Eustigmatophyceae

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Marek Eliáš, Raquel Amaral, Karen P. Fawley, Marvin W. Fawley, Yvonne Němcová, Jiří Neustupa, Pavel Přibyl, Lilia M. A. Santos, and Tereza Ševčíková

Abstract

Eustigmatophyceae (eustigmatophytes) are a distinct lineage of ochrophyte (stramenopile) algae with a relatively small number (~30) of described species, but with evidence for a substantial taxonomic diversity yet to be explored. Eustigmatophytes are all unicellular coccoid algae, usually spherical or ovoid, but sometimes with a more distinctive shape (e.g., stipitate, tetrahedral, or with branched projections). Most eustigmatophytes live in freshwater, but some are common in terrestrial habitats and one subgroup is mostly marine. Reproduction

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M. Eliáš (✉) • T. Ševčíková
Department of Biology and Ecology and Institute of Environmental Technologies, Faculty of Science, University of Ostrava, Ostrava, Czech Republic
e-mail: marek.elias@osu.cz; t.sevcikova1@gmail.com

R. Amaral • L.M.A. Santos
Coimbra Collection of Algae (ACOI), Department of Life Sciences, University of Coimbra, Coimbra, Portugal
e-mail: raquel.amaral@bot.uc.pt; rf.amaral@gmail.com; liliamas@ci.uc.pt

K.P. Fawley • M.W. Fawley
School of Mathematical and Natural Sciences, University of Arkansas at Monticello (UAM), Monticello, AR, USA
e-mail: fawley@uamont.edu; fawleym@uamont.edu

Y. Němcová • J. Neustupa
Department of Botany, Faculty of Science, Charles University in Prague, Prague, Czech Republic
e-mail: yvonne.nemcova@natur.cuni.cz; jiri.neustupa@natur.cuni.cz; neustupa@math.cas.cz

P. Přibyl
Centre for Algology and Biorefinery Research Centre of Competence, Institute of Botany, Czech Academy of Sciences, Třeboň, Czech Republic
e-mail: pavel.pribyl@ibot.cas.cz

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occurs primarily via autosporogenesis, but many members of this class form zoospores with an anterior mastigoneme-bearing flagellum and a (sometimes missing) posterior bare flagellum. Sexual reproduction has not been directly observed, but genomic evidence suggests its presence in some species. Eustigmatophytes are distinguished from other ochrophytes by a suite of cytological features (not all are necessarily present in all taxa): a pigmented lipidic body (reddish globule), a swelling at the base of the anterior flagellum associated with an extraplastidial stigma (eyespot), lamellate vesicles (with a putative reserve product), and plastids without a girdle lamella and lacking continuity with the nuclear envelope. Also characteristic is the lack of chlorophyll *c* and violaxanthin as the dominant xanthophyll. Because of their tendency to accumulate large amounts of lipids, including polyunsaturated fatty acids, eustigmatophytes are extensively used for biotechnology applications. The potential for commercial use has sparked a renewed interest in the basic biology of Eustigmatophyceae, including initiation of genome sequencing projects, although attention remains highly biased toward a single lineage comprising the genera *Nannochloropsis* and *Microchloropsis*.

Keywords

Algae • Biofuels • Genomics • PUFAs • Eustigmatophyceae • Molecular phylogenetics • *Nannochloropsis* • Ochrophyta • Stramenopiles • Taxonomy

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Summary Classification

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●●**Eustigmatophyceae *incertae sedis*** *(Botryochloropsis)*

Introduction

General Characteristics

Eustigmatophytes are a distinct group of ochrophyte (heterokont or stramenopile) algae. In the vegetative state, these organisms appear as solitary green or yellow-green coccoid cells or less frequently in loose colonies, with primarily autosporic reproduction. They may resemble some xanthophyte or chlorophyte algae but differ by a unique combination of ultrastructural and biochemical characteristics. A conspicuous characteristic of most eustigmatophyte vegetative cells is a cytoplasmic reddish globule consisting of unknown lipidic substances. Eustigmatophyte plastids lack a girdle lamella. The outer plastid membrane, a cisterna of the plastid endoplasmic reticulum, is typically not continuous with the nuclear envelope; however, this connection has been preserved in some species. Vegetative cells as well as zoospores possess vesicles containing a probable reserve material deposited in a lamellar pattern. Zoospores occasionally form in many species and bear one or two subapical flagella. One longer flagellum always has mastigonemes and a characteristic basal swelling. Typically, a red extraplastidial eyespot (stigma) is present at the extreme anterior, although some taxa recently assigned to the class do not possess this feature. Eustigmatophyceae lack chlorophylls *b* and *c*. Violaxanthin is the dominant xanthophyll and is involved in both light harvesting and in a photoprotective xanthophyll cycle. The Eustigmatophyceae is usually considered a small class, as only around 30 species in 15 genera have been described. However, recent studies have shown that many additional eustigmatophytes await recognition or description.

Occurrence

Eustigmatophytes thrive worldwide primarily in freshwater and terrestrial habitats, with the exception of the marine and brackish species of the genera *Nannochloropsis* and *Microchloropsis*. These organisms are generally inconspicuous because of their small size and are rarely a dominant component of the microbial community.

Literature

There is no recent monograph on the group. The works by Hibberd (1980, 1982, 1990) are still useful as summaries of the first phase of modern research on eustigmatophytes. Hibberd (1981) also published a thorough taxonomic revision and a formal classification of eustigmatophytes that is the starting point for current taxonomic and systematic work on the group. Santos (1996) published the most current general review of eustigmatophytes. Useful information on the morphology and ecology of eustigmatophyte species, including identification keys, can be found in algal floras and compendia (Ettl and Gärtner 1995; John 2011; Ott et al. 2015). Many eustigmatophyte species are included in older floristic works as members of the "Heterokonten" or the Xanthophyceae (Pascher 1939; Ettl 1978).

History of Knowledge

The class Eustigmatophyceae was established by Hibberd and Leedale (1971) after they investigated 12 genera of coccoid algae from the Xanthophyceae and found unique ultrastructural features (Hibberd and Leedale 1970, 1972). The pigment composition of eustigmatophytes was also important in the definition of the new class, because their signature pigments differ significantly from those in xanthophytes (Whittle and Casselton 1969, 1975a, b). The taxonomic revision of eustigmatophyte diversity by Hibberd (1981) led to the recognition of 12 species in six genera. Subsequently, the advent of molecular phylogenetic methods confirmed that the Eustigmatophyceae is a monophyletic lineage distinct from the Xanthophyceae and all other classes of ochrophytes (Bhattacharya et al. 1992; Karlson et al. 1996; Andersen et al. 1998).

Since the seminal work of Hibberd (1981), knowledge of eustigmatophyte diversity has expanded with the transfer of additional species from the Xanthophyceae (Schnepf et al. 1996; Přibyl et al. 2012; Fawley and Fawley 2017) and the descriptions of new taxa (Lubián 1982; Karlson et al. 1996; Krienitz et al. 2000; Suda et al. 2002; Trzcińska et al. 2014; Fawley et al. 2015), including four new monotypic genera (Preisig and Wilhelm 1989; Neustupa and Němcová 2001; Hegewald et al. 2007; Nakayama et al. 2015). Recent culture-based and environmental DNA cloning studies have shown that the diversity of the Eustigmatophyceae is much greater than previously expected (Fawley et al. 2014). After 2010, research on eustigmatophytes entered a new phase with the determination of the first complete genome sequences and development of tools for targeted genetic manipulation in the representative genus, *Nannochloropsis* (Kilian et al. 2011; Pan et al. 2011; Radakovits et al. 2012; Vieler et al. 2012a; Corteggiani Carpinelli et al. 2014; Wang et al. 2014, 2016).

Practical Importance

Eustigmatophytes became the focus of attention and intensive research in recent years due to the potential commercial production of biofuels and bioproducts by some species. Most biotechnology-oriented studies have been performed on the minute marine species of the genus *Nannochloropsis* (including species belonging to the recently segregated genus *Microchloropsis*; Fawley et al. 2015). There has been exponential growth in the number of research papers published on this topic in recent years (e.g., Zou et al. 2000; Krienitz and Wirth 2006; Li et al. 2011; Simionato et al. 2011; Doan and Obbard 2012; Bartley et al. 2014; Xiao et al. 2015); most of the relevant literature concerning the lipid-related biotechnological research has been reviewed by Ma et al. (2016). Briefly, *Nannochloropsis* spp. are valued primarily for the ability to produce two types of lipidic substances – neutral lipids, i.e., various forms of triacylglycerol (TAG) and long-chain polyunsaturated fatty acids (LC-PUFAs), especially eicosapentaenoic acid (EPA). Depending on the cultivation conditions, the intracellular lipid levels in *Nannochloropsis* spp. may reach up to 55–60% of dry weight biomass and may exhibit elevated content of higher saturated fatty acids, being thus suitable for transesterification to biodiesel. *Nannochloropsis* spp. have also been extensively investigated for the production of commercially valuable carotenoid pigments (Lubián et al. 2000), sterols (Suen et al. 1987; Volkman et al. 1993; Patterson et al. 1994), and vitamin E (α-tocopherol; Durmaz 2007). *Nannochloropsis* spp. may also be used as cell reactors for the production of heterologous proteins (Chen et al. 2008) and have long been used as a food source in aquaculture (Duerr et al. 1998; Pfeiffer and Ludwig 2007; Patil et al. 2007; Ferreira et al. 2009).

Industrial use of other genera of the Eustigmatophyceae has been investigated to a lesser extent. *Monodopsis subterranea* (= *Monodus subterraneus*) and *Trachydiscus minutus* both produce large quantities of EPA (Cohen 1994; Hu et al. 1997; Qiang et al. 1997; Lu et al. 2001; Iliev et al. 2010; Řezanka et al. 2010; Cepák et al. 2014; Jo and Hur 2015). Members of the closely related genera *Vischeria* and *Eustigmatos* have also been noted for producing EPA, but also high amounts of β-carotene, and their lipid metabolism also make them promising biofuels producers (Volkman et al. 1999; Li et al. 2012a, b; Aburai et al. 2013; Zhang et al. 2013; Gao et al. 2016). Eustigmatophytes in general may also be regarded as promising antioxidant sources, for example, as documented by a recent survey of various strains from the Coimbra Collection of Algae (ACOI; Assunção et al. 2016).

Environmental bioremediation uses may also be envisaged for eustigmatophytes. For example, cells of *Microchloropsis gaditana* (as *Nannochloropsis gaditana*) were reported to accumulate practically 100% of the copper or zinc present in the medium (Moreno-Garrido et al. 2002). The eustigmatophyte strain nak-9, subsequently described as *Vacuoliviride crystalliferum* (Nakayama et al. 2015), was reported to exhibit a high efficiency in eliminating radioactive caesium from the medium by cellular accumulation (Fukuda et al. 2014). Inoculation of rice grown in hydroponic conditions with *Nannochloropsis* sp. ameliorated the impact of arsenic toxicity on plant growth (Upadhyay et al. 2016). Using municipal wastewater as a source of nutrients for cultivation of *Nannochloropsis* sp. for biotechnological applications was also tested (e.g., Jiang et al. 2011).

Habitats and Ecology

Habitat preferences may differ substantially even within eustigmatophyte lineages and numerous transitions between freshwater and terrestrial habitats seem to have occurred during eustigmatophyte evolution. A transition to the marine environment probably occurred only once, in the lineage leading to an ancestor of the genera *Nannochloropsis* and *Microchloropsis*. An ancestor of the freshwater species *Nannochloropsis limnetica* must then have secondarily transitioned from marine to freshwater habitats (Fawley and Fawley 2007). Little is currently known about possible biogeographical patterns exhibited by eustigmatophyte species.

Members of the closely related genera *Vischeria* and *Eustigmatos* have been frequently isolated from soils (Petersen 1932; Vischer 1945; Neustupa and Němcová 2001) and from various subaerial habitats, such as tree bark (Nakano et al. 1991; Neustupa and Škaloud 2010), rocks (Czerwik-Marcinkowska and Mrozinska 2009), or desert crusts (Flechtner et al. 1998; Büdel et al. 2009), but they are also found in freshwater (Ott et al. 2015). The genus *Monodopsis* also occurs in soils worldwide as well as in freshwater (Ettl and Gärtner 1995; Ott et al. 2015). *Pseudellipsoidion edaphicum* was isolated from soil in Central Europe, whereas the related species *Pseudocharaciopsis ovalis* was reported from both soil and freshwater habitats (Neustupa and Němcová 2001).

Most members of the clade *Goniochloridales* (Fig. 1) and some other eustigmatophytes (e.g., *Pseudotetraëdriella kamillae*) are freshwater phytoplankton or associated with vegetation in freshwater (Ettl 1978; Schnepf et al. 1996; Přibyl et al. 2012; Fawley et al. 2014). The predominantly marine genus *Nannochloropsis* also comprises a freshwater species, *N. limnetica*, a member of picoplankton communities in lakes and ponds (Krienitz et al. 2000; Fietz et al. 2005; Fawley and Fawley 2007). An extremely abundant population of *N. limnetica* dominating the phytoplankton community and reaching up to 5.7×10^9 cells l^{-1} was reported in a hypertrophic lake in Germany (Krienitz et al. 2000), and it seems to be abundant primarily in periods of cold water (Fawley and Fawley 2007).

Mesotrophic and eutrophic lakes and ponds with neutral or slightly basic pH have proved to be a rich source of phylogenetically diverse eustigmatophyte strains (Fawley et al. 2014). On the other hand, many members of the Eustigmatophyceae are found associated with vegetation in acidic conditions and *Sphagnum* bogs (Ott et al. 2015; Karen and Marvin Fawley, pers. observation). *Chlorobotrys regularis* is frequently found in low-pH habitats such as *Sphagnum* bogs (Hibberd 1974), but some strains of *Chlorobotrys* isolated and held in the ACOI collection were isolated from lakes and ponds. Two unidentified eustigmatophytes were recorded by sequencing environmental 18S rDNA libraries from a peat bog in Switzerland (Lara et al. 2011; Fig. 1). Most recently, *Tetraëdriella subglobosa*, originally found by Pascher (1930) in acidic pools in Czechoslovakia, proved to be a

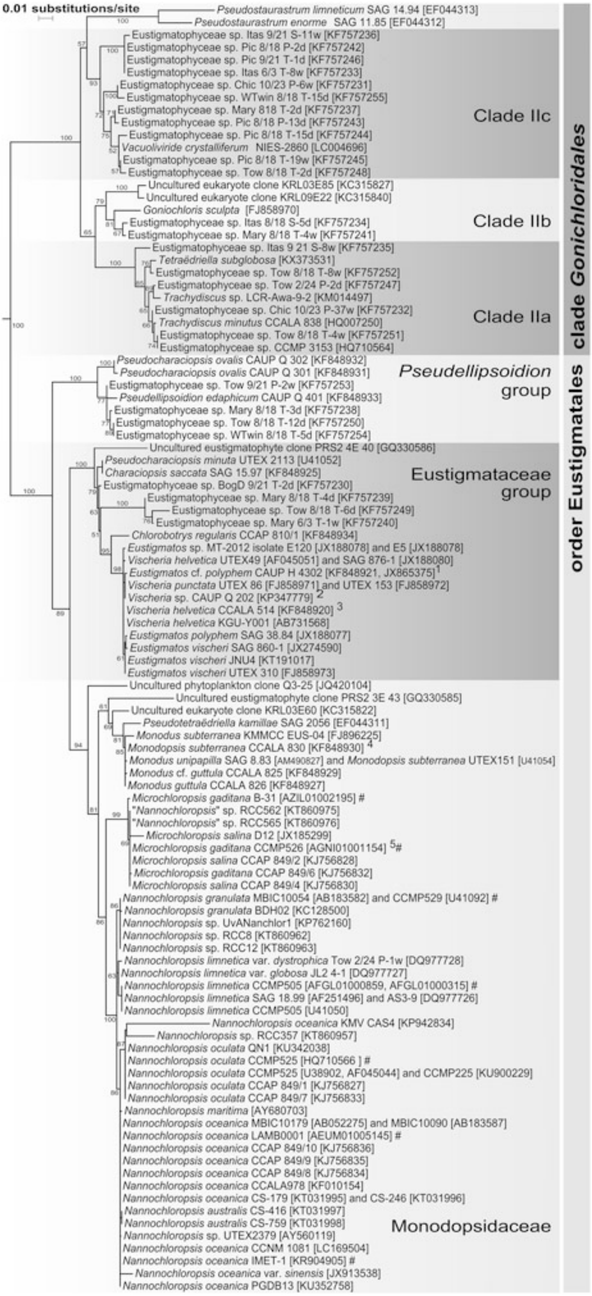

Fig. 1 The phylogenetic diversity of Eustigmatophyceae deduced from 18S rRNA gene sequences. The tree was inferred using the maximum likelihood method (RAxML) from an alignment of nearly all available eustigmatophyte 18S rRNA gene sequences plus sequences from selected non-eustigmatophyte ochrophytes used as an outgroup (not shown). The main eustigmatophyte

eustigmatophyte upon its re-isolation from an acidic pool near the shore of Lake Mácha, Czech Republic (Fawley and Fawley 2017).

The only known eustigmatophytes inhabiting marine or brackish habitats belong to the picoplanktonic genera *Nannochloropsis* (including also the freshwater species *N. limnetica*) and *Microchloropsis*. These species can form blooms in rock pools and enriched or polluted waters (Ryther 1954; Bourrelly 1958). An extensive bloom of *Microchloropsis* (=*Nannochloropsis*) *gaditana* was reported from brackish Comacchio lagoons in Italy (Andreoli et al. 1999a), whereas *Nannochloropsis granulata* was encountered as a large-scale bloom-causing species in China (in Bohai Sea and Yellow Sea; Zhang et al. 2015).

Eustigmatophytes may also be found in various less usual habitats. Frost et al. (1997) reported an unidentified eustigmatophyte endosymbiont living inside the freshwater sponge *Corvomeyenia everetti*. An unknown eustigmatophyte was detected in a wastewater treatment pond by sequencing an environmental library of the *rbc*L gene (Ghosh and Love 2011). A *Nannochloropsis* species related to *N. limnetica* was reported from a permanently ice-covered lake in Antarctica (Bielewicz et al. 2011). *Trachydiscus minutus* was found to constitute a dominant planktonic alga in a eutrophic cooling pond of a nuclear power plant (Přibyl et al. (2012). A new eustigmatophyte, *Eustigmatos calaminaris*, was recently described from Zn- and Pb-loaded calamine mine spoils (Trzcińska et al. 2014). Finally, *Vacuoliviride crystalliferum* was isolated from green-colored sediment in a bottle of glue (Nakayama et al. 2015).

Characterization and Recognition

General Appearance

Eustigmatophytes are spherical, polyhedral, stellate, ovoid, fusiform, or discoid in shape and vary in size generally between 2 and 25 μm in the longest dimension, although some species may have much larger cells (for example, some *Characiopsis*-like organisms, personal observation). The morphological diversity

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Fig. 1 (continued) lineages are annotated following the scheme proposed by Fawley et al. (2014). The number sign (#) marks strains for which nuclear genome sequence has been reported (see Table 1). Number in superscript at some taxa indicate that taxa (strains) with identical 18S rRNA gene sequences exist that were not included in the figure: ^{1}*Eustigmatos vischeri* CCAP 860/7 [KJ713283]; ^{2}*Chloridella neglecta* SAG 48.84 [KF848924] and *Eustigmatos magna* CCMP387 [U41051]; ^{3}*Eustigmatos polyphem* CAUP Q 102 [KF848922], *Vischeria stellata* SAG 33.83 [KF848919], *Chloridella simplex* CCALA 279 [KF848923], and "*Ophiocytium majus*" CCAP 855/1 [AM490835]; ^{4}*Monodus guttula* CCALA 828 [KF848928], *Monodus* sp. CAUP D 901 [KF848926], and *Monodopsis* sp. MarTras21 [KP347780]; ^{5}*Microchloropsis gaditana* MBIC10418 [AB052271], MBIC10063 [AB183586], CCMP527 [AFGN01000274] #, *Microchloropsis salina* CCMP537 [AF045049] #, and CCMP1776 [AFGQ01000729, AFGQ01000649] #

of eustigmatophytes is documented in Fig. 2. Most vegetative cells are free floating, but *Pseudocharaciopsis* spp. and other *Characiopsis*-like algae may normally or occasionally be attached. Eustigmatophytes form green or yellow-green cultures, so some may be confused with coccoid members of the Xanthophyceae or Chlorophyta (green algae). However, eustigmatophytes can be distinguished by the presence of a red-orange pigmented body in the cytoplasm that is especially prominent in older vegetative cells. This lipidic body also has a characteristic yellow fluorescence when excited with ultraviolet light (Přibyl et al. 2012). Some eustigmatophytes also have highly sculpted cell walls (Santos and Santos 2001; Přibyl et al. 2012; Fawley et al. 2014; Fawley and Fawley 2017), although careful examination is required to detect the sculpting on small cells. Because many xanthophytes were described before the recognition of the distinctions between the two classes, some taxa currently classified as Xanthophyceae will likely be reassigned to the Eustigmatophyceae upon more detailed study.

Vegetative Cell Structure

Careful light and, for most species, electron microscopical observations of eustigmatophyte vegetative cells (Hibberd and Leedale 1972; Lee and Bold 1973; Hibberd 1974; Preisig and Wilhelm 1989; Santos and Leedale 1995; Schnepf et al. 1996; Neustupa and Němcová 2001; Hegewald et al. 2007; Přibyl et al. 2012; Nakayama et al. 2015) have revealed key morphological and ultrastructural characteristics for the whole group, as well as features specific to particular taxa. Most eustigmatophytes contain a single-lobed parietal plastid (Fig. 2); however, multiple plastids have been observed in *Pseudellipsoidion* (Neustupa and Němcová 2001), *Pseudocharaciopsis ovalis* (Hibberd 1981), and *Pseudotetraëdriella* (Hegewald et al. 2007). The plastid lamellae are evenly spaced (Fig. 3a–c) and are composed of three unappressed thylakoids. Additional thylakoids commonly appear between some of the lamellae, particularly at the edge of the plastid, forming granum-like stacks. The plastids do not contain a girdle lamella, and the longitudinal lamellae terminate close to the plastid envelope (Fig. 3a–c). The plastid envelope consists of four membranes with the outermost representing the plastid endoplasmic reticulum (PER). Connection of the PER with the nuclear envelope, otherwise a general characteristic of ochrophyte algae, has been reported only from the genera *Nannochloropsis* (incl. *Microchloropsis*) and *Monodopsis* (Antia et al. 1975; Santos and Leedale 1995; Karlson et al. 1996; Krienitz et al. 2000; Suda et al. 2002).

Pyrenoids are present in vegetative cells of several species, but never in the zoospores. In some eustigmatophytes, the pyrenoid is polyhedral (*Vischeria* spp., *Eustigmatos* spp., *Chlorobotrys regularis*) or globular (*Pseudocharaciopsis minuta*) and separated from the plastid itself by a narrow stalk (Fig. 3b), whereas the genera *Monodopsis* and *Vacuoliviride* form a bulging pyrenoid without a stalk (Santos and Leedale 1995; Nakayama et al. 2015). A pyrenoid has been reported for two species of the genus *Nannochloropsis* (Antia et al. 1975), but its presence could not be confirmed by subsequent studies (Santos 1996). The organism studied by

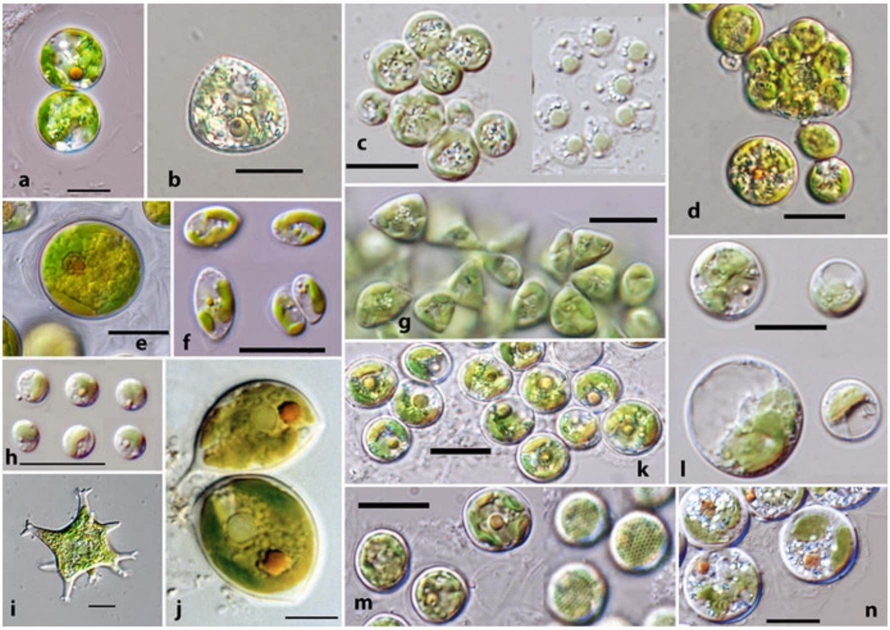

Fig. 2 Light micrographs of representative Eustigmatophyceae. (**a**) *Chlorybotrys* sp. UP3 5/31-7m (Eustigmataceae). (**b**) *Goniochloris sculpta* SAG 29.96 (Clade IIb). (**c**) Unidentified strain Mary 8/18 T-4d (Clade Ia); vegetative cells (*left*) and zoospores (*right*). (**d**) *Pseudellipsoidion edaphicum* CAUP Q 401 (*Pseudellipsoidion* group). (**e**) *Eustigmatos polyphem* (Eustigmataceae group). (**f**) *Monodus unipapilla* Skal1 4/27-2w (Monodopsidaceae). (**g**) Unidentified strain Itas 8/18 S-5d (Clade IIb). (**h**) *Nannochloropsis limnetica* CCMP 2271 (Monodopsidaceae). (**i**) *Pseudostaurastrum* sp. strain 10174 (*Goniochloridales*). (**j**) *Characiopsis acuta* ACOI 456 (Eustigmataceae group). (**k**) Unidentified strain Pic 8/18 T-15d (Clade IIc). (**l**) Unidentified strain Pic 9/21 T-1d (Clade IIc). (**m**) Unidentified strain Chic 10/23 P-37 (Clade IIa), illustrating wall sculpting (*right*). (**n**) Unidentified strain WTwin 8/18 T-15d (Clade IIc). *Bars* = 10 μm

Mohammady et al. (2004) under the name *Nannochloropsis salina* and exhibiting a prominent pyrenoid with a starch envelope is undoubtedly a green alga. The pyrenoid matrix of eustigmatophytes appears finely granular or homogeneous and is not penetrated by either plastid lamellae or individual thylakoids (Fig. 3b). The plates of refractive material that can be seen lying against the faces of the pyrenoids in the light microscope are represented in sections by flattened vesicles containing material that appears very finely lamellate after fixation. Smaller vesicles with apparently identical contents, called lamellate vesicles or refractile granules, also lie freely in the cytoplasm of both the vegetative cells and zoospores (Fig. 3g). These highly characteristic structures of all eustigmatophytes (Santos 1996) probably represent a storage material in the form of a β-1-3-linked polysaccharide (Schnepf et al. 1996).

Mitochondria contain tubular cristae as in other stramenopiles (Fig. 3c, f). A mitochondrion-dividing ring reminiscent of those known from the red alga

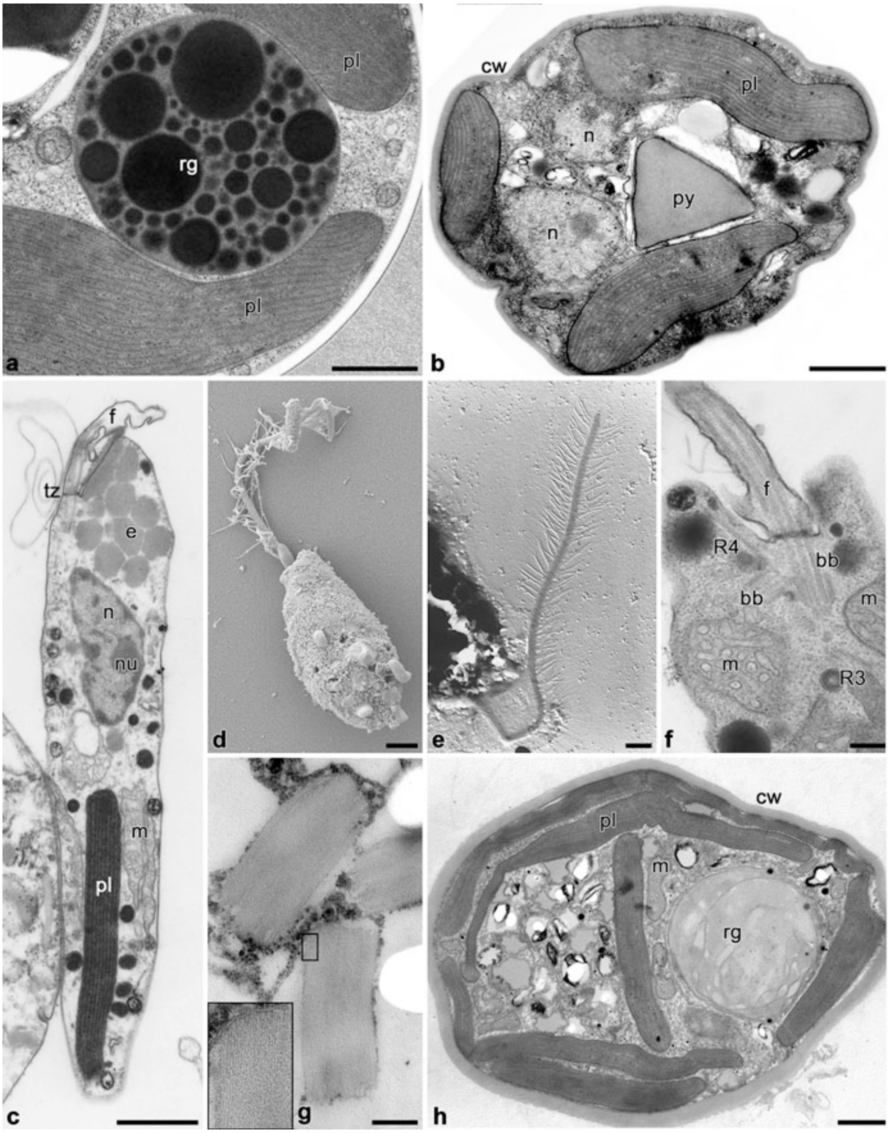

Fig. 3 Cell ultrastructure in the Eustigmatophyceae. (**a**) Detail of a plastid (without the girdle lamella) and a reddish globule of *Vischeria helvetica* (*pl* plastid, *rg* reddish globule). (**b**) Section of a vegetative cell of *Eustigmatos magna*, presumably on a way to cytokinesis (note the two nuclei; *cw* cell wall, *n* nucleus, *p* pyrenoid, *pl* plastid). (**c**) Zoospore of *Vischeria helvetica* (*e* eyespot, *f* flagellum, *m* mitochondrion, *n* nucleus, *nu* nucleolus, *pl* plastid, *tz* transitional zone of the flagellum). (**d**) Zoospore of *Trachydiscus minutus* in a scanning electron microscope. (**e**) Detail of the flagellum bearing mastigonemes, *Vischeria stellata*. (**f**) Longitudinal section of the flagellar apparatus of *Vischeria stellata* uniflagellate zoospore showing basal bodies with R3 and R4 flagellar roots; R1 and R2 are not visible on the section (*f* flagellum, *m* mitochondrion, *R3* flagellar root 3, *R4* flagellar root 4). (**g**) Lamellate vesicles containing material that appears very finely lamellate after

Cyanidioschyzon merolae has been described in the eustigmatophyte *Nannochloropsis oculata* (Hashimoto 2004). The nucleus is more or less spherical (Fig. 3b) but is relatively inconspicuous in the light microscope and can usually be clearly identified only in younger cells. Much more conspicuous is a vacuole with granular contents exhibiting Brownian movement. A highly characteristic structure of most eustigmatophytes, especially of older cells, is the so-called reddish globule (e.g., Fig. 2a, b, j, n). Its color varies from pale yellow-brown to dark red-brown and becomes larger and darker with age. In some species, the reddish globule is composed of a number of smaller droplets (Fig. 3a), but it is homogeneous in *Chlorobotrys regularis* (Hibberd 1974) or with a core less electron dense than the rest of the globule in *Vacuoliviride crystalliferum* (Nakayama et al. 2015). Prior to reproduction, the reddish globule does not divide but is completely inherited by one of the daughter autospores (Neustupa and Němcová 2001). A unique, rod- or V-shaped crystalline structure associated with the reddish globule was observed in *Vacuoliviride crystalliferum* (Nakayama et al. 2015).

The cell wall of eustigmatophytes is in one piece (Fig. 3b, h), but more than one layer can be seen, especially in older cells. The cell wall is generally smooth, but ornamentation with sculpting is seen in some members of the *Goniochloridales* (Fig. 2b, m; Přibyl et al. 2012; Fawley et al. 2014; Fawley and Fawley 2017). The composition of the eustigmatophyte cell wall has not been thoroughly investigated for most taxa, but it seems to be composed primarily of cellulose (Okuda et al. 2004; Vieler et al. 2012a). Recently, the cell wall of *M. gaditana* was studied in great detail and found to exhibit a bilayer structure consisting of a cellulosic inner wall protected by an outer hydrophobic alganean layer comprising long, straight-chain, saturated aliphatic hydrocarbons with ether cross-links (Scholz et al. 2014). The genus *Chlorobotrys* is unique in having a refractile wall exhibiting a high degree of flexibility and in being surrounded by concentric mucilaginous layers separated by tripartite membrane-like structures (Fig. 2a), probably composed of pectic materials with very little cellulose (Hibberd 1974). Biomineralization of manganese on the stalk surface was observed in *Pseudocharaciopsis minuta* (Wujek 2012).

Zoospore Structure

Eustigmatophyte genera, with the exception of *Nannochloropsis*, *Microchloropsis*, *Monodopsis*, *Chlorobotrys*, *Vacuoliviride*, and *Tetraëdriella*, are known to produce naked, somewhat amoeboid, oval, or lageniform (flask-shaped) zoospores, with one or two subapically inserted flagella (Figs. 2c and 3c–f). The zoospores generally harbor a single anteriorly positioned nucleus, a single plastid always without a pyrenoid, one or more mitochondria, a number of vesicles with lamellate or spiral

Fig. 3 (continued) fixation (enlarged lower-left figure), *Pseudocharaciopsis ovalis*. (**h**) Section of a vegetative cell of *Pseudocharaciopsis ovalis* (*cw* cell wall, *pl* plastid, *m* mitochondrion, *rg* reddish globule). (**a**–**e**): bar = 1 μm; (**f**–**g**): bar = 0.2 μm; (**h**): bar = 1 μm content, and a Golgi body (Hibberd and Leedale 1972; Lee and Bold 1973; Preisig and Wilhelm 1989; Santos and Leedale 1992; Schnepf et al. 1996).

The zoospores of some eustigmatophytes bear just one emergent flagellum (Fig. 3c–f), but at least in those species studied by electron microscopy a second bare kinetosome (basal body) lies closely associated with the one subtending the flagellum. A pair of unequal flagella was found in the zoospores of *Pseudocharaciopsis* spp. (Lee and Bold 1973; Hibberd 1981; Neustupa and Němcová 2001) and *Botryochloropsis similis* (Preisig and Wilhelm 1989), where the posterior flagellum is shorter, very narrow, and not readily apparent in the light microscope. Except for a short proximal part, the posterior flagellum exhibits a simplified structure of the axoneme consisting of only two central microtubules. The single flagellum of the unimastigote zoospores and the anterior flagellum in bimastigote zoospores bear tripartite tubular hairs (mastigonemes) of the same type as found in other ochrophytes (Fig. 3d, e) and exhibit a characteristic basal swelling appressed against the anterior region of the cell body containing the eyespot, if present (Hibberd and Leedale 1972; Santos and Leedale 1991; Schnepf et al. 1996).

The eyespot (stigma) is by far the most conspicuous feature of the eustigmatophyte zoospores and is often considered to be characteristic for the group. However, it is reportedly absent from zoospores of all members of the clade *Goniochloridales* studied so far (*Pseudostaurastrum limneticum*, *Goniochloris sculpta*, and *Trachydiscus minutus*; Schnepf et al. 1996; Přibyl et al. 2012) and also from one member of the Eustigmatales (*Pseudotetraëdriella kamillae*; Hegewald et al. 2007). The eyespot is red-orange in color and its size differs depending on the species (being very large and filling almost the whole of the extreme anterior end of the zoospore in some eustigmatophytes). It lies outside and quite separate from the plastid (Fig. 3c). It is composed of a number of osmiophilic globules of variable size, and neither the eyespot as a whole nor the globules are membrane bound. One large D-shaped droplet lies closely against the cell membrane opposite to the flagellar swelling. The basal swelling/eyespot complex is a probable photoperceptive system with the eyespot serving to enhance contrast. The identity of the actual photoreceptor substance is unknown, but it is thought to emit a green autofluorescence observed in the basal swelling/eyespot region upon excitation with a blue-violet light (Santos et al. 1996).

The transitional region between the basal body and the axoneme consists of a transverse partition and a transitional helix with three to five gyres surrounding the proximal few nanometres of the central pair of the axoneme (Fig. 3c). Flagellar roots have been reconstructed for zoospores of *Vischeria stellata* (Santos and Leedale 1991). Roots R1 and R2 originate on the opposite sides of the flagellum-bearing basal body and run anteriorly supporting the anterior part of the cell associated with the flagellar swelling. Root R3 is attached with dense material between the basal bodies and runs close to the plasma membrane down to the posterior end of the cell, whereas root R4 extends from the bare basal body. The fibrous rhizoplast connects the basal bodies and extends deep into the cell, where it splits into several branches spreading over the nuclear surface (Santos and Leedale 1991).

Reproduction and Life Cycle

Eustigmatophytes reproduce primarily by the formation of autospores (Fig. 2d, f). There may be two D-shaped or four or eight polyhedral autospores per autosporangium; in the more elongate species, the autospores are arranged more or less longitudinally within the parent cell wall before release. Details of cytokinesis in eustigmatophytes have yet to be worked out, but nuclear and plastid division were followed at the ultrastructural level in *Nannochloropsis oculata* (Murakami and Hashimoto 2009). The nucleus divides by closed mitosis, and the inner nuclear envelope constricts and pinches off before the outer nuclear envelope.

An alternative reproduction mode in eustigmatophytes is zoosporogenesis. Some species produce zoospores in relatively small quantities, and the genera *Nannochloropsis*, *Microchloropsis*, *Monodopsis*, *Vacuoliviride*, and *Chlorobotrys* (Hibberd 1974; Antia et al. 1975; Santos 1996; Nakayama et al. 2015) even appear to lack this ability completely. On the other hand, reproduction solely by zoospores was described in *Pseudostaurastrum limneticum* (Schnepf et al. 1996) and *Pseudotetraëdriella kamillae* (Hegewald et al. 2007). Factors inducing zoosporogenesis have not been systematically investigated, but zoospore production in *Trachydiscus minutus* is induced by darkness, suppressed by light, and depends on the temperature (Přibyl et al. 2012). In *Characiopsis*-like eustigmatophytes, zoospores are formed briefly after subculturing old cultures (personal observation). Profound morphological variability in vegetative cell shape, including formation of thick-walled resting cells, was reported as part of the life cycle of *Pseudocharaciopsis ovalis* and *Vischeria* sp. strains (Neustupa and Němcová 2001). Similarly, Fietz et al. (2005) observed thick-walled resting stages in *Nannochloropsis limnetica* germinating by releasing a single cell.

Sexual reproduction is unknown in eustigmatophytes and was suggested to be absent in *Nannochloropsis oceanica* based on evidence from genome sequencing (Pan et al. 2011). However, analyses of the genome sequence of two strains of *Microchloropsis* (=*Nannochloropsis*) *gaditana* unveiled a suite of genes encoding homologs of proteins involved in meiosis, including the meiosis-specific proteins Spo11, Hop1, Hop2, Mnd1, Dmc1, and Msh5 (Radakovits et al. 2012; Corteggiani Carpinelli et al. 2014). This suggests the possible existence of a cryptic sexual cycle in this species.

Genomics, Molecular Biology, and Biochemistry

Knowledge of eustigmatophyte biology at the biochemical and molecular level is heavily biased toward the genus *Nannochloropsis* (sensu *lato*), with very limited information available for other eustigmatophytes. A major change in this field came with the advent of eustigmatophyte genome sequencing, which yielded data now being explored by in silico analyses and inspiring direct experimental research. The latter has been boosted by development of a suite of methods of genome manipulations for different species of *Nannochloropsis* and *Microchloropsis*. Exogenous DNA can now be readily introduced into the cells by electroporation and integrated with high efficiency and specificity into the nuclear genome by homologous recombination with the target region (Chen et al. 2008; Kilian et al. 2011; Radakovits et al. 2012; Vieler et al. 2012a; Li et al. 2014a; Kaye et al. 2015; Iwai et al. 2015). These techniques enable gene knockout, overexpression, or expression of modified or foreign genes in *Nannochloropsis* or *Microchloropsis* genomes. For example, overexpression of an endogenous Δ12 desaturase driven by a stress-inducible promoter led to enhanced deposition of LC-PUFAs in TAG, demonstrating the power of genetic manipulations for improving the biotechnological utility of eustigmatophytes (Kaye et al. 2015). Moog et al. (2015) used predicted localization signals of several authentic proteins of *N. oceanica* to drive tagged variants of green fluorescent protein (GFP) into different compartments of transformed *N. oceanica* cells. This study thus opens up new possibilities for cell biological research of eustigmatophytes. Most recently, CRISPR/Cas9 technology has been successfully applied to *N. oceanica* for targeted genome editing (Wang et al. 2016).

Below, general characteristics of eustigmatophyte nuclear and organellar genomes are briefly reviewed, and examples of interesting insights into the molecular fabric of eustigmatophyte cells enabled by in silico analyses of genome data are discussed. An overview of the most important aspects of the eustigmatophyte metabolism as unveiled in recent years by a combination of in silico and direct experimental approaches is then provided.

Nuclear Genomes

Thanks to the efforts of several research groups utilizing the increasingly available next-generation sequencing technologies, more or less complete nuclear genome sequences have become available for most *Nannochloropsis* species (the only exception being the recently described *Nannochloropsis australis*) and for both *Microchloropsis* species (Table 1). Genome sequences for more multiple strains have even been reported for *Nannochloropsis oceanica* and *Microchloropsis gaditana*. The reported genome size varies from ~25 to ~35 Mbp, and the number of predicted genes ranges from ~6,600 to ~12,000. The differences in these values within the two genera and even between strains of the same species are striking and may partly reflect technical issues stemming from different strategies employed for sequencing, assembly, and annotation of the genomes. These uncertainties notwithstanding, *Nannochloropsis* and *Microchloropsis* species apparently belong to the category of algae with small genomes (for comparison, see, e.g., Kim et al. 2014), with relatively high gene density and low intron density (Radakovits et al. 2012; Vieler et al. 2012a; Corteggiani Carpinelli et al. 2014; Wang et al. 2014). Whether this can be generalized to eustigmatophytes as a whole is presently unknown.

The most complete information about genome organization is available for *M. gaditana* B-31, which was estimated to have 30 chromosomes based on the number of putative telomeric ends identified in the assembly (Corteggiani Carpinelli et al. 2014) and for *N. oceanica* IMET1, which was suggested to have 22 chromosomes using pulsed-field gel electrophoresis (Wang et al. 2014). The telomeric

Table 1 Sequenced nuclear genomes of eustigmatophytes. Note that the species here assigned to the genus *Microchloropsis* are treated as species of the genus *Nannochloropsis* in the respective references and database records

Species	Genome assembly size (Mbp)	Number of predicted genes	References
Nannochloropsis granulata CCMP529	30.1	8,060	Wang et al. (2014)
Nannochloropsis limnetica CCMP505	33.5	?	Xu et al. unpublished (GenBank accession number AFGL00000000.1)
Nannochloropsis oceanica CCMP1779	28.7	11,973	Vieler et al. (2012a)
Nannochloropsis oceanica CCMP531	35.5	7,268	Wang et al. (2014)
Nannochloropsis oceanica IMET1	30.1	9,915	Wang et al. (2014)
Nannochloropsis oceanica LAMB0001	27.6	6,639	Pan et al. (2011)
Nannochloropsis oceanica OZ-1	28.0	?	Xu et al. unpublished (GenBank accession number AFGK01000000.1)
Nannochloropsis oculata CCMP525	34.5	7,254	Wang et al. (2014)
Microchloropsis gaditana B-31	26.3	10,486	Corteggiani Carpinelli et al. (2014)
Microchloropsis gaditana CCMP526	29.0	8,892	Radakovits et al. (2012)
Microchloropsis gaditana CCMP527	25.6	?	Xu et al. unpublished (GenBank accession number AFGN00000000.1)
Microchloropsis salina CCMP537	26.9	6,562	Wang et al. (2014)

repeat of *M. gaditana* B-31 corresponds to the “human-type” motif TTAGGG (Corteggiani Carpinelli et al. 2014), but whether it is common in eustigmatophytes in general is uncertain, because direct testing of the presence of this telomeric sequence in *Eustigmatos polyphem* and *Vischeria punctata* by Southern hybridization failed to confirm this (Fulnečková et al. 2013). Based on the analysis of Pan et al. (2011), *Nannochloropsis oceanica* LAMB0001 is monoploid (haploid), while the ploidy of other eustigmatophytes with sequenced genomes was not investigated closer. An analysis of gene orientation along the *M. gaditana* genome revealed a pattern suggesting frequent deployment of a single bidirectional promoter to control the expression of two neighboring genes with head-to-head orientation (Jinkerson et al. 2013)

Comparative analyses of the gene complements of *Nannochloropsis* and *Microchloropsis* spp. revealed a surprising level of differences. Corteggiani Carpinelli et al. (2014) clustered genes annotated in genomes of two strains of *Microchloropsis (=Nannochloropsis) gaditana* and two strains of *Nannochloropsis oceanica* and found that only ~4600 clusters comprise homologs present in all four strains, although the total number of annotated genes were between ~9000 and ~11,000 in each strain. This was not only because of differences between the two species, as the strains of the same species also differed in the presence/absence of hundreds of genes. An analysis by Wang et al. (2014) including six strains and five *Nannochloropsis-Microchloropsis* species found an even smaller set of ~2700 core genes shared by all the taxa, whereas the pan-genome, i.e., the totality of all clusters of homologous genes and gene singletons in the six strains was a surprising ~38,000 genes. While these numbers are certainly impacted by genome annotation artifacts, it seems well established that there is considerable diversity within the *Nannochloropsis-Microchloropsis* group at the level of gene repertoire (Wang et al. 2014), which is in stark contrast to the low differences between the strains and species in their 18S rRNA gene sequences (Fig. 1). The evolutionary origin and functional significance of this diversity is yet to be worked out.

In addition to these general aspects of the gene content of eustigmatophyte genomes, many crucial insights into the molecular underpinnings of various structures and processes in eustigmatophyte cells have been obtained by in silico analyses of the sequenced genomes. Analyses of different functional gene categories generally show standard sets of genes expected for a unicellular alga, although relative enrichment of some gene categories, including genes related to lipid metabolism, organic acid metabolism, and stress response, was noted (Radakovits et al. 2012; Vieler et al. 2012a; Corteggiani Carpinelli et al. 2014; Wang et al. 2014). Most attention has been paid to investigating genes related to metabolism, which is discussed in a separate section below. Here, some of the more interesting findings concerning other aspects are highlighted.

Above all, genome analyses provided some insights into regulatory and signaling processes in eustigmatophyte cells. For example, several studies addressed the repertoire of transcription factors and found that the MYB family is the dominant group in eustigmatophytes, whereas some families common in many other eukaryotes, e.g., homeobox and MADS-box genes, are missing (Vieler et al. 2012a; Hu et al. 2014; Thiriet-Rupert et al. 2016). Eustigmatophytes proved to possess the core components of the machinery for RNA-mediated silencing, including Argonaute, Dicer, and RNA-dependent RNA polymerase (Corteggiani Carpinelli et al. 2014). This suggests the ability to employ RNA interference as a defense mechanism against parasitic genetic elements (transposons or viruses), but the machinery may also be involved in processing of miRNAs and deploying them for regulation of endogenous gene expression, as putative miRNA genes were identified in the *N. oceanica* genome (Vieler et al. 2012a). Eustigmatophyte genomes also harbor homologs of blue light receptors common in eukaryotes in general (cryptochromes) or specific for ochrophytes (aureochromes), suggesting the ability to sense blue light in the environment (Vieler et al. 2012a; Thiriet-Rupert et al. 2016).

Analyses of the *N. oceanica* genome by Vieler et al. (2012a) also led to a discovery that subsequently proved to be important concerning the evolution and function of mitochondria in eukaryotes. These authors pointed to the fact that the nuclear genome encodes homologs of bacterial MinC and MinD proteins with predicted mitochondrial targeting signals and speculated that these might be novel components of mitochondrial division machinery, given the known function of Min proteins in bacterial cell division. Leger et al. (2015) subsequently showed that not only eustigmatophytes but also a number of other eukaryotic lineages possess a previously unnoticed mitochondrial Min system (comprised of MinC, MinD, and MinE proteins) apparently inherited from the bacterial ancestor of mitochondria and presumably involved in regulating mitochondrial division mediated by the mitochondrial FtsZ protein. This example suggests that eustigmatophytes may prove useful as model organisms for investigating general questions of eukaryotic molecular and cell biology.

Organellar Genomes

In contrast to the nuclear genome sequences so far restricted only to the genera *Nannochloropsis* and *Microchloropsis*, organellar genomes have been surveyed more broadly in eustigmatophytes. Specifically, sequences of both organellar genomes have been published not only for most *Nannochloropsis* species (except *N. australis*) and for both *Microchloropsis* species (Radakovits et al. 2012; Wei et al. 2013; Corteggiani Carpinelli et al. 2014; Starkenburg et al. 2014) but also for three species from different branches of the eustigmatophyte phylogeny: *Monodopsis* sp. MarTras21 (representing a sister lineage of the *Nannochloropsis-Microchloropsis* group), *Vischeria* sp. CAUP Q 202 (representing the more distantly related Eustigmataceae group), and *Trachydiscus minutus* (a representative of the clade *Goniochloridales*) (Ševčíková et al. 2015, 2016; Yurchenko et al. 2016). This sampling allows for inferring not only the general characteristics of eustigmatophyte organellar genomes but also their evolutionary plasticity within the group.

Eustigmatophyte mitochondrial genomes (mitogenomes) are conventional in their architecture (circular-mapping molecules), size (from ~38 to ~46 kbp), and gene content (26–29 tRNA genes, 3 rRNA genes, and 37–40 protein coding genes plus nonconserved ORFs specific for particular eustigmatophyte subgroups) (Starkenburg et al. 2014; Ševčíková et al. 2016). An early study proposed that eustigmatophyte mitochondria use the standard genetic code, in contrast to the mitochondria of superficially similar xanthophytes employing a deviant genetic code with the codon AUA coding for methionine rather than isoleucine (Ehara et al. 1997). This has been corroborated by full genome sequencing, which also revealed the presence of a gene for the Ile-tRNA cognate to the AUA codon in eustigmatophyte mitogenomes (Ševčíková et al. 2016). *Trachydiscus minutus* and members of the *Nannochloropsis-Microchloropsis* lineages independently lost the nucleus-encoded mitochondrion-targeted translation termination factor mRF2 and consequently do not use UGA as a termination codon, but no indication of UGA being reassigned in these taxa as a sense codon (as in mitochondria of many other eukaryotes) was found. An unusual feature of eustigmatophyte mitogenomes is the presence of the *atp1* gene. This is an ancestral condition retained also by non-ochrophyte stramenopiles such as oomycetes, whereas all other ochrophyte classes have lost the mitochondrial *atp1* gene and the Atp1 protein is encoded by a nuclear copy. Eustigmatophyte mitogenomes also uniquely share a truncated *nad11* gene encoding only the C-terminal part of the Nad11 protein, while the N-terminal part is encoded by a separate gene in the nuclear genome. Whereas the gene order of most eustigmatophyte mitogenomes is highly similar, the *Vischeria* sp. CAUP Q 202 genome has been extensively reshuffled, coinciding with the loss of several mitochondrial genes and accelerated evolution of mitochondrial gene sequences in the *Vischeria* lineage (Ševčíková et al. 2016).

All sequenced eustigmatophyte plastid genomes (plastomes) are typical circular-mapping molecules (from ~115 to ~126 kbp in size) with short and long single-copy regions separated by inverted repeats, with the number of genes subsumed to the inverted repeat somewhat differing between the species (Starkenburg et al. 2014; Yurchenko et al. 2016). Their gene content is highly similar (25–28 tRNA genes, 3 rRNA genes, the *ssrA* gene for tmRNA, 124–128 genes coding for typical conserved plastid proteins, and a varying number of nontypical or nonconserved genes) and generally resembles that of other ochrophytes, with several notable exceptions. Firstly, eustigmatophytes plastomes are interesting in that they possess the gene *ycf49*, so far additionally found only in plastomes of cyanidiophyte red algae and the glaucophyte *Cyanophora paradoxa* (Ševčíková et al. 2015). Secondly, the gene for the ClpC protein has been split into three separate genes, encoding the N-terminal domain and the two AAA+ domains as separate polypeptides that presumably assemble into a functional protein (Starkenburg et al. 2014; Ševčíková et al. 2015; Yurchenko et al. 2016). The split of the N-terminal domain is shared with the sole-sequenced plastome of a chrysophyte, *Ochromonas* sp. CCMP1393, supporting the notion that Eustigmatophyceae and Chrysophyceae are related ochrophyte lineages (Ševčíková et al. 2015). Thirdly, the plastomes of *Vischeria* sp. CAUP Q 202 and *Monodopsis* sp. MarTras21 were surprisingly found to harbor a six-gene cluster (inserted between the *ycf54* and *rpl21* genes) acquired from a bacterial donor via horizontal gene transfer (Yurchenko et al. 2016). In silico analyses of these genes revealed that they constitute a novel putative operon, denoted *ebo*, which is quite widespread in bacteria and encodes enzymes of an uncharacterized pathway of secondary metabolism. The significance of the *ebo* operon for eustigmatophyte biology is not yet clear, but its presence in members of two main subgroups of the Eustigmatales indicates it must have been acquired early in eustigmatophyte evolution and secondarily lost in the *Nannochloropsis*-*Microchloropsis* lineage (Yurchenko et al. 2016).

Metabolism

For most eustigmatophyte species, biochemical analyses have been generally restricted to the composition of plastid pigments (Whittle and Casselton 1975a; Preisig and Wilhelm 1989; Santos 1996; Schnepf et al. 1996; Karlson et al. 1996;

Krienitz et al. 2000; Lubián et al. 2000; Suda et al. 2002). The group is unique among ochrophyte algae in that no form of chlorophyll *c* is detectable by HPLC. All species contain β-carotene. Violaxanthin is the major xanthophyll along with vaucheriaxanthin (-ester) and sometimes other minor forms (zeaxanthin, canthaxanthin, astaxanthin), but fucoxanthin, diadinoxanthin/diatoxanthin, or heteroxanthin are not detected. Violaxanthin is both a light-harvesting pigment (Owens et al. 1987; Keşan et al. 2016) and a component of the xanthophyll cycle protecting the photosynthetic apparatus against an excess of light via non-photochemical fluorescence quenching (Lubián and Montero 1998; Gentile and Blanch 2001; Bína et al. 2017). The major light-harvesting antenna of eustigmatophytes, homologous to the better-known diatom FCP (Fucoxanthin Chlorophyll Protein), is accordingly called VCP (Viola−/Vaucheriaxanthin Chlorophyll Protein) (Sukenik et al. 2000; Carbonera et al. 2014; Litvín et al. 2016). Very recently, the molecular architecture and subunit composition of the photosystem I (PSI) supercomplex were reported for two species of the *Nannochloropsis*-*Microchloropsis* group, revealing unprecedented features of the PSI antenna complexes (Basso et al. 2014; Alboresi et al. 2017; Bína et al. 2016).

Carbon metabolism in eustigmatophytes appears fairly standard and includes glycolysis and gluconeogenesis, the Krebs cycle, oxidative and reductive pentose phosphate pathway, as well as the glyoxylate cycle (Radakovits et al. 2012; Vieler et al. 2012a). Analyses of the predicted gene complements in *M. gaditana* and *N. oceanica* suggested the operation of several carbon-concentration mechanisms in these algae that would enable both C_3- and C_4-type carbon assimilation (Radakovits et al. 2012; Vieler et al. 2012a). Genes for enzymes of polysaccharide metabolism were also annotated in eustigmatophyte genomes and include those for biosynthesis and degradation of both the main cell wall component, i.e., cellulose, and the main storage polysaccharide, i.e., a β-1,3-glucan (chrysolaminarin) (Vieler et al. 2012a; Corteggiani Carpinelli et al. 2014; Scholz et al. 2014). Genes predicted to encode enzymes responsible for the synthesis of sulfated fucans were also identified, suggesting that like in some other ochrophytes, these polysaccharides may also be present in the eustigmatophyte cell wall (Corteggiani Carpinelli et al. 2014). Precursors for isoprenoid biosynthesis are formed solely by the plastid-located non-mevalonate (DOXP) pathway, as no homologs of enzymes of the cytosolic mevalonate pathway were found in the *M. gaditana* genome (Radakovits et al. 2012). Several vitamin B12-dependent enzymes were found to be encoded by the *M. gaditana* genome, suggesting that vitamin B12 may be beneficial or even essential for eustigmatophyte growth under some conditions (Jinkerson et al. 2013).

A new frontier in eustigmatophyte research was defined by the recent identification of phytohormones, specifically abscisic acid (ABA), cytokinins (CKs), and gibberellin (GA), in *Nannochloropsis oceanica* (Lu et al. 2014; Lu and Xu 2015). The *N. oceanica* genomes encode homologs of enzymes mediating the synthesis of ABA and CKs in plants, and the pathways of ABA and CK synthesis are transcriptionally up- and downregulated, respectively, upon nitrogen depletion.

CKs stimulate cell cycle progression in *N. oceanica* whereas ABA acts as a growth repressor, indicating an antagonistic role of the two regulators in response to nitrogen deprivation. Like some other algae and many anaerobic non-photosynthetic protists, *Nannochloropsis* and *Microchloropsis* have genes encoding the enzyme [FeFe]-hydrogenase (*hydA*) as well as factors involved in hydrogenase maturation (*hydE*, *hydF*, and *hydG*; Radakovits et al. 2012; Vieler et al. 2012a). In agreement with these *in silico* insights, *N. oceanica* was found to produce H_2 when grown at anaerobic conditions and supplied with an abiotic electron donor (Vieler et al. 2012a), but the actual physiological role of hydrogenase in eustigmatophytes remains unknown.

Of all metabolic pathways in eustigmatophytes, the most attractive for researchers have been those concerning the synthesis and degradation of fatty acids and lipids. The significance of these metabolic processes in eustigmatophytes is immediately apparent from the fact that the complement of genes encoding enzymes of lipid metabolism is markedly expanded in *Nannochloropsis* and *Microchloropsis* genomes compared to other algae (Radakovits et al. 2012; Wang et al. 2014). Multiple paralogs are found for many of the enzymes, and phylogenetic analyses suggested that the expansion could have partly originated from acquisition of new genes by horizontal gene transfer (Wang et al. 2014).

This genetic constitution underpins the long-known ability of eustigmatophytes to accumulate large amounts of neutral lipids, i.e., forms of TAG with varying profiles of esterified fatty acids (Ma et al. 2016). TAG accumulates in lipid droplets in eustigmatophyte cells. In *Nannochloropsis* sp., an abundant hydrophobic lipid droplet surface protein (LDSP) was characterized (Vieler et al. 2012b). It is unique in its primary sequence but is structurally similar to other lipid-droplet-associated proteins (oleosins) from other organisms. Physiological experiments established stress factors as the main trigger for TAG accumulation, with TAG accumulation serving as a carbon sink under conditions limiting cell growth. Nitrogen limitation stands out as the most effective factor. Recent studies provided a detailed view of changes in gene expression and the activity of different biochemical pathways leading to an increased TAG production upon nitrogen depletion (Li et al. 2014b; Meng et al. 2015). High light intensity also stimulates TAG production, and the molecular mechanism of this phenomenon was recently studied in fine detail using a combination of transcriptomic, lipidomic, and metabolomic approaches (Alboresi et al. 2016).

Not only the production of TAG as such but also certain features of the metabolism of fatty acids make eustigmatophytes highly attractive for biotechnological exploitation. This concerns primarily the ability to synthesize substantial amounts of nutritionally valuable LC-PUFAs. Of these, the most important is EPA (C20:5 n-3), which is found not only in the highly studied *Nannochloropsis*-*Microchloropsis* group but seems to be abundant in eustigmatophytes in general (Cohen 1994; Volkman et al. 1999; Řezanka et al. 2010; Ma et al. 2016). The actual EPA content in eustigmatophyte cells varies considerably depending on environmental conditions such as nutrient status, salinity, light intensity, or temperature (Sukenik 1991, Cohen

1994, Lu et al. 2001, Hoshida et al. 2005, Khozin-Goldberg and Cohen 2006, Pal et al. 2011). EPA is mostly present in the membrane lipids (glyco- and phospholipids) (Cohen 1994; Khozin-Goldberg and Cohen 2006; Vieler et al. 2012a; Ma et al. 2016), but in *T. minutus*, its considerable amounts were identified in TAG as well (Řezanka et al. 2011). An analysis of the *N. oceanica* genome revealed a complete set of genes encoding membrane-bound ER-localized desaturases, namely, putative Δ9-, Δ12-, Δ6-, Δ5-, and ω3-desaturases, which implies the synthesis of EPA outside of the plastid and its import into the plastid for glycolipid synthesis (Vieler et al. 2012a). Arachidonic acid (C20:4 n-6) is also present in smaller quantities in at least some eustigmatophytes (Řezanka et al. 2014). The fatty acid profile of members of the *Vischeria/Eustigmatos* group proved to be unusual due to a high concentration of long-chain hydroxy fatty acids (Volkman et al. 1999).

Systematics

Although eustigmatophytes were sometimes considered as a separate division or phylum Eustigmatophyta (Hibberd 1981, 1990; Ettl and Gärtner 1995; John 2011) or as a taxon (named Eustigmatales) with no explicitly assigned taxonomic rank (Adl et al. 2012), most often they have been treated as the class Eustigmatophyceae within the broadly defined phylum (division) Ochrophyta (Heterokontophyta) (Santos and Leedale 1991; Andersen 2004; Cavalier-Smith and Chao 2006; Graham et al. 2008; Yang et al. 2012; Ševčíková et al. 2015). The formal taxonomic scheme for eustigmatophytes established by Hibberd (1981) recognized a single order, Eustigmatales, divided into four families (Eustigmataceae, Chlorobotrydaceae, Pseudocharaciopsidaceae, Monodopsidaceae). Each family was characterized by a unique combination of character states concerning the presence/absence of zoospores, number of flagella, presence/absence of mucilage, and cell shape. One additional family, the Loboceae, was established to accommodate the newly described alga *Pseudotetraëdriella kamillae* (Hegewald et al. 2007).

However, the "one order/five families" system is incongruent with the phylogenetic relationships within the Eustigmatophyceae and cannot accommodate the diversity as revealed by recent sampling that has yielded a large number of uncharacterized or unidentified isolates (Fig. 1). Above all, the traditional classification does not capture the division of eustigmatophytes into two phylogenetically deeply diverged lineages (Fig. 1). The first lineage includes all eustigmatophyte taxa known to Hibberd (1981) and hence can be equated to his order Eustigmatales. The second lineage comprises taxa that were recognized as eustigmatophytes or described only in the past 25 years, so it naturally constitutes a new candidate eustigmatophyte order. However, in order to formally erect the order based on the International Code of Nomenclature for algae, fungi, and plants (Melbourne Code; http://www.iapt-taxon.org/nomen/main.php), the family-level classification of this group needs to be resolved. Hence, some of us employed the International Code of

Phylogenetic Nomenclature (PhyloCode; https://www.ohio.edu/phylocode/index.html) as an alternative to describe the second principal eustigmatophyte group as the clade *Goniochloridales* (Fawley et al. 2014).

A comprehensive classification of the two main eustigmatophyte groups that would be consistent with the phylogenetic relationships as revealed by molecular phylogenetic analyses is yet to be worked out. Some of the existing families and genera have proven to be para- or polyphyletic, and taxa need to be established to accommodate some newly recognized or described lineages. However, extensive revisions of many taxa are hampered by the lack of cultures corresponding to type species. As mentioned above, further work is also required to resolve the concept of families in the *Goniochloridales.* One to several separate families are conceivable based on the current picture of the phylogenetic diversity of the group (Fig. 1). In the Eustigmatales, three main lineages are apparent that can perhaps be conveniently recognized as three families. One of them fits the delimitation of the family Monodopsidaceae, provided that the family Loboceae, proposed to accommodate *P. kamillae* (Hegewald et al. 2007), is abandoned to avoid paraphyly of Monodopsidaceae. In fact, the formal description of Loboceae is invalid according to the International Code of Nomenclature for algae, fungi, and plants, as the name is not typified by a genus name. The current circumscription of the families Eustigmataceae, Chlorobotrydaceae, and Pseudocharaciopsidaceae appears to be too narrow from the phylogenetic point of view (Fig. 1). They may possibly be merged into a single monophyletic family characterized by the presence of a bulging pyrenoid connected to the plastid with a narrow stipe. On the other hand, a new family needs to be established for a clade comprising *Pseudellipsoidion edaphicum* and *Pseudocharaciopsis ovalis* together constituting a strongly supported third monophyletic clade of Eustigmatales (Fig. 1). To facilitate communication about the eustigmatophyte phylogenetic diversity before formal taxonomic revisions of the eustigmatophyte families are completed, informal names were proposed for different (presumably) monophyletic subgroups of both Eustigmatales and *Goniochloridales* (Fawley et al. 2014). For simplicity, these informal groups are only indicated in Fig. 1 and are not discussed further.

The following overview of eustigmatophyte classification includes all genera and species previously assigned to this group on the basis of ultrastructural, biochemical, and/or molecular evidence.

Order Eustigmatales

Genus *Eustigmatos* D.J. Hibberd 1981

Type species: *Eustigmatos vischeri* D.J. Hibberd

Very similar to *Vischeria* but the cell wall is always smooth and featureless. Four species (*E. vischeri*, *E. magnus*, *E. polyphem*, *E. calaminaris*) very closely related to or intermixed with *Vischeria* spp. in molecular trees (Fig. 1), suggesting that the genus *Eustigmatos* should be merged with the genus *Vischeria*.

Genus *Vischeria* Pascher 1938

Type species: *Vischeria stellata* (Chodat) Pascher (basionym: *Chlorobotrys stellata* Chodat).

The cells isodiametric, the zoospores elongate lageniform, with a single emergent flagellum, the cell wall typically raised into projections or ridges. Three species (*V. stellata*, *V. punctata*, *V. helvetica*) studied in detail and confirmed as eustigmatophytes (Hibberd 1981), nine other species described by Pascher (1939) are yet to be reinvestigated.

Genus *Chlorobotrys* Bohlin 1901

Type species: *Chlorobotrys regularis* (West) Bohlin (basionym: *Chlorococcum regulare* West).

The cells occur in pairs or colonies, surrounded by lamellate mucilage. Zoospores not observed. One species, *C. regularis*, was confirmed as a eustigmatophyte (Hibberd 1974); several other described species (Ettl 1978) are yet to be studied in detail.

Genus *Pseudocharaciopsis* K.W. Lee and H.C. Bold 1974

Type species: *Pseudocharaciopsis texensis* K.W. Lee & Bold, considered a junior synonym of *Pseudocharaciopsis minuta* (A.Braun) Hibberd (basionym: *Characium minutum* A.Braun ex Kützing)

The cells ovoid/ellipsoidal and capable of producing a stipe. The zoospores with two emergent flagella. 18S rRNA gene sequences determined for the two *Pseudocharaciopsis* species (*P. minuta* and *P. ovalis*) indicate that this genus is polyphyletic (Fig. 1). In addition, a strain identified as *Characiopsis saccata*, hence representing the genus *Characiopsis* currently classified in the class Xanthophyceae, is closely related to *P. minuta* (Fig. 1). The taxonomy of the genera *Pseudocharaciopsis* and *Characiopsis* is thus in an urgent need of revision (see also below).

Genus *Monodopsis* D.J. Hibberd 1981

Type species: *Monodopsis subterranea* (J.B. Petersen) D.J. Hibberd (basionym: *Monodus subterranea* J.B. Petersen)

Unicellular forms with spherical, ovoid, ellipsoid, or cylindrical cells, 5–10 μm in diameter. Zoospores not observed. One validly described species (*M. subterranea*). The combination *Monodopsis unipapilla* was mentioned in the literature (Santos and Leedale 1995; Santos 1996) but not yet validly published, for a species originally known as *Monodus unipapilla* and closely related to *M. subterranea* (Fig. 1). Other species of the traditionally xanthophyte genus *Monodus* may need reclassification to *Monodopsis*. One such candidate is *Monodus guttula*, nominally represented by two strains with sequenced 18S rRNA gene (Fig. 1) whose identification yet need to be critically examined.

Genus *Pseudotetraëdriella* E. Hegewald 2007

Type species: *Pseudotetraëdriella kamillae* E. Hegewald & J. Padisák

The vegetative cells with four lobes, zoospores elongate-ovate with one emergent flagellum and without an eyespot, globular resting spores. So far monotypic.

Genus *Nannochloropsis* D.J. Hibberd 1981

Type species: *Nannochloropsis oculata* (Droop) D.J. Hibberd (basionym: *Nannochloris oculata* Droop)

The cells spherical, ovoid, ellipsoid, or cylindrical, <5 μm in maximum dimension. Zoospores not observed. Previously included two species now classified in the separate genus *Microchloropsis* (see below). The five formally described species occur in marine habitats (*N. oculata*, *N. granulata*, *N. oceanica*, *N. australis*) or in freshwater (*N. limnetica* with several varieties described; Fawley and Fawley 2007). One more species, "*Nannochloropsis maritima*", appeared recently in the literature (Hu et al. 2013) and is represented by an 18S rDNA sequence in GenBank (accession number AY680703), but it has not been formally described.

Genus *Microchloropsis* M.W. Fawley, I. Jameson & K.P. Fawley 2015

Type species: *Microchloropsis salina* (D.J. Hibbard) M.W. Fawley, I. Jameson & K.P. Fawley (basionym: *Nannochloropsis salina* D.J. Hibberd)

Cells small (2–8 μm), cylindrical or rarely spherical with a single parietal chloroplast. Pyrenoids absent. Some cells with an orange or red lipid body free in the cytoplasm. Refractive granules or short rods usually present in the cytoplasm. Reproduction by autospore production. Young autospores usually hemispherical but become cylindrical or spherical as they mature. Two described species (*M. salina* and *M. gaditana*).

Genus *Pseudellipsoidion* J. Neustupa and Y. Němcová 2001

Type species: *Pseudellipsoidion edaphicum* J. Neustupa & Y. Němcová

The vegetative cells with ellipsoidal and globular morphology, zoospores of irregular or oval shape with one emergent flagellum. Pyrenoid absent. So far monotypic.

Clade *Goniochloridales*

Genus *Goniochloris* Geitler 1928

Type species: *Goniochloris sculpta* Geitler

Vegetative cells dorsoventrally flattened and irregularly triangular in frontal view, with the cell surface sculptured. Reclassification of this genus from the Xanthophyceae to the Eustigmatophyceae is based on the 18S rRNA gene sequence from the type species *G. sculpta* (Fig. 1; Přibyl et al. 2012). The taxonomic assignment of the number of remaining known *Goniochloris* species (Ettl 1978) awaits further studies.

Genus *Pseudostaurastrum* Chodat 1921

Type species: *Pseudostaurastrum enorme* (Ralfs) Chodat (basionym: *Staurastrum enorme* Ralfs)

Tetrahedral or plate-like cells with typically four branched projections. Two species (*P. enorme*, *P. limneticum*) have been studied by modern methods confirming their classification within the Eustigmatophyceae; a few other described species (Ettl 1978) are very likely related given the highly characteristic morphology.

Genus *Trachydiscus* H. Ettl 1964

Type species: *Trachydiscus lenticularis* H. Ettl

Disc-shaped cells with the cell surface sculptured by numerous cell wall protuberances (warts, bulges, or papillae). Reclassification of this genus from the Xanthophyceae to the Eustigmatophyceae is based on a cytological, biochemical, and molecular genetic evidence for the species *Trachydiscus minutus* (Fig. 1; Přibyl et al. 2012). However, the additional six known *Trachydiscus* species, including the type species, are morphologically rather different from *T. minutus*, so it must yet to be confirmed whether they (and hence formally the genus *Trachydiscus* as such) belong to the Eustigmatophyceae and specifically to the *Goniochloridales*.

Genus *Tetraëdriella* Pascher 1930

Type species: *Tetraëdriella acuta* Pascher

Cells pyramidal or tetragonal with walls ornamented by regularly arranged rows of depressions. The genus *Tetraëdriella* is traditionally classified in Xanthophyceae (Ettl 1978) but is here included in eustigmatophytes, specifically as a member of Goniochloridales, based on the recent reinvestigation of the species *Tetraëdriella subglobosa* including evidence from its 18S rRNA gene sequence (Fawley and Fawley 2017; see also Fig. 1). The morphological features of other *Tetraëdriella* species, including the types species, suggest that they are related to *T. subglobosa.* Ultrastructural and molecular evidence for eustigmatophyte affinity of several other *Tetraëdriella* species was presented at a conference (Santos and Santos 2001) or mentioned in the literature (Ott et al. 2015), but the actual data are yet to be published.

Genus *Vacuoliviride* T. Nakayama, T. Nakamura, A. Yokoyama, T. Shiratori, I. Inouye & K.-I. Ishida 2015

Type species: *Vacuoliviride crystalliferum* T. Nakayama, T. Nakamura, A. Yokoyama, T. Shiratori, I. Inouye, and K.-I. Ishida

Vegetative cells solitary, nonmotile, spherical to ellipsoidal, 6–30 μm in diameter, and covered by smooth cell wall. Cells include refractile granules, a large vacuole, and a reddish globule, frequently with a rod- to V-shaped crystalline structure, one to several greenish chloroplasts possessing bulging pyrenoid with longitudinal slit. Cells reproduce by 2–8 autospores. So far monotypic.

Eustigmatophyceae *Incertae sedis*

Genus *Botryochloropsis* H.R. Preisig and C. Wilhelm 1989

Type species: *Botryochloropsis similis* H.R. Preisig and C. Wilhelm

Spherical cells in mucilage aggregated in irregular colonies, zoospores with two emergent flagella. Pyrenoid absent. No molecular data (and no authentic culture) are available for this genus, so its exact position within eustigmatophytes is unknown. So far monotypic.

Additional Eustigmatophytes

The actual diversity of eustigmatophytes is not restricted to the taxa listed above. The many unidentified strains that have been assigned to the Eustigmatophyceae based on their 18S rDNA (Fawley et al. 2014; Fig. 1) and *rbc*L (Prior et al. 2009) sequences illustrate the extent of the hitherto unnoticed diversity of this class. Many of these strains will probably prove to represent new taxa (species and even genera), but it is possible that others can eventually be identified as previously described species after a detailed scrutiny is carried out. Indeed, several algal taxa, traditionally classified in Xanthophyceae, have been occasionally considered as candidate members of Eustigmatophytes. For example, Hibberd (1981) admitted a possibility that *Pleurochloris commutata*, the type species of the genus *Pleurochloris*, may be a eustigmatophyte based on certain morphological features of vegetative cells and zoospores. Interestingly, most of the currently known eustigmatophytes were previously classified as members of the xanthophyte family Pleurochloridaceae comprising a large number of genera and species that have not been studied by modern methods. It is very likely that many additional members of the Pleurochloridaceae will eventually be reclassified to Eustigmatophyceae.

One of the taxa of Pleurochloridaceae with possible eustigmatophyte affinities is the genus *Chloridella* Pascher. 18S rRNA gene sequences were obtained from two strains nominally representing two species, *Chloridella neglecta* (the type species of the genus) and *Chloridella simplex*, showing that both algae belong to the tight *Vischeria/Eustigmatos* cluster (Fawley et al. 2014; Fig. 1). This led Ott et al. (2015) to classify *Chloridella* as a eustigmatophyte genus. However, neither of the two strains is authentic, and their morphology has not yet been properly studied to check the identification as provided in the respective culture collections. Whereas *C. simplex* indeed resembles *Eustigmatos* species (reportedly differing by the lack of zoospores), *C. neglecta* is more reminiscent of *Pleurochloris meiringensis*, a confirmed member of Xanthophyceae (Andreoli et al. 1999b). Thus, classifying *Chloridella* as a eustigmatophyte is premature, and critical revision of this genus is needed to clarify its circumscription and phylogenetic position. A few more genera (e.g., *Gloeobotrys*, *Gloeoskene*, or *Merismogloea*) were listed as candidate eustigmatophytes by Ott et al. (2015) based on the fact that they had been proposed to be synonymous with established eustigmatophytes or that some of their members (but not type species themselves) had been demonstrated to be eustigmatophytes.

Evidence for additional eustigmatophytes was informally presented at conferences, but bona fide publication of the data is still missing. Specifically, ultrastructural and molecular data were obtained from several strains from the ACOI collection identified as different species of the genus *Characiopsis*, indicating their eustigmatophyte nature (Santos and Santos 2001; Amaral et al. 2011, 2015). Indeed, *Characiopsis* was listed as a genus of eustigmatophytes by Ott et al. (2015), but this was based on an assumption that the alga known as *Pseudocharaciopsis minuta* (and belonging to Eustigmatophyceae; Fig. 1) is in fact the type species of *Characiopsis* Borzì. However, as discussed in detail by Hibberd (1981), the type species of the genus may actually be *Characiopsis borziana* Lemmermann, whose phylogenetic position remains unknown. Dashiell and Bailey (2009) announced a new eustigmatophyte genus ("*Microtalis*") with two new species and strains labeled "*Microtalis aquatica* Bailey,JC" and "*Microtalis reticulata* Bailey,JC" that are available from the National Center for Marine Algae and Microbiota (CCMP3153 and CCMP31547, respectively). Sequences of several genes from the former strain were published by Yang et al. (2012) and indicate that "*Microtalis aquatica*" is closely related to *Trachydiscus minutus* and several unidentified strains (Fig. 1). As mentioned above, *T. minutus* morphologically differs significantly from the type species of the genus *Trachydiscus*, so treating the clade including *T. minutus* and *M. aquatica* as a new genus may prove substantiated when characterization of the strains CCMP3153 and CCMP31547 is eventually published.

A few taxa have been assigned to the Eustigmatophyceae in error. *Ophiocytium maius* (strain CCAP 855/1) was suggested to be a eustigmatophyte based on its *coxI* sequence (Ehara et al. 1997). However, it has proven to be a misidentified member of the *Vischeria/Eustigmatos* cluster (Fig. 1); the genuine *O. maius* (strain SAG 855-1) is undoubtedly a xanthophyte (Maistro et al. 2009). Ott and Oldham-Ott (2003) included the genus *Ellipsoidion* in the Eustigmatophyceae, apparently because early reports on eustigmatophytes featured an alga (strain CCAP 822/1) then identified as *Ellipsoidion acuminatum* (Hibberd and Leedale 1970, 1971, 1972). However, Hibberd (1981) reexamined the strain and eventually identified it as *Characiopsis ovalis*, basing his taxonomic revision of the species name to *Pseudocharaciopsis ovalis* (Chodat) Hibberd. Thus, there is at present no evidence that *E. acuminatum* or the type species of the genus *Ellipsoidion*, *E. anulatum* Pascher, are eustigmatophytes; both species and the whole genus thus formally remain in the Xanthophyceae. Molecular data that were published for a single confidently identified *Ellipsoidion* species, the authentic strain of *Ellipsoidion parvum*, showed that this strain is a green alga conspecific with *Neocystis brevis* (Eliáš et al. 2013). Hence, the actual identity and phylogenetic position of the genus *Ellipsoidion* remain highly uncertain.

Maintenance and Cultivation

Eustigmatophytes presently maintained in culture collections were originally isolated using a wide variety of techniques. Single-cell isolation is possible for all the larger forms. Terrestrial and some freshwater species grow well on nutrient agar, and plating may also be used (Prior et al. 2010; Fawley et al. 2014). Agar plate techniques have been used to isolate the many new stains of freshwater Eustigmatophyceae that are presented in Fig. 1. For the minute forms, particularly when occurring in blooms, dilution techniques work well.

Cultures of freshwater and terrestrial species generally grow well in a wide variety of mineral media or in biphasic soil/water cultures where they may survive for many months, even years. Clonal cultures of most strains are available and stocks are most conveniently maintained on nutrient agar slants containing dilute (e.g., Chu No. 10) or rich (e.g., Bold's Basal Medium) media (Nichols 1973). Two of the authors have had success isolating and maintaining Eustigmatophyceae on the high-nutrient medium, WH+ (Fawley et al. 1990; Fawley et al. 2014), and the low-nutrient medium originally designed for chrysophytes, DYIV (Andersen et al. 1997). The ACOI Collection of Algae holds ca. 80 strains of eustigmatophytes (Santos and Santos 2004) that have been kept for 15 years in liquid Desmidiacean medium M7 (for chemical composition, see Schlösser 1994), with a controlled pH of 6.4–6.6. This is a suitable medium for all strains, but relatively slow growth is observed for sensitive genera, namely, *Pseudostaurastrum*, *Tetraëdriella*, *Chlorobotrys*, and *Goniochloris*. A new medium composed of a one tenth dilution of WH+ with 0.1 g^{-1} MES buffer at pH 5.5 (Karen and Marvin Fawley, unpublished) is proving effective for isolating Eustigmatophyceae from acid environments. Marine forms grow easily in standard seawater media such as Erdschreiber or ASP2 (Provasoli et al. 1957), either as standing liquid batch cultures or on agar slants for stock cultures. These forms tolerate a wide range of salinity and a half-normal salinity or even a freshwater medium is usually more convenient for maintenance of stocks. Cryopreservation has been successfully tested for several eustigmatophyte species (Osório et al. 2004; Gwo et al. 2005).

Evolutionary History

There is no fossil record known for eustigmatophytes; hence, reconstruction of their evolutionary origin and diversification has relied solely on comparative analyses of morphological, biochemical, and molecular characters. All these characters firmly place eustigmatophytes into a broader group of ochrophyte (or heterokontophyte) algae, which in turn form a prominent clade within stramenopiles (or heterokonts) (Santos and Leedale 1991; Andersen et al. 1998; Andersen 2004). Phylogenetic analyses of multigene matrices generally indicate that Eustigmatophyceae are a sister group of a clade comprising Chrysophyceae (incl. Synurophyceae) and

Synchromophyceae, altogether forming the group Limnista (Yang et al. 2012; Ševčíková et al. 2015). However, the recent analysis of stramenopile phylogeny based on a 245-protein dataset and including sequences from *Nannochloropsis gaditana* as a representative eustigmatophyte placed this organism closer to Raphidophyceae and the PX clade (Phaeophyceae plus Xanthophyceae), although with unconvincing statistical support (Noguchi et al. 2016). The phylogenetic position of eustigmatophytes among ochrophytes thus needs to be further tested.

Phylogenetic relationships within eustigmatophytes have been investigated primarily with the aid of 18S rRNA gene sequences, which confirmed eustigmatophyte monophyly (Andersen et al. 1998) and revealed the existence of two principal deeply separated subgroups, *Goniochloridales* and Eustigmatales (Fig. 1; Přibyl et al. 2012; Fawley et al. 2014). Relationships within the *Goniochloridales* are not yet clear; however, four clades within this lineage are supported by analysis of the 18S rRNA gene sequence data (Fig. 1). Within the latter subgroup, three major lineages can be recognized (their names here follow the nomenclature introduced by Fawley et al. 2014): Eustigmataceae group, Monodopsidaceae, and *Pseudoellipsoidion* group. Each group is well supported by analyses of 18S rRNA gene sequences, but their relative branching order needs to be established using a higher number of molecular markers. The phylogenetic position of *Botryochloropsis similis* is unknown given the absence of molecular data. However, a characteristic combination of morphological features documented for this species by Preisig and Wilhelm (1989), specifically the presence of an eyespot, zoospores with two flagella, and plastids with no pyrenoid, suggests that *B. similis* possibly belongs to the *Pseudellipsoidion* group.

Even though incomplete, the phylogenetic tree reconstructed for eustigmatophytes enables some inferences about evolutionary trends in this group. First, eustigmatophytes apparently emerged in a freshwater or terrestrial habitat and frequent transitions between these two habitat types seem to have occurred during their evolution, whereas the *Nannochloropsis* lineage secondarily moved to the sea, from which the species *N. limnetica* came back to the freshwater. Second, since an eyespot is altogether absent in all species of the *Goniochloridales* clade investigated so far (Schnepf et al. 1996; Přibyl et al. 2012) as well as in *Pseudotetraëdriella kamillae* (Hegewald et al. 2007), it is actually possible that the characteristic extraplastidial eyespot, regarded as one of the defining features of the whole eustigmatophyte class, arose only after some eustigmatophyte lineages had diverged. Third, zoospores were presumably lost independently in the lineages leading to *Nannochloropsis* and to *Monodopsis*, since they have been retained by *Pseudotetraëdriella kamillae* (Hegewald et al. 2007). Fourth, up to three independent losses of the continuity between the plastid ER and the nuclear envelope can be inferred to have occurred (in the *Goniochloridales*, in the Eustigmatophyceae group, and in the *Pseudellipsoidion* group). Fifth, species with zoospores with a single emergent flagellum (*Vischeria/Eustigmatos* cluster, *Pseudotetraëdriella kamillae*, members of the *Goniochloridales* with zoospore morphology investigated in detail, and potentially also *Pseudellipsoidion edaphicum*) do not form a monophyletic grouping, which indicates multiple independent losses of the posterior flagellum. Altogether, the evolutionary history of eustigmatophytes appears complex and heavily influenced by homoplasy. A more detailed reconstruction remains a task for the future, with the very real possibility of a greatly expanded number of taxa included in the class.

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