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Flavoaurantia, a new genus of the Chlorobotryaceae (Eustigmatales, Eustigmatophyceae) with the single species, *Flavoaurantia irregularis*

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Abstract: An algal strain with the morphological characteristics of the Eustigmatophyceae was isolated from a small pond in Itasca State Park, Minnesota, USA. Phylogenetic analyses of the DNA sequences from the nuclear 18S rDNA and the plastid rbcL loci both placed the strain in the order Eustigmatales and family Chlorobotryaceae. The new strain was not clearly allied with any lineage within the Chlorobotryaceae. Morphological features as determined by both light microscopy and transmission electron microscopy were typical for the Eustigmatales, except for the presence of an irregular surface of the cell wall (including the presence of stipitate or digitate projections on some cells) and the production of yellow–orange inclusions in the cytoplasm of cells in stationary–phase cultures. Upon transfer to fresh media, these inclusions are subsequently expunged from the cells during autosporulation. Based on these features and the results of phylogenetic analysis, we describe *Flavoaurantia irregularis* gen. et sp. nov.

Keywords: Chlorobotryaceae, Eustigmatales, Eustigmatophyceae, new genus, new species, phylogeny

Introduction

The Eustigmatophyceae are a group of ochrophyte algae that inhabit diverse environments such as oceans, eutrophic lakes, peat bogs and soil biotic crusts (Eliáš et al. 2016) and have also been found in the extreme conditions of acidic geothermal sites (Hsieh et al. 2018) and perennially ice–covered lakes in Antarctica (Bielewicz et al. 2011). Many eustigmatophytes are small, nearly spherical, or ellipsoidal organisms, which have few features that distinguish them from other algal classes, such as Xanthophyceae, Trebouxiophyceae and Chlorophyceae, by light microscopy. As a result, research on the diversity of these interesting organisms has been limited until quite recently. However, the Eustigmatophyceae are of interest because of their high content of oil and polyunsaturated fatty acids (Eliáš et al. 2016), and the potential commercial use of these organisms has led to an increase in the study of the diversity and taxonomy of the Eustigmatophyceae.

Early taxonomic studies of the Eustigmatophyceae recognized the single order, Eustigmatales, which included the commercially important genus *Nannochloropsis* (Droop) D.J.Hibberd (now *Nannochloropsis* and *Microchloropsis* M.W.Fawley, I.Jameson et K.P. Fawley; Fawley et al. 2015), and the genera *Vischeria* Pascher (now including *Eustigmatos* D.J.Hibberd; Kryvenda et al. 2018) and *Monodopsis*, D.J.Hibberd (Hibberd 1981; Hibberd & Leedale 1971). The discovery of many new, unnamed eustigmatophyte strains led to the expansion of the Eustigmatophyceae with the ordinal level lineage, Clade *Goniochloridales* (Fawley et al. 2014; Eliáš et al. 2016; Fawley et al. 2021; Přibyl & Procházková 2022), and a new understanding of the diversity within the Eustigmatales (Eliáš et al. 2016; Kryvendra et al. 2018; Fawley et al. 2019; Amaral et al. 2020; Amaral et al. 2021; Barcytė et al. 2022). Initial characterizations of unidentified eustigmatophyte strains and metabarcode surveys targeting the rbcL locus have revealed that there are likely thousands of species among these lineages with many genera awaiting taxonomic studies (Fawley et al. 2021).

Most recently, Barcytė et al. (2022) have revised the taxonomy of the so–called "Eustigmataceae group" (Fawley et al. 2014), and have shown that this lineage

within the Eustigmatales is properly referred to the family Chlorobotryaceae instead of the Eustigmataceae, as established by HIBBERD (1981). The Chlorobotryaceae comprises a number of genera in addition to *Chlorobotrys* Bohlin, including *Vischeria*, *Characiopsis* Borzì and the new genera *Neustupella* Barcytė, Němcová et Eliáš and *Lietzensia* Barcytė et al. (BARCYTĖ et al. 2022).

In this study, we examined an algal strain isolated from a small, slightly eutrophic pond in Itasca State Park, Minnesota, USA. Using DNA sequence analysis, light microscopy and transmission electron microscopy, we determined that this strain represents a new lineage in the Chlorobotryaceae, which we describe as the new genus *Flavoaurantia* with the single species *F. irregularis*.

Materials and Methods

Site description/Strain isolation. Tychoplankton samples were collected from Picnic Pond, a shallow, slightly eutrophic pond in Itasca State Park, Minnesota, USA (47.2403° N, 95.2024° W) on 9 August 2016 (see FAWLEY et al. 2004 for water chemistry information). New Eustigmatophyceae strains were isolated from a sample that was spread on an agar plate of M6.8 medium (FAWLEY & FAWLEY 2017) and incubated at room temperature under continuous cool–white fluorescent light. The strain studied here was designated Pic 8/9 T–4m6.8 and maintained on M6.8 agar.

Phylogenetic analysis. DNA was extracted using the technique of FAWLEY & FAWLEY (2004), which was modified by the addition of a step to disperse any mucilage that might adhere to the cells. In this method, we harvested the cells by centrifugation, added 200 µl of DNA extraction buffer and agitated the suspension with a MiniBeadBeater (BioSpec Products, Inc. Bartlesville, OK, USA) at high speed for 10 to 15 s. We pelleted the cells again by centrifugation and added new buffer and proceeded with the normal method of FAWLEY & FAWLEY (2004). The 18S rRNA gene was amplified using the Polymerase Chain Reaction (PCR) with the primers 18LX and NSIX using PCR conditions described in FAWLEY & FAWLEY (2004). The plastid rbcL gene was amplified with primers Eu–rbcL–F1 and Eu–rbcL–R1 (FAWLEY et al. 2015). DNA sequencing was performed as described in FAWLEY et al. (2015), with sequencing done by Sequetech Corp. (Mountain View, California, USA). Sequence reads were joined using the Staden Package 2.0.0b8 (BONFIELD et al. 1995) and alignments were constructed in MEGA11 (TAMURA et al. 2021). The alignment used for rbcL analysis was that of BARCYTĖ et al. (2022), with the alignment trimmed to the length of the sequence available for Pic 8/9 T–4m6.8. The possible relationship of uncultured eustigmatophytes and Pic 8/9 T–4m6.8 was evaluated by adding the environmental amplicon sequence variants (ASVs) from FAWLEY et al. (2021) to the alignment, after removing the chimeric sequences detected by BARCYTĖ et al. (2022). The 18S rDNA analysis used the full alignment of the Eustigmatophyceae given in FAWLEY et al. (2019), with the addition of sequences from AMARAL et al. (2020; 2021) and BARCYTĖ et al. (2022).

Maximum likelihood phylogenetic analyses were performed with IQ–TREE v.2.2.0 (MINH et al. 2020), using the GTR+F+I+G4 model for rbcL and the TIM2+F+I+G4 model for 18S rDNA, as selected by TreeFinder (KALYAANAMOORTHY et al. 2017) and bootstrap analyses with 500 iterations. The analysis of rbcL data was also performed with the sequence partitioned into the first, second, and third bases of the codons. For the alignment that included the environmental ASVs, the ML analysis was performed with MEGA11 using the GTR model. Phylogenetic trees were visualized with iTOL (LETUNIC & BORK 2021) and finished using Inkscape (Inkscape.org) and Paint.net (getpaint.net).

Light Microscopy. For differential interference contrast (DIC) microscopy, we used a Nikon E600 microscope (Nikon, Melville, New York, USA) equipped with 60× (NA 1.4) and 100× (NA 1.4) objectives. Digital images and measurements were captured with an Olympus SC180 camera system (Olympus, Waltham, Massachusetts, USA) with CellSens imaging software. Strain Pic 8/9 T–4m6.8 was grown in liquid M6.8 medium at 20 °C with illumination of about 20 $\mu M.m^{-2}.sec^{-1}$ and a 14:10 light:dark cycle and typically examined within 10 days of inoculation. Older cultures that produced the yellow–orange color were also examined, as well as the recovery to normal green color that occurred after transfer to fresh medium. We attempted to induce the production of zoospores using the conditions described in FAWLEY et al. (2015) and BARCYTĖ et al. (2022).

Electron Microscopy. For transmission electron microscope observations, samples were fixed for 5 hours at 5 °C in a 2% glutaraldehyde solution in 0.05 $mol.l^{-1}$ phosphate buffer, postfixed for 2 hours at 5 °C in 1% osmium tetroxide in 0.05 $mol.l^{-1}$ phosphate buffer, and postfixed overnight at 5 °C in 1% uranyl acetate in methanol. After dehydration through an ethanol series (70%, 96%, 100%), cells were embedded in Spurr's medium (SPURR 1969) over propylene oxide. Ultrathin sections cut with a diamond knife on an Ultracut E (Reichert–Jung, Wien, Austria), were poststained with lead citrate and examined with a JEOL 1011 TEM (JEOL Ltd., Tokyo, Japan). Photomicrographs were taken using a Veleta CCD camera with image analysis software (Olympus Soft Imaging Solution GmbH).

Morphology of strain Pic 8/9 T–4m6.8 was compared to algae placed in the Xanthophyceae in several references, especially ETTL (1987) and PASCHER (1925, 1930, 1937–1939).

Results

Maximum likelihood phylogenetic analyses of both nuclear 18S rDNA and plastid rbcL sequence data (Figs 1–2) place the strain Pic 8/9 T–4m6.8 in the family Chlorobotryaceae, with strong bootstrap support. The relationship of Pic 8/9 T–4m6.8 to other genera within the Chlorobotryaceae, however, is not clear. Analyses of rbcL data, both without (Fig. 2) and with partitioning (not shown), suggest that our strain is allied with the genus *Lietzensia* (with modest bootstrap support) whereas analysis of 18S rDNA sequence data does not support this relationship. The rbcL sequence of Pic 8/9 T–4m6.8 differs from that of *Lietzensia* by 106 substitutions out of 1398 bases in the alignment, clearly enough to suggest a new genus.

Phylogenetic analysis of the rbcL sequence and the 370 base metabarcoding data from FAWLEY & FAWLEY (2021) indicated that the most similar rbcL haplotype

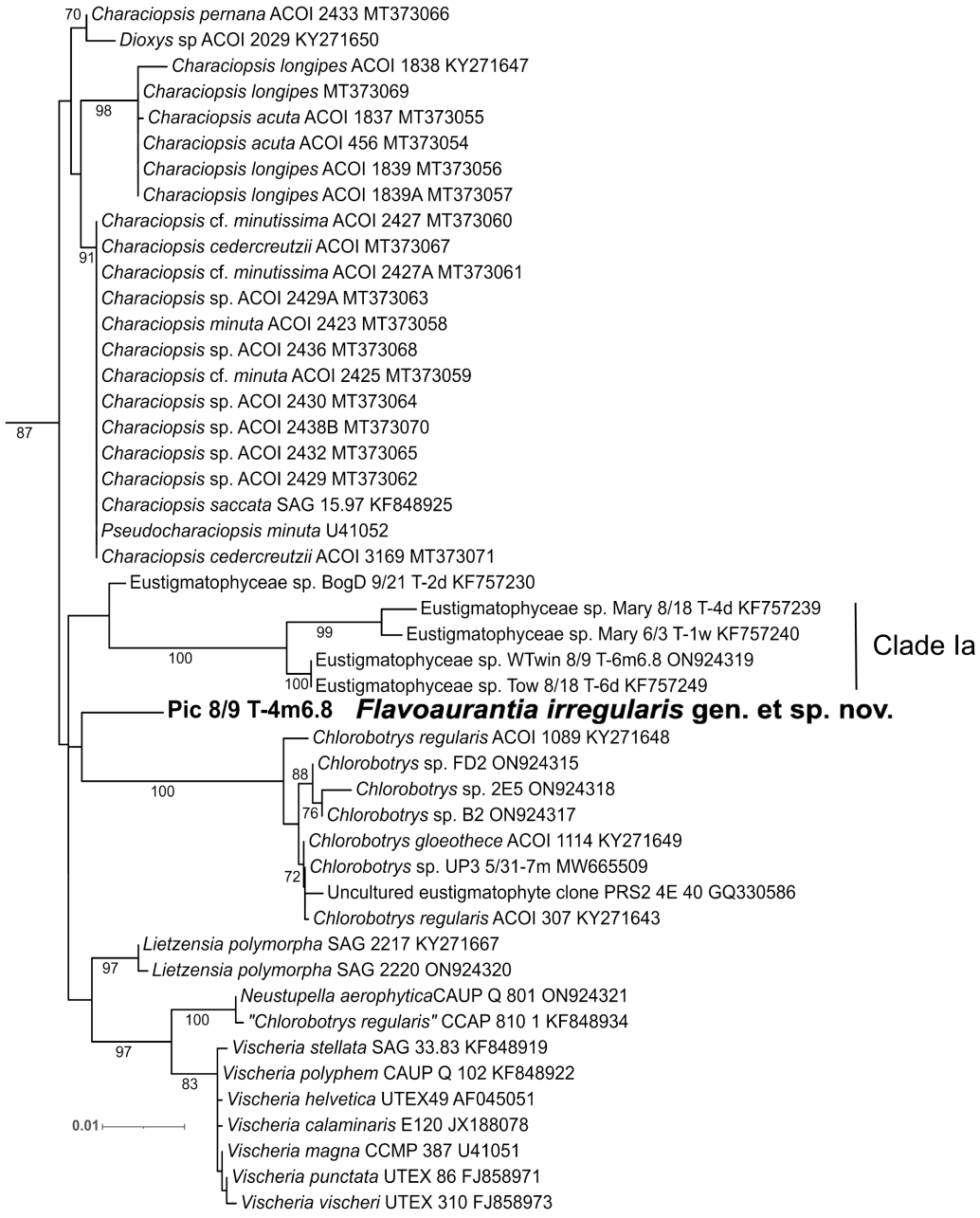


Fig. 1. Results of maximum–likelihood analysis of 18S rDNA sequences, showing only the results for the family Chlorobotryaceae. Bootstrap values (500 iterations) 70 or greater are given below the nodes.

from the environmental samples had 19 differences or about 95% similarity. This haplotype was present in five of the samples (UVA Wise central pond ASV 278; UVA Wise retention pond 1991 ASV 134; UVA Wise retention pond parking lot ASV 7; UVA Wise retention pond puddle ASV 129 and Wise County reservoir ASV 131). However, phylogenetic analysis of these data (Fig. S1) does not support a close relationship between Pic 8/9 T–4m6.8; instead, they are nested within *Characiopsis*. A different possible relative to Pic 8/9 T–4m6.8 is suggested by the ML results, with a single sequence (Wise retention pond 1991 ASV 498) possibly allied with our strain and *Leitzensia polymorpha*. However, this ASV differs from the Pic 8/9 T–4m6.8 sequence and the *L. polymorpha* sequence by 24 and 22 substitutions, respectively, or only about a 94% similarity.

Light microscopy of strain Pic 8/9 T–4m6.8 (Figs 3–13) revealed normal features of the Eustigmatophyceae. Cells were mostly spherical with diameters ranging from about 5 µm to nearly 20 µm and were either solitary or in small aggregates or chains. Some smaller elliptical cells were also present; these cells may be early in the development of mature cells from autospores (Fig. 3). A few large cells, sometimes with very thick cell walls,

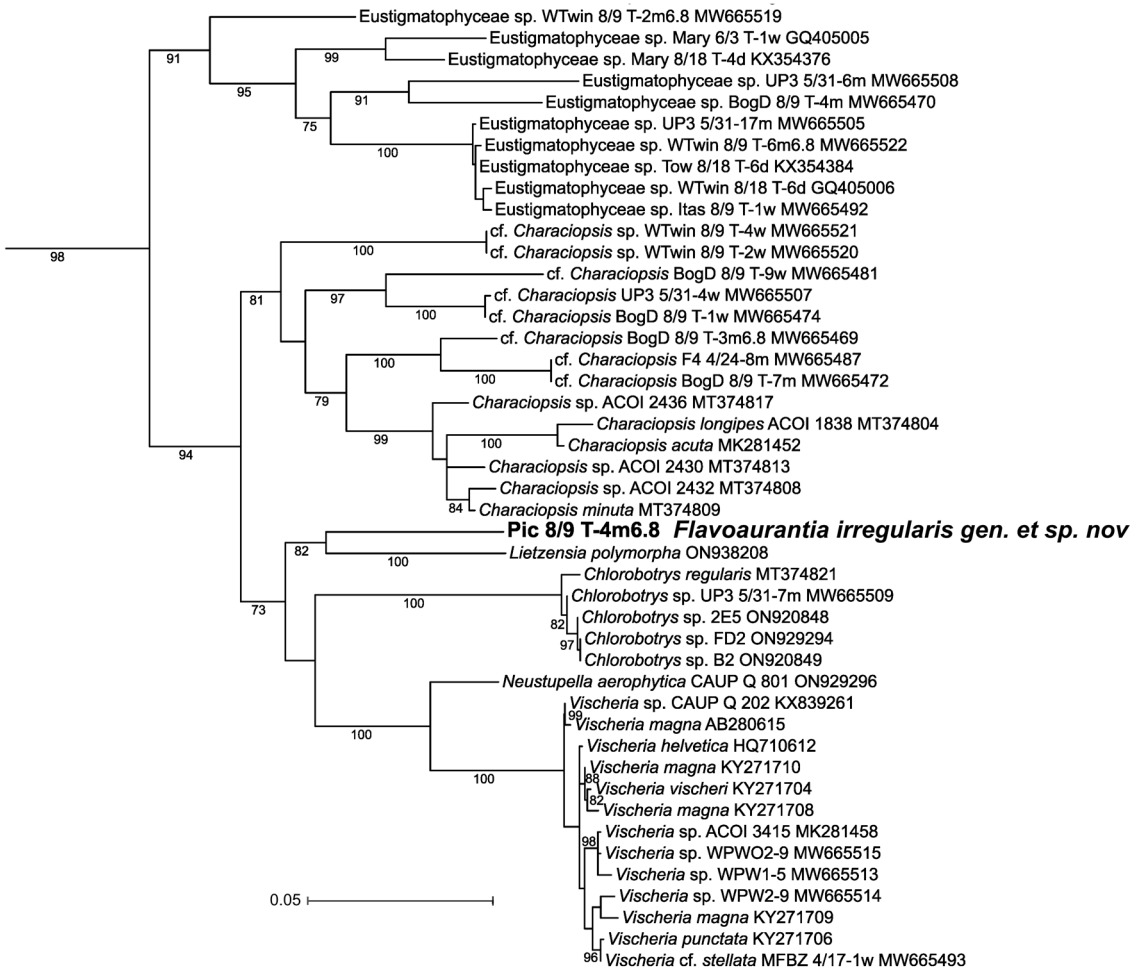
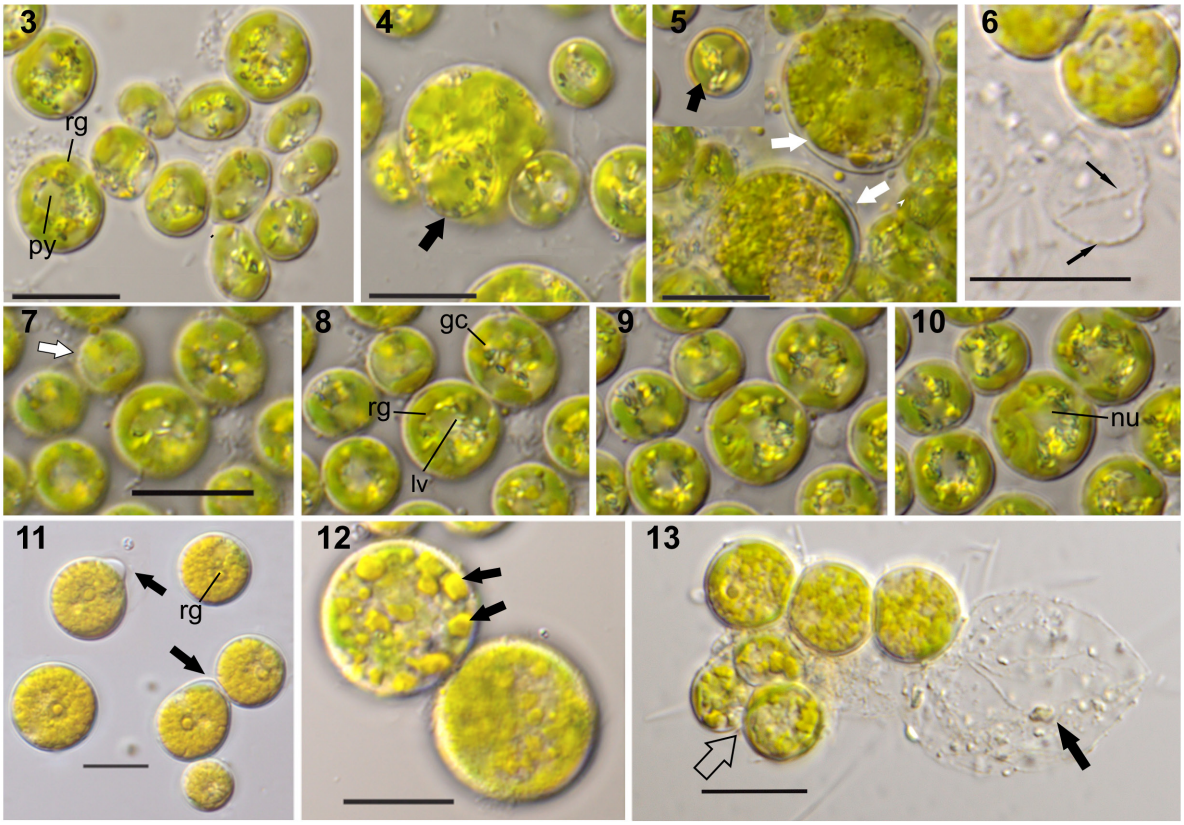


Fig. 2. Results of maximum–likelihood analysis of rbcL sequences without partitioning, showing only the results for the family Chlorobotryaceae. Bootstrap values (500 iterations) 70 or greater are given below the nodes.

were also present (Figs 4–5). The large cell in Fig. 4 may be undergoing autospore production. Reddish globules, lamellate vesicles and crystalline guanine granules that are typical for the Eustigmatophyceae were present (Figs 3, 8, 11). The nucleus was difficult to identify with LM, but is visible as a gray body surrounded by vesicles and crystals (Fig. 10). The cell walls of some cells were pigmented, suggesting mineralization, possibly iron, in the cell wall (Fig. 5). The uppermost image (Fig. 7) in a Z–stack series (Figs 7–10) revealed irregular surfaces on the cell walls that are also visible in Fig. 12 and on the discarded mother cell wall in Fig. 6. The cells in Fig. 12 have a blurred appearance due to the presence of thin digitate structures. The Z–stack series also revealed a single lobed parietal plastid (Fig. 7–10). Large, angular pyrenoids that bulged from the plastids were present (Fig. 3). Some cells in stationary phase cell cultures had bulges on the cell walls (Fig. 11) of unknown function; perhaps these thick areas of the wall can form connections between cells or attachments to substates. Cells from old cultures typically possessed many inclusions of yellow–orange material enclosed in vesicles (Fig. 12). When cells from an old culture were transferred to new medium, they lost their yellow–orange color over several generations, apparently by expunging the vesicles and other material (frequently including the reddish globule) during autospore production (Fig. 13). There were no obvious layers of mucilage surrounding cells as in *Chlorobotrys*, nor is there an obvious vacuole, such as those seen in some *Vischeria* species (KRYVENDA et al. 2018). However, under some conditions our strain produced copious thin mucilage when grown on agar. Reproduction was by the production of 2–4 autospores (Fig. 13); no zoospores were observed.

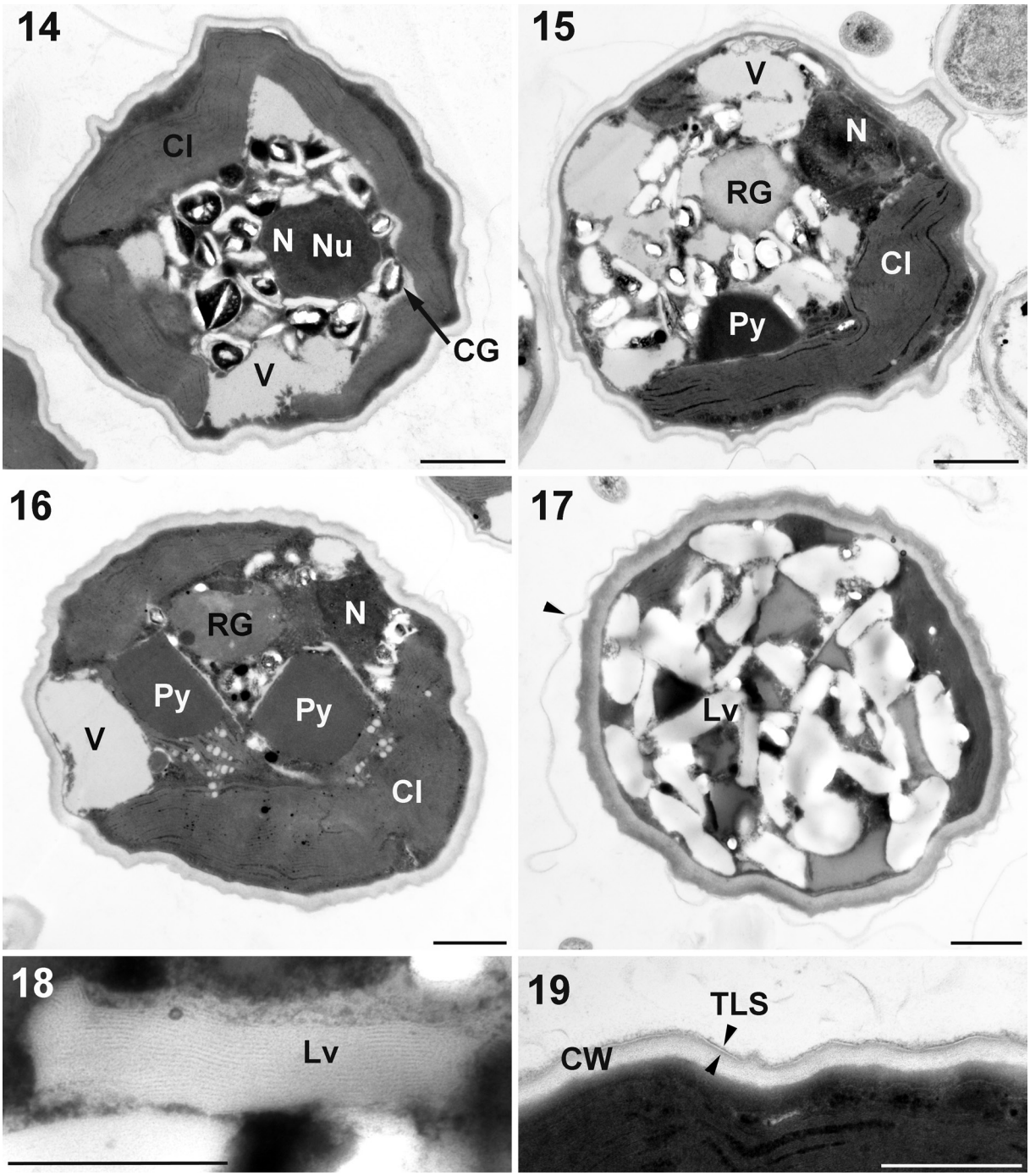
Transmission electron microscopy confirmed the parietal localization of the plastid without the girdle lamella (Figs 14–17). The conspicuous angular pyrenoid (Figs 15–16) is adjacent to the concave part of the chloroplast. Loose thylakoids were observed in close proximity to the pyrenoid but did not penetrate the pyrenoid matrix (Fig. 16). The nucleus, sometimes with a visible nucleolus, is usually located centrally. Fig. 14 shows the nucleus sur-



Figs 3–13. Light micrographs of *Flavoaurantia irregularis*, gen. et sp. nov.: (3) spherical and smaller ellipsoidal cells, showing the angular pyrenoid (py) and reddish globule (rg); (4) large cell (black arrow) forming autospores; (5) thick, smooth cell walls on large cells (white arrows) and mineralization of the cell wall in a single small cell (black arrow); (6) discarded mother cell wall with irregularities in the cell wall clearly visible (black arrows) and two cells at the top of the figure joined together to form a short chain; (7–10) z–stack of vegetative cells which clearly shows the two largest cells joined together and the single lobed plastid, (7) irregularities in the rough surface of the cell wall (white arrow), (8) guanine crystal (gc), reddish globule (rg) and lamellate vesicle (lv), (10) nucleus (nu) surrounded by inclusions; (11) cells from a culture in stationary phase with obvious reddish globules (rg) and bulges in the cell wall (black arrows); (12) yellow–orange vesicles (black arrows) in cells from an old culture that also have cell walls with small digitate or stipitate projections; (13) autospores from a culture that was started from a very old culture showing a yellow–orange vesicle among many particles that were expelled during autospore formation; the three vegetative cells are joined together forming a short chain and an autosporangium is indicated by the open arrow. Scale bars 10 μm.

rounded by crystalline guanine granules. Hard crystals often detach from the TEM ultrathin sections, leaving small holes. Light gray vesicles may be identical to the yellow–orange vesicles observed in the light microscope. A reddish globule, located in the center of the cell, has a regular shape and homogeneous content (Figs 15–16). The cell in the stationary growth phase is filled with numerous vesicles containing reserve substance, including lamellate vesicles (Fig. 17). A detail of a lamellate vesicle is shown in Fig. 18. The vesicles contain material that appears very finely lamellate after fixation. The cell wall (Fig. 19) consists of two layers: 1) an inner layer, probably cellulosic, 70–90 nm, and 2) an outer layer with a trilaminar structure; a dark–light–dark domain (TLS, about 8 nm). The trilaminar layer of the cell wall appears to be relatively resistant, as the TLS remnants of the older cell walls are preserved and visible on ultrathin sections (e.g., Fig. 17). The cell walls are typically uneven, with stipitate or digitate projections on some cells (Figs 14, 15). A structure resembling a short stipe is occasionally present (Fig. 15).

Comparison to published descriptions of Xanthophyceae (Ettl 1978; Pascher 1937–1939) revealed Xanthophyceae genera with some features similar to Pic 8/9 T–4m6.8. Species of *Gloeochloris* Pascher and *Gloeobotrys* Pascher produce ample mucilage somewhat similar to the mucilage occasionally produced by Pic 8/9 T–4m6.8. However, their cells have smooth walls and lack pyrenoids in contrast to the pyrenoids and irregular wall thickness of Pic 8/9 T–4m6.8 cells. *Trachycystis subsolitaria* Pascher has cell walls that vary in thickness, producing a sculpted appearance very similar to that of Pic 8/9 T–4m6.8, but *Trachycystis* does not have pyrenoids and Pascher (1937–1939) clearly states that there are no red oil bodies or crystals in this genus. Species of *Asterogloea* Pascher also have a thin gelatinous mucilage and cell wall sculpting; however, the sculpting is a very regular pattern (in contrast to the irregular thickening of Pic 8/9 T–4m6.8) and the cells do not have pyrenoids.



Figs 14–19. TEM photomicrographs of *Flavoaurantia irregularis*, gen. et sp. nov.: (14–16) ultrastructure of actively growing vegetative cells, (14) the cell with a parietal chloroplast, and a centrally located nucleus surrounded by crystalline guanine granules, (15) the cell with distinct reddish globule surrounded by crystalline guanine granules, parietal chloroplast with bulging pyrenoid and a lateral nucleus, (16) the cell with two conspicuous angular pyrenoids; (17) ultrastructure of the cell in the stationary growth phase filled with numerous vesicles containing reserve substance, including lamellate vesicles, TLS remnants of the older cell walls are marked by an arrowhead; (18) a detail of a lamellate vesicle; (19) the cell wall consisting of two layers: an inner and an outer layer with trilaminar structure – between two arrowheads; (CG) crystalline guanine, (Cl) chloroplast, (CW) cell wall, (LV) lamellate vesicle, (N) nucleus, (Nu) nucleolus, (Py) pyrenoid, (RG) reddish globule, (V) vesicle probably identical to the yellow–orange vesicles observed in the light microscope. Scale bars 1 µm (14–17); 0.5 µm (18–19).

Descriptions of new taxa:

***Flavoaurantia* Alexandre, Němcová, M.W. Fawley et K.P. Fawley gen. nov. (Figs 3–19)**

Description: Cells spherical or mostly so. Cell wall with irregular ridges and pits. A single parietal chloroplast with an angular pyrenoid. Reproduction by autospores. This genus comprises a separate phylogenetic lineages of the Chlorobotryaceae.

Type species: *Flavoaurantia irregularis* Alexandre, Němcová, M.W. Fawley et K.P. Fawley sp. nov.

Etymology: The genus name is derived from a combination of the Latin, *flavus* (yellow) and *aurantius* (orange).

***Flavoaurantia irregularis* Alexandre, Němcová, M.W. Fawley et K.P. Fawley sp. nov. (Figs 3–19)**

Description: Solitary cells typically 5.0–20.0 µm in diameter that may form loose aggregate or short chains; uninucleate; spherical, or mostly so. Cell walls with irregular ridges and pits and sometimes forming small digitate or stipitate extensions, especially in old cultures. Small stipe–like structures sometimes present. Cells with a single parietal chloroplast with a large, bulging angular pyrenoid. No obvious vacuole is present. Cells from older cultures with a yellow–orange color due to presence of pigmented globules in the cytoplasm. Reproduction by formation of two or four autospores. Sexual reproduction or zoospores not observed.

Holotype: Freeze–dried sample of strain Pic 8/9 T–4m6.8 deposited in the Arkansas State University Herbarium (STAR), State University, Arkansas, USA and cryopreserved in a metabolically inactive state in liquid nitrogen at the National Center for Marine Algae and Microbiota at Bigelow Laboratory for Ocean Sciences, East Boothbay, Maine, USA as strain CCMP3758.

Type locality: Slightly eutrophic pond in Itasca State Park, Minnesota, USA, 47.2403° N, 95.2024° W.

Etymology: The specific epithet *irregularis* refers to the uneven ridges and pits on the cell walls.

DNA sequence data: GenBank accessions 18S rDNA: PQ149852; rbcL: PQ316420.

Discussion

Phylogenetic analyses of both 18S rDNA and rbcL place the new genus *Flavoaurantia* in the recently emended family Chlorobotryaceae of the Eustigmatales (Barcytė et al. 2022). *Flavoaurantia* is in many ways similar to other members of the Eustigmatophyceae order Eustigmatales, and specifically the family Chlorobotryaceae. Ultrastructure analysis indicated the presence of common features of the Eustigmatales, such as the angular pyrenoid bulging from the plastid, lamellate vesicles in the cytosol, and crystalline bodies (Eliáš et al. 2016). These crystalline bodies have been identified as guanine crystals that function as large capacity nitrogen storage in other members of the Eustigmatophycae (Mojzeš et al. 2020). *Flavoaurantia* differs from many known members of the Chlorobotryaceae by the presence of large vesicles or globular inclusions of a yellow–orange colored material, especially in old cultures. It is not uncommon for some members of the genera *Vischeria* (e.g. Kryvenda et al. 2019) and *Characiopsis* (Amaral et al. 2021) to take on a yellow or orange color as the culture ages, but they do not often have the same type of vesicles that are seen in *Flavoaurantia*. In *Flavoaurantia*, this pigmented material is lost from the cells after several generations of rapid growth in fresh media. During these periods of growth, the cells appear to expunge large amounts of material during autospore formation, including the yellow–orange material. The reddish globule that is characteristic of Eustigmatophyceae can also be lost in this way, as was previously described by Nakayama et al. (2015) and Gee et al. (2024).

An unusual feature of *F. irregularis* is the presence of small stipitate or digitate protrusions on the cell wall, sometimes forming a dense covering on the cell, as well as a short thick area that is found in some cells that may function as a stipe or a connection between cells. Whether or not the presence of these cell wall features is a diagnostic character for the genus *Flavoaurantia* requires the discovery and characterization of other species that can be assigned with confidence to this genus as well as additional closely related Eustigamatophyceae. Cells of *F. irregularis* sometimes have bulging cell walls that almost appear to represent budding, similar to cells of *Lietzensia polymorpha* Barcytė et al. reported by Barcytė et al. (2022). The irregular surface of the cell walls on many cells of *F. irregularis* is also seen on *Lietzensia polymorpha* and *Neustupella aerophytica* Barcytė, Němcová et Eliáš (Barcytė et al. 2022).

Although analyses of both 18S rDNA and rbcL sequence data place our new genus and species, *Flavoaurantia irregularis*, in the Eustigmatophyceae family Chlorobotryaceae, the position of the new genus within the family based on the analyses of these two loci is somewhat incongruent. Analysis of rbcL sequence data suggests that *F. irregularis* and the recently described taxon, *Lietzensia polymorpha*, form a lineage that diverged early in the Chlorobotryaceae whereas results from analysis of the 18S rDNA data do not suggest this alliance. Instead, the 18S rDNA results place *F. irregularis* and *L. polymorpha* as separate monotypic lineages with no support for alliances with other recognized lineages in the family. Although these results leave us with uncertainties concerning the position of *Flavoaurantia* in the Chlorobotryaceae, they do not affect the determination of *Flavoaurantia* as a new eustigmatophyte genus. The distinguishing features of *Flavoaurantia,* including both the yellow–orange color of older cultures and the small stipitate and digitate features of the cell walls, have not been observed for *Lietzensia* (Kryvenda et al. 2018; Barcytė et al. 2022). Additional diversity in the Chlorobotryaceae that may ultimately be useful in resolving the taxonomy of this lineage has been detected by analysis of environmental metabarcoding (Fawley et al. 2021; Barcytė et al. 2022), although those organisms have not yet been cultured.

The description of the new genus, *Flavoaurantia,* provides a new, possibly early–branching lineage in the Chlorobotryaceae that can be used to better understand the evolution and phylogeny of the family and the order Eustigmatales. Future research should include genomic studies of *Flavoaurantia* as well as members of other unstudied lineages in the order.

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Supplementary material

The following supplementary material is available for this article:

Fig. S1. Phylogenetic analysis of the Chlorobotryaceae using rbcL sequence data. The analysis includes metabarcoding data from the Fawley et al. (2021). Sequences were shortened to the 370 base pairs of the ASVs.

This material is available as part of the online article (http://fottea.czechphycology.cz/contents)

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