora und Algenvegetation der Fjeldgegenden um Abisko in Schwedisch-Lappland. Nova Acta Regiae Societatis Scientiarum Upsaliensis, Series 4, 18(3): 333.

HOLOTYPE: Tab. LXV, fig. 12-13 in Skuja 1964.

EPITYPE (designated here to support holotype): strain ACOI 2424 permanently preserved in a metabolically inactive state (cryopreserved in liquid nitrogen), deposited at Coimbra Collection of Algae (ACOI), University of Coimbra.

ETYMOLOGY: The genus name is a tribute to the Mondego river, the largest entirely Portuguese river that runs through the city of Coimbra, since all strains were isolated from its basin. *Munda* is a Roman name for Mondego, meaning clarity and purity.

REMARKS: Some genetic diversity among the four strains assigned to *Munda* is apparent from *rbc*L gene sequences. Three of them (ACOI 2424, ACOI 2424A, ACOI 2424B) have identical *rbc*L sequences and can be unambiguously identified as *Characiopsis aquilonaris*, whereas the forth does not fit the description of this species that well and differs from the other three strains at four positions of the *rbc*L gene, so it may represent a separate species and is hence cautiously identified as *Munda* sp. ACOI 2428.

REFERENCE MOLECULAR DATA (GenBank accession numbers): 18S rRNA gene – MN389513, *rbc*L gene – MN401191.

2.6. Conclusions

Our expanded sampling and the analysis of *rbc*L gene sequences in addition to 18S rRNA gene sequences corroborate the former *Pseudellipsoidion* group as a robustly monophyletic familial lineage within the Eustigmatales, here formalized as the family Neomonodaceae. We established a new genus, *Neomonodus*, to hopefully provide a final taxonomic home for the

species introduced to science as *Monodus ovalis* and subsequently moved to different genera, the most recent being the polyphyletic genus *Pseudocharaciopsis*. By obtaining the first ultrastructural and molecular data from *Characiopsis minima* and *Characiopsis aquilonaris* we demonstrated that these algae are eustigmatophytes, further enriching the diversity of this class at the expense of xanthophytes. At the same time, we show that the genus *Characiopsis*, as presently conceived, is polyphyletic, which we partly solve by erecting two new genera, *Characiopsiella* and *Munda* in the Neomonodaceae to include *Characiopsiella minima* and *Munda aquilonaris*. Our study thus takes an important step towards modern classification of eustigmatophytes. Further work on the Neomonodaceae has to be done to clarify the taxonomic significance of the genetic diversity apparent within individual genera and a comprehensive reassessment of the large genus *Characiopsis* is needed to resolve its identity and scope.

3.

Characiopsis Borzì belongs to the Eustigmatophyceae

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3.1. Abstract

Characiopsis, established by Borzì in 1895, is the largest genus traditionally classified in the class Xanthophyceae. However, *Characiopsis*-like algae studied over the last five decades by transmission electron microscopy and molecular phylogenetics have all proved to belong to a different class, the Eustigmatophyceae. Despite this, *Characiopsis* is still treated as a xanthophyte taxon by most resources on algal taxonomy, partly because of uncertainties concerning the identity of the type of the genus. Here we document the morphology of 20 morphologically diverse, and mostly previously unstudied *Characiopsis* isolates to document their morphology and establish their phylogenetic position by 18S rRNA and *rbc*L gene sequence data. We demonstrate that all these algae constitute a single clade within the eustigmatophyte subgroup referred to as the Eustigmataceae group. From careful reexamination of previous taxonomic accounts concerning the genus *Characiopsis* we conclude that its type is undoubtedly *Characiopsis minuta* (Braun) Borzì (basionym *Characium minutum* Braun). To account for the loss of the holotype of this species, we designate a neotype and also a supporting epitype (a cryopreserve culture of one of the studied strains). Our results thus convincingly show that the genus *Characiopsis* must be transferred from the Xanthophyceae to the Eustigmatophyceae. Furthermore, its specific assignment to the Eustigmataceae group is consistent with our observation of a pyrenoid in most of the strains studied, which distinguishes these algae from pyrenoid-less species previously classified in the genus *Characiopsis* but recently accommodated in the newly erected genera *Neomonodus*, *Characiopsiella*, and *Munda* in the eustigmatophyte family Neomonodaceae. We additionally confirm the previous suggestion that *C. minuta* is closely related to, if not conspecific with, *Pseudocharaciopsis texensis* K.W.Lee & Bold, the type of the genus *Pseudocharaciopsis*, which is thus rendered a junior synonym of *Characiopsis*. Altogether, our work significantly improves the classification of a charismatic yet poorly known group of algae.

3.2. Introduction

The current concept of the class Xanthophyceae (yellow-green algae), one of the most prominent lineages of ochrophyte algae, is been largely inherited from the pre-molecular era of phycology. According to the most recent general treatment of the class by Maistro et al. (2017), it comprises about 600 described species in over 90 genera. However, as the authors emphasize, the traditional classification into orders, families and genera is not congruent with insights from molecular phylogenetics and critical revision of the xanthophyte systematics is needed. The problem, however, is not only in the inaccurate internal classification of the group, but also in the fact that the Xanthophyceae as presently circumscribed is a polyphyletic taxon including organisms that are not directly related to the "core" of the class. Various traditional xanthophytes, as presented in the most recent monographic account by Ettl (1978), were later shown to be representatives of unrelated groups, such as Chlorarachniophyta (Hibberd and Norris 1984) and green algae (Gärtner and Schragl 1988, Darienko et al. 2010, Eliáš et al. 2013). Other taxa may still be misplaced in the Xanthophyceae and a selection of those are the subject of this paper.

Most important for an improved definition of the Xanthophyceae was the realization that some of its traditional members constitute a group of their own, formalized as the class Eustigmatophyceae by Hibberd and Leedale (1970, 1971). Over the years the number of taxa moved from xanthophytes to eustigmatophytes has been growing and as a result, more than half of the presently known ~35 eustigmatophyte species were originally placed in the Xanthophyceae (Hibberd 1981, Eliáš et al. 2017, Amaral et al. 2020). Eustigmatophytes differ from xanthophytes by a suite of features concerning the ultrastructure and pigment composition and are readily separated by molecular phylogenetics (Eliáš et al. 2017). However, the majority of traditional xanthophytes have not yet been studied using these

modern approaches and it is likely that a substantial proportion of them proves to be hitherto unrecognized eustigmatophytes when investigated properly.

The genus *Characiopsis* Borzì is the largest among all genera formally classified in Xanthophyceae (Ettl, 1978). It was established by Borzì (1895) for accommodating species originally classified in the chlorophyte genus *Characium* Braun in Kützing 1849, but differing from *bona fide* members of the genus by accumulating oil as the reserve material rather than intraplastidial starch. Additional characters used by Borzì for distinguishing *Characiopsis* from *Characium* included the presence of a fewer number of chloroplasts and the absence of a pyrenoid. Six species were transferred by Borzì from *Characium* to *Characiopsis*, with *Characium minutum* Braun in Kützing, in its new combination called *Characiopsis minuta* (Braun) Borzì, designated by Borzì as the type species of the genus. Subsequent work by Lemmermann (1914), Pascher (1925, 1938), Ettl (1960, 1977, among other studies), Pizarro (1995) and others have substantially expanded the genus *Characiopsis*, partly by transferring additional species from *Characium*, but mostly by describing completely new species. The work of the previous generations of phycologists has historically generated some 190 names of *Characiopsis* species or their forms and varieties (Index Nominarum Algarum; https://ucjeps.berkeley.edu/ina). AlgaeBase, a key resource of taxonomic information for algae (Guiry and Guiry, 2019), presently lists 89 *Characiopsis* species flagged as accepted, together with 16 other species names of uncertain status or considered to be synonyms (https://www.algaebase.org/search/genus/detail/?genus_id=43814).

Interestingly, none of the *Characiopsis* species studied by modern methods has so far been confirmed as xanthophytes; instead, they have all been demonstrated to belong to Eustigmatophyceae. The first such case was initially investigated under the name *Ellipsoidion acuminatum* and shown to exhibit the typical cytological features of eustigmatophytes (Hibberd & Leedale 1970, 1972). The alga was later reidentified by Hibberd (1981) as

Characiopsis ovalis (Chodat) Chodat and thereafter treated as a new combination *Pseudocharaciopsis ovalis* (Chodat) Hibberd in the eustigmatophyte genus *Pseudocharaciopsis* Lee & Bold. Hibberd (1981) additionally proposed that *Pseudocharaciopsis texensis* Lee & Bold, demonstrated to be a eustigmatophyte on the basis of its ultrastructure (Lee and Bold 1973), is in fact synonymous with *C. minuta*, leading him to create a new combination *Pseudocharaciopsis minuta* (Braun) Hibberd. Molecular data confirmed that both *Pseudocharaciopsis* species belong to eustigmatophytes, but revealed that they are not directly related to each other, rendering the genus polyphyletic (Fawley et al. 2014). The 18S rRNA gene was subsequently sequenced from strains assigned to *Characiopsis saccata* N. Carter, *Characiopsis acuta* (Braun) Borzì, and *Characiopsis longipes* (Braun) Borzì (Fawley et al. 2014, Kryvenda et al. 2018), which proved to be eustigmatophytes closely related to *P. minuta*, and the placement of one of these strains in eustigmatophytes was further confirmed by a phylogenetic analysis of its plastid genome sequence (Ševčíková et al. 2019). However, no morphological data were provided for these strains and their identification was not verified, making the taxonomic implications of these findings uncertain.

Using molecular phylogenetics and transmission electron microscopy (TEM) we have recently studied several *Characiopsis*-like isolates, including strains identified as *Characiopsis minima* Pascher and *Characiopsis aquilonaris* Skuja (Amaral et al. 2020). Again, they were found to be eustigmatophytes, falling into a broader clade together with *P. ovalis* and another alga, *Pseudellipsoidion edaphicum* Neustupa et Němcová. This clade, previously known as the *Pseudellipsoidion* group (Fawley et al. 2014), was formalized as the new family Neomonodaceae, with a new genus *Neomonodus* created for (*Pseudo*)*characiopsis ovalis*. In addition, *C. minima* was transferred into a new genus *Characiopsiella* and *C. aquilonaris* was moved to the new genus *Munda* (Amaral et al. 2020). This work thus improved the classification of *Characiopsis*-like algae by removing the polyphyly of the genus *Pseudocharaciopsis*, and by finding a home for two species from the apparently polyphyletic genus *Characiopsis*. Nevertheless, some key

questions remain open: what is the actual position of the genus *Characiopsis* and what is its relationship to the genus *Pseudocharaciopsis*?

To pursue the answer, we present morphological and molecular characterization of 20 *Characiopsis* strains, which have either not been studied before, or for which only molecular data have been reported so far. We combine the new findings with a discussion of the formal taxonomy of *Characiopsis* to conclude that this genus belongs to eustigmatophytes rather than xanthophytes and that *Pseudocharaciopsis* is a junior synonym of *Characiopsis*.

3.3. Materials and Methods

Algal cultures and light microscopy

All strains selected for the study (Table 3.1.) were obtained from the Coimbra Collection of Algae (ACOI). The strains had previously been identified at ACOI based on light microscopy observations and attributed to the genus *Characiopsis* according to Ettl (1978), Pizzaro (1995), and original sources (when accessible to us) for species not covered by these two monographs. In addition, the strain ACOI 307 (*Chlorobotrys regularis*) was used to obtain the *rbc*L gene sequence (Genbank accession number MT374821) in order to improve the sampling for a phylogenetic analysis. The strains were cultivated in liquid Desmideacean Medium (Schlösser 1994), pH 6.4-6.6, at 20°C, under a light intensity of 10 μmol photons m^{-2} s^{-1} (12:12 h photoperiod) provided by cool white fluorescent lamps. Morphological evaluation of the cells was performed using a Leica DMRB microscope with conventional light microscopy or DIC microscopy, using 60x and 100x PLAN APO objectives. Micrographs were acquired with a Nikon DS-Fi2 digital camera. Cell size was accessed by using the digital image analysis software NIS 4.60 (Isaza).

Transmission electron microscopy

For TEM, the cell suspension was fixed for 150 min with 2.5% glutaraldehyde in phosphate buffer (0.05 M pH 6.8), with glutaraldehyde subsequently washed out with the same buffer by centrifugation for 5 min at 2,000 rpm. The cell suspension was embedded in 2% agar and post-fixed in 1% osmium tetroxide solution (prepared 1:1 (v/v) with the same phosphate buffer) for 2 h. The fixative was then washed out three times successively by addition of the buffer and centrifugation (5 min at 2,000 rpm). Samples were dehydrated in an ethanol series (70%, 80%, 95% and 100%), each for 15 min. Samples were then embedded into a sequence of a mixture of ethanol and Spurr's resin (33%, 50% and 66%) for 1 h and finally into 100% Spurr's resin and kept overnight in a desiccator. Resin blocks were cut with an ultramicrotome (Ultracut E, Reichert-Jung) and ultrathin sections were mounted on copper grids and stained with 2% uranyl acetate and 0.2% lead citrate. Samples were examined in a FEI-Tecnai G2 Spirit Bio Twin electron microscope. Direct preparations of zoospores were obtained by fixing a drop of zoospore suspension on a formvar/carbon-coated grid in 2% osmium tetroxide vapor, drying at room temperature and shadowcasting with gold/palladium.

PCR amplification and DNA sequencing

Cells were collected by centrifugation of 2 ml of culture at 14,000 rpm and disrupted using a mixer mill (MM200, Retsch, Haan, Germany) and glass beads for 5 min. Genomic DNA was extracted using an Invisorb® Spin Plant Mini Kit (Stratek). PCR was performed with MyTaq™ Red DNA Polymerase (Bioline, United Kingdom) or Supreme NZYTaq II 2x Green Master Mix (Nzytech, Portugal), under the following conditions: denaturation at 95°C for 2 min, 35 cycles of 95°C for 30 sec, 52°C for 30 sec, 72°C for 2.5 min, and final extension at 72°C for 5 min. PCR products from amplification of the 18S rRNA and *rbc*L genes were

purified using a GenElute™ PCR Clean-Up Kit (SIGMA). Sequences were obtained using a BigDye® Terminator v3.1 Cycle Sequencing Kit (ThermoFisher scientific) and analysed using the 3130xl Genetic Analyzer in the DNA Sequencing Laboratory of the Faculty of Science, Charles University (Prague). Primers used for obtaining full sequences of the 18S rRNA gene included the amplification primers 18S-F and 18S-R and internal sequencing primers according to Katana et al. (2001). Primers used for amplification of *rbc*L were EU-rbcL-F1 or eustigrbcL-F and the reverse primer EU-rbcL-R1 (Amaral et al. 2020) or alternatively a combination of the forward DPrbcL7 (Jones et al. 2005) and reverse NDrbcL8 (Daugbjerg and Andersen 1997). For sequencing reactions, the amplification primers were used along with the newly designed sequencing primers (Amaral et al. 2020). Sequencing reads were assembled with SeqAssem (SequentiX, http://www.sequentix.de/software_seqassem.php) and manually edited by visual inspection of sequencing chromatograms. Sequences were trimmed to exclude primer regions.

Phylogenetic analyses

The complete dataset for analyses of the 18S rRNA gene sequences included a total of 129 sequences and consisted of the 18 newly obtained sequences of *Characiopsis* strains, and a selection of 15 sequences from phylogenetically diverse ochrophytes to provide an outgroup. The sequences were aligned with MAFFT 7.429 (Katoh and Frith 2012, Katoh and Standley 2013), using the "Add" option and a preexisting alignment used in a previous study (Amaral *et al.*, 2020). Redundant sequences were removed in BioEdit version 7.0.5 (Hall 1999; http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and the resulting final alignment was trimmed with trimAl v1.4.rev6 by removing columns with more than 20% gaps (Capella-Gutiérrez et al. 2009; https://www.genome.jp/tools/ete/), leaving 1759 positions for tree inference. For the *rbc*L gene analysis, a selection of 74 eustigmatophyte sequences available

from GenBank (retaining one sequence per described species for non-*Characiopsis* representatives) and the 20 newly obtained sequences were aligned with MAFFT 7.429. The termini of the alignment were trimmed in BioEdit to remove positions with a high percentage of missing data, leaving 1347 positions. Trees were inferred using the maximum likelihood (ML) method implemented in RAxML (8.2.12) at the Cyberinfrastructure for Phylogenetic Research (CIPRESS) Portal (http://www.phylo.org/sub_sections/portal) (Miller et al., 2010) using the strategy of Stamatakis et al. (2008) for obtaining the highest likelihood tree. The evolutionary model used was the default GTR+Γ. Bootstrap analyses was performed with the rapid bootstrapping procedure, with the adequate number of replicates detected by the program itself ("halt bootstrapping automatically" option); the number of bootstrap replicated for each tree is specified in the respective figure legends. Trees were drawn with the aid of the iTOL tool (Letunic and Bork 2016; https://itol.embl.de/).

3.4. Results

General morphology of *Characiopsis* spp. strains

Vegetative cells of the *Characiopsis* spp. investigated are light green, with cell shapes varying from ovoid, fusiform (acute), ellipsoidal to cylindrical (Figs 3.1. to 3.22.). Cell size varies from very small species or strains with a size range of 12-29 × 3-14 μm (rarely up to 63 μm long) to larger species 32-43 × 10-12 μm (rarely up to 84 μm long); old cells are wider and round up. All strains exhibit cell polarity given by an attaching stipe or disc (stipitate cells) positioned at the posterior end of the cell. The stipe is an extension of the cell wall and is usually short (Fig. 3.7.), but in some cases long and thin (Fig. 3.18.). An orange-brownish accumulation on the stalk of the cells may occasionally be observed (Fig. 3.17.), which possibly corresponds to mineral deposits containing manganese and other elements, as reported for *Pseudocharaciopsis minuta* (Wujek 2012). On the apical end, cells may be round

(Fig. 3.11.) or acute (Figs. 3.3. and 3.6.) and often display a translucid tip (Figs. 3.12. and 3.18.). *Characiopsis* cell wall is smooth and continuous with the stalk and the apical tip (Fig. 3.18.). Sometimes there was a visible thickening of the cell wall at the apical end and/or at the base (Fig. 3.22.) and in some cases although it seems like a thickening, it looks more like a distinctive refringent portion of the cell wall (Fig. 3.12.), a feature also mentioned for *Characiopsis naegelii* Braun a long time ago by Carter (1919).

Young cells display one to two parietal chloroplasts (Fig. 3.16.) or several in older cells. A globular, bulging pyrenoid was observed with light microscopy (Figs 3.4., 3.11. and 3.21.), as a refractile body projecting from the chloroplast. This structure was clearly seen in all investigated strains except for ACOI 2438, ACOI 3169, and ACOI 2436. A narrow stalk attaching the pyrenoid to the chloroplast was observed in sections (Fig. 3.24.), in accordance with previous reports for *Pseudocharaciopsis minuta* (Lee and Bold 1973, Santos 1996). The pyrenoid matrix is devoid of thylakoids and is surrounded by the characteristic flattened lamellate vesicles (Fig. 3.24.). What appears to be a multiple-stalked pyrenoid may be seen in some sections (Fig. 3.25.).

The two most distinctive eustigmatophyte organelles were found in the cells, the reddish globule (Figs 3.2., 3.12., 3.18.) composed of several adjacent droplets not bound by any membrane (Fig. 3.26.) and refractive lamellate vesicles scattered in the cytoplasm (Fig. 3.26.). Other eukaryotic cell organelles were detected in TEM sections, such as a Golgi body lying next to the nucleus (Fig. 3.26.) and mitochondria with tubular cristae (Fig. 3.24.). In larger cells more than one nucleus was present (Fig. 3.23.), agreeing with previous studies of *Munda* sp. ACOI 2424 (Amaral et al. 2020), *Neomonodus ovalis* (syn. *Characiopsis ovalis*) (Poulton 1926) and *Characiopsis saccata* (Carter 1919). A connection between the chloroplast endoplasmic reticulum and the nuclear envelope could not be seen with confidence in any strain investigated by TEM

and may be absent as generally seems to be the case in eustigmatophytes (Fig. 3.26.; Eliáš et al. 2017). Reserves in the form of oil droplets were occasionally observed (Fig. 3.13.).

As is also generally true in eustigmatophytes (Eliáš et al. 2017, Amaral et al. 2020), only asexual reproduction was observed in *Characiopsis* spp. Abundant zoospore production (more than ten per zoosporangia) was observed in the strains ACOI 2432 (Fig. 3.6.) and ACOI 2430. Despite their small size, zoospores were occasionally observed by light microscopy swimming with a visible long flagellum. Formation and release of four autospores per mother cell was observed (Figs 3.14. and 3.19.).

Identification and specific characteristics of *Characiopsis* spp. strains

The original identification of the investigated strains at ACOI, was re-evaluated, leading to revised identification in some cases. Ten strains could be matched with reasonable confidence to known *Characiopsis* species, whereas the other ten strains displayed characteristics precluding their unambiguous identification at the species level. Identification and main morphological characteristics of the strains studied by us are summarized in Table 3.1.

Several strains were initially considered as candidates for an alga matching the original verbal description of *Characium minutum* by Braun (in Kützing 1849), later documented by him by a drawing (Braun 1855; Fig. 3.1.). Of these, the strain ACOI 2423 seems to best fit the characteristics of the alga observed by Braun and is thus identified here as *Characiopsis minuta*. The cells are long, acute or frequently with a tip, and with a short stipe at the opposite end. They may be slightly curved on one or both ends and become wider and larger in older cultures (Figs 3.2.-3.4.). The cell size ranges from 12 to 29 (less frequently up to 42) μm in length and from 3 to 14 μm in width. The strain ACOI 2425 (Fig. 3.5.) is generally similar to ACOI 2423, but cells are longer and thinner than ACOI 2423. Owing to these differences, we cautiously refer to it as *Characiopsis* cf. *minuta*.

The strain ACOI 2432 would fit the morphological characteristics of *C. minuta* but is noticeably larger (Table 3.1.). The formation of more than 10 small zoospores per zoosporangium was observed in this strain (Fig. 3.6.). Cells of the strain ACOI 2438B are also larger than those of *C. minuta* and most of them have a round rather than pointed apex (Fig. 3.7.). The strains ACOI 2429 (Fig 3.8.), ACOI 2429A (Fig 3.9.) and ACOI 2430 (Fig. 3.10.) also somewhat resemble *C. minuta* in morphology but are wider and have a rounder cell end (Fig. 3.10.). Unambiguous identification to the species level based on all sources available to us proved impossible for the strains ACOI 2432, 2438B, 2429, 2429A, and 2430, so they are all referred to as *Characiopsis* sp.

The strain ACOI 2433 exhibits oval vegetative cells with a round apex and a short stipe (Fig. 3.11.). It seems to be readily identifiable as *Characiopsis pernana* Pascher. Cells of the strains ACOI 2427 and ACOI 2427A are small and wide, with a rounded apex, or sometimes acute and with a short stipe on the opposite end (Fig. 3.20.). They resemble *Characiopsis minutissima* but are significantly larger: 13-24(56) × 4-7(18) μm and 13-18(31) × 4-6(14) μm respectively, compared to the 6-9 × 4-6 μm reported for *C. minutissima* (Ettl 1978). Therefore, we refer to them as to *Characiopsis* cf. *minutissima*. One group of six strains is characterized by the presence of a long thin stipe. Two of them, ACOI 456 (Figs 3.12.-3.14.) and ACOI 1837 (Fig. 3.15.), have oval cells with a tip. These characteristics fit the description of *Characiopsis acuta*. The strains ACOI 1838, 1839, 1839A, and 2438 have longer oval (oblong) acute cells, with or without a tip and the stipe being particularly long (6-12 μm) (Figs 3.16. and 3.17.; ACOI 1839 and 1839A are not shown). These "typical" cells indicate that the strains correspond to the species *Characiopsis longipes*, although we note that other cell morphologies were observed to co-exist in the cultures.

The 18S rRNA gene sequence from the strain ACOI 481 has been reported before (Kryvenda et al. 2018) and its replica SAG 15.97 (Fawley et al. 2014) was assigned to

Characiopsis saccata, but without providing any data on the morphology of the strains. The cells are large, much longer than they are wide, with a short stipe at the base and rounded at the opposite end (Fig. 3.21.). Some cells are straight, but the majority exhibits curved ends and widening of one or both ends of the cell or even a contorted shape. Rarely, cells with a triangular form were found, too. Many reddish globules were found in the cells (Fig.3.21.). The cells differ from *C. saccata* in the fact that most cells do not have an acute end and also contorted cells are frequently seen, whereas the *C. saccata* cells depicted by Carter (1919) are acute and not contorted although some are curved (Carter, 1919). Owing to these doubts, we refer to ACOI 481 (SAG 15.97) as *Characiopsis* cf. *saccata*. In the case of strains ACOI 2434 and ACOI 3169, the cells are cylindrical, long and large, with a rounded apex. A thickening of the cell wall was sometimes observed in ACOI 3169 (Fig. 3.22.). Altogether, we confirm the initial identification of these two strains as *Characiopsis cedercreutzii*. The strain ACOI 2436, isolated from the same field sample as ACOI 2434, has shorter and wider cells (Table 3.1.), so we leave it unidentified to the species level.

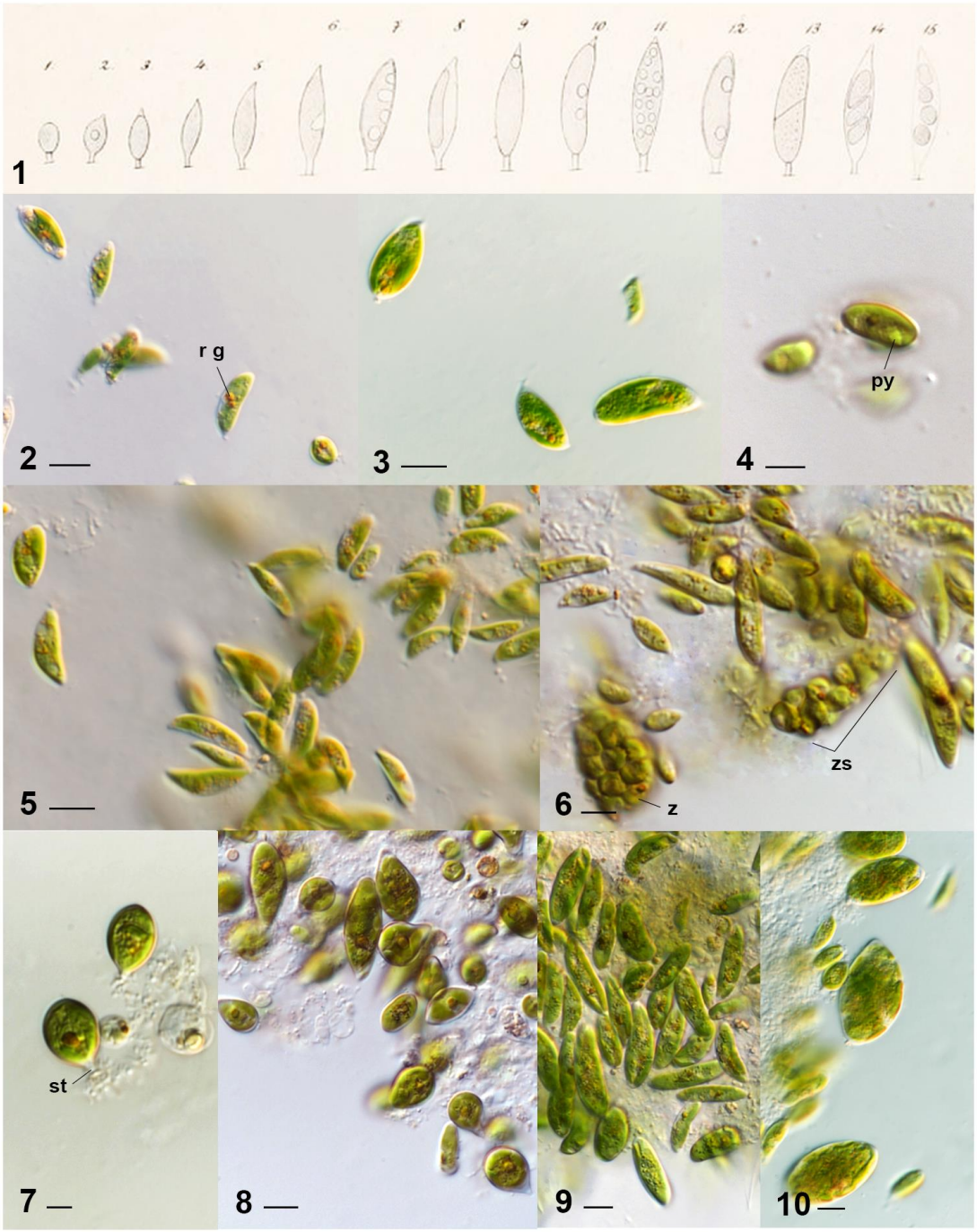
Table 3.1. *Characiopsis* strains studied and their characteristics. Cell morphology refers to the cell forms that predominate in the strains studied, with the caveat that the morphology can be quite diverse in the same culture. Dimensions of the cell body and stipe correspond to length x width (the general range is indicated, with exceptional values in brackets).

Strain (ACOI number)	Revised identification	Initial identification[a]	Locality, habitat and year of collection	Cell morphology	Dimensions (µm)	Pyrenoid[c]	18S rRNA[d]	*rbc*L[d]
456	*C. acuta*	*C. acuta*	Quiaios, Lagoa das Braças, plankton, 1991	cell body: oval apex: beak, stipe: long and thin	cell body: 17-26 × 5-8 stipe: 3-6	LM	**MT373054**	**MK281452**
1837	*C. acuta*	*C. acuta*	Quiaios, Lagoa das Braças, plankton, 1991	cell body: oval apex: beak, stipe: long and thin	cell body: 15-28 × 6-10 stipe: 4-7	LM, TEM	**MT373055**	**MT374803**
2434	*C. cedercreutzii*	*C. cedercreutzii*	Coimbra, Sta. Cruz park, water mine, 2002	cell body: cylindrical apex: rounded stipe: short	cell body: 10-40(80) × 4.5-12 stipe: 1-2	LM	**MT373067**	**MT374818**
3169	*C. cedercreutzii*	*C. cedercreutzii*	Coimbra, Botanic Garden (medical school), , plant squeezing, 2009	cell body: cylindrical apex: rounded stipe: short	cell body: (30)20-55(75) × (6)11-23(33) stipe: 1-2	-	**MT373071**	**MT374814**
1838	*C. longipes*	*C. longipes*	Castelo Branco, reservoir, 1997	cell body: oblong apex: acute or beak stipe: long and thin	cell body: 16-28(84) × 5-20(53) stipe: 7-12	LM	KY271647	**MT374804**

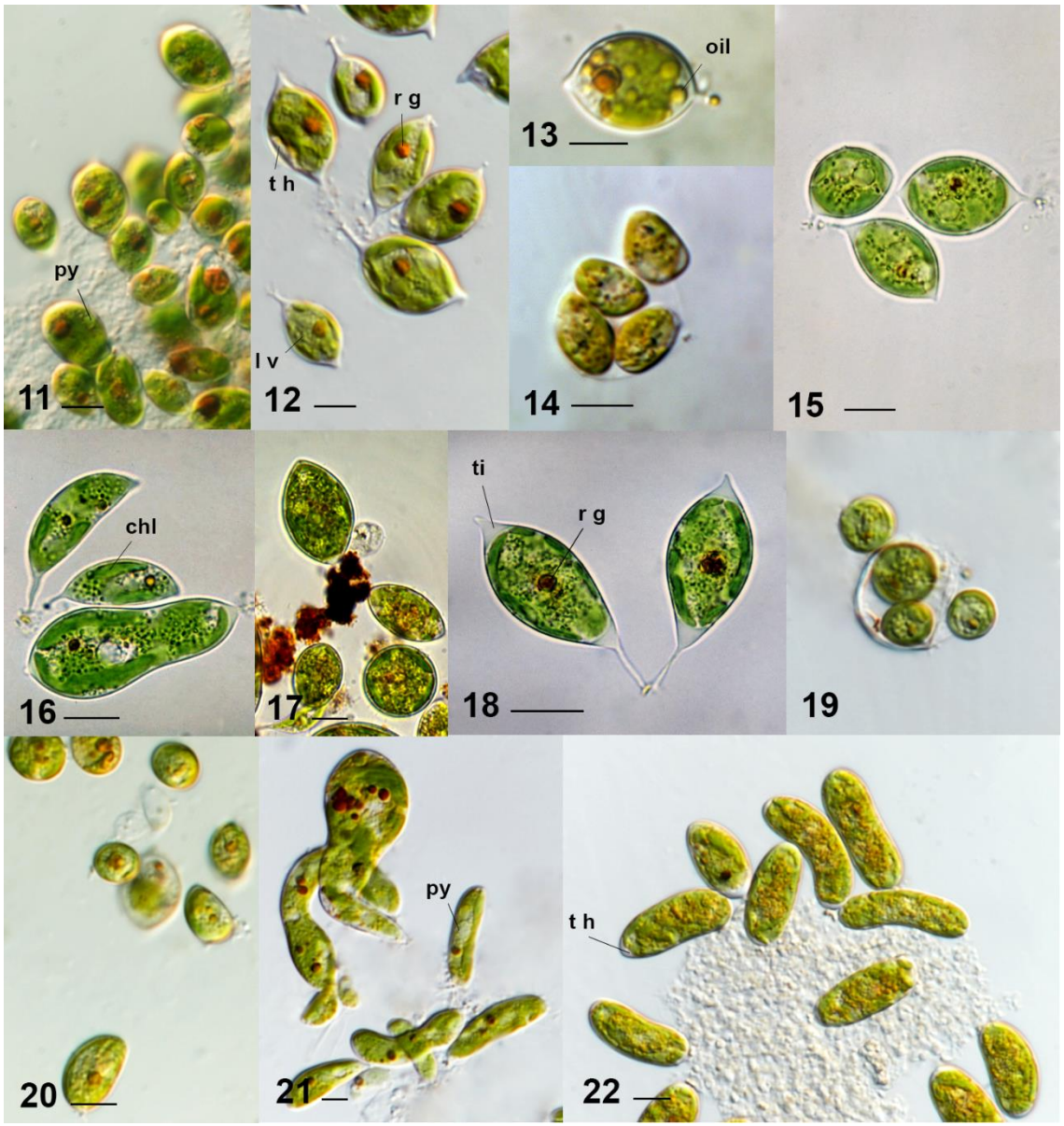
1839	*C. longipes*	*C. longipes*	Abrantes, lago norte da Lagoa da Murta, plant squeezing, 2003	cell body: oblong apex: round, acute or beak stipe: long and thin	cell body: 22-33(39) × 10-22(24) stipe: 5-11	LM	**MT373056**	**MT374805**
1839A	*C. longipes*	*C. longipes*	Abrantes Campo militar, lago norte da Lagoa da Murta, plant squeezing, 2003	cell body: oblong apex: round acute or beak stipe: long and thin	cell body: 21-33 × 6-20(26) stipe: 4-11	LM	**MT373057**	**MT374807**
2438	*C. longipes*	*C. longipes*	Abrantes, Campo militar, lagoa do meio, plankton, 2003	cell body: oblong apex: acute or beak stipe: long and thin	cell body: 15-23 × 5-20 stipe: 6-11	-	**MT373069**	**MT374806**
2423	*C. minuta*	*C. minuta*	Beja, S. Domingos pyrite mine lagoon, 2000	cell body: long apex: acute, beak or round stipe: short	cell body: 12-29 (42) × 3-14 stipe: 1-2	LM, TEM	**MT373058**	**MT374809**
2425	*C.* cf. *minuta*	*C. minuta*	Casal Novo do Rio, old river branch, plankton, 2000	cell body: long apex: acute, sometimes beak stipe: short	cell body: 14-28 (63) × 3.5-9(27) stipe: 1-2	LM	**MT373059**	**MT374810**
2432	*Characiopsis* sp.	*Characiopsis* sp.	Coimbra, Mosteiro de Sta. Clara, flowervase, 2001	cell body: long apex: acute or beak stipe: short	cell body: (14) 24-35(50) × 8-13(18) stipe: 1-2	LM	**MT373065**	**MT374808**
2429	*Characiopsis* sp.	*C. minuta?*	Paúl da Tornada (marsh), plankton, 2000	cell body: large or long apex: round or acute stipe: short	cell body: 13-21(45) × 6-12(18) stipe: 1-2	LM	**MT373062**	**MT374811**
2429A	*Characiopsis* sp.	*C. minuta?*	Paúl da Tornada (marsh), plankton, 2000	cell body: large or long apex: round or acute stipe: short	cell body: (17)27-30(37) × 4-9 stipe: 1-2	LM	**MT373063**	**MT374812**

2427	*C.* cf. *minutissima*	*C. minutissima?*	Casal Novo do Rio, old river branch, plankton, 2000	cell body: oval to oblong apex: round or acute stipe: short	cell body: 13-24(56) × 4-7(18) stipe: 1-2	LM	**MT373060**	**MT374820**
2427A	*C.* cf. *minutissima*	*C. minutissima?*	Casal Novo do Rio, old river branch, plankton, 2000	cell body: oval or oblong apex: round or acute stipe: short	cell body: 13-18(31) × 4-6(14) stipe: 1-2	LM	**MT373061**	**MT374816**
2433	*C. pernana*	*C. pernana*	Quiaios, Lagoa da Vela, plankton, 2001	cell body: oval apex: round stipe: short	cell body: 10-17(23) × 8-14 stipe: 1-2	LM	**MT373066**	**MT374802**
481	*C.* cf. *saccata*	*C. saccata*	Montemor-o-Velho, rice field, 1974	cell body: very long, angled apex: rounded or rarely acute stipe: short	cell body: (12)32-43(70) × 10-12(15) stipe: 1-2	LM, TEM	KF848925	**MT374815**
2430	*Characiopsis* sp.	*Characiopsis* sp.	Paúl da Tornada (marsh), plankton, 2000	cell body: short apex: round or acute stipe: short	cell body: 20-23(63) × 4-15(41) stipe: 1-2	LM	**MT373064**	**MT374813**
2436	*Characiopsis* sp.	*C. cedercreutzii*	Coimbra, Sta. Cruz park, canal, 2002	cell body: drop-like, some cylindrical apex: round stipe: short	cell body: 22-30(63) × 12-23(32) stipe: 1-2	-	**MT373068**	**MT374817**
2438B	*Characiopsis* sp.	*C. longipes*	Abrantes, Campo militar, lagoa do meio, plankton, 2003	cell body: large apex: round stipe: short	cell body: 19-38 × 5-21 stipe: 1-2	LM	**MT373070**	**MT374819**

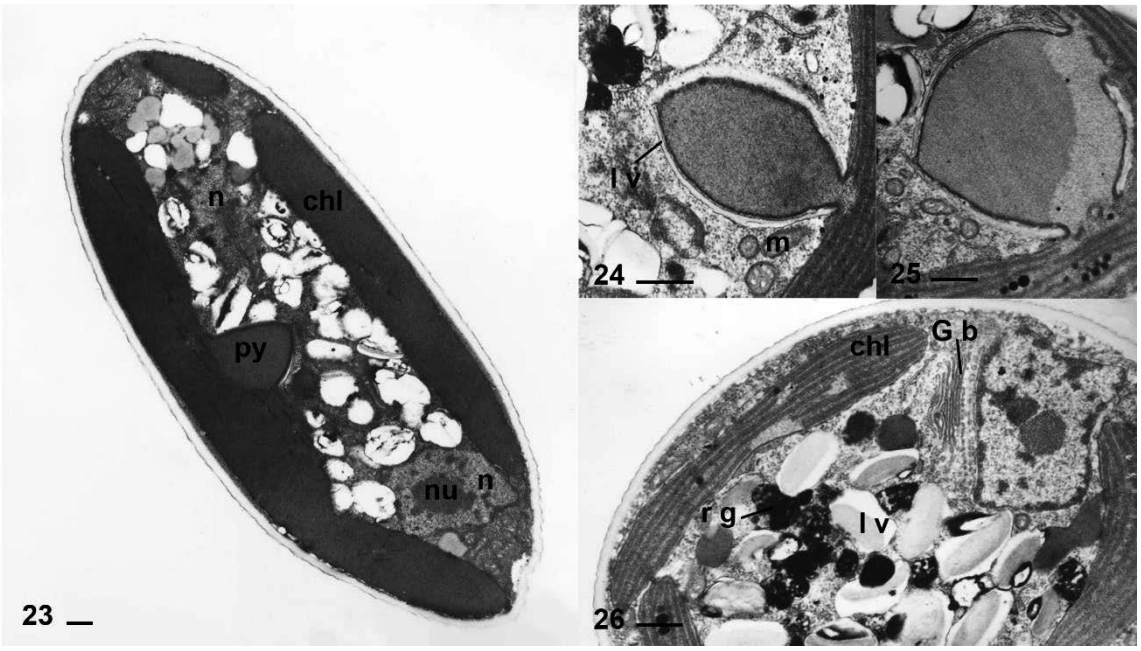
[a] Species identification of the strain as provided by the former ACOI curator Fátima Santos. [b] cell length (without stipe) × width, stipe length. [c] pyrenoid observation by light microscopy (LM) or transmission electron microscopy (TEM). [d] GenBank accession numbers for 18S rRNA and *rbc*L sequences (newly reported sequences highlighted in boldface).



Figures 3.1-3.10. *Characiopsis* strains with morphology similar to *Characiopsis minuta* and unidentified *Characiopsis* strains. Fig. 3.1. *Characium minutum* (adapted from Braun, 1855). Fig. 3.2-3.4. *Characiopsis minuta* ACOI 2423. Fig. 3.5. *Characiopsis* cf. *minuta* ACOI 2425. Fig. 3.6. *Characiopsis* sp. ACOI 2432. Fig. 3.7. *Characiopsis* sp. ACOI 2438B. Fig. 3.8. *Characiopsis* sp. ACOI 2429. Fig. 3.9. *Characiopsis* sp. ACOI 2429A. Fig. 3.10. *Characiopsis* sp. ACOI 2430. Pyrenoid (py), reddish globule (r g), zoospore (zoo), zoosporangium (z s). Photos 3.1-3.10 DIC 100x APO. Scale bars: 10 μm.



Figures 3.11-3.22. *Characiopsis* strains with morphology unlike that of *Characiopsis minuta*. Fig. 3.11. *Characiopsis pernana* ACOI 2433. Figs. 3.12-3.14. *Characiopsis acuta* ACOI 456. Fig. 3.15. *Characiopsis acuta* ACOI 1837. Fig. 3.16. *Characiopsis longipes* ACOI 1838. Fig. 3.17. *Characiopsis longipes* ACOI 1838. Fig. 3.18. *Characiopsis longipes* ACOI 2438. Fig. 3.19. *Characiopsis longipes* ACOI 1839_9. Fig. 3.20. *Characiopsis* cf. *minutissima* ACOI 2427A. Fig. 3.21. *Characiopsis* cf. *saccata* ACOI 481. Fig. 3.22. *Characiopsis cedercreutzii* ACOI 3169. Apical tip (ti), chloroplast (chl), lamellate vesicles (lv), oil droplets (oil), pyrenoid (py), reddish globule (rg), stipe (st), cell wall thickening (th). Photos 3.11.-3.14. and 3.19.-3.22. DIC 100x APO. Scale bars: 10 μm.



Figures 3.23-3.26. TEM sections of *Characiopsis* vegetative cell. Fig. 2.23. *C. minuta* ACOI 2423. Figs. 2.24-2.26. *C.* cf. *saccata* ACOI 481. Chloroplast (chl), Golgi body (G b), lamellate vesicles (l v), mitochondrion (m), nucleous (n), nucleolous (nu), pyrenoid (py), reddish globule (r g). Scale bars: 1 μm.

Molecular phylogeny of the *Characiopsis* strains

Altogether we report 18 new 18S rRNA gene sequences and 20 new *rbc*L gene sequences (Figs. 3.27. and 3.28.). Together with the previously published data, both 18S rRNA and *rbc*L gene sequences are now available for 20 *Characiopsis* strains. The topology of the 18S rRNA tree (Fig. 3.27.) closely recapitulates the results of other recent analyses of this phylogenetic marker (Kryvenda et al. 2018, Ševčíková et al. 2019, Amaral et al. 2020). Eustigmatophytes are divided into two principal lineages, the clade *Goniochloridales* (Fawley et al. 2014) and the order Eustigmatales. The latter includes four main, strongly supported lineages, two corresponding to formally recognized families, Monodopsidaceae (Hibberd 1981) and Neomonodaceae (Amaral et al. 2020), one informally called the Eustigmataceae group

(Fawley et al. 2014), and the final one represented by a single cultured member, *Paraeustigmatos columelliferus* CAUP Q 701 (Fawley et al. 2019). All 20 strains investigated in this study are placed in the Eustigmatacae group in a broader unsupported clade that also includes four previously sequenced *Characiopsis* strains and representatives of three additional nominal genera, *Pseudocharaciopsis minuta* UTEX 2113, *Dioxys* sp. ACOI 2029, and several *Chlorobotrys* isolates. Two subclades with strong or at least medium support emerge within this broader clade, one comprising all *C. acuta* and *C. longipes* strains and the other including most other strains except for *C. pernana* ACOI 2433 and *Dioxys* sp. ACOI 2029. The sequences of different strains within the two subclades are completely identical.

The tree obtained using eustigmatophyte *rbc*L sequences (Fig. 3.28.) is likewise congruent with previous similar analyses (Ševčíková et al. 2019; Amaral et al. 2020), and divides eustigmatophytes into the same main lineages as the 18S rRNA gene tree. All of the newly investigated strains, together with the previously sequenced *C. acuta* ACOI 456, constitute a single, strongly supported clade (bootstrap value of 99%) within the Eustigmataceae group, which we hereafter call the *Characiopsis* clade. More genetic variation is recorded in the *rbc*L gene compared to the 18S rRNA gene, allowing for better resolved relationships both within the Eustigmataceae group and the *Characiopsis* clade. Thus, the so-called clade Ia comprised of several unidentified isolates (Fawley et al. 2014) is positioned with strong support as a lineage sister to the other representatives of the Eustigmataceae group for which the *rbc*L gene sequence is available. *Chlorobotrys regularis* ACOI 307, newly sequenced by us to improve the sampling of the Eustigmataceae group, belongs to a moderately supported group together with the genus *Vischeria* (including the former *Eustigmatos* species; Kryvenda et al. 2018), whereas the *Characiopsis* clade may be specifically related to the unidentified isolate BogD 9/21 T-2d, although this relationship is supported only by a moderate bootstrap value (70%).

The internal structure of the *Characiopsis* clade in the *rbc*L tree is more elaborate than the corresponding part of the 18S rRNA tree, although many deep branches lack statistical support (Fig. 3.28.). Strains of *C. acuta* and *C. longipes* constitute a clearly delimited strongly supported (99% bootstrap) clade separated from other *Characiopsis* strains by a long stem branch. Some sequence heterogeneity is apparent among *C. longipes* strains, with ACOI 1838 being separated from the other three strains. Another noticeable grouping comprises ACOI 481 (*Characiopsis* cf. *saccata*), ACOI 2436 (*Characiopsis* sp.) and ACOI 2434 and 3169 (*C. cedercreutzii*), whose *rbc*L sequences are identical. This cluster may be specifically related (80% bootstrap support) to the two strains referred to as *C.* cf. *minutissima* (ACOI 2427 and 2427A), and together with them may belong to an even more inclusive group (78% bootstrap support) additionally embracing four unidentified *Characiopsis* strains. Three of these strains share identical *rbc*L sequences and may therefore be conspecific. The *rbc*L sequences of ACOI 2423 (*C. minuta*) and ACOI 2425 (*C.* cf. *minuta*) are also identical.

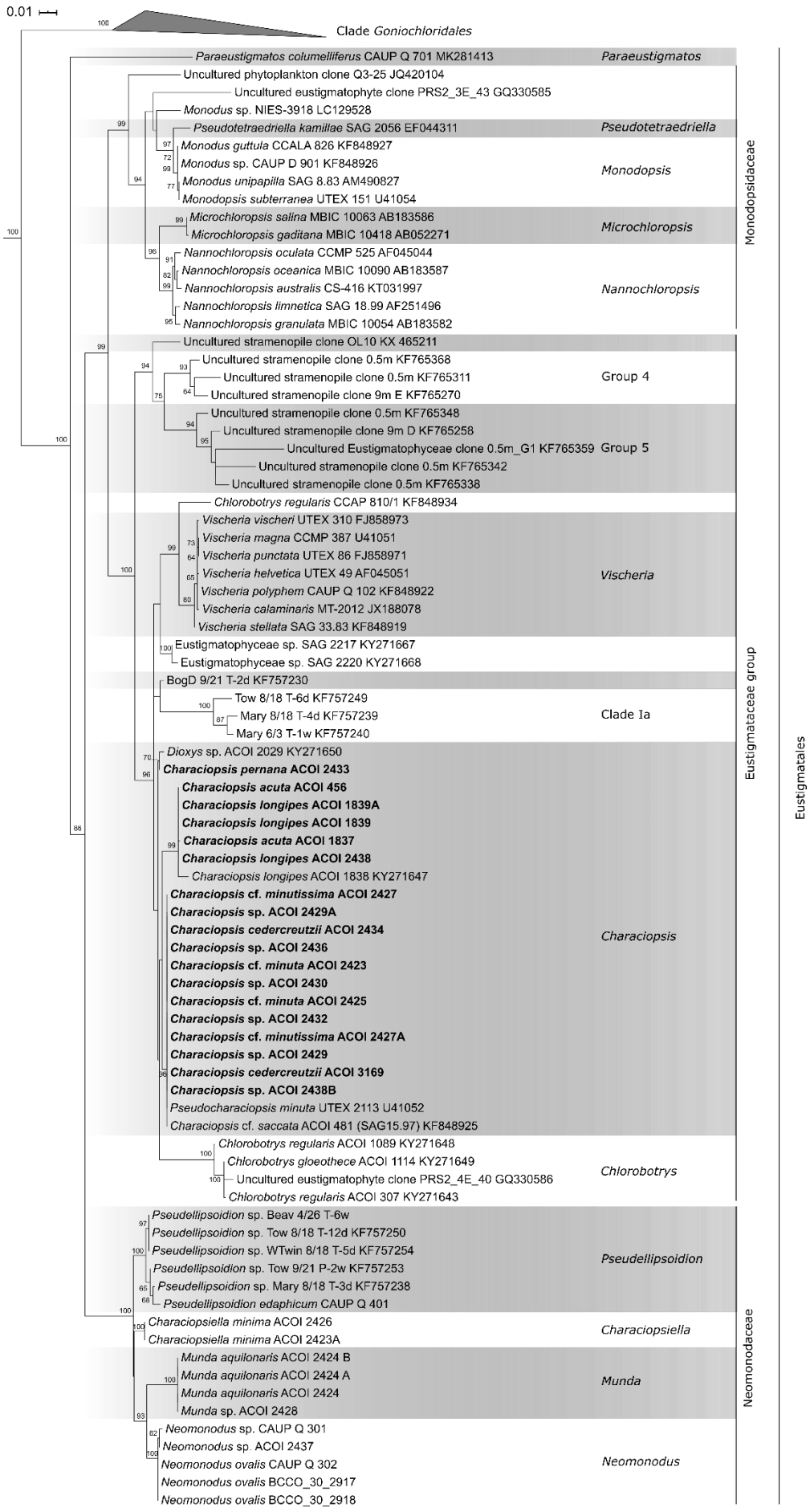

0.01
Clade Goniochloridales
Paraeustigmatos columelliferus CAUP Q 701 MK281413
Paraeustigmatos
Uncultured phytoplankton clone Q3-25 JQ420104
Uncultured eustigmatophyte clone PRS2_3E_43 GQ330585
Monodus sp. NIES-3918 LC129528
Pseudotetraedriella kamillae SAG 2056 EF044311
Pseudotetraedriella
Monodus guttula CCALA 826 KF848927
Monodus sp. CAUP D 901 KF848926
Monodus unipapilla SAG 8.83 AM490827
Monodopsis subterranea UTEX 151 U41054
Monodopsis
Microchloropsis salina MBIC 10063 AB183586
Microchloropsis gaditana MBIC 10418 AB052271
Microchloropsis
Nannochloropsis oculata CCMP 525 AF045044
Nannochloropsis oceanica MBIC 10090 AB183587
Nannochloropsis australis CS-416 KT031997
Nannochloropsis limnetica SAG 18.99 AF251496
Nannochloropsis granulata MBIC 10054 AB183582
Nannochloropsis
Monodopsidaceae
Uncultured stramenopile clone OL10 KX 465211
Uncultured stramenopile clone 0.5m KF765368
Uncultured stramenopile clone 0.5m KF765311
Uncultured stramenopile clone 9m E KF765270
Group 4
Uncultured stramenopile clone 0.5m KF765348
Uncultured stramenopile clone 9m D KF765258
Uncultured Eustigmatophyceae clone 0.5m_G1 KF765359
Uncultured stramenopile clone 0.5m KF765342
Uncultured stramenopile clone 0.5m KF765338
Group 5
Chlorobotrys regularis CCAP 810/1 KF848934
Vischeria vischeri UTEX 310 FJ858973
Vischeria magna CCMP 387 U41051
Vischeria punctata UTEX 86 FJ858971
Vischeria helvetica UTEX 49 AF045051
Vischeria polyphem CAUP Q 102 KF848922
Vischeria calaminaris MT-2012 JX188078
Vischeria stellata SAG 33.83 KF848919
Vischeria
Eustigmatophyceae sp. SAG 2217 KY271667
Eustigmatophyceae sp. SAG 2220 KY271668
BogD 9/21 T-2d KF757230
Tow 8/18 T-6d KF757249
Mary 8/18 T-4d KF757239
Mary 6/3 T-1w KF757240
Clade 1a
Eustigmataceae group
Dioxys sp. ACOI 2029 KY271650
Characiopsis pernana ACOI 2433
Characiopsis acuta ACOI 456
Characiopsis longipes ACOI 1839A
Characiopsis longipes ACOI 1839
Characiopsis acuta ACOI 1837
Characiopsis longipes ACOI 2438
Characiopsis longipes ACOI 1838 KY271647
Characiopsis cf. minutissima ACOI 2427
Characiopsis sp. ACOI 2429A
Characiopsis cedercreutzii ACOI 2434
Characiopsis sp. ACOI 2436
Characiopsis cf. minuta ACOI 2423
Characiopsis sp. ACOI 2430
Characiopsis cf. minuta ACOI 2425
Characiopsis sp. ACOI 2432
Characiopsis cf. minutissima ACOI 2427A
Characiopsis sp. ACOI 2429
Characiopsis cedercreutzii ACOI 3169
Characiopsis sp. ACOI 2438B
Pseudocharaciopsis minuta UTEX 2113 U41052
Characiopsis cf. saccata ACOI 481 (SAG15.97) KF848925
Characiopsis
Chlorobotrys regularis ACOI 1089 KY271648
Chlorobotrys gloeothece ACOI 1114 KY271649
Uncultured eustigmatophyte clone PRS2_4E_40 GQ330586
Chlorobotrys regularis ACOI 307 KY271643
Chlorobotrys
Pseudellipsoidion sp. Beav 4/26 T-6w
Pseudellipsoidion sp. Tow 8/18 T-12d KF757250
Pseudellipsoidion sp. WTwin 8/18 T-5d KF757254
Pseudellipsoidion sp. Tow 9/21 P-2w KF757253
Pseudellipsoidion sp. Mary 8/18 T-3d KF757238
Pseudellipsoidion edaphicum CAUP Q 401
Pseudellipsoidion
Characiopsiella minima ACOI 2426
Characiopsiella minima ACOI 2423A
Characiopsiella
Munda aquilonaris ACOI 2424 B
Munda aquilonaris ACOI 2424 A
Munda aquilonaris ACOI 2424
Munda sp. ACOI 2428
Munda
Neomonodus sp. CAUP Q 301
Neomonodus sp. ACOI 2437
Neomonodus ovalis CAUP Q 302
Neomonodus ovalis BCCO_30_2917
Neomonodus ovalis BCCO_30_2918
Neomonodus
Neomonodaceae
Eustigmatales

Figure 3.27. Phylogeny of Eustigmatophyceae based on sequences of the 18S rRNA gene, showing the Eustigmatales. The phylogeny shown was inferred using maximum likelihood method implemented in RAxML (employing GTR+Γ substitution model) with bootstrap analysis followed by thorough search for the ML tree. Bootstrap values higher than 50 are shown. Labels at terminal leaves comprise the strain updated taxonomic name followed by the collection reference number and the GenBank accession number. New sequences are highlighted in boldface. The tree was rooted using 15 sequences from stramenopile algae sampled from GenBank. The outgroup is omitted and the ordinal clade *Goniochloridales* is shown collapsed for simplicity.

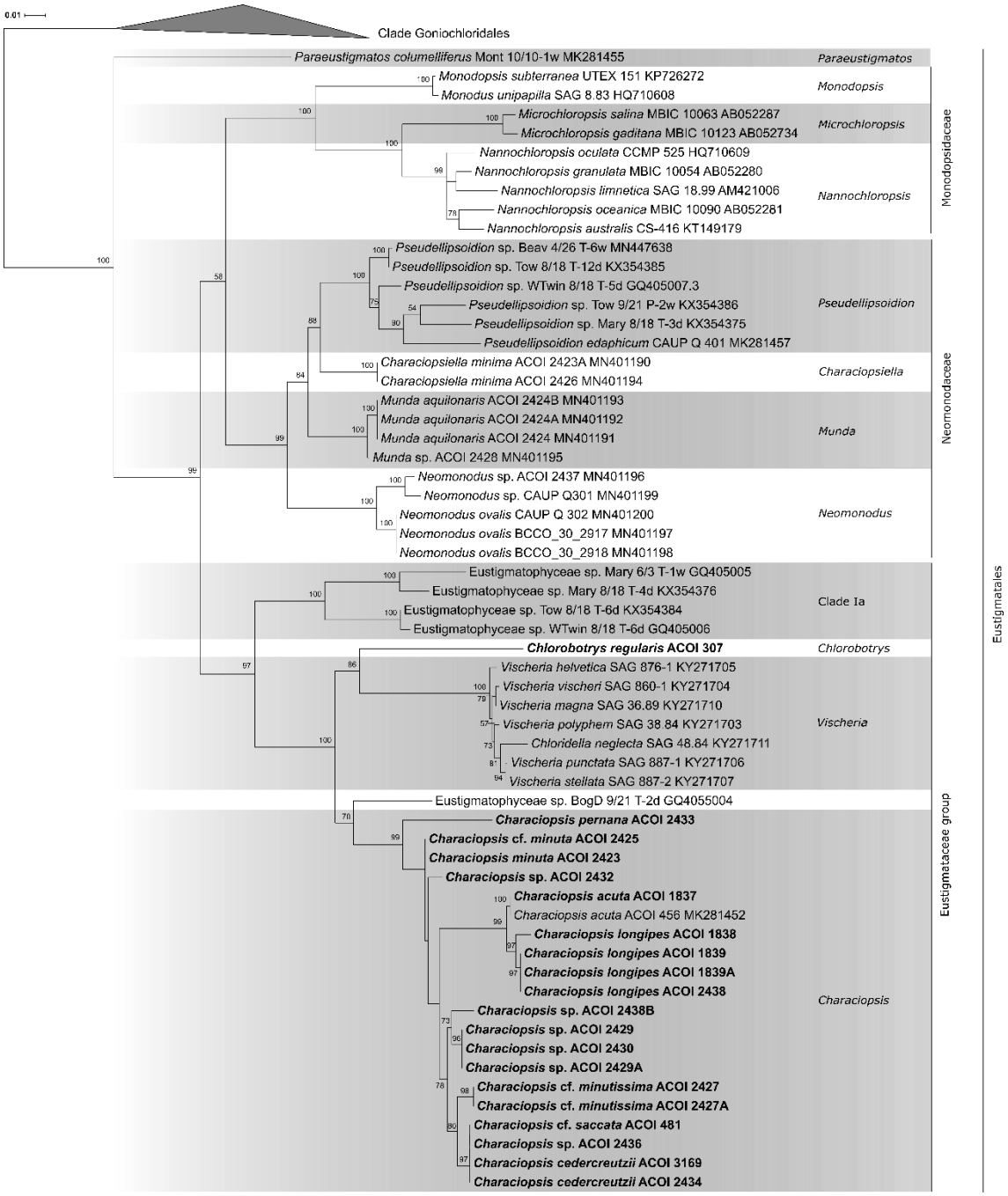


Figure 3.28. Phylogeny of Eustigmatophyceae based on *rbc*L gene, showing the Eustigmatales. The phylogeny shown was inferred using maximum likelihood method implemented in RAxML (employing GTR+Γ substitution model) with bootstrap analysis followed by thorough search for the ML tree. Bootstrap values higher than 50 are shown. Labels at terminal leaves comprise the strain updated taxonomic name followed by the collection reference number when applicable and the GenBank accession number. New sequences highlighted in boldface. The tree was rooted at the ordinal clade *Goniochloridales* which is shown collapsed for simplicity.

3.5. Discussion

Identity of the genus *Characiopsis*

Morphological and molecular data is provided for a series of algal strains identified as particular species of the genus *Characiopsis* or at least fitting the general characteristic morphology of this genus. All the strains were proven to be eustigmatophytes, specifically members of the Eustigmataceae group. This position is also consistent with the phylogenetic analysis of concatenated plastid genome-encoded proteins including a single representative of the *Characiopsis* clade, *C. acuta* ACOI 456 (Ševčíková et al. 2019). Whereas the eustigmatophyte nature of these algae is undeniable, the question arises of whether this also implies that the genus *Characiopsis* as such should be transferred from its current taxonomic home, the class Xanthophyceae, to the class Eustigmatophyceae.

The answer relies on resolving the actual identity of the type of the genus. However, what is to be considered the type of *Characiopsis* Borzì has become a matter of controversy in the literature. When establishing the genus, Borzì explicitly stated this (Borzì 1895, p. 154): "Tuttavia una forma sulla quale non parmi possano cadere di dubbi e che con contezza debba assumersi come tipo del nuovo genere *Characiopsis* è il *Characium minutum* di Al. Braun". In the same publication Borzì provided drawings of an alga he observed and identified as *C. minutum* Braun, designated by him with the new combination *Characiopsis minuta*. However, Lemmermann (1914) had an opportunity to study the original specimen used by Braun to describe *C. minutum* and based on this he concluded that the alga documented by Borzì is a different species, which he described as *Characiopsis borziana* Lemmermann. In light of this, Silva (1979) interpreted the typification of *Characiopsis* as follows (p. 40): "In my opinion a genus should be typified with material at hand, whether or not the author misidentified the type with a previously described species. Accordingly, I consider *C. borziana* the type of its genus". This interpretation was adopted by Hibberd (1981), who considered it in agreement

with the intention of Article 10.1 of the International Code of Botanical Nomenclature, and later also by Pizarro (1995).

However, after years of debates by authorities on botanical nomenclature (McNeill 1981), a modified version of Article 10.1 appeared in the 1983 edition of the *Code* and remains the same in the currently valid edition of the International Code of Nomenclature for algae, fungi, and plants (Shenzhen Code) (Turland et al. 2018). The Article specifically states this: "The type of a name of a genus or of any subdivision of a genus is the type of a name of a species .... For purposes of designation or citation of a type, the species name alone suffices, i.e. it is considered as the full equivalent of its type". Hence, according to the current meaning of the *Code* the type of the genus name *Characiopsis* is the type of the species name *Characium minutum* Braun, regardless of what exactly Borzì had at hand and identified as *C. minutum*. Braun's description of the species is not accompanied with an illustration and does not explicitly specify a type (Kützing 1849, p. 892). However, Lemmermann (1914) mentioned Braun's "Original exemplare von *Ch. minuta*" in Berlin Herbarium that he could study (see above), which can be considered the holotype of *C. minutum* Braun. Unfortunately, this specimen no longer exists and was most likely destroyed during the World War II (Dr. Nélida Abarca, Botanic Garden and Botanical Museum Berlin, personal communication). Hence, following Article 9.16 of the *Code*, we here designate a neotype for *C. minutum* Braun. Specifically, we select Braun's original drawing of *C. minutum* published by him in 1855 and reprinted here as Fig. 3.1. It then follows that this drawing represents the type of the genus name *Characiopsis*.

Characiopsis minuta is an eustigmatophyte

The key step towards final resolution of the question of which lineage should be called *Characiopsis* and where it fits phylogenetically is to investigate an alga that can unambiguously

be identified as Braun's *C. minutum*. Several strains from our set were considered as possible candidates, including those initially identified by the ACOI curator as *C. minuta* (ACOI 2423 and ACOI 2425) and "*Characiopsis minuta*?" (ACOI 2429 and 2429A). A careful reevaluation of the morphology of these strains led us to conclude that the strain ACOI 2423 best fits the characteristics of *C. minuta* and can be used as a basis for further taxonomic reasoning concerning the genus *Characiopsis*.

Another strain was previously proposed to represent *C. minuta*. Hibberd (1981) discussed in detail the morphology of the authentic strain of the eustigmatophyte alga *Pseudocharaciopsis texensis* Lee & Bold and concluded that it can be identified as conspecific of *Characiopsis minuta*. Given his conviction that *Characiopsis* is typified by *C. borziana* (see above) and that the genus should stay in Xanthophyceae, he created a new combination *Pseudocharaciopsis minuta*, with *P. texensis* as its junior synonym. The sequence of the 18S rRNA gene of this strain (held in the UTEX collection as the culture UTEX 2113) was obtained by Andersen et al. (1998) and confirms that this alga is indeed closely related to the studied *Characiopsis* strains, including the proposed candidate for *C. minuta*, the strain ACOI 2423 (Figs 3.2.-3.4. and 3.27.). In fact the 18S rRNA sequences of these two strains differ by only two one-nucleotide indels, and inspection of a multiple alignment of available eustigmatophyte sequences revealed that the differences map into conserved regions of the gene, with the UTEX 2113 sequence being the one that deviates from the conserved pattern (not shown). Considering that this sequence was obtained by manual sequencing on a polyacrylamide gel (Andersen et al. 1998), a less accurate procedure than the current implementations of the Sanger method, the differences between these sequences and those obtained from other eustigmatophytes might possibly be artefacts. Unfortunately, the strain is no longer available from the UTEX collection, and an equivalent strain, held in the CCAP collection with the reference number 864/1 (see Hibberd, 1981) is likewise lost (overgrown by a green alga; details not shown). It is therefore not possible to sequence its 18S rRNA gene once more,

nor to determine the sequence of its *rbc*L gene to get a more precise understanding of its relationship to strains investigated in the present study. Nevertheless, the evidence available is compatible with a notion that the strain ACOI 2423 is closely related, if not conspecific, with what Hibberd interpreted as *C. minuta*. Consequently, our results strengthen the argument for the name *P. texensis* being considered synonymous to *C. minuta*.

To stabilize the meaning of the name *Characiopsis minuta*, and thus to anchor the definition of the genus *Characiopsis*, below we designate an epitype to support the neotype of *Characium minutum* (Braun's drawing, see above), whose practical use as a reference for identification of the species is inherently limited. The strain ACOI 2423 is morphologically the closest among all available *Characiopsis* strains to the alga reported by Braun as *Characium minutum* and we have confidence in its identification as the same species. Hence, a metabolically stable cryopreserved material derived from a living culture of this strain is here designated as the epitype of *Characium minutum* (=*Characiopsis minuta*).

Characiopsis is an eustigmatophyte genus, *Pseudocharaciopsis* is its junior synonym

Based on the above data and arguments, the question of the identity of the type of the genus *Characiopsis* is resolved. Also, with the convincingly demonstrated position of the type in a particular lineage of eustigmatophytes, the debate on whether the genus should be classified in Xanthophyceae or Eustigmatophyceae seems to be closed. However, Hibberd (1981) discussed the morphological features of the type species of the genus *Characiopsis*, *C. borziana*, and considered the possibility that like *C. minuta*, it may also be a eustigmatophyte, even possibly congeneric with *Pseudocharaciopsis* species. Should this prove to be the case, he proposed that the "transfer of the name *Characiopsis* from the Tribophyceae to the Eustigmatophyceae could be prevented either by conserving *Characiopsis* with an altered type

or by proposing the name as a *nomen rejiciendum* on the grounds that it had been widely and persistently used for a taxon not including its type" (Hibberd 1981, p. 109). So, when confronted with the situation *de facto* envisaged by Hibberd, should we consider implementing his formal taxonomic act to preserve *Characiopsis* as a genus of xanthophyte algae and *Pseudocharaciopsis* as an independent genus in eustigmatophytes?

One argument pointing against this advice is that all representatives of the traditionally circumscribed genus *Characiopsis* studied so far by modern methods prove to be eustigmatophytes rather than xanthophytes. In addition to *C. minuta*, this was previously demonstrated for three such species, *C. ovalis*, *C. minima*, and *C. aquilonaris*, which are however not directly related to the "main" *Characiopsis* clade (see also Figs 3.27. and 3.28.) and each have been placed in its own newly erected genus (*Neomonodus*, *Characiopsiella*, and *Munda*, respectively; Amaral et al. 2020). The present study adds four known morphologically recognized (Table 3.1.) and up to nine genetically delimited *Characiopsis* species (see below) that all belong to eustigmatophytes and are specifically related to *C. minuta*. Hence, at the moment there is no strong evidence for any nominal *Characiopsis* species being a xanthophyte. So, Hibberd's proposal currently lacks any real biological reason. With the present knowledge of *Characiopsis*-like algae we can thus conclude that *Characiopsis* is a genus embracing a set of closely related eustigmatophyte algae in the Eustigmataceae group and that *Pseudocharaciopsis* is its junior synonym.

This conclusion does not necessarily imply that all algae presently classified in the genus *Characiopsis* must belong to Eustigmatophyceae. In this regard it is interesting to consider a note mentioned by Lee and Bold in their paper describing *P. texensis*: "The writers have in their collection two strains of *Characiopsis*. It was of interest to ascertain whether these also should be assigned to the new genus *Pseudocharaciopsis*. However, both light and electron microscopy indicated that they are not eustigmatophycean algae" (Lee & Bold 1973, p. 37). Unfortunately,

no further details on these strains seem to have been published by the authors and it is unclear whether they represented xanthophytes or yet another algal group. While the morphological characters documented for the various *Characiopsis* species are generally insufficient to determine their actual affiliation, some species seem unlikely to belong to Eustigmatophyceae and some may indeed be xanthophytes instead. For example, zoospores in *Characiopsis elegans* and *Characiopsis galeata* were depicted with a laterally positioned eyespot located in the chloroplast (Ettl 1956), which contrasts with the characteristic zoospore structure in eustigmatophytes featuring an extraplastidial anterior eyespot associated with the base of the long flagellum (Hibberd 1981). A renewed culturing effort will hopefully enable reevaluation of a broader set of *Characiopsis* species with molecular and other modern methods of algal systematics, which may ultimately unveil *Characiopsis*-like species belonging to xanthophytes or other classes outside eustigmatophytes. Should such species be found, they will have to be transferred from *Characiopsis* to a new genus or genera. Also worthy of attention are those strains which cannot be identified to the species level based on the available literature. Some old literature sources are very difficult or impossible to find, which originates that some strains retain the possibility of representing already known species. These taxonomic obscurities also represent novel transferences of *Characiopsis* species to the Eustigmatophyceae or on the contrary, will remain unsolved until a taxonomic decision is achieved.

Phylogenetic delimitation of the genus *Characiopsis*

Phylogenetic analyses of both 18S rRNA and *rbc*L gene sequences generally support the existence of a eustigmatophyte subgroup including *C. minuta* and a suite of genetically more or less differentiated strains, naturally interpreted as the genus *Characiopsis* (Figs 3.27. and 3.28.). The *rbc*L tree shows the genus as a strongly supported clade well separated from other eustigmatophyte lineages. In contrast, the 18S rRNA gene tree does not exhibit an equivalent

clade due to a cluster of *Chlorobotrys* sp. sequences nested among *Characiopsis* sp. sequences. An analogous grouping, denoted "*Pseudocharaciopsis/Chlorobotrys/Dioxys*-clade", was also retrieved in a previous study with a much poorer sampling of the *Characiopsis* diversity (Kryvenda et al. 2018). The position of the *Chlorobotrys* cluster in the 18S tree, in fact not supported by the bootstrap analysis (Fig. 3.27.), is incongruent with the position of a representative of this cluster (*C. regularis* ACOI 307) in the *rbc*L tree, where it is placed (with a rather high bootstrap value of 86%) sister to the genus *Vischeria* (Fig. 3.28.). Instead, the unidentified strain Bog 9/21 T-2d may be more closely related to *Characiopsis* according to *rbc*L data.

The 18S rRNA gene appears to have an insufficient signal for resolving the branching order at the base of the Eustigmataceae group, and even for demonstrating the monophyly of *Characiopsis*. The latter problem may stem from the noticeably lower rate of evolution of the 18S rRNA gene in *Characiopsis* spp. as compared to most other members of the Eustigmataceae group: note the short branches of the *Characiopsis* sequences and their identity or high similarity even in species that are well differentiated by morphology and *rbc*L sequences. As a result, probably only few synapomorphic mutations have accumulated in the 18S rRNA gene of the *Characiopsis* lineage, allowing for robust inference of its monophyly. While the *rbc*L gene phylogeny and morphological characters collectively provide sufficient support for the delimitation of the genus *Characiopsis* as a monophyletic entity within the Eustigmataceae group, multigene analyses including lineages currently represented only by 18S rRNA data (such as the lineage comprised of the two unidentified strains SAG 2217 and 2220) are required to better understand the phylogenetic position of *Characiopsis* among its closest relatives.

Interestingly, the 18S rRNA tree suggests that specific relatives of the nominal *Characiopsis* strains (including the authentic *Pseudocharaciopsis texensis* strain reinterpreted as *C. minuta*, see above) may also include the strain ACOI 2029 assigned as an unidentified species to the

genus *Dioxys* (Fig. 3.27.). The respective sequence was obtained by Kryvenda et al. (2018), but the authors did not provide information on the morphology of *Dioxys* sp. ACOI 2029, so the validity of the identification remains uncertain. However, the genus *Dioxys* Pascher exhibits clear resemblance to *Characiopsis* owing to the presence of a stipe, and the probable specific relationship of the two genera is reflected by their classification into the informal "*Characiopsis*-Gruppe" in the family Characiopsidaceae (Ettl, 1978). It cannot be ruled out that some *Dioxys* species in fact belong phylogenetically to the *Characiopsis* clade and should be reclassified accordingly. If this also concerned the type species, *Dioxys incus* Pascher, *Dioxys* (described in Pascher 1932) would have to be reconsidered as a junior synonym of *Characiopsis*. Future investigations of the *Dioxys* sp. ACOI 2029 strain will help test these possibilities.

Interestingly, the *Characiopsis*-like morphology is not restricted to a single evolutionary lineage of eustigmatophytes, as several species historically classified as *Characiopsis* are found in a group distantly related to the *Characiopsis* clade that was recently defined as the new family Neomonodaceae. These species, now placed in three different genera *Neomonodus, Characiopsiella* and *Munda* (Figs 3.27. and 3.28.), were noted to share a morphological feature discriminating them from *Characiopsis* and *Pseudocharaciopsis* species placed into the Eustigmataceae group: the absence of a pyrenoid (Amaral et al. 2020). Indeed, we observed a pyrenoid with light microscopy in nearly all studied (*bona fide*) *Characiopsis* strains (Table 3.1.), further strengthening the case that the presence or absence of a pyrenoid is a phylogenetically informative character in algae with a *Characiopsis*-like morphology. TEM sections may clarify if this structure is present in the three *Characiopsis* strains in which a pyrenoid could not be discerned under light microscopy. If confirmed, the absence of a pyrenoid would be a recently evolved feature of these strains, as they are all closely related (most likely conspecific) with strains that do have it (Fig. 3.28., Table 3.1.).

Diversity in the genus *Characiopsis*

Whereas the 18S rRNA gene has proven to be slowly evolving in *Characiopsis* and hence not particularly informative about the (phylo)genetic diversification within the genus (Fig. 3.27.), *rbc*L gene sequences demonstrate considerable diversity (Fig. 3.28.). A natural question is how this diversity translates into formal classification, i.e. delimitation of species within the genus. Considering the degree of differences in *rbc*L sequences between different nominal species in other eustigmatophyte genera, up to ten separate species seem to have been captured by the present sampling of the genus *Characiopsis* (Fig. 3.28.). Some interesting conclusions can be drawn when the molecular data are combined with morphological observations of the strains.

Firstly, morphologically similar strains may prove to be genetically different, as is the case with the set of strains more or less reminiscent of *C. minuta*, and the strains identified as *C. longipes*. Thus, it is possible that new *Characiopsis* species need to be recognized to properly reflect the actual diversity within the genus. Secondly, the *Characiopsis* cultures usually exhibit a range of different cell morphologies, also depending on the age of the culture. This makes it difficult to match the organisms to the species descriptions provided by previous authorities, which were typically derived from observing the algae in natural samples and thus could not really capture the actual morphological plasticity the species exhibits in reality. Finally, morphologically distinguishable strains may be genetically that close as to be possibly conspecific. Most notable is the case of a cluster of four strains with identical *rbc*L sequences (Fig. 3.28.), two of which identified as *C. cedercreutzii*, one resembling *C. saccata* and the fourth without clear species assignment (Figs 3.21. and 3.22.). We really do not understand whether this reflects the insufficiency of even the *rbc*L gene to discriminate closely related yet distinct species, or whether we have encountered a case of considerable morphological plasticity whereby the same species may look different depending on minor genetic or epigenetic differences.

Indeed, the life cycles of eustigmatophyte algae including *Characiopsis* are yet to be clarified, and we cannot, for instance, exclude the possibility that some of the eustigmatophytes exhibit alternation of generations, i.e. vegetative phases occurring at two different ploidy levels and potentially differing in their morphology. Another factor potentially impacting the appearance of the algae in culture are biotic interactions with co-cultivated microorganisms (bacteria, fungi etc.; note that the strains studied are not in axenic cultures). In this regard it is interesting to note that some eustigmatophytes, including two strains of *C. acuta* studied in this paper (ACOI 456 and ACOI 1837), were recently shown to harbour endosymbiotic bacteria representing a new genus of the family Rickettsiaceae (*Candidatus* Phycorickettsia; Yurchenko et al. 2018). To what extent the presence of the endosymbiont in the algal host influences its morphology is presently unknown, but some effects would not be surprising. With these arguments in mind we refrain from herein proposing taxonomic changes such as description of new species or synonymization of existing species, since we feel our understanding of the biology and phylogenetic diversity of *Characiopsis* is presently insufficient to make such an effort substantiated and well founded in the data.

3.6. Conclusions

This study makes an important step in a scientific endeavor that started in the middle of the 19th century and will certainly continue in the future. By analyzing in detail, the convoluted taxonomic history of the genus *Characiopsis* and its type, we convincingly demonstrate that *Characiopsis* is a name to be used for a taxon belonging to Eustigmatophyceae rather than Xanthophyceae. This implies formal reclassification of over 80 currently accepted nominal *Characiopsis* species (including *C. minima* and *C. aquilonaris* recognized as eustigmatophytes in another recent study and placed in newly erected genera; Amaral et al. 2020), as a result of which the number of described eustigmatophyte species instantaneously more than doubles.

Nevertheless, as discussed above, not all species presently placed in *Characiopsis* are necessarily related to the core of the genus and some may really not belong to eustigmatophytes. In addition, our work suggests that the nominal species diversity of *Characiopsis* may be inflated due synonymy resulting from artefacts of the history of study of these algae or from misinterpreted morphological plasticity of individual species. Renewed culturing effort, combined with modern "omics" approaches, will be instrumental in improving our knowledge of the genus *Characiopsis*. Indeed, a genome survey has been recently conducted for *C. acuta* ACOI 456, yielding a complete plastid genome sequence (Ševčíková et al. 2019) and much more, including genome data from its *Phycorickettsia* endosymbiont (Eliáš et al., unpublished results). The present work establishes a useful framework for future exploration of the biological mysteries of this fascinating algal group.

Formal taxonomy

***Characiopsis minuta* (Braun) Borzì**

BASIONYM: *Characium minutum* Braun in Kützing 1849

HETEROTYPIC SYNONYM: *Pseudocharaciopsis minuta* (Braun) Hibberd

NEOTYPE (designated here): Figure Tab V, F in Braun (1855), reprinted here as Fig 3.1.

EPYTYPE (designated here to support the neotype): strain ACOI 2423 permanently preserved in a metabolically inactive state (cryopreserved in liquid nitrogen), deposited at ACOI – Coimbra Collection of Algae, University of Coimbra.

NOTE: While we consider the illustration cited above a neotype, it is formally possible that it should instead be designated as a lectotype. The latter would become appropriate should it be demonstrated that Braun in reality prepared the illustration before the publication of the species description in 1849. Such a possibility cannot be ruled out, but since Braun (1855,

p. 46) mentions his observations of *C. minutum* in 1851 and 1854, i.e. after the first encounter of the species in 1848, it is likely the drawing published in 1855 postdates the description in 1849. Hence, neotypification rather than lectotypification seems to be the appropriate act with the evidence available at the moment.

4.

Phylogeny of *Pseudostaurastrum* (Eustigmatophyceae)

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4.1. Abstract

The diversity of the class Eustigmatophyceae has been progressively revealed with molecular studies combined with morphological data, performed in cultivated strains from culture collections and new isolates or field material. The taxonomy of the class is presently composed of two ordinal clades, the Eustigmatales and the clade *Goniochloridales*. The genus *Pseudostaurastrum* is a member of the clade *Goniochloridales* with sequences from two strains consistently present in recent eustigmatophyte phylogenies. The ACOI culture collection holds a significant number of *Pseudostaurastrum* strains therefore a unique resource to enable the needed studies to clarify its taxonomy and phylogeny. In this study, twenty-four different strains form an independent lineage based on both 18S rRNA and rbcL gene phylogenies. The whole clade is resolved in five internal clades, four comprised by strains with a distinctive morphology of *P. lobulatum*, *P. limneticum*, *P. enorme* and *P. hastatum*. These species are therefore considered as eustigmatophytes and their taxonomy is discussed.

4.2. Introduction

Tetrahedral and polyhedric eustigmatophytes have long been under taxonomic turbulence, mostly revolving around the chlorococcacean genus *Tetraedron,* established in 1845 by Kützing with the type species *T. regulare* Kützing (1845). The name *Pseudostaurastrum* was introduced as a section of *Tetraedron* by Hansgirg (1888) due to the branched, angled processes of the cell, resembling those of the desmidiacean genus *Staurastrum*. The observation of cell characteristics from field material suggesting that these algae were heterokonts rather than chlorophytes was later realized by Chodat (1921). This author conserved the name *Taetraedron* for the tetrahedral chlorophyte species and established the new genus *Pseudostaurastrum* (Hansgirg) Chodat for accommodating the organisms from his sample that included the species *P. enorme* (Ralfs) Chodat, *P. lobulatum* (Nageli) Hansgirg, *P. armatum, P. gracile* (Reinsh) Hansgirg and *P. hastatum*

(Reinsh) Chodat. He noticed considerable morphological plasticity among *P. enorme* and expressed his taxonomic opinion that these different cells merely corresponded to different morphologies of *P. enorme* and were not to be described as independent species, although he recommended further studies with isolates from a single cell (Chodat 1921). *Pseudostaurastrum enorme* (Ralfs) Hansgirg *in* Chodat was the designated type species for the genus. The transfer of these *Tetraedron* species to *Pseudostaurastrum* was not immediately adopted by phycologists and some authors listed some as *Tetraedron hastatum* (Reinsh) Hansgirg and *Tetraedron gracile* (Reinsh) Hansgirg (e.g Smith 1926).

Another genus derived from *Tetraedron* is *Isthmochloron* created by Skuja (1948) to accommodate heterokont cells with a central constriction like the isthmus of desmids. This genus was considered superfluous by Bourrely (1951) who suggested that *Isthmochloron* and *Pseudostaurastrum* should be merged into one, the name *Pseudostaurastrum* having taxonomic priority. He also considered that other genera with similar morphology like *Tetraedriella*, *Tetragoniella*, *Tetrakenton* and *Goniochloris* should be included in *Pseudostaurastrum* as sections.

Although acknowledged as related taxonomically, the integration of these genera into *Pseudostaurastrum* was later questioned and the taxonomy clarified according to the available data (Fott and Komárek 1960). The older name *Pseudostaurastrum* was considered the valid attribution for the genus and emended to exclude the genera merged by Bourrely (1951). Fott and Komárek (1960) also considered that the original type *P. enorme* could not be used due to undefined limits, since Chodat (1921) had included several species in it. They therefore described new species of *Pseudostaurastrum* and considered that, in the absence of a valid type attribution by Chodat (1921), the valid description of the genus was that given by Skuja (1949), however Skuja used the name *Isthmochloron.* Therefore, Fott and Komárek (1960) emended the genus to *Pseudostaurastrum* (Hansgirg) Chodat emend. Skuja, according to Skuja's description and keeping the priority name and considered the species *P. hastatum* (Reinsh)

Chodat indicated by Skuja (1948) as the nomenclatural type. Besides this species, the genus comprised 3 other species, namely *P. lobulatum* (Naegeli) Chod., *P. trispinatum* (West) Skuja and *P. enorme* (Ralfs) Hansg. (Fott and Komárek, 1960).

Bourrelly adopted Fott and Komárek's taxonomy of *Pseudostaurastrum* in his Flora of Freshwater Algae (Bourrelly, 1981) and gave descriptions of genera removed from of *Pseudostaurastrum*, namely *Tetraedriella*, *Tetraplektron* (=*Tetrakentron*), *Goniochloris*, but leaving *Tetragoniella* omiss. In Ettl´s flora (1978), *Pseudostaurastrum* comprised *P. enorme*, *P. hastatum* and *P. limneticum* while other two species listed by Fott and Komárek (1960) appeared included in the genus *Isthmochloron* as *I. trispinatum* (West) Skuja and *I. lobulatum* (Nageli) Skuja (Ettl 1978).

Slight morphological differences found in the cells had originated the description of different varieties of some *Tetraedron* species, such as *T. hastatum* (Reinsch) Chodat var. *palatinum* and *T. hastatum* var. *hastatum* (Smith 1926). These were not perceived as *Pseudostaurastrum* varieties (Starmach 1968, Ettl 1978) until later, when both were found in field material together with *P. limneticum* (Krienitz and Heynig 1992). These authors also described a new combination *P. planctonicum* (Smith) Krienitz and Heynig by transferring *Tetraedron planctonicum* Smith. Currently there were five recognized species of *Pseudostaurastrum,* namely *P. enorme*, *P. hastatum*, *P. lobulatum*, *P. trispinatum*, *P. limneticum* and *P. planctonicum* (Krienitz and Heynig 1992).

Although the class Eustigmatophyceae was established in the early 1970´s (Hibberd and Leedale 1970, 1971, 1972), the first *Pseudostaurastrum* species to be considered as an eustigmatophyte was *P. limneticum* (Borge) Chodat (Schnepf et al. 1995/96). While studying how cell shape is developed after zoospore settling, the eustigmatophyte nature of this organism was unambiguously proven by ultrastructure and pigment composition.

The use of molecular methods has progressively enlightened the phylogeny of eustigmatophytes and altered the classic single order taxonomic scheme long adopted for the class (Hibberd 1981) to a two-order systematics (Fawley et al. 2014). The first 18S rRNA

gene sequences obtained from *Pseudostaurastrum* (*P. enorme* SAG 11.85 and *P. limneticum* SAG 14.94) and included in phylogenetic analysis were reported in 2007 (Hegewald et al 2007). The authors noted the deeply diverged lineage formed by both *Pseudostaurastrum* strains in relation to other known eustigmatophytes. Later molecular studies evidenced their eustigmatophyte nature and position in a deeply diverged lineage (Přibyl et al 2012), that was expanded and described as a second ordinal clade of the Eustigmatophyceae named clade *Goniochloridales* (Fawley et al. 2014). The *Pseudostaurastrum* clade was recently enriched with sequences of two other strains of the same species (*P. enorme* ACOI 2426 and *P. limneticum* ACOI 1860 (Kryvenda et al. 2018).

Based on these molecular studies, *Pseudostaurastrum* is listed as an eustigmatophyte genus comprising two species, *P. enorme* and *P. limneticum*, with *P. enorme* as the type species (e.g. Eliáš et al. 2017, Ott et al. 2015). As mentioned above, this type species of *Pseudostaurastrum* was considered invalid by Fott and Komárek (1960) that elected *P. hastatum* (Reinsh) Chodat as the type species. Since the original strain is not available from any culture collection, any eustigmatophyte found and identified as *P. hastatum* may formally be designated as the type material of *P. hastatum*.

The present study includes molecular and morphological data on ACOI *Pseudostaurastrum* isolates, thus contributing to clarify the taxonomy and phylogeny of this poorly known eustigmatophyte genus.

4.3. Materials and Methods

Algal cultures and microscopy

A total of 19 freshwater strains held at the Coimbra Collection of Algae were studied (Table 4.1.). The strains had previously been identified at ACOI based on light microscopy

observations and attributed to the genus *Pseudostaurastrum* according to Ettl (1978). The strains were cultivated in liquid Desmideacean Medium (Schlösser 1994) pH 6.4-6.6, at 20 °C, a 12:12 h, under a light intensity of 10 μmol photons.m^{-2}.s^{-1} provided by cool white fluorescent lamps. Morphological evaluation of the cells was performed using a Leica DMRB microscope with conventional light microscopy or DIC microscopy, using 60x and 100x PLAN APO objectives. Micrographs were acquired with a Nikon DS-Fi2 digital camera. Cell size was accessed by using the digital image analysis software NIS 4.60 (Isaza).

Table 4.1. The studied strains with indication of their ACOI reference number, origin and collection year. ACOI - Coimbra Collection of Algae, Portugal.

Species	ACOI nr.	Origin	Collecting Year
Pseudostaurastrum cf. *lobulatum*	2780 ni	plankton, lagoa das Braças, Quiaios, Portugal	1998
Pseudostaurastrum lobulatum	2442_A3ni	canal, Sta. Olaia, Montemor-o-Velho, Portugal	1996
Pseudostaurastrum enorme	2785 ni	plankton, barragem da Erva da Fome, Serra da Estrela, Portugal	1997
Pseudostaurastrum enorme	2026	plankton, barragem da Erva da Fome, Serra da Estrela, Portugal	1997
Pseudostaurastrum enorme	568	mud, Diogo Vaz, Ilha de S. Tomé, São Tomé e Príncipe	1992
Pseudostaurastrum limneticum	3132 ni	plankton, paúl da Tornada, Portugal	2000
Pseudostaurastrum limneticum	1860	plankton, barragem da Agolada, Portugal	1996
Pseudostaurastrum limneticum	1861	plankton, barragem da Agolada, Portugal	1996
Pseudostaurastrum hastatum	2441	plankton, barragem do Monte da Barca, Portugal	1996
Pseudostaurastrum sp.	2028	plankton, barragem de Toulica, Castelo Branco, Portugal	2007

Pseudostaurastrum cf. *hastatum*	2551 ni	plankton, barragem da Agolada, Portugal	1996
Pseudostaurastrum hastatum	2337 ni	canal, Sta. Olaia, Montemor-o-Velho, Portugal	1996
Pseudstaurastrum hastatum	2441_1ni	plankton, barragem da Agolada, Portugal	1996
Pseudostaurastrum hastatum	2441_2ni	plankton, barragem da Agolada, Portugal	1996
Pseudostaurastrum hastatum	2441_3ni	plankton, barragem da Agolada, Portugal	1996
Pseudostaurastrum sp.	2622 ni	plankton, barragem de Toulica, Castelo Branco, Portugal	2007
Pseudostaurastrum cf. *hastatum*	2440 Ani	plankton, barragem da Agolada, Portugal	1996
Pseudostaurastrum hastatum	2419 ni	plankton, barragem da Agolada, Portugal	1996
Pseudostaurastrum cf. *hastatum*	2593 ni	plankton, barragem da Agolada, Portugal	1996

PCR amplification and DNA sequencing

Cells were collected by centrifugation of 2 ml culture at 14000 rpm and disrupted using a mixer mill (MM200, Retsch, Haan, Germany) for 5 minutes. Genomic DNA was extracted using Invisorb® Spin Plant Mini Kit (Stratek). Primers used for obtaining full sequences of the 18S rRNA gene were the amplification primers 18S-F, 18S-R and internal sequencing primers 402-23F, 895-916F, 1323-44F and 416-37R, according to Katana et al. (2001). PCR products from amplification of the 18S rRNA and *rbc*L genes were purified using GenElute™ PCR Clean-Up Kit (SIGMA). For some strains double bands were obtained in the PCR, in these cases it was repeated in a larger volume and the fragment was excised from the gel and purified using GenElute™ Gel Extraction Kit. Primers used for amplification of *rbc*L gene were newly designed EU-rbcL-F1 ATGTTTCAATCTGTAGAAGAAAG and

EU-rbcL-R1 CCTTGTGTTAATCTCACTCTTC and a partial sequence was obtained with EU-rbcL-F1. Sequences were achieved with BigDye® Terminator v3.1 Cycle Sequencing Kit (ThermoFisher scientific) and analyzed by Macrogen service. Sequencing reads were assembled with SeqAssem (SequentiX, http://www.sequentix.de/software_seqassem.php) and manually edited by visual inspection of sequencing chromatograms.

Phylogenetic analysis

The complete dataset for analysis of the 18S rRNA gene sequences included a total of 149 sequences and consisted of the 19 newly obtained sequences of *Pseudostaurastrum* strains, and a selection of 15 sequences from phylogenetically diverse ochrophytes to provide an outgroup. The sequences were aligned with MAFFT 7.429 (Katoh and Frith 2012, Katoh and Standley 2013), using the "Add" tool and a preexisting alignment used in a previous study (Amaral et al, in revoew). Redundant sequences were removed in Bioedit version 7.0.5 (Hall 1999; (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and the resulting final alignment was trimmed with trimAl v1.4.rev6 by removing columns with more than 20% gaps (Capella-Gutiérrez et al. 2009; https://www.genome.jp/tools/ete/). The 19 new *rbc*L gene sequences from *Pseudostaurastrum* were aligned with MAFFT "Add" to a previously obtained alignment comprising 74 eustigmatophyte sequences available from GenBank (retaining one sequence per described species for non-*Pseudostaurastrum* representatives) and the *Characiopsis* strains (Amaral et al. resubmitted). The termini of the alignment were trimmed in BioEdit to remove positions with a high percentage of missing data.

Trees were inferred using the maximum likelihood (ML) method implemented in RAxML-HPC BlackBox (8.2.10) at the Cyberinfrastructure for Phylogenetic Research (CIPRESS) Portal (http://www.phylo.org/sub_sections/portal/) (Miller et al. 2010) using the strategy of Stamatakis et al. (2008) for obtaining the highest likelihood tree. The evolutionary model

used was the default GTR+Γ. Bootstrap analysis was performed with the rapid bootstrapping procedure, with the adequate number of replicates detected by the program. The graphic tree was obtained with iTOL (https://itol.embl.de/), and for simplicity the phylogenies obtained are given with collapsed Eustigmatales (Figure 4.1. and Figure 4.2.).

4.4. Results and discussion

Phylogenetic analyses

The topology emerging from eustigmatophyte backbone obtained with 18S rRNA gene phylogeny shows two deeply diverged ordinal lineages, one corresponding to the Eustigmatales (shown collapsed for simplicity) and a second ordinal clade *Goniochloridales*, where the studied strains are positioned (Fig. 4.1.). The diversity of clade *Goniochloridales* agrees with previous reports (Fawley et al. 2014, Nakayama et al. 2015, Eliáš et al. 2017, Fawley and Fawley 2017, Kryvenda et al. 2018, Amaral et al. 2020, Amaral et al. resubmitted). It includes strains of the genera *Trachydiscus*, *Tetraedriella, Goniochloris* and *Vacuoliviride* distributed together with unnamed strains in Clade IIa, Clade IIb and Clade IIc, and also the independent fully resolved lineage *Pseudostaurastrum*. The clade comprised by *Pseudostaurastrum* strains was previously shown to be a sister lineage to Clade IIc (Fawley et al. 2014, Nakayama et al. 2015, Eliáš et al. 2017, Fawley and Fawley 2017). A recent deployment with other sequences changed the topology to include three new lineages positioned out of these clades, one consists of single leaf *Goniochloris tripus* ACOI 1855 and two independent fully resolved lineages, each comprising two sequences of *Tetraedriella* and *Goniochloris* (Kryvenda et al. 2018). The newly added *Tetraedriella* and *Goniochloris* sequences show a paraphyletic nature of both genera, since other strains of these genera are positioned elsewhere in the Goniochloridales. The above-mentioned topology remained unchanged with the addition of the studied *Pseudostaurastrum* strains in the present study (Fig. 4.1) and it is now known to

include also one of the new lineages, Group 1, revealed by an environmental study (Villanueva et al. 2014). The larger group comprising these lineages and the *Pseudostaurastrum* strains is sister to Clade IIc. The taxonomic level of the lineages apparent in this order is still under consideration since its deployment comprised until now mostly unnamed strains with undescribed morphology. A possible interpretation of Clades IIa, IIb and IIc at the family rank could be considered but the phylogeny emerged from the present study shows that Clade IIc is clustered with Group 1, the paraphyletic group of *G. tripus* / *T. verrucosa* / *T. tumidula* and also *Pseudostaurastrum*. The phylogeny indicates that *Pseudostaurastrum* corresponds to a deeply diverged, fully supported independent lineage. It is presently apparently positioned at the family rank but it is not yet possible to determine this position with certainty until more diversity is added to the phylogeny of this eustigmatophyte order. It is therefore advisable to consider Clade IIc as an independent lineage separated from the others with 82% bootstrap support and also Group 1, with a moderate support of 72%. The paraphyletic group of organisms composed by *Goniochloris* and *Tetraedriella* poses a challenge yet to be clarified, further studies comprising more strains are required in order to unambiguously determine the phylogeny and taxonomy of these two genera.

The internal phylogeny given by 18S rRNA gene for the *Pseudostaurastrum* clade (Fig. 4.1.) shows that both sequences which consistently feature in eustigmatophyte phylogenies are positioned in single leaves with no bootstrap support, with *P. enorme* SAG 11.85 isolated from all the remaining sequences and *P. limneticum* SAG 14.96 positioned internally to one clade comprising three strains with *P. enorme* morphology. There are 5 fully supported internal clades composed of the studied strains. One diverged lineage is comprised by the studied strain *P.* cf. *lobulatum* ACOI 2780ni with Genebank sequences from *Pseudostaurastrum* sp. CCALA 10174 and *Pseudostaurastrum* sp. ACOI 2027, here named P. LOBULATUM CLADE. A second clade is composed of three sequences from *P. enorme*, two sequences from ACOI 2026 (one sampled from Genebank and another newly sequenced in the present study) and

a third one from ACOI 2785ni, named P. ENORME CLADE. A third internal clade is the only one composed of strains identified as different *Pseudostaurastrum* species, specifically *P. limneticum* ACOI 3132ni and *P. enorme* ACOI 568, named P. LOBULATUM/ENORME CLADE. A fourth internal clade comprises two sequences from *P. limneticum* ACOI 1860 (one sampled from Genebank and another newly sequenced in the present study), and *P. limneticum* ACOI 1861, named P. LIMNETICUM CLADE. The fifth clade is composed of sequences from strains with *P. hastatum* morphology and undetermined *Pseudostaurastrum* sp. strains, named P. HASTATUM CLADE.

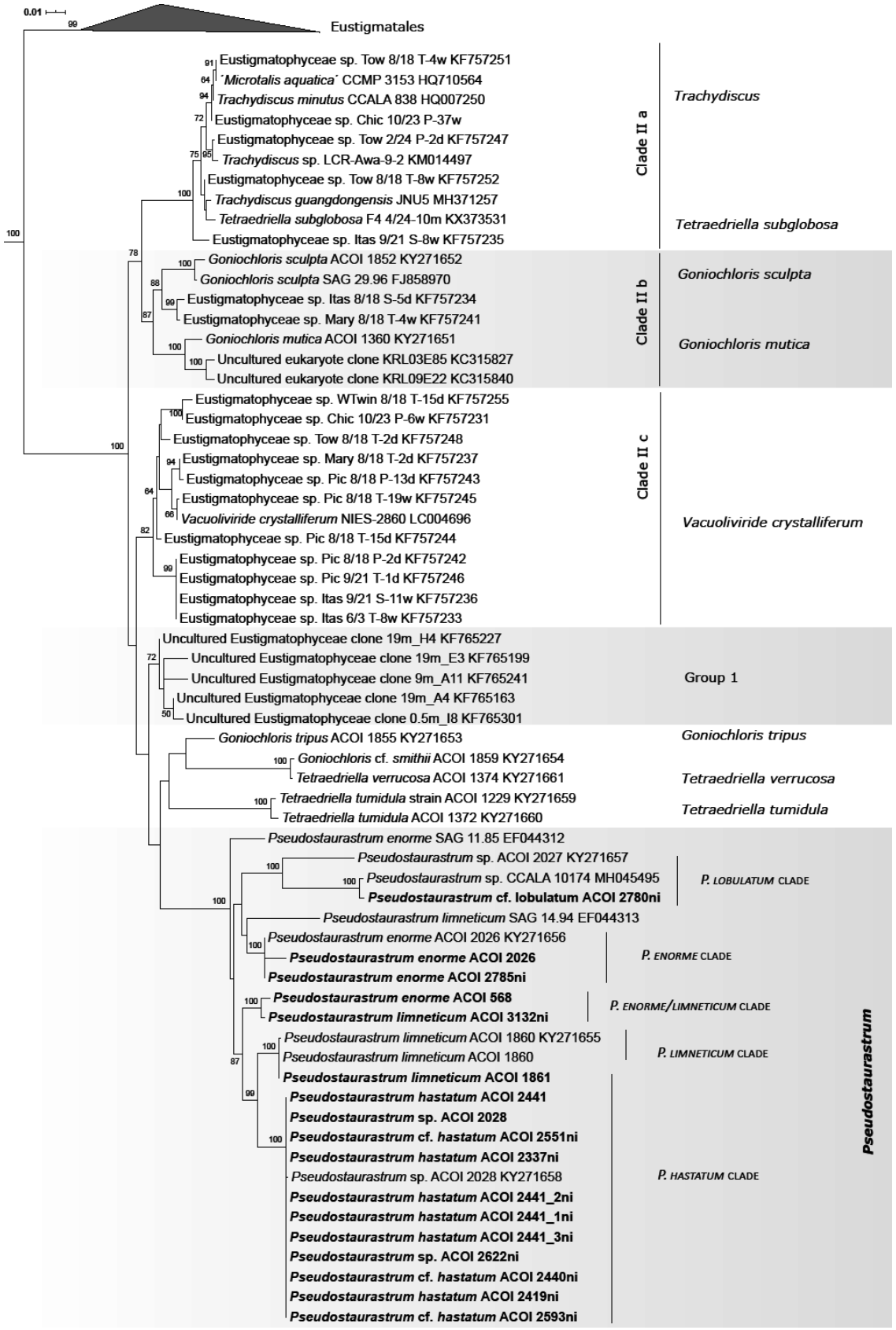

Figure 4.1. Phylogeny of Eustigmatophyceae based on sequences of the 18S rRNA gene, showing clade *Goniochloridales*. The phylogeny shown was inferred using maximum likelihood

method implemented in RAxML (employing GTR+Γ substitution model) with bootstrap analysis followed by thorough search for the ML tree. Bootstrap values higher than 50 are shown. Labels at terminal leaves comprise the strain taxonomic name followed by the collection reference number and the GenBank accession number. New sequences are highlighted in boldface. The tree was rooted using 15 sequences from stramenopile algae sampled from GenBank. The outgroup is omitted and the order Eustigmatales is shown collapsed for simplicity.

The phylogeny given by *rbc*L gene analysis corroborates that all *Pseudostaurastrum* strains form a single deeply diverged lineage within clade *Goniochloridales* with full bootstrap support (Fig. 4.2.). The internal topology generally agrees with 18S rRNA gene analysis (Fig. 4.1.). The position of *P. enorme* SAG 11.85 and *P. limneticum* SAG 14.96 is undetermined because *rbc*L sequences are not available in GenBank. The lineage named as P. LOBULATUM CLADE shown in 18S rRNA phylogeny is also present in *rbc*L phylogeny with 99% bootstrap support. It is represented by *Pseudostaurastrum* cf. *lobulatum* ACOI 2780ni and an additional strain *P. lobulatum* ACOI 2442_A3, from which an 18S rRNA sequence was not obtained. The second lineage apparent from 18SrRNA gene analysis named P. ENORME CLADE, is also represented in the *rbc*L tree as an independent lineage represented by ACOI 2785ni. The other three clades correspond exactly to those shown with 18S rRNA gene phylogeny, with no substantial internal resolution.

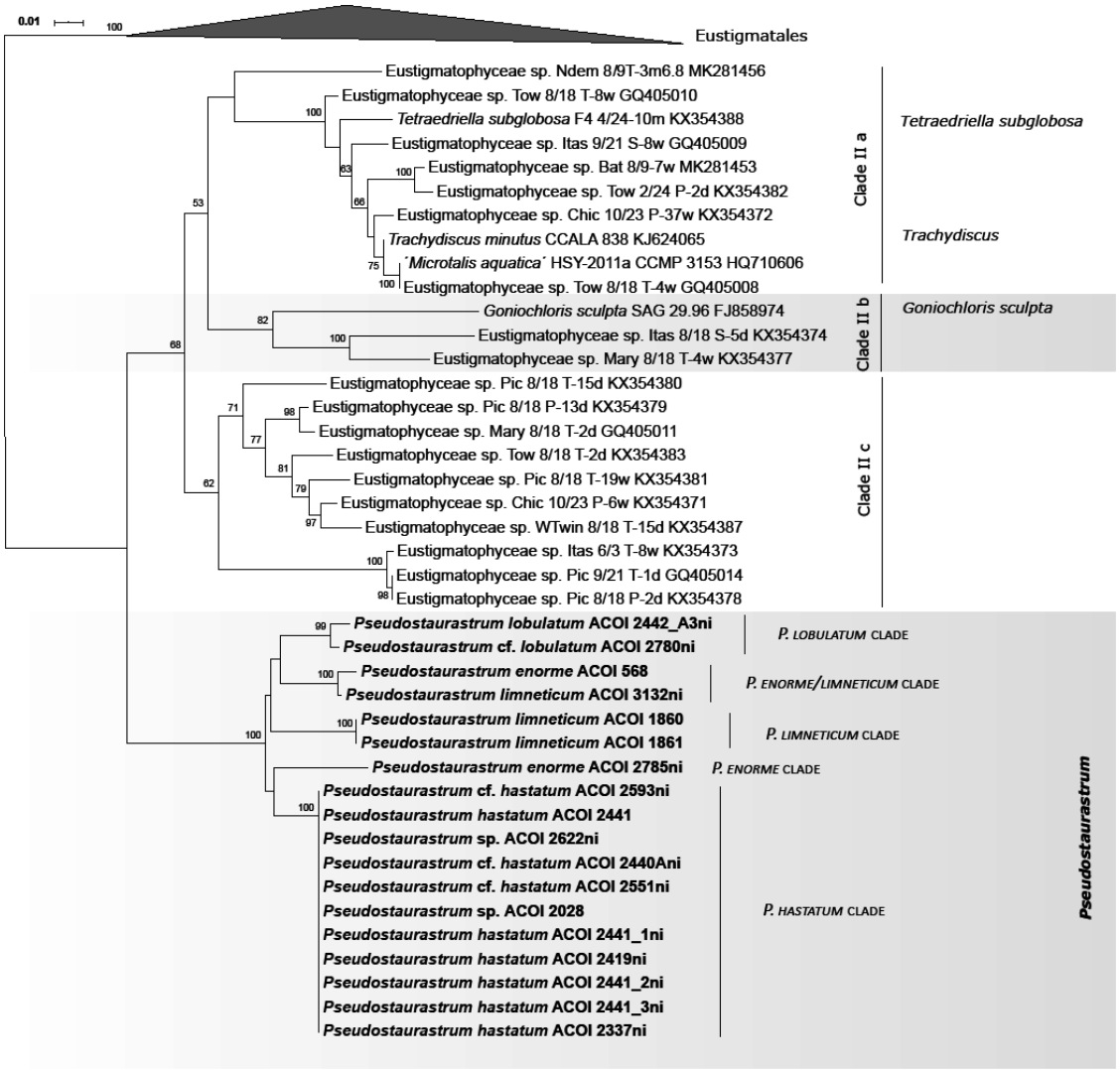

Figure 4.2. Phylogeny of Eustigmatophyceae based on *rbc*L gene, showing clade *Goniochloridales*. The phylogeny shown was inferred using maximum likelihood method implemented in RAxML (employing GTR+Γ substitution model) with bootstrap analysis followed by thorough search for the ML tree. Bootstrap values higher than 50 are shown. Labels at terminal leaves comprise strain name and number. New sequences are highlighted in boldface. The tree was rooted at the ordinal clade Eustigmatales, which is shown collapsed for simplicity.

Morphology and light microscopy observations

The cells of studied *Pseudostaurastrum* species are free-floating, with a tetrahedral, polyhedral or cruciform shape, with marked lobes prolonged by arms or processes (Fig. 4.3. c) bearing

spines at the end (Fig. 4.3. g), which may be simple, bifurcated or up to three times dichotomically branched. The young cells are filled with chloroplasts and the lateral walls are concave and then they become straight and finally convex, in older cells (Fig. 4.3. h). The plastids are disc shaped (Fig. 4.3. a) and numerous, usually parietal. No pyrenoid was detected in the observed organisms by light microscopy. The lamellate vesicles, also known as refractive granules, typically seen in all eustigmatophytes (Santos 1996), were possible to observe scattered throughout the cytoplasm due to their characteristic refringent behavior under light microscopy (Fig. 4.3. k). A reddish globule, also typical of eustigmatophytes, is present in all studied *Pseudostaurastrum* cells (Fig. 4.3.). It is colored red, contrarily to the more orange-like color which originated the expression "reddish globule", used for eustigmatophytes (Fig. 4.3. c). In older cells, up to four globules may be present.

The clades shown by the phylogenetic analysis (Fig. 4.1. and 4.2.) have a correspondence to the morphology of the strains, with most clades formed by strains assigned to the same taxa. The strains positioned in P. LOBULATUM CLADE (Fig. 4.1. and Fig. 4.2.) have tetrahedral cells with lobes ending in a spine, usually bifurcated (Fig. 4.3. a and b), in agreement with the original descriptions by Nägeli (1849). Other morphologies may be found in culture simultaneously, such as cells with longer lobes or with the cell body longer and with a less regular shape, seen in ACOI 2780ni *P.* cf. *lobulatum* (Fig. 4.3. c)., which originated some doubt in its identification based only on morphological data. This diversity in culture is possibly explained by morphological plasticity, already acknowledged in other eustigmatophytes (Amaral et al. 2020, Amaral et al. resubmitted).

P. enorme cells are lobed, not symmetrical, showing a constriction on the lateral sides. Broad lobes ending in short spines, which may be simple or branched (Fig. 4.3. d). The cell is occupied by one or two large parietal plastids and one or more large red globule is seen at the center of the cell (Fig. 4.3. d). The P. ENORME CLADE is composed by ACOI 2785ni,

represented in both 18S rRNA gene and *rbc*L gene phylogenies (Fig. 4.3. e) and also by two sequences of ACOI 2026 (Fig. 4.3. d), one of the sequences was released to GenBank by Kryvenda et al. (2018) in supplementary material of a study dedicated to *Eustigmatos*/*Vischeria*, and another was obtained in the present study. Both have the cell morphology of *P. enorme* but display significant morphological diversity in culture, also reported by Chodat (1921) and Ralfs (1848).

The similarities between *P. enorme*, *P. limneticum* and *P. planctonicum* are noted by Krienitz and Heynig (1992) who evidence that *P. enorme* has short processes and a large cell body contrasting with the latter two, which have longer processes, with *P. planctonicum* in the middle of the other two regarding these two relative measurements. Observations of both cultures comprising P. ENORME/LIMNETICUM CLADE, *P. enorme* ACOI 568 (Fig. 4.3. f) and *P. limneticum* ACOI 3132ni (Fig. 4.3. g), show that many cells of ACOI 568 are large with short processes but many others are intermediate. The same happens with ACOI 3132ni, where cells with long processes and a small cell body are found but also many are intermediate. These differences may reflect either morphological plasticity or alternatively, the older cell changing to larger cell body with an inversion of the cell to convex form (Fig. 4.3. h). It may also be the case that those intermediate forms may correspond to *P. planctonicum*, this clade is therefore composed of strains with uncertain identity.

P. limneticum ACOI 1860 and ACOI 1861 (Fig. 4.3. i) cell have long processes and smaller cell body than ACOI 3132ni. The cells are tetrahedral or cruciform and have long processes with bifurcated ends. There is some variation on the depth of the bifurcation of the four arms of the cell, also noted by Smith (1926). The cell body may be quite thin or larger, with some diversity noted in this aspect, also reported by Shnepf et al. (1995/96). Both strains were sampled from a dam (Table 4.1.), which agrees with previous observations that species

is not so common and that it occurs mainly in nutrient-rich water bodies (Lang et al. 2014). This is a coherent clade here named P. LIMNETICUM CLADE.

Pseudostaurastrum hastatum or undetermined *Pseudostaurastrum* sp. strains (Figures 4.1 and 4.2) form a very consistent clade with both gene phylogenies, here named P. HASTATUM CLADE. Cells have four processes, arranged in a tetrahedic (Fig. 4.3. j) or plane (Fig. 4.3. l) configuration. Cells have sides concave from the apex of one process to the apex of the next (Fig. 4.3. k – bottom left-hand cell), which may become much less marked in older cells (Fig. 4.3., k – bottom right-hand cell). The processes may be simple, ending in a spine (Figure 4.3. l) or they may exhibit branching, usually bifid (Fig. 4.3. m). One or more red globules are seen, usually at the center of the cell or one on each side of the cell (Fig. 4.3. l).

The morphological plasticity reported for most strains is consistently seen in other cultured eustigmatophytes and has been attributed to serial sub-culturing or morphological adaptations to the *in vitro* condition (Amaral et al. 2020, Amaral et al. resubmitted).

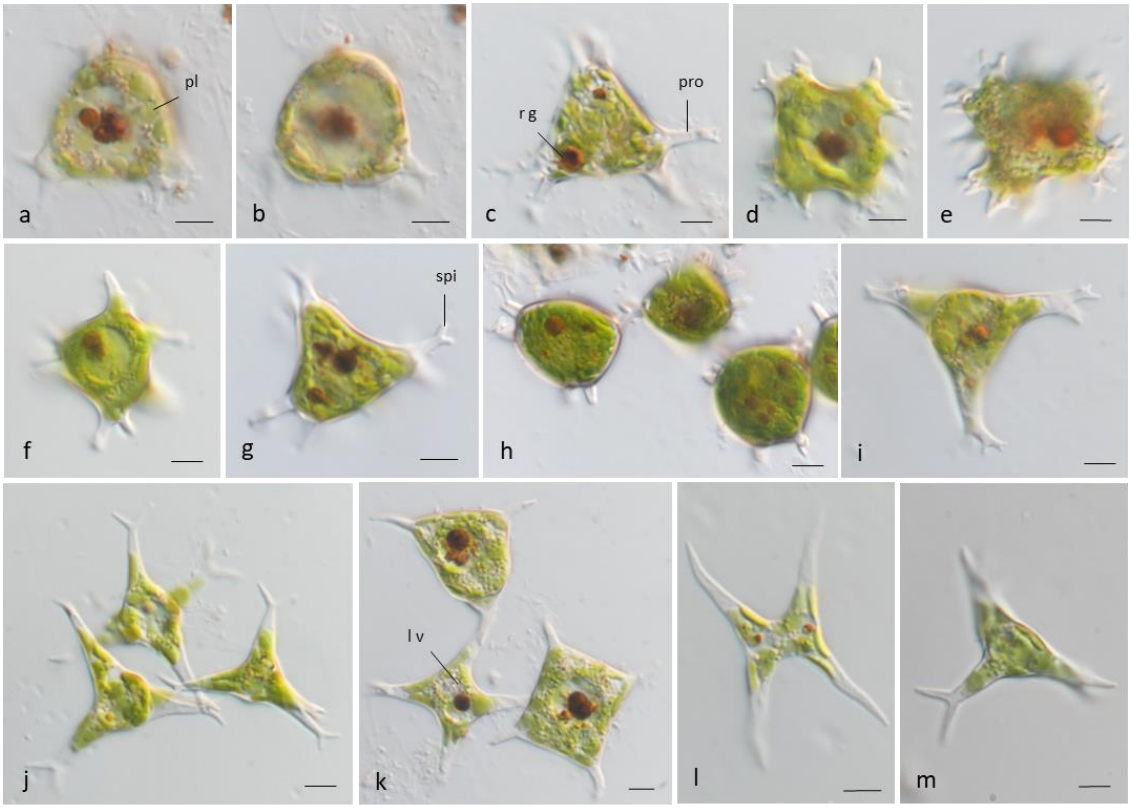

Figure 4.3. *Pseudostaurastrum* strains observed with light microscopy. a) and b) *P. lobulatum* ACOI 2442_A3ni; c) *P.* cf. *lobulatum* ACOI 2780ni; d) *P. enorme* ACOI 2026; e) *P. enorme* ACOI 2785ni; f) *P. enorme* ACOI 568; g) and h) *P. limneticum* ACOI 3132ni; i) *P. limneticum* ACOI 1861, j) *P. hastatum* ACOI 2419ni, k) *P. hastatum*?, l) and m) *Pseudostaurastrum* sp. ACOI 2028. pl – plastid, r g – red globule, pro – processes or arms, spi – spines at end of the processes, l v – lamellate vesicles. Scale bar 10 μm.

Taxonomic considerations on *Pseudostaurastrum*

The species *Pseudostaurastrum lobulatum* (Nägeli) Chodat, *Pseudostaurastrum enorme* (Ralfs) Hansgirg and *Pseudostaurastrum hastatum* Chodat were listed by Chodat (1921) as morphological variants of *Pseudostaurastrum enorme* (Ralfs) Hansgirg. The name *Pseudostaurastrum lobulatum* is most likely derived from *Tetraedron lobulatum* (Nägeli) Hansgirg. However, Chodat did not formally clarify the taxonomic genus transfer of these three species. Hansgirg (1888) had previously considered *T. lobulatum, T. hastatum* and *T. enorme* as species belonging to section *Pseudostaurastrum* Hansgirg of genus *Tetraedron* Kutzing (Hansgirg

1888) and Chodat decided to use the name of the section to the new genus *Pseudostaurastrum* (Chodat 1921). The first name of the species *Pseudostaurastrum lobulatum* (its basyonim) is *Polyedrium lobulatum* Nägeli, established by Nägeli (1849) for accommodating organisms observed and drawn by him. The original descriptions and drawing of *Polyedrium lobulatum* resemble some cells of *Pseudostaurastrum enorme* (Ralfs) Chodat (Chodat 1921), which explains the opinion of Chodat (1921) (see above). Skuja considered this organism as a member of *Isthmochloron*, *I. lobulatum* (Nägeli) Skuja (Skuja 1948) but the genus *Isthmochloron* was later considered superfluous and *I. lobulatum* was rendered a synonym of *Pseudostaurastrum lobulatum* (Fott and Komárek 1960). No other reports were found for this species since then so to our knowledge these ACOI strains, collected in 1996 and 1998, (Table 4.1.) are the most recent occurrences.

The basionym of *Pseudostaurastrum enorme* is *Staurastrum enorme* Ralfs, described by Ralfs (1848) as a Desmidiaceae. A later study proved that it was not a desmidiacean species but a member of the xanthophycean genus *Polyedrium* so *Staurastrum enorme* was rendered a synonym of *Polyedrium enorme* (Ralfs) Reinsh but with some doubts due to the singularity of the cell morphology Reinsh (1867). This species was later included by Hansgirg (1888) in the section *Pseudostaurastrum* as *Tetraedron enorme* (Ralfs) Hansgirg and then raised to the genus *Pseudostaurastrum* by Chodat (1921). It was expected that the acknowledged strain *P. enorme* SAG 11.85 would cluster together with these *P. enorme* strains but instead, it forms a distinct single-leaf lineage diverged from all other *Pseudostaurastrum* strains included in the 18S rRNA tree (Figure 3.1.). The available photos from the strain file available from the SAG culture collection shows a morphology characteristic of *Pseudostaurastrum enorme* (http://sagdb.uni-goettingen.de/detailedList.php?str_number=11.85). Also, one strain with *P. enorme* morphology ACOI 568 (Figure 4.3. f) is included in a third clade with *P. limneticum* ACOI 3132ni. It is the only molecular group in this study which does not group strains with the same species name (and therefore, with similar morphology). The fact that *P. enorme* strains

are distributed through the internal topology of the *Pseudostaurastrum* clade may be explained by the diverse morphologies found in culture, also reported by Chodat (1921) and Ralfs (1848), which makes it difficult to unambiguously determine the species based only in morphology-based methods. Some older cells of *P. limneticum* ACOI 3132ni, like with all concave-sided species, turn into a convex lateral shape and round-up (Figure 4.3. h), sometimes showing branched spines, which gives them a morphology similar to some *P. enorme* cells. It is important to find more *P. enorme* strains in order to study them and to understand how diverse this species is and to what extent its phylogeny has a correspondence to morphology.

The species name *P. limneticum* was based on its basionym *Tetraedron limneticum* Borge (1906) and many authors (Schnepf et al. 1995/96, Hegewald et al. 2007, Přibyl et al. 2012 and Lang et al. 2014) considered it automatically transferred to *Pseudostaurastrum* by Chodat (1921) and refer to this species as *P. limneticum* (Borge) Chodat although it is not included among the *Pseudostaurastrum* species listed by Chodat (1921). Krienitz and Heinig (1992) refer to *T. limneticum* as its predecessor species but they write *P. limneticum* (Borge) Chodat ex Wojciechowsky 1971 without a reference to the later publication. It was not possible to track it down, so the authority is here kept as *P. limneticum* (Borge) Chodat although with some doubts. *P. limneticum* was the first species of *Pseudostaurastrum* to be considered as an eustigmatophyte (Schnepf et al. 1995/96). The similarities between *P. enorme*, *P. limneticum* and *P. planctonicum* are noted by Krienitz and Heynig (1992) (see above) who transferred *Tetraedron planctonicum* (Smith) to the Xanthophyceae and made a new combination *Pseudostausrastrum planctonicum* (Smith) Krienitz and Heynig.

The large clade composed by twelve strains named *P. hastatum* or *Pseudostaurastrum* sp. with no species assignment (Figure 4.1. and 4.2.) is quite diverse in cell morphologies. In *P. hastatum* strains the majority of cells are tetrahedral with concave side wall and long processes

ending with branched spines but there may be different cells in culture (Figure 4.3. j), in accordance to Smith (1926). This diversity of cells in culture is noted in some studied strains namely *P.* cf. *hastatum* ACOI 2593ni and *Pseudostaurastrum* sp. 2028 (Figure 4.3. k and l), ACOI 2440 Ani where these tetrahedral cells occur together with cells with no branches at the end of the processes, similar to the cells described for *Tetraedron arthrodesmiforme* Woloszynska (1914). In some strains it is possible to observe cells with a marked constriction of the cell wall and a planar arrangement of the cell processes, resembling *Tetraedron constrictum* Smith (1926). The cell characteristics which served for distinguishing *Tetraedron* strains were the space orientation of the processes and if they possess branched ends (Smith 1926). These characteristics were used to distinguish the tetrahedral *T. hastatum* from the plane cells of *T. constrictum* and *T. arthrodesmiforme* which could also display a cruciform arrangement and differed from the other two in not possessing branched processes. If the above-mentioned strains could unambiguously be identified as *T. constrictum* and *T. arthrodesmiforme* then these two species could be transferred to *Pseudostaurastrum* but it is not possible due to doubts stemming from the diversity seen in culture, maybe caused by morphological plasticity. The studied strain which most resembles the available descriptions for *P. hastatum* (Reinsch in Chodat 1921) is ACOI 2419ni (Figure 4.3. j).

4.5. Conclusions

Like most eustigmatophytes, *Pseudostaurastrum limneticum* was transferred from the Xanthophyceae to the Eustigmatophyceae (Schnepf 1995/96) and the present study shows molecular and morphological data which support the transfer of *P. lobulatum, P. enorme* and *P. hastatum* to the Eustigmatophyceae as well. Such as observed with other recently transferred taxa (Neomonodaceae), the strains exhibit some degree of morphological diversity in culture, which causes trouble in using only morphological data for species

identification. Segregating characteristics historically used for tetrahedral genera included the cell wall ornamentations such as hollows, dots or reticulated structures, their arrangement in loose or dense, regularly or irregularly throughout the cell surface (Bourrelly 1981, Hegewald et al. 1983, Krienitz and Heynig 1992).

Tetrahedral and polygonal members of clade *Goniochloridales* are indeed fragile organisms with a difficult maintenance by sub-cultivation and *in vitro* maintenance. This particularity had been noted by other authors for *Tetraedriella*, *Goniochloris* and *Pseudostaurastrum* species, which were studied in natural samples since they did not survive after isolation (Hegewald et al. 1983). This observation possibly justifies the lesser extent of studies in this ordinal clade of eustigmatophytes, which are becoming a very relevant part of eustigmatophyte diversity and wait for dedicated studies to clarify their phylogeny and taxonomy.

With this study the clade *Goniochloridales* is broadened with more strains and the eustigmatophyte genus *Pseudostaurastrum* is now composed of *P. limneticum, P. enorme, P. lobulatum* and *P. hastatum.*

5.

Eustigmatophyte phylogeny overview

The topology emerging from 18S rRNA eustigmatophyte backbone phylogeny comprising all known strains consistently shows two deeply diverged ordinal clades (Figures 2.1, 3.27 and 4.1.), also evident with *rbc*L gene analysis (Figures 2.2, 3.28. and 4.2.). This topology agrees with current interpretations of a two-order taxonomic scheme for the Eustigmatophyceae (Fawley et al. 2014, Nakayama et al. 2015, Eliáš et al. 2017, Fawley and Fawley 2017, Kryvenda et al. 2018, Amaral et al. 2020). Eustigmatophyte phylogeny is heavily supported in molecular data, leaving no doubt it includes two ordinal clades and seven familial clades. The most current taxonomic scheme is summarized in Table 5.1..

Table 5.1. Taxonomy of the Eustigmatophyceae, based on the most comprehensive molecular datasets based on 18S rRNA gene phylogeny (Eustigmatales in Figure 3.27 and clade *Goniochloridales* in Figure 4.1.). Higher taxa named as group or clade denote informal names adopted since their original descriptions until further studies formally validate the clade names.

Order	Families	Genera
Eustigmatales	Eustigmataceae group	*Vischeria* (syn. *Eustigmatos*)
		Clade Ia
		Chlorobotrys
		Characiopsis (syn. *Pseudocharaciopsis*)
	Monodopsidaceae	*Monodopsis*
		Pseudotetraëdriella
		Nannochloropsis
		Microchloropsis
	Neomonodaceae, fam. nov.	*Neomonodus*, comb. nov.
		Pseudellipsoidion
		Characiopsiella, gen. nov.
		Munda, gen. nov.

Goniochloridales clade	clade IIa	*Trachydiscus*
		Tetraedriella subglobosa
		´Microtalis aquatica´
	clade IIb	*Goniochloris* (*G. sculpta*)
		Goniochloris (*G. mutica*)
	clade IIc	*Vacuoliviride*
		Goniochloris (*G. tripus*)
	Pseudostaurastrum clade	*Pseudostaurastrum*
Incertae sedis	*Incertae sedis*	*Botryochloropsis*
		Tetraedriella (*T. tumidula*)
		Tetraedriella (*T. verrucosa*)

Studies using metabarcoding of environmental samples (Lara et al. 2011, Nikouli et al. 2013, Villanueva et al. 2014) originated the release to Genbank of 18S rRNA gene sequences from uncultured eustigmatophytes. Sequences from PRS2_4E_40, PRS2_3E_43 (Lara et al. 2011) are positioned in order Eustigmatales (Figure 3.27.). Also, a large set of short eustigmatophyte 18S rRNA sequences covering a region of the 18S rRNA gene slightly over 500 bp were derived from an environmental study in African lake Challa (Villanueva et al. 2014). Most of these sequences are from eustigmatophytes, distributed in groups by the currently acknowledged families. The set of sequences named as ´Group 2´ includes a large number of sequences specifically affiliated to *Paraeustigmatos columelliferus*, in the Eustigmatales, showing some diversity of this clade. ´Group 3´encompasses a set of sequences which are positioned at genus-level sublineage of Neomonodaceae. ´Group 4´ and ´Group 5´ are related and form a basal lineage to the Eustigmataceae group. Additionally, an independent environmental study originated many sequences related to *Microchloropsis* (Alves-

de-Souza et al. 2017). Some sequences were found to be included in clade *Goniochloridales*, such as ´Group 1´ or the above-mentioned large dataset study (Villanueva et al. 2014), originating a novel sublineage (see supplementary material Figure S1). Other environmental sequences are positioned in this ordinal clade (Nikouli et al. 2013), with KRL03E85 and KRL09E22 specifically related to *Goniochloris* (Figure 2.1).

These sequences are not from cultivated organisms so a clarification of their identity is compromized by the lack of live material for observation and morphological study leading to formal species determinations or new descriptions. Also, the sequences are short, covering a small region of 18S rRNA gene. However, they provide enough evidence that Eustigmatophyte diversity is much larger than that already estimated by dedicated studies.

The comprehensive phylogenies given by 18S rRNA gene analyses in the present study revealed that the majority of cultured eustigmatophytes are positioned in the Eustigmatales, with 70 sequences (and 12 uncultured environmental sequences) (Figure 3.27), compared to 55 positioned in clade *Goniochloridales* (and 7 uncultured environmental sequences) (Figure 4.1). The descriptions below are based on the most comprehensive phylogenies, obtained for each ordinal clade, within the present study.

5.1. Order Eustigmatales

The most current and comprehensive phylogenies of the order Eustigmatales was achieved by the addition of the *Characiopsis* sequences, with the 18S rRNA gene based backbone of the order given in Figure 3.27, complemented with the *rbc*L gene phylogeny for a more detailed view of the internal topology of this order, given in Figure 3.28.

The phylogeny of the Eustigmatales, given by 18S rRNA gene analysis, shows it is comprised by a deeply diverged lineage composed only of *Paraeustigmatos columelliferus* CAUP Q701, and

by three families, the Monodopsidaceae *sensu* Hibberd (1981), the Eustigmataceae group (Fawley et al. 2014) and the recently described Neomonodaceae (Chapter 2; Amaral et al., 2020). The familial clades are resolved with high bootstrap support (>99% with 18S rRNA gene analysis and >87% with *rbc*L gene analysis) (Figures 3.27. and Figure 3.28., respectively).

Family Monodopsidaceae

The Monodopsidaceae is resolved with 99% bootstrap support (Fig. 3.27.) and includes two internal clades agreeing with previous reports (Kryvenda et al. 2018). One is composed by *Pseudotetraedriella kamillae* SAG 2056 (Fig. 5.1. A) and by *Monodopsis subterranea* UTEX 151 and related strains (Fig. 5.1. B). The uncultured sequences from environmental sampling PSR2 3E 43 and Q3-25 are also positioned in this clade, in separate single leaf lineages. The second is a coherent clade composed by two groups of the *Microchloropsis* and *Nannochloropsis* strains (Figure 5.1. C and D).

Familial clade Eustigmataceae group

The Eustigmataceae group is a fully resolved lineage (Fig. 3.27.) It is generally interpreted at the family level and it has been informally named as the Eustigmataceae group until there is a study dedicated to formally declaring it as a family.

Some uncultured sequences are present in this familial clade, including the uncultures clone OL10 and also two Groups of the above-mentioned large dataset environmental study, Group 4 and Group 5 (Villanueva et al. 2014).

Considering the cultivated organisms, the Eustigmataceae group is composed by five well resolved internal clades. One comprises the *Vischeria* clade, composed by a cluster of *Vischeria* strains (Figure 5.1. E) and some strains previously known as *Eustigmatos*, now a synonym of

Vischeria (Kryvenda et al. 2018). It also includes a single strain named *Chlorobotrys regularis* CCAP 810/1. This strain is positioned isolated from other *Chlorobotrys* strains, morphological studies are therefore needed to confirm or exclude its resemblance with *Chlorobotrys*.

A second clade is fully resolved within the Eustigmataceae group, it is composed by two unnamed eustigmatophytes SAG 2217 and SAG 2220. This finding contradicts previous reports showed that the unnamed strain SAG 2217 was related to the *Vischeria* clade (Kryvenda et al. 2018). A careful morphological evaluation may clarify the identity of these organisms.

A third clade is composed by unnamed strains has been informally named Clade Ia, it is not yet clear if one of these strains Bog 9/21 T-2d is a different taxon since it is positioned without support with the others (Fawley et al. 2014).

The fourth clade within the Eustigmataceae group was initially composed by *Pseudocharaciopsis minuta* UTEX 2113 and *Characiopsis saccata* SAG 15.97 (Fawley et al. 2014). The latter strain was derived from ACOI 481, it was incorporated in the SAG collection in the nineties and given the collection number SAG 15.97. This explains the identical 18S rRNA gene sequence of ACOI 481 and SAG 15.97 (Kyvendra et al. 2018). A recent study released new sequences from *Characiopsis acuta* ACOI 456 (Figure 5.1. F) and *C. acuta* ACOI 1837 as well as *Characiopsis longipes* ACOI 1838 and *Dioxys* sp. ACOI 2029 but no morphological data or taxonomic considerations were made. The clade received the informal name of *Pseudocharaciopsis/Chlorobotrys/Dioxys* clade (Kyvendra et al. 2018). Its diversity was expanded with work stemming from this thesis (Chapter 3; Amaral et al., resubmitted) by the addition of sequences from ACOI *Characiopsis* strains (Figure 3.27.). The 18S rRNA phylogeny shows that *P. minuta*, *C. saccata*, the other *Characiopsis* and *Dioxys* are positioned together with the type *Characiopsis minuta* in a paraphyletic cluster which was named *Characiopsis* (Figure 5.1. F and G). The eustigmatophyte nature of the strain named *Dioxys* is clear and based on 18S

rRNA gene phylogeny, it is related to *Characiopsis pernana* ACOI 2433 (Fig. 3.27.). If a morphological re-evaluation of this strain reveals similar morphological characteristics to *Characiopsis* then its taxonomic identification must be considered. On the contrary, if it is proven to be similar to the type *Dioxys incus* Pascher 1932 then a re-evaluation of this genus and the species name *Characiopsis pernana* is required.

The fifth clade is a deep fully resolved lineage, comprising strains with *Chlorobotrys* morphology (Figure 5.1. H and I). *C. gloeothece* ACOI 1114 and *C. regularis* ACOI 1089 and ACOI 307 are positioned with an uncultured eustigmatophyte sequence PRS2 4E 40, which was previously unrelated with any other eustigmatophyte (Fawley et al. 2014).

The Neomonodaceae, fam. nov.

The third familial clade, informally called the *Pseudellipsoidion* group for some time, was described as a new family of eustigmatophytes, the Neomonodaceae (Figure 5.1. J-O). The Neonomodaceae comprises four fully resolved genera, the free living *Pseudellipsoidion* and the stipitate *Nemonodus*, *Characiopsiella* and *Munda* (Chapter 2; Amaral et al. 2020). The description of this new family expanded considerably the Eustigmatales diversity, with three new genera (*Neomonodus*, *Characiopsiella* and *Munda*).

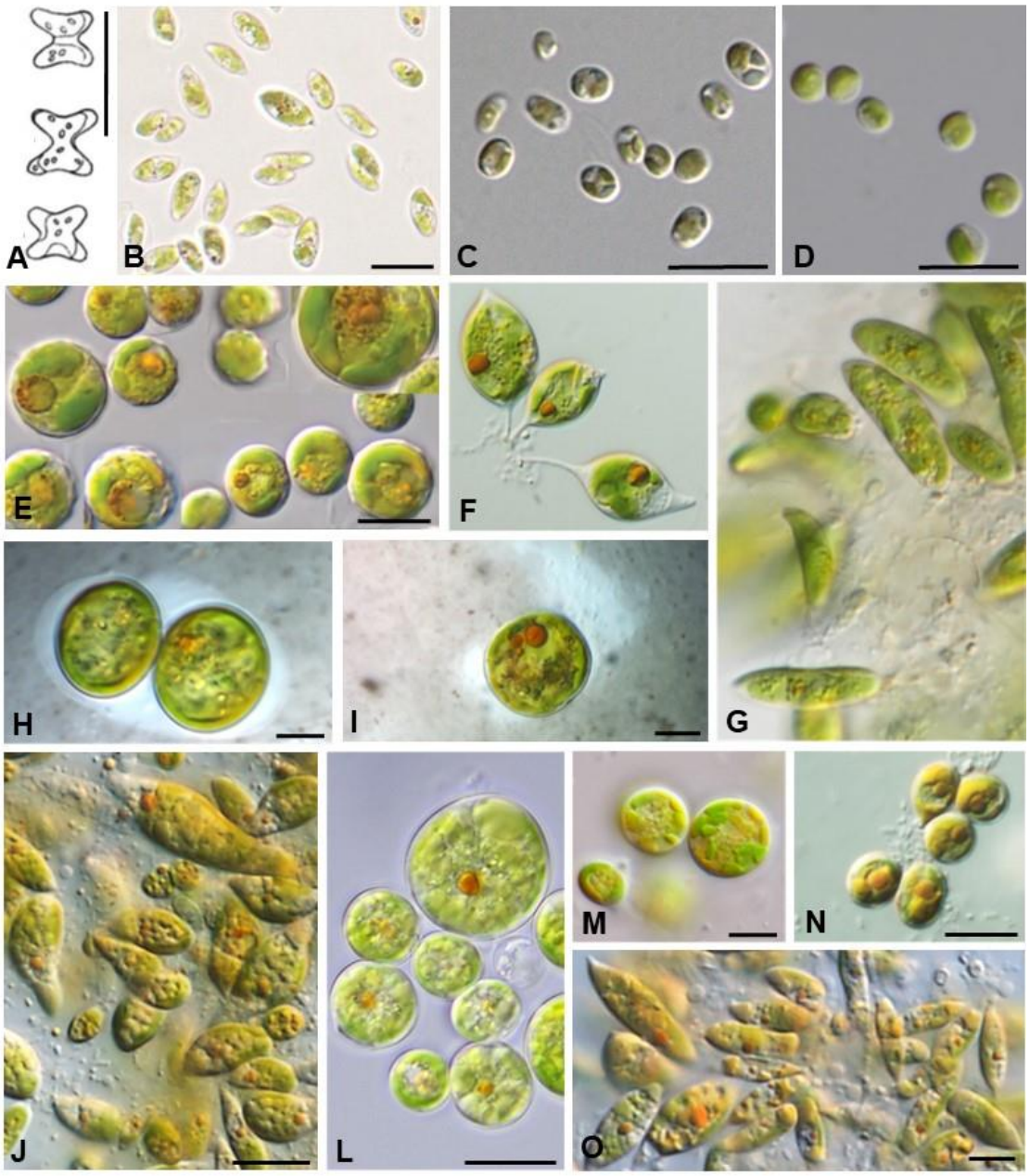

Figure 5.1. Vegetative cells of the Eustigmatales, family Monodopsidaceae (A–D), familial *clade Eustigmataceae* (E–I), family Neomonodaceae (J–O). A) *Pseudotetraedriella kamillae* SAG 2056 (adapt. Hegewald et al. 2007); B) *Monodopsis unipapilla* SAG 8.83 (adapt. sagdb.uni-goettingen.de); C) *Microchloropsis salina* SAG 40.85 (adapt. sagdb.uni-goettingen.de); D) *Nannochloropsis limnetica* SAG 18.99 (adapt. sagdb.uni-goettingen.de); E) *Vischeria stellata* SAG 33.83 (adapt. Kryvenda et al. 2018) F) *Characiopsis acuta* ACOI 456; G) *Characiopsis* sp. ACOI 2429A; H) *Chlorobotrys gloeothece* ACOI 1114; I) *Chlorobotrys* sp. ACOI 3952 ni; J) *Neomonodus* sp. ACOI 2437; L) *Pseudellipsoidion edaphicum* CAUP Q401; M) *Pseudellipsoidion* sp. Mary 8/18 T-3d; N) *Characiopsiella minima* ACOI 2426; J); O) *Munda* sp. ACOI 2428; Scale 10 μm.

5.2. The ordinal clade *Goniochloridales*

The most current and comprehensive phylogenies of the ordinal clade *Goniochloridales* was achieved by the addition of the *Pseudostaurastrum* sequences as a result of the present study. The 18S rRNA gene phylogeny provides the most current backbone of the order (Fig. 4.1.), complemented with the *rbc*L gene phylogeny for a more detailed view of the internal topology of this ordinal clade (4.2.).

The topology given by comprehensive 18S rRNA gene analysis (Figure 4.1.) shows that the second ordinal clade within eustigmatophyte phylogeny, informally known as clade *Goniochloridales*, generally agrees with previous reports where its diversity was described including *Trachydiscus*, *Tetraedriella, Vacuoliviride, Goniochloris* and *Pseudostaurastrum* (Fawley et al. 2014, Nakayama et al. 2015, Eliáš et al. 2017, Fawley and Fawley 2017, Amaral et al. 2020; Amaral et al. resubmitted). The clade *Goniochloridales* has been gradually deployed since its first report by Fawley et al. (2014). It originally comprised 32 strains, distributed by 4 clades, one deeply diverged lineage composed by two *Pseudostaurastrum* strains and the other 3 clades received informal working names Clade IIa, Clade IIb and Clade IIc until they receive taxonomic treatment (Fawley et al. 2014). The reason underlying the use of these names stems from the fact that a deeply diverged lineage within the Eustigmataceae group was given the working name Clade Ia in the same study.

Familial Clade IIa

Strains initially comprising Clade IIa were six unnamed isolates and also *Trachydiscus minutus* CCALA 838 (Fig. 5.2. A) (Fawley et al. 2014). The strain *'Microtalis aquatica'* CCMP3153, described as an eustigmatophyte by Dashiell and Bailey (2009), was already known as a deeply diverged lineage from Eustigmatales (Yang et al. 2012). It was later proved to belong to clade *Goniochloridales*, in Clade IIa (Nakayama et al. 2015) together with the founder strains. Two

Trachydiscus strains were recently added to this clade, *Trachydiscus* sp. (Eliáš et al. 2017, Fawley and Fawley 2017) and *Trachydiscus guangdongensis* (Fig 5.2. B) (Gao et al. 2019). Surprisingly, a rather narrow sampling of five sequences was used by the authors for inference of the phyogenetic position of the later organism, while there were several Goniochloridales sequences already present in GenBank by then. The comprehensive phylogeny given in Fig. 4.1. confirms this position and shows a full bootstrapp support for this molecular clade, with some internal resolution although in not very deeply diverged branches (Fig. 4.1). The internal topology observed with *rbc*L gene phylogeny (Fig. 4.2.) reveals a diverged lineage constituted only by the unidentified Ndem 8/9T/3m6.8, standing out from the remaining strains which constitute a molecular group corresponding to a genus within the familial Clade IIa. This implicates that the taxonomic status of *Trachydiscus* and *Tetraedriella* must be revised based on further molecular and morphological data.

Familial Clade IIb

The second familial clade, named Clade IIb, is sister to Clade IIa (Fig. 4.1. and Fig. 4.2.). It was first described as comprising *Goniochloris sculpta* SAG 29.96, two unnamed isolates and two sequences from uncultured strains (Fawley et al. 2014). It was recently deployed with sequences from *Goniochloris sculpta* ACOI 1852 and *Goniochloris mutica* ACOI 1360 (Kryvenda et al. 2018). These strains form a moderately supported clade (87% bootstrap) and the branching given by 18S rRNA gene phylogeny shows some resolution with three possible internal clades at the genus level (Figure 4.1.). Two of these sub-clades were confirmed with *rbc*L gene analysis (Figure 4.2.). The clade as a whole is comprised by *Goniochloris* together with unnamed strains, one is composed of two *G. sculpta* strains and the two uncultured eustigmatophytes are positioned together with *G. mutica* (Fig 5.2., E) (Figure 4.1.). There is a possibility that these may correspond to species level clades and that the whole clade may

correspond to genus *Goniochloris* but a thorough study of this genus is required for testing this possibility.

Familial Clade IIc

Clade IIc was initially comprised by eleven unnamed strains (Fawley et al. 2014) and then *Vacuoliviride crystalliferum* NIES 2860 (Fig 5.2. F) was described and its position was found in this clade (Nakayama et al. 2015). The 18SrRNA gene phylogeny shows that it consists of a well resolved clade (82% bootstrapp) at the genus rank (Fig. 4.1.). The internal resolution revealed with *rbc*L gene analysis shows at least three diverged internal clades (Fig. 4.2.). A morphological inspection of the unnamed strains may reveal if they resemble *Vacuoliviride*, which might originate a clarification of the taxonomy of this clade.

Incertae sedis

Five strains are positioned with uncertainty. *Goniochloris tripus* ACOI 1855 is positioned as a single leaf and the other strains are positioned in two groups, one comprising two sequences of *Tetraedriella tumidula* and another composed by *Goniochloris* sp. and *Tetraedriella verrucosa*. These sequences were released in supplementary material of a study concerning the taxonomic status of the *Eustigmatos/Vischeria* cluster (Kryvenda et al. 2018), without any morphological study. Considering that *Tetraedriella subglobosa* (Fig 5.2. C) is positioned away in Clade IIa and that other *Goniochloris* strains are positioned in Clade IIb, it is important that these genera have their taxonomic status revised in a dedicated study combining molecular with morphological data.

Pseudostaurastrum

The two *Pseudostaurastrum* strains *P. enorme* SAG 11.85 and *P. limneticum* SAG 14.94 were first included in eustigmatophyte phylogenies in a study focused on another organism (Hegewald et al. 2007) and featured unaltered in all following phylogenetic studies thereafter, despite the addition of new Goniochloridales members (Přibyl et al. 2012, Fawley et al. 2014, Nakayama et al. 2015, Eliáš et al. 2017, Fawley and Fawley 2017). Only recently, it was expanded with *Pseudostaurastrum* strains and named as *Pseudostaurastrum* clade (Kryvenda et al. 2018). This clade is fully resolved and may be interpreted at a family level with at least five fully supported internal clades, shown by both phylogenies given by 18S rRNA gene (Figure 4.1.) and *rbc*L gene (Figure 4.2.). However, its sister branch is comprised by *Goniochloris tripus* and *Tetraedriella verrucosa* and *Tetraedriella tumidula* is not yet fully clarified, since it is unsupported. It still seems unclear which molecular groups may originate taxonomically valid families of the Goniochloridales. However, since the group of the *Pseudostaurastrum* strains form a fully supported, highly diverged lineage, the whole clade is most likely to be considered at the family level, which in that case should be named as *Pseudostauraceae* because it composed only by *Pseudostaurastrum* strains.

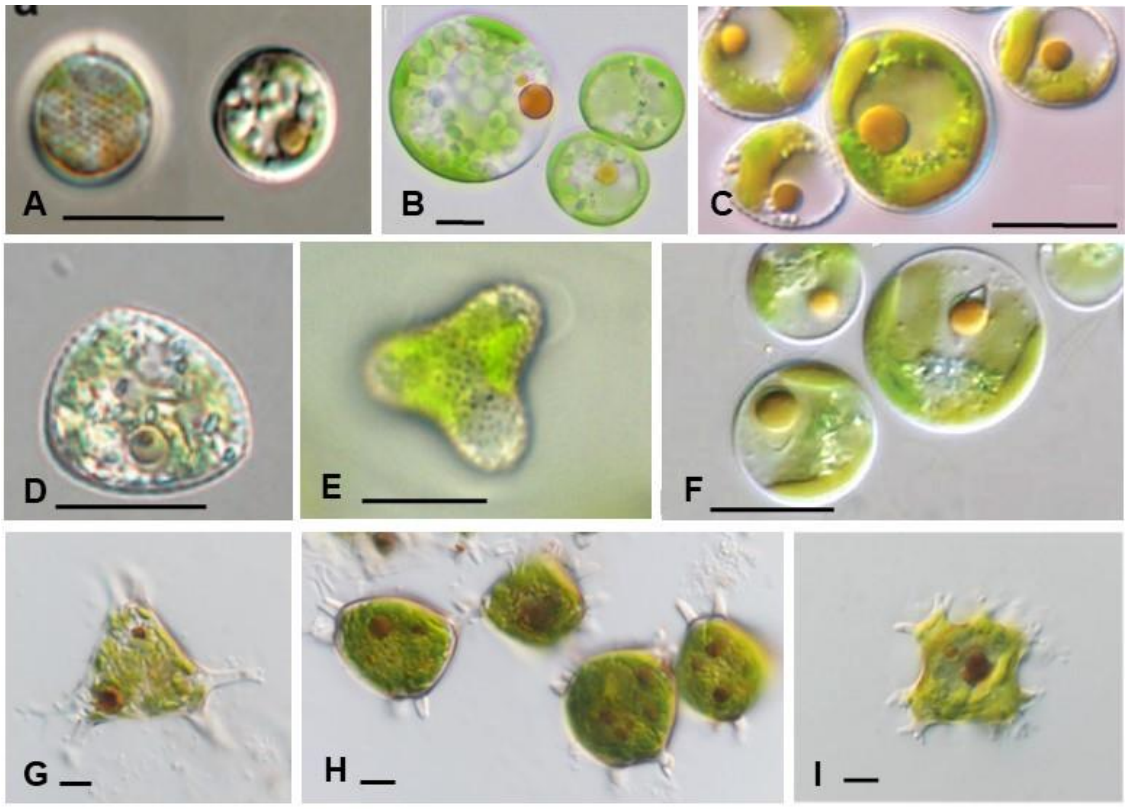

Figure 5.2. Vegetative cells of the clade *Goniochloridales*, Clade II a (A–C), Clade II b (D–E), Clade II c (F), clade *Pseudostaurastrum* (G–I). A) *Trachydiscus minutus* CCALA 838 (adapt. Přibyl et al. 2012); B) *Trachydiscus guangdongensis* JNU5 (adapt. Gao et al. 2019); C) *Tetraedriella subglobosa* F4 4/24-10m (adapt. Fawley and Fawley 2017); D) *Goniochloris sculpta* SAG 29.96 (adapt. Eliáš et al. 2017); E) *Goniochloris mutica* (adapt. Guiry in Guiry and Guiry 2019); F) *Vacuoliviride crystalliferum* NIES-2860 (adapt. Nakayama et al. 2015); G) *Pseudostaurastrum* cf. *lobulatum* ACOI 2780ni; H) *Pseudostaurastrum limneticum* ACOI 3132ni; I) *Pseudostaurastrum enorme* ACOI 2026. Scale 10 μm.

6.

Pigments of eustigmatophytes

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6.1. Abstract

Pigments are a fundamental part of the microalgal cell, with chlorophylls at the center of photosynthesis and carotenoids as accessory pigments for function protection. The pigment profile of the Eustigmatophyceae was one of the singularities which originated the segregation of this class of organisms from the Xanthophyceae. Recent findings indicate that eustigmatophyte carotenoids are interesting compounds with laboratory -scale proven health-promoting effects. In this work, extracts of 27 strains belonging to different taxa were prepared and analyzed by HPLC-DAD. Results showed a typical eustigmatophyte pigment profile present in all strains, namely chlorophyll a, violaxanthin, vaucheriaxanthin, β-carotene, and other minor carotenoids. Violaxanthin was the most abundant pigment, achieving half the total pigment content in *Monodopsis unipapilla* ACOI 2938. It was the most abundant carotenoid in two studied *Vischeria* strains, representing around 70% of carotenoids in both strains. The second major carotenoid was vaucheriaxanthin, and the highest amount of this carotenoid was found in *Characiopsis saccata* ACOI 481 and *Characiopsis* cf. *minuta* ACOI 2423. The β-carotene was found in all studied strains, with the highest production of this commercially important carotenoid detected in *Pseudostaurastrum* sp. ACOI 2419ni and *Characiopsis acuta* ACOI 1837. To our best knowledge, the pigment profile of eustigmatophytes with a stipitate *Characiopsis*-like morphology has not been characterized previously. The results further strengthen the potential use of eustigmatophyte strains as sources of naturally derived carotenoids, with nutritional applications.

6.2. Introduction

During the photosynthetic process, microalgae are exposed to high oxygen and radical stress that may cause photo-oxidative damage (Hamed 2016). This may be controlled by the pigments involved in the process namely chlorophylls and carotenoids (Larkum 2016).

Chlorophylls are a part of the light harvesting complexes in the chloroplasts. The molecule consists of a conjugated cyclic tetrapyrrole with a fifth isocyclic ring (and often an esterified long-chain alcohol) coordinated to a central magnesium ion (Wright and Jeffrey 2006). Along with its prominent role in the electron transfer chain during photosynthesis, another relevant feature attributed to chlorophyll *a* is the considerable antioxidant activity related to lipidic oxidation protection (Lanfer-Marquez et al. 2005). Carotenoids are lipophilic terpenoid molecules which include two classes, carotenes and xanthophylls (Choudhury and Behera 2001). Many of these carotenoids are involved in light harvesting, with absorbance of light in the blue and green regions of the electromagnetic spectrum (420 to 550 nm) where they bridge the gap between chlorophyll absorption bands (Wright and Jeffrey 2006). The primary function of carotenoids is to protect the cell from photoxidation. Since carotenes, such as β-carotene, have lower lying triplet states they act as antioxidants by quenching the reactive triplet state of chlorophyll and singlet oxygen. Xanthophylls act indirectly by quenching excited singlet state of chlorophyll ($^1Chl^*$) thus promoting lower $^3Chl^*$ formation (Choudhury and Behera 2001).

The nutritive value of microalgal biomass and/or extracts has been explored by the biotechnological community and the pigments are a part of its nutritional value, already proven at lab scale (Koyande et al. 2019). Chlorophylls have been indicated as having anti-inflammatory, antimutagenic and anticarcinogenic activity (Assunção et al. 2019). Microalgal carotenoids on the other hand, are already used in human nutrition especially due to their antioxidant activity (Koller et al. 2014, D'Alessandro and Antoniosi Filho 2016) as well as cancer, cardiovascular and chronic diseases prevention (Rao and Rao 2007, Matos et al. 2017). β-carotene, astaxanthin, zeaxanthin and lutein are the most studied carotenoids extracted from microalgae for nutritional applications (Undayan et al. 2017, Vidyashankar et al. 2017).

The unique pigment profile of the Eustigmatophyceae is due to the lack of chlorophyll *b* and *c* and the presence of violaxanthin as the major carotenoid (Whittle and Casselton 1969, Hibberd and Leedale 1970, 1972, Whittle and Casselton 1975, Antia et al. 1975). Recent findings suggest, however, that a small amount of chlorophyll c may be present but in such low amounts that it is almost undetectable and may have been overlooked in the past (Přybil el al 2012, Stoyneva-Gärtner et al. 2019a). Violaxanthin, vaucheriaxanthin ester (designated as vaucheriaxanthin for simplicity), and β-carotene are the major carotenoids found and other minor xanthophylls (zeaxanthin, canthaxanthin, lutein, astaxanthin, antheraxanthin, among others) may also be detected in microalgae (Whittle and Casselton 1975, Antia and Cheng, 1982, Nobre et al. 2012, Li et al. 2012a, Eliáš et al. 2017, Stoyneva -Gärtner et al. 2019a). Violaxanthin and vaucheriaxanthin are the most abundant carotenoids in eustigmatophytes and β-carotene is invariably present (Whittle and Casselton 1975, Brown 1987, Preisig and Wilhelm 1989, Arsalane et al. 1992, Schnepf et al. 1995/96, Li et al. 2012a, Wang et al. 2018, Stoyneva-Gärtner et al. 2019a). Violaxanthin has a role in light-harvesting of microalgae (Owens et al. 1987) and is part of the non-photochemical quenching xanthophyll cycle together with antheraxanthin and zeaxanthin, known as the violaxanthin cycle, a photoprotective mechanism of the photosynthetic apparatus (Lubián and Montero 1998, Wright and Jeffrey 2006, Larkum 2016). Vaucheriaxanthin is the second most abundant carotenoid in eustigmatophytes, it was considered as a diagnostic pigment for the Eustigmatophyceae since the first studies on this class (Whittle and Casselton 1975). Recent studies revealed lutein as a novel pigment for the Eustigmatophyceae, also minor xanthophylls in higher amounts than usually reported (e.g. antheraxanthin) and rarely reported xanthophylls for eustigmatophyceae such as lutein and luteoxanthin (Stoyneva-Gärtner et al. 2019a). The unique pigment profile of Eustigmatophyceae was initially an important information for taxonomic reasons, including the differences which originated the segregation from the Xanthophyceae (Hibberd and Leedale 1970). Eustigmatophytes are

considered as interesting sources of biotechnologically derived carotenoids for nutritional applications (Lubián et al. 2000, Li et al. 2012b, Wang et al. 2018) with some genera already characterized and studied such as *Nannochloropsis*/*Microchloropsis* (Antia et al. 1975, Antia and Cheng 1982, Brown 1987, Lubián and Montero 1998, Lubián et al. 2000, Nobre et al. 2012), *Vischeria* (syn. *Eustigmatos*) (Stoyneva-Gärtner et al. 2019a, Wang et al. 2018, Li et al. 2012a, Li et al. 2012b, Whittle and Casselton 1975) *Monodopsis* (Arsalane et al. 1992, Whittle and Casselton 1975), *Pseudostaurastrum* (Schnepf et al. 1995/96) and *Trachydiscus* (Přybil et al. 2012); yet, no stipitates (*Characiopsis*-like morphology) were studied for their pigmentary content before. With this study we aimed to consolidate the known typical pattern for eustigmatophytes by determining the pigment profile of different genera of eustigmatophytes, specially the stipitate strains also studied by molecular methods for taxonomic inference.

6.3. Materials and Methods

Culture of strains

Twenty-seven ACOI eustigmatophytes were selected for the study, namely the Neomonodaceae strains (Amaral et al. 2020), *Characiopsis* strains (Amaral et al. resubmitted), and representatives of other genera were *Vischeria* sp. (syn. *Eustigmatos*) ACOI 4864ni, *Vischeria helvetica* ACOI 299, *Tetraplektron* sp. ACOI 2650ni, *Monodopsis unipapilla* ACOI 2938, *Giniochloris sculpta* ACOI 1852, *Dioxys* sp. ACOI 2029 and *Pseudostaurastrum* sp. 2419ni.

The cultivation process started with a scale-up of the strains from an aliquot to a larger volume in order to obtain dense cultures in 300 mL Erlenmeyer flasks. The cultures were established with a methodology consisting in the use of a controlled pre-culture as inoculum for obtaining a normalized beginning of the culture growth.

The preparation of the pre-culture was performed by adding 100 ml of a dense culture of the selected strain to 100 mL M7 culture medium (Schlösser 1994) at pH 6.4-6.6, followed by homogenization and batch cultivation for 15 days. The culture was established by inoculating pre-culture (after homogenization) in fresh culture medium at a 1:3 ratio, to a final volume of 400 mL, in order to achieve approximate cell density of ~10^6 cell/mL. The cultures were cultivated for 9 days under a light intensity of 11 μmol.photons.m^{-2}.s^{-1}, a photoperiod of 16:8 h of light: dark and a temperature of 23 °C and constantly mixed with air bubbling, 0.5^{-1} L.min^{-1}.

Extraction of pigments and preparation of extracts

Each culture was homogenized and an aliquote of 45 mL taken for analysis. The cells were harvested by centrifugation at 4500 rpm during 15 minutes at room temperature and the supernatant was discarded. An amount of 0,1 g wet biomass was distributed by two Falcon tubes and frozen at - 80° C. Two independent extractions were performed, one using DMF (anhydrous, SIGMA Aldrich) and another using MeOH (VWR, HPLC grade). A volume of 2 mL solvent was added to the corresponding pellet, resuspended by manual shaking, followed by ultrasound bath treatment (35 kHz, 240 W, 1 % liquid detergent added to the water), for 1 min. in dim light. The tubes were placed at 4 °C for and left overnight at 4 °C.

The extracts were combined and centrifuged for 12000 rpm during 5 minutes for removal of the whole content of cells, membranes and other large particles. The extract was recovered and filtered through a Whatman nylon/propilene housing syringe filter with 0.45 μm pore. The samples were prepared for analysis by adding 100 ml of the internal standard (chlorophyll b) to 900 ml extract into an amber vial. A different vial was prepared without the internal standard, for a confirmation of the absence of chlorophyll b from the extract. Since the Eustigmatophyceae have only chlorophyll a, chlorophyll b (from spinach, HPLC

grade, SIGMA) was used as an internal standard to co-elute with the extract, in order to provide an internal control of the identity of the strains as belonging to this family.

The samples were immediately analyzed in order to avoid the degradation of the pigments. Each sample was analyzed three times (three injections), the results are presented as averaged values (with n=3) except for *Neomonodus* sp. ACOI 2437 and *Munda* sp. ACOI 2428 (n=2) for which one of the replicates was withdrawn due to technical problems.

Extractions and sample preparation were conducted in dim light and the tubes with extracts were wrapped in aluminum foil in order to avoid the possible photo-oxidation of the extracted pigments.

Standards of chlorophyll a (from spinach, HPLC grade, SIGMA-Aldrich) and β-carotene (Type II, synthetic HPLC grade SIGMA-Aldrich), were injected and their characteristics of elution with the employed HPLC-DAD solvent system were recorded, namely the retention time (t_r), maximum wavelength of absorption and band measurements for III/II% ratio calculations.

Analysis by HPLC-DAD

The samples were analyzed in an analytical Elite Lachrom HPLC-DAD system with L-2455 Diode Array Detector, L-23000 Column Oven (RP-18 end capped column), L-2130 Pump and a L-2200 Auto Sampler. For elution a solvent gradient method was performed with a flow rate of 1 $ml.min^{-1}$ with a three solvent combination gradient (Table 6.1.) including one ion pair reagent according to Wright et al. (1991).

Table 6.1. Solvent system for analysis and for column conditioning before shutdown. Solvent A - 80:20 methanol: ammonium acetate 1M (aq., pH 7.2) (double Milli-Q water, v/v), Solvent B - 90:10 acetonitrile: water (2 x MilliQ water, v/v), Solvent C - ethyl acetate (HPLC grade), 2x MiliQ – water twice through MilliQ system.

Time (min)	Solvent A	Solvent B	Solvent C	2x MilliQ	Flow rate (ml/min)
a. analytical protocol					
0	0	100	0	0	1
1	100	0	0	0	1
5	0	100	0	0	1
19	0	20	80	0	1
22	0	100	0	0	1
25	100	0	0	0	1
30	100	0	0	0	1
b. column cleaning and shutdown protocol					
0	0	100	0	0	1
3	0	80	0	20	1
7	0	50	0	50	1
15	0	10	0	90	1
25	0	50	0	50	1
35	0	80	0	20	1
50	0	100	0	0	1
60	0	100	0	0	1
61	0	100	0	0	0.8
65	0	100	0	0	0.6
67	0	100	0	0	0.4
69	0	100	0	0	0.2
70	0	100	0	0	0

The analytical method was slightly changed to include an initial step start with Solvent B, which is equivalent to the last step of column cleaning protocol. This prevents the unwanted start of the ionic-pair solvent being sent to the column by pump A, a technical specificity of the software.

A photodiode array detector was used for pigment detection and data were acquired three-dimensionally (absorbance-time-wavelength) in the wavelength range of 300-800 nm. Chromatograms were analyzed with EZChrom Elite software and pigments were identified based on authentic standards for chlorophyll a and β-carotene and the others by comparing

ultraviolet-visible spectral and chromatographic characteristics with those in literature, including the % III/II band ratio for carotenoids, which is obtained by measuring the height of the third and second bands comparing to the valley between them (Roy et al. 2011).

Preliminary method optimization process

The above described methods were preceded by preliminary tests for the determination of the best approach through the work chain, from the first step of cultivation to the final step of column conditioning after analysis.

The tested steps included: 1) the use of wet or dry biomass; 2) a maximum time the biomass could be stored at -80 °C before analysis; 3) the type of extraction solvents used, acetone, MeOH, MeOH+DMF (DMF alone was never tried because it may damage the internal structure of the separating column); 4) the time of extraction of the combined extracts; 5) sample preparation procedures until a clear extract is achieved, with no loss of pigment composition during the process (preliminary centrifugation step and type of used filter and pore width); 6) the HPLC run method; 7) the column conditioning method for removal of ionic-pair running solvents.

Statistical Analysis

Experimental data are expressed as mean ± SD (standard deviation). Student-Newman-Keul´s test using one-way ANOVA was performed using a statistical analysis software package Statistica 7.0.61. Unless stated otherwise, all affirmations refer to statistically significantly different values $p<0.05$.

6.4. Results

The detected pigments were consistent in their elution order (Fig. 6.1.), with violaxanthin (Figure 6.3.), vaucheriaxanthin (Figure 6.4.), β-carotene (Figure 6.5.) and chlorophyll a (Figure 6.8.) present in all strains.

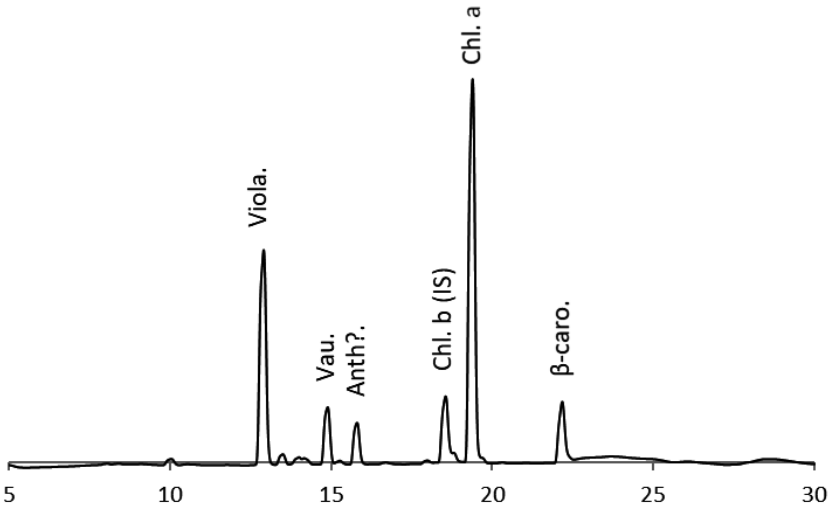

Figure 6.1. Chromatogram showing the elution of the pigment content in an extract of *Characiopsis* sp. ACOI 2423A. Viola. – violaxanthin, Vau. – vaucheriaxanthin, Anth?. – possibly antheraxanthin, Chl. b (IS) – chlorophyll b internal standard co-eluted with extract, Chl. a – chlorophyll a, β-caro. – β-carotene.

An overview of the individual contribution of each pigment to the total pigment content is expressed in average % obtained from all studied strains (Fig. 6.2. left). Chlorophyll is found as the most abundant pigment, with 43% contribution to the total pigment %, followed by the carotenoid violaxanthin with a contribution of 26%, vaucheriaxanthin with 12%, β-carotene with 10%, and other carotenoids with 9% contribution. Considering the carotenoids contribution to total carotenoid % (Fig. 6.2. right), it is evident that violaxanthin is the major carotenoid with 45% contribution to total carotenoids, followed by vaucheriaxanthin with 23%, β-carotene with 19%, other carotenoids account for a smaller fraction of 13% total carotenoids.

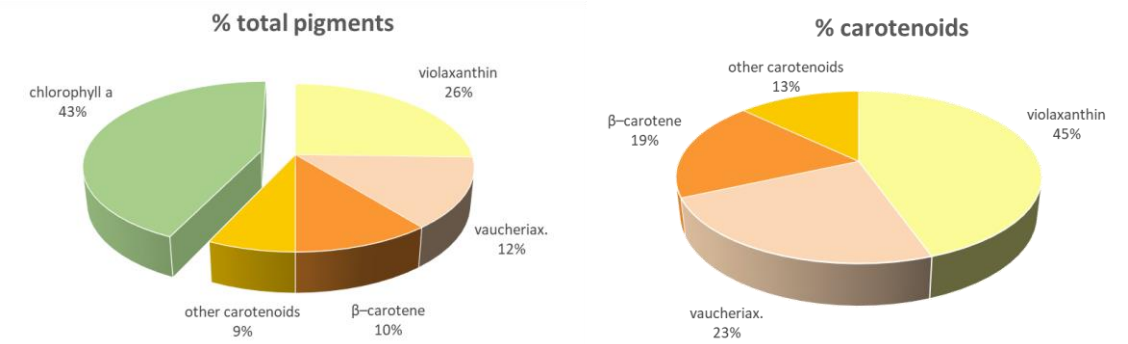

Figure 6.2. Contribution of each detected pigment to the total content (left) and contribution of each carotenoid to the total carotenoid content (right), expressed as average % of all strains.

The higher values of each pigment detected in the studied Eustigmatophyceae were obtained in different strains. The highest value determined for violaxanthin was 48% total pigment in *Monodopsis unipapilla* ACOI 2438 (Table 6.2.) ($p<0.05$), for vaucheriaxanthin it was 19% total pigment in *Characiopsis saccata* ACOI 481 and *Characiopsis* cf. *minuta* ACOI 2423 (Table 6.3.) ($p<0.05$), for β-carotene it was 24% in *Pseudostaurastrum* sp. ACOI 2419ni (Table 6.4.) ($p<0.05$) and for chlorophyll was 54% total pigment content detected in *Characiopsis* sp. ACOI 2429 and 52% in *Characiopsis cedercreutzii* ACOI 3169 and *Characiopsis longipes* ACOI 2438 (Table 6.6.) ($p<0.05$).

A minor carotenoid was detected based on the retention time and absorption spectrum shape (the maxima were not always detected due to the low amount of this pigment), with a proposed identification as antheraxanthin (Fig. 6.6.). Although the most abundant and relevant carotenoids could be detected and quantified, these minor carotenoids could not be identified based solely on the comparison with literature values and others were present in such very low amounts, so that a proper identification could not be made (Table 6.5.).

6.4.1. Carotenoids

i. VIOLAXANTHIN

The major pigment violaxanthin (Fig. 6.3.) was identified based on the retention time, absorbance wavelength maxima and band ratio (%III/II), and further compared to published references specific for eustigmatophytes (Antia et al. 1975, Whittle and Casselton 1975, Antia and Cheng 1982, Preisig and Wilhelm 1989, Schnepf et al. 1995/96, Roy et al. 2011, Wang et al. 2018).

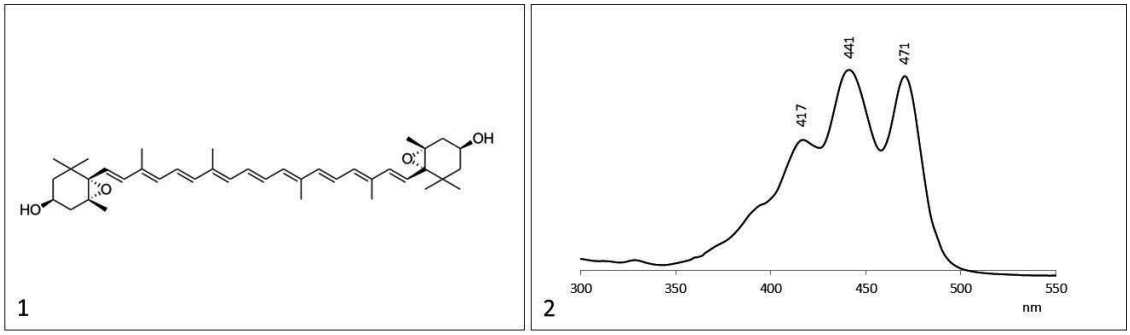

Figure 6.3. 1) Chemical structure of violaxanthin, IUPAC: $C_{40}H_{56}O_4$. IUPAC: (3 S,5R,6S,30S,50R,60S)-5,6:50,60-Diepoxy-5,6,50,60-tetrahydro-b,b-carotene-3,30-diol, 2) Absorption spectrum of violaxanthin obtained from strain *Characiopsis* sp. ACOI 2423A.

The violaxanthin peaks eluted with t_r= 12.5, 12.7 or 12.8 min with an absorption band displaying vibronic resolution with wavelengths λ_{max} 417, 441 and 471 nm (Table 6.2.).

The highest percentage values of violaxanthin were determined for *Monodopsis unipapilla* ACOI 2938 with 48% of the total pigments present in the extract, these representing a 66% contribution to the total fraction of carotenoids. The second highest value was obtained in *Vischeria* sp. ACOI 4864ni and *V. helvetica* ACOI 299 with violaxanthin accounting for 40% and 36% of total pigments respectively. The highest contribution of violaxanthin to total carotenoids was achieved in these two strains with 69% and 70% respectively ($p<0.05$).

The lowest value determined for violaxanthin was found in *Characiopsis* cf. *minuta* ACOI 2423 accounting for 7% of all pigments and contributing to only 9% of carotenoids present in the extract (Table 6.2.). It is worth noting that this strain is the richest in "other carotenoids", with the highest portion identified as possible violaxanthin derivatives (Table 6.5.). This shows that there is a possibility that the total violaxanthin content may be underestimated. The same applies to *Characiopsis* sp. ACOI 2438B, also among the lower values determined of violaxanthin.

There were 13 strains with violaxanthin occupying more than 25% of the total pigment content in the extract. Furthermore, violaxanthin is the most abundant carotenoid found, representing more than 50% of all carotenoids in those extracts. In the other strains, although the violaxanthin amount does not reach half of the total carotenoid content of extracts, it is never lower than 20% of the total carotenoid content (Table 6.2.).

It is evident from Table 6.2. that eustigmatophytes which are not stipitates (the stipitates are *Characiopsis, Neomonodus*, *Characiopsiella* and *Munda*), are the richest in violaxanthin content, with 28-48%. Next, the stipitates belonging to Neomonodaceae *Neomonodus*, *Characiopsiella* and *Munda* have the highest content of violaxanthin with 24 to 28 % of the total of pigments, accounting for almost half of the total of carotenoid content (47-57%). The only exception is *Characiopsiella minima*, with higher violaxanthin content of 31% of all pigments, accounting for 62% of carotenoids.

Table 6.2. Violaxanthin detected in the studied strains. RT- retention time, % total pigm.- area of the violaxanthin compared to total pigments, % total carot. – area of violaxanthin compared to total carotenoid content, % III/II – band ratio, λ_{max} - absorbance wavelength maxima. Values with different letters in the same column are significantly different ($p<0.05$).

Strain	ACOI nr.	RT (min.)	% total pigm.		% total carot.		% III/II	λ_{max} (nm)		
Monodopsis unipapilla	2938	12.8 ± 0.01	**48**	a	**66**	a	85	417	441	471
Vischeria sp. (syn. *Eustigmatos*)	4864ni	12.8 ± 0.01	**40**	b	**69**	b	91	417	441	471
Vischeria helvetica	299	12.8 ± 0.01	**36**	c	**70**	b	92	417	441	471
Tetraplekton sp.	2650ni	12.8 ± 0.00	**33**	d	**61**	c	87	417	441	471
Goniochloris sculpta	1852	12.8 ± 0.00	**31**	e	**57**	d	87	417	441	471
Characiopsiella minima	2426	12.7 ± 0.01	**31**	e	**62**	c	92	417	441	471
Dioxys sp.	2029	12.8 ± 0.01	**28**	f	**56**	d	92	417	441	471
Pseudostaurastrum sp.	2419ni	12.8 ± 0.00	**28**	f	**46**	e	90	417	442	470
Neomonodus sp.	2437	12.7 ± 0.01	**28**	f	**57**	d	89	417	441	471
Characiopsiella minima	2423A	12.8 ± 0.06	**28**	f	**56**	d	92	417	441	471
Munda aquilonaris	2424A	12.8 ± 0.07	**27**	g	**53**	f	92	417	441	471
Munda sp.	2428	12.7 ± 0.01	**26**	g	**49**	g	91	417	441	471
Munda aquilonaris	2424	12.7 ± 0.02	**25**	g	**50**	g	90	417	441	471
Munda aquilonaris	2424B	12.8 ± 0.04	**24**	h	**47**	e	92	417	441	471
Characiopsis sp.	2429	12.8 ± 0.00	**24**	h	**53**	f	77	417	441	471
Characiopsis saccata	481	12.8 ± 0.00	**23**	i	**33**	h	87	417	441	471
Characiopsis cedercreutzii	2434	12.5 ± 0.01	**21**	j	**42**	i	89	417	441	471
Characiopsis cedercreutzii	3169	12.5 ± 0.00	**21**	j	**43**	i	89	418	441	471
Characiopsis acuta	456	12.8 ± 0.01	**21**	j	**34**	h	86	417	441	471
Characiopsis minutissima	2427A	12.8 ± 0.01	**21**	j	**39**	j	86	417	441	471
Characiopsis longipes	2438	12.5 ± 0.01	**19**	k	**40**	j	87	417	441	470
Characiopsis pernana	2433	12.8 ± 0.00	**18**	k	**33**	h	93	417	441	471
Characiopsis longipes	1839	12.5 ± 0.00	**17**	l	**31**	h	89	417	441	470
Characiopsis acuta	1837	12.5 ± 0.01	**14**	m	**27**	k	89	421	441	470
Characiopsis sp.	2430	12.8 ± 0.01	**14**	m	**24**	l	90	417	441	471
Characiopsis sp.	2438B	12.8 ± 0.01	**14**	m	**22**	l	87	417	441	471
Characiopsis cf. *minuta*	2423	12.5 ± 0.01	**7**	n	**9**	m	83	416	442	470

ii. VAUCHERIAXANTHIN

Also an abundant carotenoid, vaucheriaxanthin (Fig. 6.4.) was identified based on the retention time (t_r), absorbance wavelength maxima and band ratio (% III/II), comparing to published references specific for eustigmatophytes (Roy et al. 2011, Schnepf et al. 1995/96).

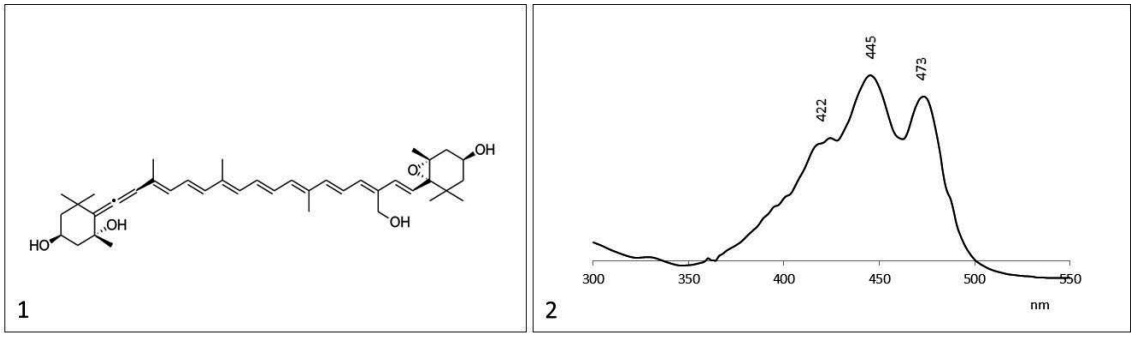

Figure 6.4. 1) Chemical structure of vaucheriaxanthin molecule, $C_{40}H_{56}O_5$. IUPAC: (3 S,5R,6R,30S,50R,60S)-50,60-Epoxy-6,7-didehydro-5,6,50,60-tetrahydro-b,bcarotene-3,5,30,190-tetrol, 2) Absorption spectrum of vaucherixanthin detected in strain *Characiopsis* sp. ACOI 2423A.

The vaucheriaxanthin peak eluted at t_r= 14.5 or 14.8 minutes with λ_{max}= 422, 445 and 473 nm (Table 6.3.).

The highest percentage value of vaucheriaxanthin was determined for *Characiopsis* cf. *saccata* ACOI 4864ni and *Characiopsis* cf. *minuta* ACOI 2423 accounting for 19% of total pigments in both strains, representing 28% and 25% contributions to the total carotenoids respectively. The higher contribution to total carotenoid content was 35%, determined in *Characiopsis cedercreutzii* ACOI 2434, which was second rank in the most abundant extracts in vaucheriaxanthin. The lowest values determined were found in *Characiopsiella minima* ACOI 2423A and *Monodopsis unipapilla* ACOI 2938, with the values of 7 and 8 % of all pigments

respectively (not statistically different, $p<0.05$), representing 15 and 11% of total carotenoids present in the extract respectively.

Eustigmatophyte stipitates *Characiopsis*, *Munda* (except *Munda* sp. ACOI 2428) and *Neomonodus* are the richest in vaucheriaxanthin with values higher than 13% total pigment ($p<0.05$) representing 33-35% total carotenoid, whereas *Munda* sp. ACOI 2428, *Vischeria*, *Dioxys*, *Tetraplektron*, *Pseudostaurastrum*, *Goniochloris* and *Monodopsis* and *Characiopsiella* have a vaucheriaxanthin content lower than 11% of the total of pigments content, although it represents a considerable fraction of the total carotenoids (11-23%).

Table 6.3. Vaucheriaxanthin detected in the studied strains. RT- retention time, % total pigm. – area of the vaucheriaxanthin compared to total pigments, % total carot. – area of vaucheriaxanthin compared to total carotenoid content, % III/II – band ratio, λ_{max} - absorbance wavelength maxima. Values with different letters in the same column are significantly different ($p<0.05$).

Strain	ACOI nr.	RT (min.)	% total pigm.	% total carot.	% III/II	λ_{max} (nm)		
Characiopsis saccata	481	14.8 ± 0.01	**19** a	**28** b	72	422	445	473
Characiopsis cf. *minuta*	2423	14.5 ± 0.01	**19** a	**25** c	70	423	445	473
Characiopsis cedercreutzii	2434	14.5 ± 0.00	**18** b	**35** d	71	422	445	473
Characiopsis sp.	2438B	14.8 ± 0.00	**17** b	**28** b	70	421	445	473
Munda aquilonaris	2424	14.8 ± 0.01	**16** c	**31** e	73	422	445	473
Characiopsis cedercreutzii	3169	14.5 ± 0.01	**16** c	**33** f	72	422	444	473
Characiopsis minutissima	2427A	14.8 ± 0.00	**16** c	**29** b	71	422	445	473
Characiopsis sp.	2430	14.8 ± 0.01	**16** c	**27** b	70	421	445	473
Munda aquilonaris	2424A	14.8 ± 0.06	**15** c	**29** b	69	-	445	473
Munda aquilonaris	2424B	14.8 ± 0.04	**15** c	**29** b	48	423	445	473
Characiopsis pernana	2433	14.8 ± 0.00	**15** c	**26** b	73	422	445	473

Characiopsis acuta	1837	14.5 ± 0.01	**14**	d	**26**	c	70	422	444	473
Characiopsis acuta	456	14.8 ± 0.01	**14**	d	**23**	g	72	423	445	473
Neomonodus sp.	2437	14.8 ± 0.00	**13**	d	**25**	c	72	423	445	473
Characiopsis longipes	1839	14.5 ± 0.01	**13**	d	**24**	c	(65)	-	445	473
Munda sp.	2428	14.8 ± 0.01	**11**	e	**20**	h	79	423	445	473
Vischeria sp. (syn. *Eustigmatos*)	4864ni	14.8 ± 0.01	**11**	e	**19**	h	73	422	445	473
Dioxys sp.	2029	14.8 ± 0.01	**11**	e	**23**	g	73	422	445	473
Tetraplekton sp.	2650ni	14.8 ± 0.00	**11**	e	**20**	h	58	-	445	473
Vischeria helvetica	299	14.8 ± 0.01	**10**	e	**20**	h	73	422	445	473
Characiopsiella minima	2426	14.8 ± 0.00	**9**	f	**17**	i	67	423	445	473
Characiopsis longipes	2438	14.5 ± 0.00	**9**	f	**19**	h	(58)	-	445	472
Characiopsis sp.	2429	14.8 ± 0.00	**9**	f	**21**	h	66	423	445	473
Pseudostaurastrum sp.	2419ni	14.8 ± 0.01	**9**	f	**14**	a	51	-	445	471
Goniochloris sculpta	1852	14.8 ± 0.00	**9**	f	**17**	i	52	424	445	472
Monodopsis unipapilla	2938	14.8 ± 0.00	**8**	g	**11**	j	64	423	445	473
Characiopsiella minima	2423A	14.8 ± 0.05	**7**	g	**15**	a	67	-	445	473

iii. β-CAROTENE

The β-carotene (Figure 6.1.4.) was identified based on the retention time, absorbance maxima and band ratio (% III/II), comparing to the purchased standard (with t_r 22.25 minutes and λ_{max} –, 453, 478 nm) and to published values (Schnepf et al. 1995/96, Roy et al. 2011).

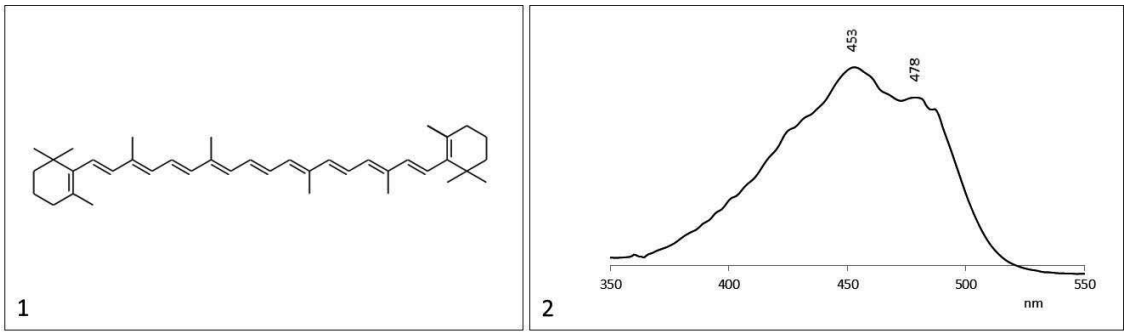

Figure 6.5. 1) Chemical structure of β-carotene $C_{40}H_{56}$, IUPAC: β, β-carotene, 2) absorption spectrum of β-carotene obtained from the strain *Characiopsis* sp. ACOI 2423A.

The β-carotene peak eluted with t_r= 21.8 or 22.1 minutes with absorbance wavelength maxima of 453 (and an additional vibronic peak at 478 nm), see Table 6.4..

The β-carotene was the second most abundant carotenoid, the highest value was obtained for *Pseudostaurastrum* sp. ACOI 2419ni, accounting for 24% of the total amount of pigments present in the extract, with a 39% contribution to total carotenoids. The second highest value was obtained in *Characiopsis acuta* ACOI 1837 with β-carotene accounting for 21% of total pigments, representing the highest contribution to total carotenoid of this study, with 40% ($p<0.05$).

The lowest value determined was found in *Vischeria helvetica* ACOI 299 accounting for 5% of all pigments with β-carotene representing 10% of all pigments present in the extract.

Table 6.4. β-carotene detected in the studied strains. t_r- retention time, % total pigm. - integrated area of peaks attributed to β-carotene compared to total pigments, % total carot. – area of β-carotene compared to total carotenoid content, % III/II – band ratio, λ_{max} - absorbance wavelength maxima. Values with different letters in the same column are significantly different ($p<0.05$).

Strain	ACOI number	t_r (min.)	% total pigm.	% total carot.	% III/II	λ_{max} (nm)
Pseudostaurastrum sp.	2419ni	22.1 ± 0.01	**24** a	**39** a	nd -	453 477

Characiopsis acuta	1837	21.8 ± 0.00	**21**	b	**40**	a	5	-	453	475
Characiopsis longipes	1839	21.8 ± 0.01	**16**	c	**30**	b	7	-	453	477
Characiopsis acuta	456	22.1 ± 0.00	**16**	d	**25**	c	11	-	454	479
Goniochloris sculpta	1852	22.0 ± 0.00	**14**	e	**26**	c	4	-	453	477
Characiopsis saccata	481	22.1 ± 0.00	**13**	f	**19**	d	10	-	454	480
Characiopsis cf. *minuta*	2423	21.8 ± 0.01	**13**	f	**17**	d	7	-	453	478
Munda sp.	2428	22.2 ± 0.00	**12**	g	**22**	e	7	-	453	478
Characiopsis longipes	2438	21.8 ± 0.00	**12**	g	**25**	c	nd	nd	nd	nd
Characiopsis sp.	2430	22.1 ± 0.01	**11**	g	**18**	d	5	-	453	477
Dioxys sp.	2029	22.1 ± 0.03	**11**	g	**22**	e	12	-	454	479
Characiopsiella minima	2426	22.2 ± 0.00	**11**	g	**21**	e	7	-	453	478
Monodopsis unipapilla	2938	22.0 ± 0.01	**10**	h	**13**	f	8	-	453	479
Munda aquilonaris	2424A	22.1 ± 0.04	**9**	h	**18**	d	nd	-	453	478
Munda aquilonaris	2424B	22.1 ± 0.02	**9**	h	**17**	d	7	-	453	478
Characiopsis pernana	2433	22.1 ± 0.00	**9**	h	**16**	d	12	-	454	479
Characiopsis minutissima	2427A	22.1 ± 0.01	**9**	h	**16**	d	9	-	453	479
Characiopsis sp.	2438B	22.1 ± 0.01	**9**	h	**14**	g	3	-	453	477
Characiopsiella minima	2423A	22.1 ± 0.04	**9**	h	**15**	g	9	-	453	479
Characiopsis cedercreutzii	2434	21.8 ± 0.01	**8**	h	**15**	g	8	-	453	477
Characiopsis cedercreutzii	3169	21.8 ± 0.01	**8**	h	**17**	d	9	-	453	477
Characiopsis sp.	2429	22.1 ± 0.00	**8**	h	**17**	d	5	-	453	479
Munda aquilonaris	2424	22.2 ± 0.01	**7**	i	**13**	f	10	-	453	479
Vischeria sp. (syn. *Eustigmatos*)	4864ni	22.1 ± 0.00	**7**	i	**12**	f	16	-	454	480
Neomonodus sp.	2437	22.2 ± 0.00	**6**	j	**12**	f	7	-	453	478
Tetraplekton sp.	2650ni	22.1 ± 0.01	**6**	j	**11**	h	nd	-	453	-
Vischeria helvetica	299	22.1 ± 0.01	**5**	k	**10**	h	15	-	454	480

iv. OTHER CAROTENOIDS

The other carotenoids present in the studied extracts (Table 6.5.) may be divided into three categories:

i) a minor carotenoid possibly identifiable as antheraxanthin

A minor carotenoid eluted in all strains at t_r 15.5, 15.7 or 15.8 min, with a vibronically resolved absorption band with wavelength peaks at 422, 445 and 473 nm with some minor variations on the wavelength absorption maxima observed (Figure 6.6). The measured parameters indicate it is most likely antheraxanthin (Roy et al. 2011), yet this cannot be undoubtedly established with the present study.

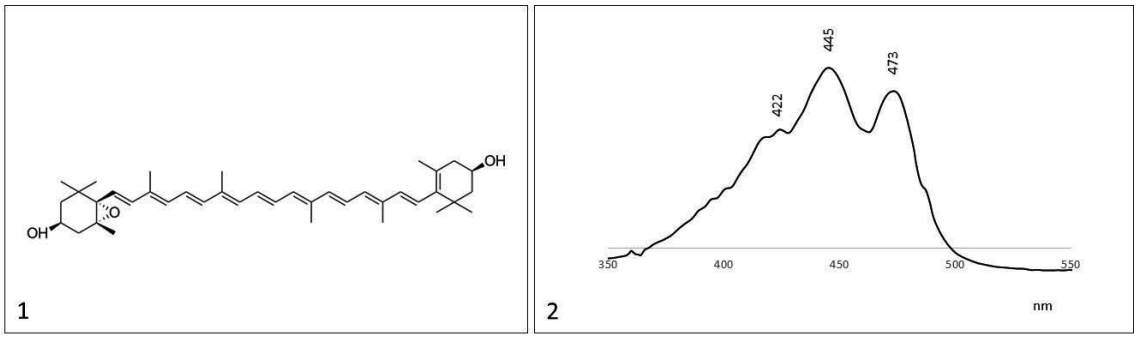

Figure 6.6. 1) Chemical structure of antheraxanthin, $C_{40}H_{56}O_3$, IUPAC: (3 S,5R,6S,30R)-5,6-Epoxy-5,6-dihydro-b,b-carotene-3,30-diol, 2) absorption spectra of antheraxanthin obtained from the strain of *Characiopsis* sp. ACOI 2423A.

The highest value for this pigment was found for *Characiopsiella minima* ACOI 2423A with 6 % of the total pigment content, with contribution of 11% of the total carotenoid content (Table 6.5.).

ii) undetermined carotenoids

These carotenoids are present in such low amounts that it was not possible to measure the absorbance bands and therefore their identification was compromised and not possible based

only in their retention time. An undetermined carotenoid was found to elute at t_r= 15.5 min in *Characiopsis longipes* ACOI 1839 and t_r=15.6 min. in *Characiopsis acuta* ACOI 1837; with a very small contribution of 4 and 3% of the total pigment content respectively. This carotenoid much likely corresponds to antheraxanthin, based on the t_r. Another undetermined carotenoid was found to elute around t_r= 14.9 min in *Characiopsis cedercreutzii* ACOI 2434 with a very small contribution of ~1% of the total pigment content.

iii) main carotenoid derivatives

The identification of the carotenoids was made based on the absorption wavelength maxima and t_r. However, in some cases the absorption wavelength maximum was found to correspond to a known carotenoid present in the extract, but with a different retention time than expected. One example is a carotenoid with the absorption wavelength maximum of violaxanthin, but that eluted much earlier (lower t_r value) than violaxanthin detected for example in *Characiopsis* cf. *minuta* ACOI 2423 (Table 6.5.). There are various possibilities for this behavior. These carotenoids may have suffered damage due to methodological manipulation (slight light exposure, temperature oscillations, etc.) which may lead to small structural changes with however the same fundamental chromophoric unit. The violaxanthin derivatives found in *Characiopsis* sp. *minuta* ACOI 2423 and *Characiopsis* sp. 2438B contribute largely to the high amount of "other carotenoids" which make these two strains the richest in this wider category of detected carotenoids. As previously discussed, (6.2.), if the violaxanthin derivatives are removed from the pool of undetermined carotenoids and added to the violaxanthin values, then these two strains are no longer the richest in "other carotenoids". If such would be the case, then the strain with the highest value of undetermined carotenoids would be *Characiopsis longipes* ACOI 1839.

Table 6.5. Other carotenoids detected in the studied strains. t_r- retention time, % total pigm. - integrated area of peaks attributed to the other carotenoids compared to total pigments, % total carot. – area of other carotenoids compared to total carotenoid content, % III/II – band ratio, derivative – carotenoid derivative, with altered characteristics due to manipulation, λ_{max} - absorbance wavelength maxima, violax. - violaxanthin, anther. - antheraxanthin. Values highlighted in bold are the total obtained from all fraction contributions. Values with different letters in the same column are significantly different ($p<0.05$).

Strain	ACOI nr	t_r (min.)	% total pigm.	% total carot.	% III/II	λ_{max} (nm)			Possible identification (a)
Characiopsis cf. *minuta*	2423	7.8 ± 0.01	26	34	89	417	441	470	violax.? derivative
		9.6 ± 0.01	3	4	nd	416	443	469	violax.? derivative
		10.1 ± 0.02	3	4	nd	416	443	469	violax.? derivative
		14.9 ± 0.01	2	3	nd	416	444	468	derivative
		15.5 ± 0.00	4	5	nd	416	444	468	derivative
			38 a	**50** a					
Characiopsis sp.	2438B	7.7 ± 0.00	12	20	78	417	440	470	violax.? derivative
		9.7 ± 0.01	5	8	75	416	441	470	violax.? derivative
		13.8 ± 0.01	3	4	42	423	445	472	anther.? derivative
		15.8 ± 0.01	2	4	27	-	442	467	derivative
			22 b	**36** b					
Characiopsis longipes	1839	13.1 ± 0.01	5	10	58	424	445	470	derivative
		14.5 ± 0.07	13	24	65	-	445	473	derivative
		15.5 ± 0.00	**4**	**6**	nd	nd	nd	nd	?
			22 b	**40** c					
Characiopsis saccata	481	13.4 ± 0.00	5	8	99	401	423	450	derivative
		14.1 ± 0.00	4	6	-	415	431	465	derivative
		15.2 ± 0.00	1	1	-	-	448	-	derivative
		15.4 ± 0.00	1	2	-	406	425	452	derivative
		15.8 ± 0.00	3	4	-	-	440	468	derivative
			14 c	**21** d					
Characiopsis pernana	2433	13.3 ± 0.00	3	5	19	423	447	471	derivative
		13.8 ± 0.00	2	4	-	-	446	471	derivative
		14.1 ± 0.00	3	6	-	-	442	467	derivative

		15.2 ± 0.00	2	4	55	-	444	472	anther.? derivative
		15.7 ± 0.00	4	6	54	-	444	472	antheraxanthin
			14 c	**25** e					
Characiopsis acuta	456	10.0 ± 0.02	2	4	nd	416	442	469	derivative
		15.2 ± 0.01	9	14	12	-	453	479	derivative
			11 d	**18** f					
Characiopsis sp.	2430	9.7 ± 0.01	5	8	72	415	441	469	derivative
		13.8 ± 0.01	3	4	37	423	445	471	derivative
		15.8 ± 0.01	2	4	nd.	nd.	nd.	nd.	antheraxanthin?
			10 e	**16** f					
Characiopsis minutissima	2427A	13.3 ± 0.00	2	4	23	-	423	446	derivative
		14.1 ± 0.01	3	5	-	-	445	473	derivative
		15.7 ± 0.01	4	7	51	-	444	472	antheraxanthin
			9 e	**16** f					
Characiopsis longipes	2438	7.8 ± 0.00	3	6	nd	416	444	468	derivative
		15.5 ± 0.07	5	10	nd	nd	nd	nd	antheraxanthin?
			8 e	**16** f					
Monodopsis unipapilla	2938	9.8 ± 0.01	3	5	76	416	442	469	violax.? derivative
		13.8 ± 0.01	4	5	59	-	445	472	derivative
		15.7 ± 0.00	nd	nd	nd	nd	nd	nd	antheraxanthin?
			7 e	**10** g					
Characiopsiella minima	2423A	15.7 ± 0.05	**6** f	**11** g	64	-	445	473	antheraxanthin
Munda aquilonaris	2424B	15.7 ± 0.03	**4** f	**7** h	57	-	445	473	antheraxanthin
Munda sp.	2428	15.7 ± 0.01	**4** f	**8** g	63	-	445	473	antheraxanthin
Characiopsis cedercreutzii	3169	15.5 ± 0.01	**4** f	**8** g	66	422	444	473	antheraxanthin
Characiopsis sp.	2429	15.7 ± 0.00	**4** f	**10** g	54	-	444	472	antheraxanthin
Tetraplekton sp.	2650ni	13.4 ± 0.00	**4** f	**8** g	42	424	445	470	derivative
		15.8 ± 0.01	nd	nd	nd	nd	nd	nd	antheraxanthin?
Characiopsis cedercreutzii	2434	14.9 ± 0.00	1	3	nd	-	-	-	?
		15.6 ± 0.00	3	5	nd	-	-	-	antheraxanthin?
			4 f	**8** g					
Munda aquilonaris	2424	15.7 ± 0.01	**3** g	**6** h	63	-	445	473	antheraxanthin
Neomonodus sp.	2437	15.7 ± 0.01	**3** g	**6** h	63	-	445	473	antheraxanthin

Characiopsis acuta	1837	15.6 ± 0.00	**3** f	**7** g	nd	-	-	-	?
Munda aquilonaris	2424A	15.7 ± 0.05	**nd**	**nd**	nd	nd	nd	nd	antheraxanthin?
Characiopsiella minima	2426	15.7 ± 0.00	**nd**	**nd**	nd	nd	nd	nd	antheraxanthin?
Vischeria sp. (syn. *Eustigmatos*)	4864ni	15.8 ± 0.01	**nd**	**nd**	nd	nd	nd	nd	antheraxanthin?
Vischeria helvetica	299	15.7 ± 0.01	**nd**	**nd**	nd	nd	nd	nd	antheraxanthin?
Dioxys sp.	2029	15.7 ± 0.01	**nd**	**nd**	nd	nd	nd	nd	antheraxanthin?
Pseudostaurastrum sp.	2419ni	15.7 ± 0.03	**nd**	**nd**	nd	nd	nd	nd	antheraxanthin?
Goniochloris sculpta	1852	15.7 ± 0.00	**nd**	**nd**	nd	nd	nd	nd	antheraxanthin?

[(a)]According to Roy et al. 2011.

6.4.2. Chlorophylls

i. CHLOROPHYLL B (internal control)

Naturally occurring chlorophyll b was not detected in the studied extracts. The identification of the internal standard chlorophyll b was performed by comparing with the characteristics of an isolated standard run and by comparing with literature (Roy et al. 2011).

The standard eluted with t_r= 18.5 min in all extracts, with absorption wavelength maxima at 457nm and 646 nm (Figure 6.7.), with slight variations on the band wavelength maximum values. These characteristics and the absorbance spectrum agree with the published data for this chlorophyll (Roy et al. 2011).

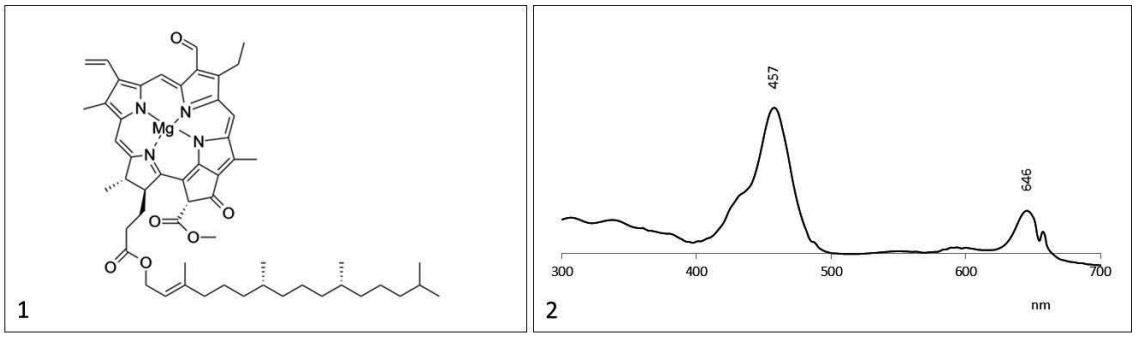

Figure 6.7. a) Chemical structure of Chlorophyll b molecule, $C_{55}H_{70}N_4O_6Mg$, IUPAC: (2 2R,17S,18S)-12-Ethenyl-7-ethyl -21,22,17,18-tetrahydro8-methanoyl-22-(methoxycarbonyl)-3,13,17 -trimethyl-21-oxo-18{2-[(2E,7R,11R)-3,7,11,15-tetramethylhexadec-2enoxycarbonyl]ethyl}cyclopenta[at] porphyrinatomagnesium(II), 2) Absorption spectrum of β-carotene detected in strain *Characiopsis* sp. ACOI 2423A.

ii. CHLOROPHYLL A

Chlorophyll a (Fig. 6.8.) was identified based on the retention time t_r= 19.32 min and absorption wavelength maxima λ_{max} = 413, 431 and 662 nm, comparing to the standard and to the literature reported values (Roy et al. 2011, Schnepf et al. 1995/96). Moreover, the presence of a degradation product was observed t_r= 8.00 min and identified as chlorophyllide a, based on published characteristics (Roy et al. 2011).

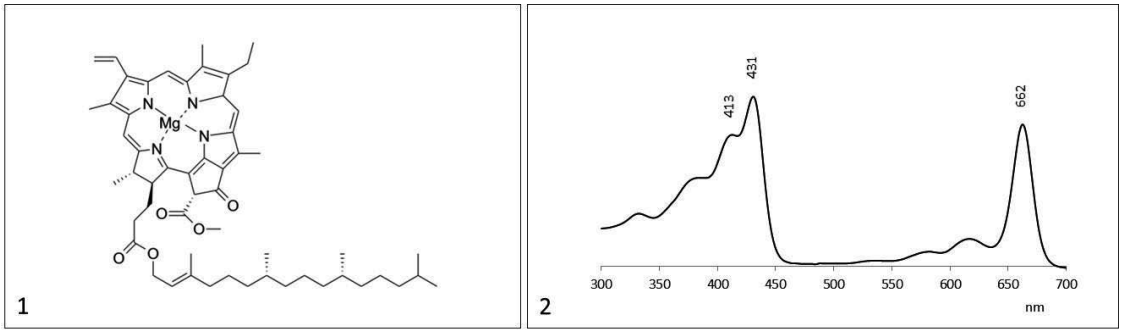

Figure 6.8. 1) Chemical structure of Chlorophyll a molecule, $C_{55}H_{72}N_4O_5Mg$, IUPAC: (2 2R,17S,18S)-12-Ethenyl-7-ethyl21,22,17,18-tetrahydro-22-(methoxycarbonyl)-3,8,13,17tetramethyl-21-oxo-18-{2-[(2E,7R,11R)-3,7,11,15tetramethylhexadec-2enoxycarbonyl]ethyl}cyclopenta[at] porphyrinatomagnesium(II), 2) Absorption spectrum of Chlorophyll a detected in strain *Characiopsis* sp. ACOI 2423A.

Chlorophyll a is the largest fraction of the total pigment content detected in the studied strains, with 43% average content in the studied strains (Fig. 6.2. left).

The highest content in chlorophyll a was detected in *Characiopsis* sp. ACOI 2429 with 54% of the total pigment, not statistically different than the value determined for *Characiopsis cedercreutzii* ACOI 3169 and *Characiopsis longipes* ACOI 2438 both with 52% total pigment. The lowest content of chlorophyll a was determined in *Pseudostaurastrum* sp. ACOI 2419ni, with 17% and in *Characiopsis* cf. *minuta* ACOI 2423 with 18% total pigment ($p<0.05$) (Table 6.6.).

The first eighteen listed strains have around half the total pigment ocupied by chlorophyll a. Chlorophyll a is considered as the only chlorophyll present in Eustigmatophyceae, with chlorophyll b and c absent as a rule. However, in some cases it does not account to 100% of the whole chlorophyll content, with a fraction corresponding to the presence of degradation of the molecule due to photooxidation products. These compounds are originated during the extraction procedure process in algae with highly active chlorophyllase enzyme (Roy et al. 2011). In the analysis of the values of chlorophyll a it is advisable that the contributions of the degradation products are included in the measurements (Roy et al. 2011, Antia et al. 1975). The calculations of the chlorophyll a therefore reflect the sum of the total chlorophyll a content, in the 12 strains where these products were detected (Table 6.6.). In most cases a chlorophyll peak eluted at t_r~ 8 min, the absorbance bands corresponded to chlorophyllide a (according to Roy et al. 2011), sometimes with doubt. Chlorophyllide a was detected in *Dioxys* ACOI 2029, *C. cedercreutzii* ACOI 2434, *M. aquilonaris* ACOI 2424, *C. acuta* ACOI 1837, *C. minutissima* ACOI 2427A, *C. longipes* ACOI 1839, *Goniochloris sculpta* ACOI 1852, *Characiopsis pernana* ACOI 2433, *Characiopsis* sp. ACOI 2430, *C. acuta* ACOI 456, *Characiopsis* sp. ACOI 2438B, *C. saccata* ACOI 481, *Characiopsis* cf. *minuta* ACOI 2423.

In *Characiopsis* sp. ACOI 2438B an unidentified derivative of chlorophyll at t_r = 10.3 min with wavelength absorption maxima λ_{max} = 431 and 658 nm was found.

Table 6.6. Chlorophyll a detected in the studied strains. t_r- retention time, Area % - integrated area of peaks attributed to chlorophyll a compared to total pigments, % total chlorophyll – area of chlorophyll a compared to total chlorophyll content. In some cases, its alteration product chlorophyllide-a was also detected, in minor amount (data presented in blue, below the chlorophyll a data for the corresponding strain). λ_{max} - absorbance wavelength maxima. Values with different letters in the same column are significantly different ($p<0.05$).

Strain	ACOI number	t_r (min.)	Area %		λ_{max} (nm)		
Characiopsis sp.	2429	19.2 ± 0.00	**54**	a	412	431	662
Characiopsis cedercreutzii	3169	19.0 ± 0.01	**52**	a	412	430	662
Characiopsis longipes	2438	19.0 ± 0.00	**52**	a	413	431	662
Dioxys sp.	2029	19.2 ± 0.01	44		412	430	662
chlorophyllide		8.0 ± 0.00	7		415	431	664
			51	b			
Characiopsis cedercreutzii	2434	19.0 ± 0.01	22		412	430	662
chlorophyllide?		8.0 ± 0.00	28		336	431	664
			50	b			
Neomonodus sp.	2437	19.3 ± 0.00	**50**	b	412	430	662
Characiopsiella minima	2426	19.3 ± 0.00	**50**	b	413	431	662
Characiopsiella minima	2423A	19.3 ± 0.04	**50**	b	412	431	662
Munda aquilonaris	2424A	19.3 ± 0.04	**49**	b	412	431	662
Munda aquilonaris	2424B	19.4 ± 0.01	**49**	b	412	431	662
Vischeria helvetica	299	19.2 ± 0.01	**49**	b	412	430	662
Munda aquilonaris	2424	19.4 ± 0.01	45		412	430	662
chlorophyllide		8.4 ± 0.06	3		414	431	664
			48	b			
Characiopsis acuta	1837	19.0 ± 0.00	17		413	430	662
chlorophyllide?		8.0 ± 0.00	31		336	431	664
			47	b			
Munda sp.	2428	19.3 ± 0.01	**47**	b	412	431	662
Characiopsis minutissima	2427A	19.2 ± 0.01	43		412	431	662
chlorophyllide		8.0 ± 0.00	3		415	432	664
			46	b			
Tetraplekton sp.	2650ni	19.2 ± 0.00	**46**	b	412	431	662

Characiopsis longipes	1839	19.0 ± 0.00	36		413	431	662
chlorophyllide		8.0 ± 0.00	9		415	431	664
			45	b			
Goniochloris sculpta	1852	19.2 ± 0.00	37		412	431	662
chlorophyllide?		8.0 ± 0.00	8		415	432	659
			45	b			
Characiopsis pernana	2433	19.2 ± 0.00	31		412	431	662
chlorophyllide		8.0 ± 0.00	13		414	431	664
			44	c			
Vischeria sp. (syn. *Eustigmatos*)	4864ni	19.2 ± 0.00	**43**	c	412	430	662
Characiopsis sp.	2430	19.2 ± 0.00	21		412	431	662
chlorophyllide?		8.0 ± 0.01	18		nd	432	664
			39	d			
Monodopsis unipapilla	2938	19.2 ± 0.01	**39**	d	412	431	662
Characiopsis acuta	456	19.2 ± 0.01	32		412	431	662
chlorophyllide		8.0 ± 0.00	6		413	431	664
			39	d			
Characiopsis sp.	2438B	19.2 ± 0.01	17		413	431	662
chlorophyllide?		7.9 ± 0.00	18		nd	432	664
?		10.3 ± 0.00	3		nd	431	658
			35	e			
Characiopsis saccata	481	19.2 ± 0.00	13		-	431	663
chlorophyllide		8.1 ± 0.00	19		417	431	663
			34	e			
Characiopsis cf. *minuta*	2423	19.0 ± 0.01	**18**	f	413	431	662
Pseudostaurastrum sp.	2419ni	19.2 ± 0.01	**17**	f	412	431	662

6.5. Discussion

When a standard is not available for pigment identification, the absorbance spectrum (including its shape and wavelength maxima) must be combined with the retention time (t_r)

and other parameters, and further compared with the literature data in order to identify the pigment with confidence. However, an important aspect that must be taken into consideration is the fact that the spectra of the pigments is solvent dependent and therefore the wavelength maxima of the pigment is dependent on the solvent used. In the case of the gradient used for the elution in the current experiments, this means that it also depends on the solvent system used for the HPLC run (Roy et al. 2011); therefore, slight differences when compared with literature data must be taken into account when identifying an eluted pigment.

It is known that the carotenoid content of eustigmatophytes varies with the strain (Wang et al. 2018, Roy et al. 2011, Schnepf et al. 1995/96, Preisig and Wilhelm 1989, Antia and Cheng 1982, Whittle and Casselton 1975, Antia et al. 1975). However, some carotenoids are present in amounts which also vary as a function of cultivation conditions such as light (Lubián and Montero 1998) and age (Antia and Cheng 1982).

The highest value of violaxanthin was found in *M. unipapilla* ACOI 2938. The second and third highest values of violaxanthin were found in the *Vischeria* strains, representing 69% and 70% of all carotenoids in both analyzed strains; higher values than found in previous reports for *Vischeria* (syn. *Eustigmatos*) where violaxanthin represented around 40% of the detected carotenoids (Whittle and Casselton 1975). Furthermore, the determined value is up to 6 times higher than the one reported in *Vischeria* (syn. *Eustigmatos*) when cultivated in high light conditions (150 $\mu mol.m^{-2}.s^{-1}$), with violaxanthin representing 10 – 14.6 % of the total carotenoid content (Li et al. 2012a). In the case of the present study, strains were cultivated with a much lower light intensity of 11 $\mu mol.m^{-2}.s^{-1}$ which may explain the higher amount of violaxanthin present in the extracts. In fact, the production of violaxanthin is inversely proportional to the exposure to higher light intensity, according to the dynamics of the violaxanthin cycle present in the eustigmatophyte, which constitutes a photoprotective mechanism of the photosynthetic apparatus (Lubián and Montero, 1998).

Another example of higher amounts of violaxanthin found in the studied strains possibly due to lower light conditions is the discrepancy found between the studied strains and a previously reported member of ordinal clade *Goniochloridales*, *Trachydiscus minutus* (Přibyl et al. 2012). The authors found violaxanthin accounts for 16% of its total pigment content, corresponding to 56% of the carotenoid content (Přibyl et al. 2012). This value is lower than the one found for majority of the strains analyzed in our study and half the value determined for members of clade *Goniochloridales*, *Goniochloris* sp. ACOI 1852 and *Pseudostaurastrum* sp. ACOI 2419ni (Table 6.2.).

Characiopsis cf. *minuta* ACOI 2423 and *Characiopsis* sp. ACOI 2438B violaxanthin content values are among the lowest of the study, representing 7% and 14% total pigment (Table 6.2.). However, there is a possibility that the compound which eluted earlier (t_r= 7.8, 9.6 and 10.1 min.) than the typical retention time expected for violaxanthin may be a violaxanthin derivative (listed in other carotenoids, see Table 6.5.). If this is the case, then the violaxanthin content is highly underestimated in these two strains and if these are accounted, the total values summed are therefore 39% (7% + 32% due to derivatives) for *Characiopsis* cf. *minuta* ACOI 2423 and 31% (14% + 17%) for *Characiopsis* sp. 2438B (Table 6.5.).

As previously mentioned, violaxanthin is known to be the most abundant carotenoid in eustigmatophytes. However, its abundancy is dependent of its counterparts of the violaxanthin cycle namely zeaxanthin and vaucheriaxanthin. This balance depends on the light conditions to which the cells are subjected (Lubián and Montero 1998). The conditions for violaxanthin production in *Vischeria* were studied and its high antioxidant capacity indicates violaxanthin it may be regarded as interestinf for nutritional purposes (Wang et al. 2018). Violaxanthin isolated from microalgae has potential pharmaceutical applications, studies reveal their anti-inflammatory (Soontormchaiboon et al. 2012) and antiproliferative properties (Pasquet et al. 2011) among others.

Considering all the studied strains, vaucheriaxanthin was the second most abundant pigment, with 12% contribution to all pigment content and representing 23% of the total carotenoid content (Figure 6.2.). This observation agrees with previous reports for the Eustigmatophyceae, with vaucheriaxanthin as the second most abundant carotenoid found in several species, sometimes detected in the same amount as violaxanthin (Antia et al. 1975, Antia et al. 1982, Arsalane et al. 1992, Lubián and Montero 1998, Lubián et al. 2000, Přybil et al. 2012). It is important to notice that vaucheriaxanthin content may vary a lot in some members of the Eustigmatophyceae. Reports on *Nannochloropsis/Microchloropsis* species, vaucheriaxanthin show it is the second major carotenoid in *N. oculata* and *M. salina* (Antia et al. 1975); but it is reported with similar predominance as violaxanthin in *N. oculata*, *M. salina* and *N. gaditana* (Lubián et al. 2000) and it is reported as the major carotenoid in *Nannochloropsis* sp. (Nobre et al. 2012). These different results achieved for members of the *Nannochloropsis/Microchloropsis* may be attributed to different cultivation conditions, especially light intensity, age of culture among other factors which are known to interfere with carotenoid accumulation in microalgae (Antia et al. 1982, Lubián and Montero 1998).

Reports on *Vischeria* (syn. *Eustigmatos*) strains (known as *Pleurochloris* in older literature) mention vaucheriaxanthin as representing around 30% of the total carotenoid content (Whittle and Casselton 1975), which is threefold the one detected in the two *Vischeria* strains analyzed, with 10 and 11% (values not statistically different, $P<0.05$). In these, vaucheriaxanthin represented around 20% of the total carotenoid content (Table 6.3.). Lower contributions of vaucheriaxanthin to the total carotenoid were recently reported in *Vischeria* (syn. *Eustigmatos*), with 7.6 – 14.1 % of the total carotenoid content (Li et al. 2012a, Stoyneva-Gärtner et al. 2019a).

Regarding the eustigmatophytes positioned in the ordinal clade *Goniochloridales*, reports for *Trachydiscus minutus* refer vaucheriaxanthin as the second major carotenoid, accounting for 16

% total pigments, contributing with 26% to the total carotenoid content (Přybil et al. 2012). This contrasts with the studied members of that order *Goniochloris* sp. ACOI 1852 and *Pseudostaurastrum* sp. ACOI 2419ni where the violaxanthin accounted for nearly half of those values, i.e., both with 9 % of the total pigment content and with 17 % and 14 % for the total carotenoid content, respectively. In previous reports for *Pseudostaurastrum limneticum* this carotenoid is noted as a major pigment, but no quantification of its content was performed (Schnepf et al. 1996). The culture conditions, methodology used, and the strain may be the cause of the above-mentioned lower values found in the studied Goniochloridales members.

The highest values of vaucheriaxanthin found for the studied strains concerns the extracts of the stipitates *Characiopsis saccata* ACOI 481 and *Characiopsis* cf. *minuta* ACOI 2423, both accounting for 19 % of the total pigments ($p<0.05$). This carotenoid was found abundant in stipitate strains such as *Characiopsis*, *Munda, Neomonodus* (Table 6.3.). If optimized conditions are established for enhanced carotenoid production, it is anticipated that other isolates from these taxa may also have a potential to generate substantial amounts of vaucheriaxanthin.

β-carotene is considered as a major carotenoid in the analyzed strains. Considering all measurements, it contributes with 10% to the total pigment content and is the third major carotenoid with 19% contribution to the total carotenoid content of all studied strains, just after vaucheriaxanthin (Figure 6.2.). This observation concurs with other reports of this carotenoid as the third most represented in eustigmatophytes namely *Monodopsis subterranea* (Arsalane et al. 1992), *Microchloropsis salina* (Antia et al. 1982, Brown 1987), *Nannochloropsis oculata* (Antia and Cheng 1982) *Pseudostaurastrum limneticum* (Schnepf et al. 1995/96), *Botryochloropsis similis* (Preisig and Wilhelm 1989), *Vischeria* (Whittle and Casselton 1975, Li et al. 2012a, Wang et al. 2018), *Trachydiscus minutus* (Přybil et al. 2012).

The highest value % of the total pigment determined for β-carotene is 24% in *Pseudostaurastrum* sp. ACOI 2419ni ($p<0.05$) (Table 6.4.). Within the studied eustigmatophytes, 12 strains have more than 10% β-carotene in the total pigment contents.

The β-carotene contribution to the total carotenoids is found to be around 40% in the case of *Pseudostaurastrum* sp. ACOI 2419ni and *Characiopsis acuta* ACOI 1837, which indicates these two strains as promising to biotechnological applications related to the production of this commercially acknowledged carotenoid. Indeed, it should be considered as a relevant production of this carotenoid comparing with reports for other eustigmatophytes, with β-carotene contributing in *Trachydiscus minutus* to 9.7% total carotenoids (Přybil et al. 2012). If the production of β-carotene is envisaged, a possible enhancement for its mass production in *Pseudostaurastrum* sp. ACOI 2419ni and *Characiopsis acuta* ACOI 1837 is the use of older cultures, since it has been proved in the eustigmatophytes that β-carotene accumulation may be higher in older cultures of *Microchloropsis gaditana* (Lubián et al. 2000), *Nannochloropsis oculata* and *Microchloropsis. salina* (Antia et al. 1982).

There are reports of β-carotene 14-17.2 % contribution to the total carotenoids in *Vischeria* strains (Whittle and Casselton 1975). A similar value was found in our studied strains *Vischeria* sp. and *V. helvetica* with 12% and 10% respectively (Table 6.4.). These values are half as much as a recent report for *Vischeria/Eustigmatos* group, which revealed 23% total carotenoids, in that study it was even higher than violaxanthin (Stoyneva-Gärtner et al. 2019a). Despite the fact that our studied strains of *Vischeria* did not display such high amounts of β-carotene, these strains have a very high content of violaxanthin (Table 6.2.) which makes *Vischeria* sp. ACOI 4864ni and *V. helvetica* ACOI 299 very interesting from a biotechnological point of view for mixed carotenoid production (violaxanthin + β-carotene). However, if *Vischeria* strains are used for massive production of β-carotene, the use of high light intensity and

deficit in nitrogen supply highly enhances production (Li et al. 2012b) and thus in this case the production of violaxanthin is compromised.

The violaxanthin cycle consists of two parts: i) the de-epoxidation of violaxanthin through the intermediate antheraxanthin to form zeaxanthin when exposed to excessive light and ii) the reverse epoxidation reaction that regenerates violaxanthin through antheraxanthin, and is induced under low light intensities or even slower in the dark (Lubián and Montero 1998). This may explain the high amounts of violaxanthin found in the strains whereas with zeaxanthin was not detected. The presence of antheraxanthin denotes that the cycle was active at the time of harvesting and extraction. Since extraction occurred with very low light intensities, if any zeaxanthin was present at the time, it is possible that it was readily converted into antheraxanthin in the reverse direction of the cycle, in order to form violaxanthin. A rare combination of high amounts of antheraxanthin as well as the presence of zeaxanthin was found in *Vischeria* strains (Stoyneva-Gärtner et al. 2019a). These values are accompanied with low violaxanthin % of total carotenoids. The used light intensity is not given by the authors, it is very likely that an excessive light was used in the cultivation of the strains, which may have started the forward reaction of the violaxanthin cycle, with its consumption to form the intermediate antheraxanthin and zeaxanthin.

Studies with *Vischeria* (syn. *Eustigmatos*) revealed a different relative composition of carotenoid content, with the second larger fraction occupied not by vaucheriaxanthin but by lutein and antheraxanthin, both around 20% total carotenoids (Stoyneva-Gärtner et al. 2019a). This value more than doubles the highest found for the pigment tentatively identified as antheraxanthin in the studied strains, which was 6% in *Characiopsiella minima* ACOI 2423A. It is not possible to compare it with the studied *Vischeria* strains since this pigment was not detected.

Some of the minor carotenoids found in the strains most likely correspond to altered molecules of the major carotenoids which may have suffered chemical change during the process of extraction. One example is the presence of violaxanthin derivatives, identifiable by their absorbance maxima and the III/II % ratio, which are similar to those of violaxanthin, but with a different retention time. This was the case for *Characiopsis* cf. *minuta* ACOI 2423 and *Characiopsis* sp. ACOI 2438A (Table 6.5).

Naturally occurring chlorophyll b was not detected in any strain, which is a characteristic absence in the Eustigmatophyceae (Whittle and Casselton 1975, Antia et al. 1975, Hibberd and Leedale 1970, 1972, Whittle and Casselton 1969). Chlorophyll b is not as prone to chemical alterations during sample preparation as chlorophyll a because it has an aldehyde at position C7 instead of a methyl group found in chlorophyll a. This difference causes chlorophyll b to display different spectral properties than chlorophyll a and a suggested additional stability towards photooxidation (Wright and Jeffrey 2006). For these two reasons it was considered as an ideal internal standard for quantitative purposes but in practice it was not very straightforward, due to dissolution problems and there were also very few companies from which to buy chlorophyll b. The use of an internal standard for quantitative purposes was therefore compromised.

Chlorophyll a derivatives may occur naturally or as a result of the extraction process. The molecule may suffer changes such as the loss the phytol chain (chlorophyllides) or it may suffer re-arrangements (epimers) or oxidation (allomers) (Wright and Jeffrey 2006). The presence of such additional products should be avoided by improving the conditions used while manipulating the extract. The use of DMF is considered as one of the best solvents for an efficient total pigment extraction. However, the extract must be immediately used for HPLC injection in order to prevent the formation of chlorophyllide a (Furuya et al. 1998). It is possible that this was the case with the strains where chlorophyllide a was detected (Table

6.6.). When these chlorophyll derivatives are detected, then these must be taken into account as contributing to the total value of chlorophyll a. Chlorophyll a is the most abundant pigment found in eustigmatophytes (Preisig and Whilhelm 1989, Lubián and Montero 1998, Lubián et al. 2000, Přybil et al. 2012, Wang et al. 2018), only surpassed by violaxanthin which may achieve very high levels, when the violaxanthin cycle is operating in reverse (Preisig and Whilhelm 1989, Wang et al. 2018).

6.6. Conclusions

All studied strains have the major pigments typical for Eustigmatophyceae: chlorophyll a, violaxanthin, vaucheriaxanthin and β-carotene. No new pigment could be detected in these studies, at least in considerable amounts to be properly identified.

Violaxanthin was the most abundant pigment in *Monodopsis unipapilla* ACOI 2938 and the most abundant carotenoid in both *Vischeria* strains representing around 70% of carotenoids in both strains, which makes them quite promising, so optimized production-oriented conditions are the next step towards the exploration of these strains for the biotechnological fields of nutrition. Regarding the content in vaucheriaxanthin, *Characiopsis saccata* ACOI 481 and *Characiopsis* cf. *minuta* ACOI 2423 were proved to be the richest strains in this pigment. Also considered as producers of commercially important carotenoids are *Pseudostaurastrum* sp. ACOI 2419ni and *Characiopsis acuta* ACOI 1837 with the highest production of β-carotene.

The undetermined carotenoids found in *Characiopsis longipes* ACOI 1839, *Characiopsis cedercreutzii* ACOI 2434 and *Characiopsis acuta* ACOI 1837, as well as the other carotenoids for which undoubtful identification was not possible, are worthy of study in order to determine their correct structure and to achieve a more comprehensive characterization of the carotenoid content of eustigmatophytes. For that purpose, there is a need to develop an extraction method which can detect pigments present in low amounts.

Carotenoids from natural sources have a historical presence in the market and are consistently regarded as health-promoting molecules. Due to the large amounts of violaxanthin found in the studied eustigmatophytes, these emblematic organisms represent a valuable source of this carotenoid for pharmaceutical and nutrition industries. Furthermore, no previous reports have been made until now regarding the characterization of the pigment content of eustigmatophyte stipitates, which value as carotenoid producers is now disclosed.

7.

General Conclusions

The present work is a contribution for the taxonomy and phylogeny of the microalgal class Eustigmatophyceae, and for the characterization of the pigment content and antioxidant capacity of extracts.

The polyphasic approach consisting on the combination of molecular methods with morphological observations originated the clarification of some taxa. The ACOI stipitate eustigmatophytes, those bearing an attachment structure, were the most studied strains. By analyzing the convoluted taxonomic history and the molecular data of the genus *Characiopsis,* it became clear that it was polyphyletic in its previous form. *Characiopsis*-like strains are now distributed through two different families, the Neomonodaceae, *fam nov.* (Chapter 2), and the Eustigmataceae group (Chapter 3). Some strains are positioned with other members of the former *Pseudellipsoidion* group and the whole clade was described as family Neomonodaceae. A new genus was established, *Neomonodus*, where *Monodus ovalis* is now taxonomically housed. Original members of the *Pseudellipsoidion* group are now formally included in genus *Pseudellipsoidion* and two novel eustigmatophyte genera were described, *Munda* and *Characiopsiella.* Strains identified as *Characiopsis aquilonaris* and *Characiopsis minima* were proved to be eustigmatophytes and are positioned in the new genera as *Munda aquilonaris* and as *Characiopsiella minima* respectively. The other strains with *Characiopsis*-like morphology are positioned in the Eustigmataceae group and were formaly described as *Characiopsis*. These advances are quite significative in the current taxonomy of the Eustigmatales, which is now composed of three formally described families. The absence of a pyrenoid in one of these lineages (Neomonodaceae) and its presence in the other "true" *Characiopsis* clade in the Eustigmataceae group, shows a taxonomic signal of this morphological aspect. Some morphological structures correlate with molecular data and their presence or absence may have evolutionary meaning. Furthermore, the presence of a pyrenoid, albeit found consistent with the discrimination of eustigmatophyte stipitate families, has already been regarded as a "remarkably capricious" character in the majority of algal classes by DJ Hibberd in his early work.

With the addition of all these stipitates, the number of described eustigmatophyte species more than doubled. Furthermore, our work evidences that the nominal species diversity of *Characiopsis* was indeed inflated due to synonymy resulting from historical artefacts or misinterpreted morphological plasticity of individual species. Renewed culturing effort, combined with modern "omics" approaches, will be instrumental to improve further our knowledge of genus *Characiopsis*. A genome survey has already been conducted for *C. acuta* ACOI 456, yielding a complete plastid genome sequence and genome data from its *Phycorickettsia* endosymbiont).

The ACOI collection of *Pseudostaurastrum* strains provided the opportunity to study the phylogeny of this genus for the first time, and its monophyly within the less extensively studied ordinal clade *Goniochloridales*. Molecular and morphological data supported the transfer of *P. lobulatum, P. enorme* and *P. hastatum* to the Eustigmatophyceae (Chapter 4). The present study is also a significative contribution to understand the diversity and phylogeny of this order.

All studied strains showed the major pigments typical for the Eustigmatophyceae: chlorophyll a, violaxanthin, vaucheriaxanthin and β-carotene (Chapter 6). No new pigment was detected, at least in considerable amounts for identification. Substantial amount of violaxanthin was detected in *Monodopsis unipapilla* ACOI 2938, *Vischeria* sp. ACOI 4864ni and *Vischeria helvetica* ACOI 299. Stipitates were determined as the top producers of vaucheriaxanthin, with the highest producer *Characiopsiella minima* 2423A. *Pseudostaurastrum* sp. ACOI 2419ni and *Characiopsis acuta* sp. ACOI 1837 showed the highest production of β-carotene. These results indicate these strains as natural sources of biotechnologically interesting carotenoids if cultivated in optimized culture conditions.

The present study contributes to the scientific endeavor of clarifying the diversity of the microalgal class Eustigmatophyceae and its biotechnological valorization. It establishes a useful framework for future exploration of the biological mysteries of this fascinating group.

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Supplementary material

Figure S.1. Phylogeny of Eustigmatophyceae based on sequences of the 18S rRNA gene including partial sequences from environmental DNA surveys. The tree was inferred using RAxML (GTR+ Γ model). A selection of representative non-eustigmatophyte ochrophytes is used as an outgroup. Bootstrap values (based on 354 rapid bootstrap replicates) are shown when higher than 50. The main eustigmatophyte clades are highlighted by different colour background. The five groups of partial sequences from uncultivated eustigmatophytes obtained by Villanueva et al. (2014) are labelled accordingly as Group 1 to Group 5.

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