Assessing phylogenetic delineations of taxa of filamentous Xanthophyceae (Stramenopiles) using DNA sequence analyses and morphology

Dissertation

in fulfillment of the requirements for the degree
Doctor rerum naturalium
of the Faculty of Mathematics and Natural Sciences
at Kiel University

submitted by

Nataliya Rybalka

Kiel 2015

First Referee: Prof. Dr. Rüdiger Schulz
Second Referee: Prof. Dr. Dietrich Ober
Date of the oral examination: October 7 2015

Approved for publication: October 7 2015
gez. Prof. Dr. Wolfgang J. Duschl, Dekan

Table of content

Summary

This thesis focuses on assessing taxonomic boundaries among the filamentous members of "yellow-green algae", i.e. the class Xanthophyceae (Stramenopiles), which are widespread microalgae in freshwater or terrestrial habitats. The scarcity of morphological characters as well as their considerable plasticity requires to employ molecular marker sequences to define monophyletic genera and species as well as for the unambiguous identification of new isolates. Molecular phylogenetics already supported a split of the filamentous Xanthophyceae in two independent subclades of Xanthophyceae corroborating the distinction of those forms exhibiting bipartite cell walls of H-shaped pieces, i.e. the Tribonemataceae with five previously described genera (Chapters 1 and 2), from those with cell walls consisting of one piece, i.e. several lineages of *Heterococcus* Chodat (Chapter 3).

The focus in Chapter 1 was on the genus *Xanthonema* Silva for which many culture strains isolated from Antarctica were available. The genetic diversity of all available culture strains of *Xanthonema* from Antarctica was assessed using the chloroplast-encoded *psb*A/*rbc*L spacer region sequences, a highly variable molecular marker, to test for endemism when compared with their closest temperate relatives. Direct comparisons of the spacer sequences and phylogenetic analyses of the more conserved *rbc*L gene revealed that current morphospecies were inadequate to describe the actual biodiversity of the group. *Xanthonema*, as currently circumscribed, was paraphyletic. Fortunately, the presence of distinctive sequence regions within the *psb*A/*rbc*L spacer, together with differences in the *rbc*L phylogeny, provided significant autoapomorphic criteria to re-define *Xanthonema* species. There was no species endemic for Antarctica, and no phylogenetic clade corresponded to a limited geographical region. However, species of *Xanthonema* may have Antarctic populations that are distinct from those of other regions because the Antarctic strain spacer sequences were not identical to sequences from temperate regions. Spacer sequences from five new Antarctic isolates were identical to one or more previously available Antarctic strains, indicating that the diversity of *Xanthonema* in Antarctica may be rather limited.

In Chapter 2 species boundaries within other genera of Tribonemataceae, i.e. *Bumilleria* Borzì, *Bumilleriopsis* Printz, *Pseudobumilleriopsis* Deason & Bold and *Tribonema* Derbès & Solier, were investigated with sequences of the *psb*A/*rbc*L spacer region as well as full *rbc*L gene sequences newly determined for 34 strains. About half of the strains represented new isolates which we attempted to identify at the species level using sequence comparisons with reference strains from public culture collections. Already visual comparisons of the *psb*A/*rbc*L spacer regions revealed that none of the morphologically defined genera were monophyletic. Rather, the studied strains were distributed on seven distinct sequence similarity groups which we propose to represent genera. The same groups were resolved, with high statistical support, in phylogenetic analyses of complete *rbc*L sequences. The

strains previously assigned to *Bumilleria*, *Bumilleriopsis* or *Pseudobumilleriopsis* on the base of morphology were separated into three *psb*A/*rbc*L spacer similarity groups and the strains of *Tribonema* were separated into four different groups. Between groups the *psb*A/*rbc*L spacers differed in length and no alignment was possible and, therefore, this discriminates genera. There were considerable spacer sequence differences within a similarity group, in particular at the more variable 5'-end of the *psb*A/*rbc*L spacer region, and they were found valuable for the unambiguous identification of species. Within a similarity group significant *psb*A/*rbc*L spacer differences imply that it consists of several species, whereas a group with almost invariant sequences is interpreted to represent only a single species. Thus the molecular data were more conservative than morphology because strains from those *psb*A/*rbc*L spacer similarity groups with little within-sequence variation have previously been assigned to several species.

In Chapter 3 a method and a reference data base for the unambiguous identification of species of *Heterococcus* which uses DNA sequence variation in markers from plastid and nuclear genomes was established. *Heterococcus* species are common and widespread in soils, especially from cold regions. They are characterized by extensively branched filaments produced when grown on agarized culture medium. Despite the large number of species described exclusively using light microscopic morphology, the assessment of species diversity is hampered by extensive morphological plasticity. Two independent types of molecular data, the chloroplast-encoded *psb*A/*rbc*L spacer complemented by *rbc*L gene and the internal transcribed spacer 2 of the nuclear rDNA cistron (ITS2), congruently recovered a robust phylogenetic structure. The 'monophyletic species concept', appropriate for microalgae without known sexual reproduction, revealed eight different species. The molecular-based species boundaries were more conservative than those defined by morphology. Within a species, almost identical genotypes were repeatedly recovered from strains of different origins. Genotypes recovered from Antarctic strains were distinct from those in temperate habitats.

Zusammenfassung

Die vorliegende Arbeit hat zum Ziel taxonomische Abgrenzungen innerhalb der fadenförmigen „Gelbgrünalgen“, Vertretern der Klasse Xanthophyceae (Stramenopiles), festzustellen. Fadenförmige Xanthophyceae sind weitverbreitete Mikroalgen im Süßwasser und in terrestrischen Lebensräumen. Da diese Algen arm an morphologischen Merkmalen sind und diese zudem eine beträchtliche Plastizität aufweisen, sind molekulare Markersequenzen notwendig, um monophyletische Gattungen und Arten zu definieren und neue Isolate dieser Algen auf Artniveau sicher zu bestimmen. Molekular-phylogenetische Analysen haben bereits eine Auftrennung der fademförmigen Xanthophyceae in zwei unabhängige monophyletische Gruppierungen unterstützt, d.h. die Trennung der Formen mit zweigeteilten Zellwänden aus H-förmigen Stücken, die Tribonemataceae mit fünf bisher beschriebenen Gattungen (Kapitel 1 und 2), von solchen mit Zellwänden aus einem Stück, d.h. mehreren Linien der Gattung *Heterococcus* Chodat (Kapitel 3).

In Kapitel 1 lag der Schwerpunkt auf der Gattung *Xanthonema* Silva, für die viele aus der Antarktis isolierte Kulturstämme zur Verfügung standen. Es wurde die genetische Diversität aller Kulturstämme von *Xanthonema* aus der Antarktis anhand der Plastiden-kodierten *psb*A/*rbc*L Spacer-Region, eines hochauflösenden molekularen Markers, erfasst, um auf einen möglichen Endemismus schließen zu können wenn sie mit ihren nächst-verwandten Stämmen aus gemäßigten Breiten verglichen wurden. Direkte Vergleiche der Spacer-Sequenzen und phylogenetische Analysen anhand der mehr konservierten *rbc*L Gensequenzen zeigten, dass die gegenwärtig morphologisch definierten Arten („morphospecies“) nicht geeignet sind, die tatsächliche Biodiversität von *Xanthonema* und *Xanthonema*-ähnlichen Xanthophyceae adäquat zu beschreiben. *Xanthonema* ist anhand der gegenwärtigen morphologisch definierten Abgrenzung paraphyletisch. Jedoch bieten das Vorhandensein distinkter Sequenzabschnitte innerhalb des *psb*A/*rbc*L Spacers zusammen mit Unterschieden in der *rbc*L-Phylogenie signifikante Autoapomorphien, d.h. gemeinsame abgeleitete Merkmale, um Arten von *Xanthonema* neu zu definieren. Es konnten keine für die Antarktis endemische Arten gefunden werden und keine phylogenetische Gruppierung entsprach einem begrenztem geographischen Gebiet. Jedoch können *Xanthonema*-Arten Antarktische Populationen, die sich von solchen anderer Regionen unterscheiden, beinhalten. Denn die Spacer-Sequenzen der Antarktischen Stämme waren nicht mit den entsprechenden Sequenzen von Stämmen temperater Regionen identisch. Jedoch waren die Spacer-Sequenzen von fünf neuen Antarktischen Isolaten identisch mit einem oder mehreren schon bislang verfügbaren Antarktischen Stämmen, was bedeutet, dass die Diversität von *Xanthonema* in der Antarktis ziemlich begrenzt sein muss.

In Kapitel 2 wurden die Abgrenzungen weiterer Gattungen der Tribonemataceae, d.h. *Bumilleria* Borzì, *Bumilleriopsis* Printz, *Pseudobumilleriopsis* Deason & Bold und *Tribonema* Derbès & Solier, anhand hochvariabler Sequencen der *psb*A/*rbc*L Spacer-Region als auch der konservierter vollständiger *rbc*L Gensequenzen, die für 34 Stämme ermittelt wurden, untersucht. Etwa die Hälfte der untersuchten

Stämme waren neue Isolate, die mithilfe von Sequenzvergleichen mit Referenzstämmen aus öffentlichen Kulturensammlungen auf Artniveau identifiziert werden sollten. Bereits visuelle Vergleiche der *psb*A/*rbc*L Spacer-Regionen zeigten, dass keine der morphologisch definierten Gattungen monophyletisch war. Vielmehr verteilten sich die untersuchten Stämme auf sieben distinkte Gruppen ähnlicher Sequenzen, für die wir vorschlagen, dass sie Gattungen repräsentieren. Die gleichen Gruppen von Stämmen wurden auch in phylogenetischen Analysen vollständiger *rbc*L Gensequenzen mit hoher statistischer Unterstützung dargestellt. Solche Stämme, die bislang aufgrund morphologischer Merkmale zu *Bumilleria*, *Bumilleriopsis* oder *Pseudobumilleriopsis* eingeordnet waren, waren anhand der *psb*A/*rbc*L Spacer-Regionen auf drei Sequenzähnlichkeitsgruppen verteilt. Stämme, die bislang *Tribonema* zugeordnet wurden, waren auf drei Gruppen aufgetrennt. Die *psb*A/*rbc*L Spacer waren zwischen diesen Gruppen so unterschiedlich in ihren Längen, dass kein Alignment möglich war, und daher grenzen sie Gattungen voneinander ab. Innerhalb einer Sequenzähnlichkeitsgruppe gab es ebenfalls beträchtliche Unterschiede, besonders an dem mehr variablen 5‘-Ende der *psb*A/*rbc*L Spacer-Region, und es zeigte sich, dass diese Unterschiede gut geeignet waren, Arten eindeutig zu identifizieren. Beträchtliche Sequenzunterschiede innerhalb einer *psb*A/*rbc*L Spacer-Ähnlichkeitsgruppe bedeuten, dass die Gruppe aus mehreren Arten besteht, während eine Gruppe mit nahezu keinen Sequenzunterschieden als eine einzige Art angesehen wird. Das bedeutet, die molekularen Daten waren konservativer als die Morphologie, denn Stämme aus solchen *psb*A/*rbc*L Spacer-Ähnlichkeitsgruppe mit nur wenigen Sequenzunterschieden waren bislang mehreren Arten zugeordnet.

In Kapitel 3 wurden eine Methode und Referenzdaten zur eindeutigen Identifizierung für Arten von *Heterococcus* erarbeitet, die auf Sequenzvariationen in Markermolekülen des Plastiden- und Kerngenoms beruhen. *Heterococcus*-Arten sind häufig und weitverbreitet in Böden, besonders der kalten Regionen. Sie bilden charakteristische stark verzweigte Fäden in Kultur auf Agar-Nährmedien. Obwohl eine große Anzahl von *Heterococcus*-Arten anhand lichtmikroskopischer Merkmale beschrieben wurde, ist die Erfassung ihrer Vielfalt wegen ihrer besonders großen morphologischen Plastizität stark eingeschränkt. Zwei unabhängige Typen von molekularen Daten, d.h. die Plastiden-kodierte *psb*A/*rbc*L Spacer-Region in Kombination mit dem *rbc*L-Gen und die kernkodierte ITS2-Region des rDNA-Cistrons, lieferten übereinstimmend dieselbe gut unterstützte phylogenetische Struktur der untersuchten *Heterococcus*-Isolate. Die Anwendung des sogenannten „monophyletischen Artkonzeptes“, passend für Mikroalgen, für die keine sexuelle Reproduktion bekannt ist, stellte acht klar voneinander differenzierte Arten heraus. Die molekular-phylogenetische begründeten Artabgrenzungen waren konservativer als die aufgrund morphologischer Merkmale. Innerhalb derselben Art wurden wiederholt nahezu identische Genotypen, die aber aus verschiedenen geographischen Ursprüngen stammten, aufgedeckt.

Introduction[1]

1. The Xanthophyceae – a class of stramenopile algae (Heterokontophyta)

Briefly, the Xanthophyceae (commonly referred to as the "yellow-green algae") is a class of phototrophic protists within the Stramenopiles which possess chlorophyll *c* as main accessory pigment, do not accumulate starch and produce motile cells with two differently shaped flagella (heterokont flagella; van den Hoek et al., 1995). Most Xanthophyceae are common freshwater and terrestrial organisms, although representatives of the genus *Vaucheria* A.P.de Candolle occur in marine and brackish water habitats as well. Currently accepted members of the class are coccoid or filamentous forms, but several genera form multinucleate (siphonous) often macroscopically visible single cells (e.g., *Botrydium* Wallroth) or filaments (e.g., *Vaucheria*). For most Xanthophyceae the only known method of reproduction is asexual and involves vegetative cell division, formation of aplanospores or zoospores. Sexual reproduction is safely established only for a single genus, i.e. the siphonous *Vaucheria* which exhibits oogamy (Rieth, 1980; van den Hoek et al., 1995).

Xanthophyceae represent biflagellate "heterokont" organisms, commonly referred to as Stramenopiles (Patterson, 1989), a well-defined monophyletic group of eukaryotes. Stramenopiles are defined by flagellated stages which possess two different flagella, an anteriorly directed one with tripartite and rigid tubular hairs (mastigonemes) in two opposite rows along the flagellum and a posteriorly directed usually smooth flagellum without hairs (Patterson, 1989; Adl et al., 2005; Yoon et al., 2009; Yang et al., 2012). With the shorter smooth flagellum an eyespot which is located within the plastid is associated as in most other stramenopile algae (see Chapter 2, Fig. 4C).

Most other shared features of stramenopile algae refer to their pigmentation and chloroplasts (plastids). As in all stramenopile algae chlorophyll *b* is absent in the Xanthophyceae which exhibit chlorophyll *c* instead. A peculiarity of the Xanthophyceae with respect to photosynthetic pigments is the absence of the brown carotenoid fucoxanthin, in contrast to most classes of photosynthetic Stramenopiles. Therefore, the color of xanthophytes is bright green to yellowish green, hence their common name "yellow green algae". Xanthophyceae like most stramenopile plastids exhibit lamellae of three stacked or appressed more or less plate like thylakoids with a spheroidal girdle lamella surrounding all the other lamellae in the plastid. However, in several filamentous Xanthophyceae a

[1] Parts of this introduction have been published as part of a book chapter (see Appendix 1):
Ott, D.W., Oldham-Ott C.K., Rybalka, N., and Friedl, T. (2015) Xanthophyte, Eustigmatophyte, and Raphidophyte Algae. In: Wehr, J.D., Sheath, R.G., Kociolek, J.P. (Eds.). *Freshwater Algae of North America: Ecology and Classification, 2nd edition.* Academic Press, Amsterdam, pp. 483-534.

girdle lamella is absent, i.e. in members of the *Bumilleriopsis* 1 group, the genus *Bumilleria*, see Chapter 2.

All photosynthetic stramenopiles acquired their plastids through secondary endosymbiosis, which is the uptake and retention of a primary algal cell by another eukaryotic lineage (Keeling, 2009). The primary algal cell involved was a red algal cell. However, recently phylogenomic evidence emerged for a third partner that was involved in the secondary endosymbiosis. These data indicate an endosymbiosis with a green alga most likely preceded the red algal endosymbiont (Moustafa et al., 2009; Tirichine and Bowler, 2011). Substantially more genes of putative green than red origin were recovered in genome analyses of stramenopile algae which, unfortunately, did not include xanthophytes. Except for a single study on phosphoribulokinase (Petersen et al., 2006) no Xanthophyceae have been considered for studies on the plastid origin in the stramenopile algae. The green genes in these algae are interpreted as the remnants of a cryptic endosymbiosis that occurred early in stramenopile algae evolution; the subsequent red algal capture would have led to the loss or replacement of most green genes via intracellular gene transfer from the new endosymbiont (Frommolt et al., 2008; Dorrell and Smith, 2011). The plastids of all stramenopile algae are so-called complex plastids with four membranes. The outermost of them is continuous with the outer nuclear membrane giving rise to a shared endomembrane system, the plastid endoplasmatic recticulum (PER), and resulting in an intimately connection of the plastid to the nucleus (Yoon et al., 2009; see also Chapter 2, Fig. 4C).

Most molecular phylogenetic analyses show the stramenopile algae as a monophyletic group within the Stramenopiles that is either derived from, or sister to, a clade of entirely non-photosynthetic Stramenopiles (Riisberg et al., 2009; Yoon et al., 2009). Sixteen classes of photosynthetic Stramenopiles have been described up to now and their monophyletic origin has been confirmed by molecular phylogenetic analyses. These studies agree on that Xanthophyceae defines an independent monophyletic lineage which is somehow closer related to six other stramenopile classes, i.e. the Aurearenophyceae, Chrysomerophyceae, Phaeophyceae, Phaeothamniophyceae, Rhaphidophyceae, and Schizocladiophyceae which form the “SI clade” of Yang et al. (2012), a subclade of the photosynthetic stramenopiles. The exact relationships within this photosynthetic subclade, however, are not well resolved so far (Kai et al., 2008; Brown and Sorhannus, 2010; Přibyl et al., 2012; Yang et al., 2012). A recent phylogeny based on multigene DNA sequences resolved three major clades within the photosynthetic Stramenopiles (Yang et al., 2012). The Xanthophyceae formed together with six other classes of photosynthetic Stramenopiles (see above) the “SI clade” and thus were rather distant to the morphologically similar Eustigmatophyceae which together with the Chrysophyceae and

Synurophyceae were in the “SII” clade. The Bacillariophyceae, diatoms, most likely the largest group of stramenopile algae, were placed in another clade, the “SIII” (Yang et al., 2012).

What is recognized as the monophyletic stramenopile algae within the Stramenopiles in molecular phylogenies now, has previously been described as a division of its own, the Heterokontophyta (van den Hoek, 1978). Only later van den Hoek included also non-photosynthetic protists, i.e. the Oomycetes, in the Heterokontophyta (van den Hoek et al., 1995). Heterokontophyta is synonymous with the Chromophyta established earlier by Christensen (1962). In addition, the terms chromophyte, Chromista, ochrophyte, and Ochrophyta have been referred to the stramenopile algae as well (Yoon et al., 2009).

2. Definition and classification of the Xanthophyceae

Definition. Because of the presence of heterokont flagella, the xanthophytes were together with the presently recognized Eustigmatophyceae (which in the past was included into the Xanthophyceae, see below) and Raphidophytes (as Chloromonadophyceae) combined into the “Heterokontae” more than hundred years ago (Luther 1899). Heterokontae Luther refers to a group of the rank of a class (Silva, 1979). Later, the name Xanthophyceae was proposed for the class by Allorge in 1930 (Allorge, 1930); it was first validly published by Fritsch in 1935 (see Fritsch, 1935; Hibberd, 1990). Principles of traditional morphological classification of xanthophytes algae were established by A. Pascher (Pascher, 1925, 1939) and with minor changes used by subsequent authors (e.g., Ettl, 1978; Matvienko and Dogadina, 1978) until today. Silva (1979) and van den Hoek (van den Hoek, 1978; van den Hoek et al., 1995) have summarized the repeated and continuous taxonomic rearrangements to which the Xanthophyceae was subjected since its establishment in the early 20th century. The Xanthophyceae are also known as the Tribophyceae (Hibberd, 1981) and earlier were given even the rank of a division “Xanthophyta” (e.g., Margulis, 1974; Matvienko and Dogadina, 1978). Description of the class Xanthophyceae went through significant changes since it has been recognized as a class distinct from the green algae. A first definition of the class as summarized by Fritsch (1935)[2] was still too broad to

[2] “Chromatophores (chloroplasts) which are yellow-green owing to the presence of an excess of the yellow xanthophyll. Starch is absent and pyrenoids are wanting or rarely evident, oil being the customary storage-product. The non-flagellate members have a cell-wall which is often rich in pectic compounds and which is frequently composed of two equal or unequal pieces overlapping at their edges. Resting stages with a silicified membrane are of common occurrence. The motile cells possess two very unequal flagella (or sometimes only one) arising from the front end. As a general rule the cells contain a number of discoid chromatophores. The majority of the members are non-flagellate, but sexual reproduction is rare and always isogamous. The most advanced forms have a simple filamentous habit.” (Fritsch 1935, p. 5)

distinguish the Xanthophyceae clearly from other nowadays recognized classes of stramenopile algae. Additional characteristics of the class have been revealed using new methods (e.g., pigment analyses, transmission electron microscopy) which became available during the second half of the 20th century (Hibberd and Leedale, 1971b; Hibberd and Leedale, 1971a; Whittle and Casselton, 1975; van den Hoek et al., 1995, and references therein). The most recent summary of general characteristics of the class Xanthophyceae which distinguishes it from other stramenopile algae has been presented by van den Hoek et al. (1995). Although still based on data obtained before the introduction of molecular phylogenetics to examine the distinction of the Xanthophyceae within the Stramenopiles, it is still regarded as generally valid. According to van den Hoek and co-authors (van den Hoek et al., 1995, p. 123-130) the main distinguishing features of Xanthophyceae are as following: 1) Organizational level. Most genera of xanthophytes are unicellular or colonial, coccoid algae, but there is also a considerable number of species in which the thalli are composed of multinucleate siphons, and a few that consist of multicellular filaments. Many non-flagellate species, however, produce flagellate cells (zoids) at some stage in their life cycles. In the zoids the flagella are inserted close to the apex of the cell. 2) Eyespot. A typical photoreceptor apparatus is present in the zoids, consisting of a swelling on the short, smooth flagellum and an eyespot lying within the chloroplast. 3) Chloroplasts and pigments. Chloroplasts are discoid and green or yellow-green. In addition to chlorophyll *a*, there are also small amounts of chlorophylls *c*1 and *c*2. The principal accessory pigments are ß-carotene, vaucheriaxanthin, diatoxanthin, diadinoxanthin, and heteroxanthin. Fucoxanthin, the brown pigment of many other stramenopile algae is absent. 4) Cell wall in vegetative cells and resting cysts. Spherical or ellipsoidal cysts are formed by some species. Each cyst is formed within a cell (it is an endogenous cyst). Its wall is impregnated with silica and consists of two unequal halves which fit tightly together. The cell wall too can be often seen to consist of two halves which overlap to some extent in the middle of the cell. The cell wall, like the cyst wall, is often impregnated with silica, although it seems to consist for the most part of cellulose microfibrils.

The yellow-green color of xanthophytes makes it not always easy to distinguish them from true green algae (Chlorophyta). Even up today several xanthophyte genera, still not subjected to molecular phylogenetic analyses, are suspected to represent green algae (Ott et al., 2015). For example, species of coccoid green algae may have originally described as xanthophytes (Darienko et al., 2010). Similarly, the genus *Heterotrichella* Reisigl appears as morphological “twin” of the green alga genera *Koliella* and *Raphidonema* and, therefore, it may represent green algae rather than xanthophytes, but this needs to be proved by molecular analyses.

With the extended pigment and ultrastructural analyses available since the second half of the 20th century and more recently molecular phylogenetics, genera and species originally described as

members of Xanthophyceae have been recognized to actually belong to other evolutionary lineages and classes of stramenopile algae, i.e. the Eustigmatophyceae, Raphidophyceae, Phaeothamniophyceae, as well as the phylum Chlorarachniophyta (Hibberd, 1990; Bailey and Andersen, 1998). Many species from the traditional xanthophycean order Chloramoebales and almost all genera of Heterogloeales have already been transferred to other stramenopile classes, e.g. Chrysophyceae and Synurophyceae, or even other phyla, e.g. the Haptophyta and Cryptophyta. A prominent case is presented by *Chlamydomyxa labyrinthuloides* W.Archer which is characterized by amoeboid or plasmodioid structures as the main stage in the life history; it was placed by A. Pascher within the Xanthophyceae, Rhizochloridales (Pascher, 1939; Ettl, 1978). DNA sequence analyses, however, revealed *Chlamydomyxa* as a close relative with the recently described class Synchromophyceae (Kai et al., 2008) which is also supported by morphology, pigment and DNA sequence analyses (Wenderoth et al., 1999; Schmidt et al., 2012). As based on DNA sequence phylogenetic analyses there are no species without cell walls and no species with flagellate cells in the vegetative phase belonging to the Xanthophyceae. This is also true for other members of the PX clade *sensu* Kai et al. (2008) of Stramenopiles, a clade which unites the Xanthophyceae with five other classes (i.e. the Aurearenophyceae, Chrysomerophyceae, Phaeophyceae, Phaeothamniophyceae, and Schizocladiophyceae). The PX clade corresponds to the "SI clade" of Yang et al. (2012), but does not include the Rhaphidophyceae. A rather peculiar case with respect to the distinction of the class Xanthophyceae refers to the coccoid forms. Despite being phylogenetically rather distant from the Xanthophyceae, members of Eustigmatophyceae are all coccoid and virtually impossible to distinguish from coccoid xanthophytes without molecular phylogenetic or detailed pigment analysis. Consequently, general conclusions about the structure, reproduction and ecology of Xanthophyceae as summarized in many books and reviews (e.g. (Ettl, 1978; Hibberd, 1990; van den Hoek et al., 1995; Andersen, 2004) need to be viewed with care because they may have been based on species which in fact were even not closer related to the Xanthophyceae *sensu stricto*. In many cases it is difficult to interpret biochemical, physiological and ecological data about Xanthophyceae correctly because identification and/or assessment of algae to this group could be doubtful in many cases. There are even several recent examples of analyses which were based on incorrect assignments, i.e. strains of clearly different origins were erroneously placed into the Xanthophyceae. For example, Lang et al. (2011) included numerous strains actually belonging to Eustigmatophyceae or other lineages of stramenopile algae into the Xanthophyceae in their study of fatty acids distribution among various groups of algae. Gigova et al. (2012) reported about the "xanthophyte" *Trachydiscus minutus* despite the same species has already been considered as a doubtful xanthophyte (Iliev et al., 2010) and later it was revealed as an eustigmatophyte (Přibyl et al., 2012). A number of coccoid species have been transferred from genera of Xanthophyceae to new genera of Eustigmatophyceae (for overview see Ott

et al., 2015, Appendix 1). Currently, there is total of 13 former genera of Xanthophyceae checked by molecular phylogenetics for their correct phylogenetic position which are recognized as actually to belong to the Eustigmatophyceae (Ott et al., 2015; Appendix 1); this already outnumbers the total number of coccoid xanthophyte genera (11; Table 1) approved by molecular phylogenetics. Therefore, with a growing number of cultures available for species of "traditional" coccoid xanthophytes, studies employing DNA sequence analyses are likely still to decrease the number of coccoid Xanthophyceae. However, there is even an example of a Xanthophyceae species which switched to another stramenopile class and then back within 10 years. *Pleurochloridella botrydiopsis* Pascher, originally assigned to the Xanthophyceae (Ettl, 1956), was transferred to the Phaeothamniophyceae based on morphological characteristics and photosynthetic pigment composition (Bailey et al., 1998), but now is regarded as occupying a more ancestral position within the Xanthophyceae based on molecular evidence (Kai et al., 2008).

The Xanthophyceae certainly is a group of stramenopile algae with still considerable potential of revealing new phylogenetic lineages and new classes. Recently, molecular phylogenetic analyses showed that the authentic strain SAG 31.83 of *Botrydiopsis pyrenoidosa* Trenkwalder, described as a species of Xanthophyceae, Mischococcales, by Trenkwalder (1975), neither is within the Xanthophyceae, nor any other currently known class of Stramenopiles (Maistro et al., 2009; Brown and Sorhannus, 2010). Also *Chrysowaernella hieroglyphica* (the latter still assigned to the Chrysomerophyceae) and *Pleurochloridella botrydiopsis* actually represent lineages which form sister-taxa with the Xanthophyceae or should be even integrated into this class (Kai et al., 2008; Přibyl et al., 2012; Yang et al., 2012).

About 600 species from ca. 100 genera of Xanthophyceae have been described mostly on the basis of morphology so far (van den Hoek et al., 1995), but the number of true yet described members of Xanthophyceae attributed to Xanthophyceae with confidence on the basis of molecular phylogenetics is dramatically lower. So far, there are no summarized data about taxa which were excluded from Xanthophyceae and moved to other classes following pigment, ultrastructural or molecular investigations. As a first summary, we compiled 18 genera comprising the three organizational levels coccoid, filamentous and siphonous which represent xanthophyte genera with confidence because they have been included into molecular phylogenetic analyses, most of them consistently through several studies and using sequences from even two or three genes (Table 1).

Classification. Traditional morphological classifications of the Xanthophyceae include six or seven orders corresponding to the level of organization of vegetative cells (Ettl, 1978; Silva, 1979): unicellular

		18S	*rbc*L	*psa* A	*rbc*L and 18S	*psa* A and *rbc*L	18S, *rbc*L and *psa* A
coccoid	*Botrydiopsis*	1, 8	4, 5	6	2, 4	6	7
	*Botryochloris**	4, 8	4		4		7
	Bumilleriopsis	8	6, 5	6	2, 4	6	7
	Chlorellidium	4, 8	6	6	4	6	7
	Excentrochloris		9				
	*Mischococcus**	8	4, 5		2, 4		7
	Ophiocytium		6	6		6	7
	*Pleurochloris**	3, 8			4		7
	Pseudobumilleriopsis						7
	*Pseudopleurochloris**	3, 8	4		4		7
	*Sphaerosorus**	1, 8	4	6	4	6	7
filamentous	*Bumilleria*		6	6		6	7
	Heterococcus	8	4, 5	6	2, 4	6	7
	Tribonema	1, 8	4, 5	6	2, 4	6	7
	Xanthonema	1	4, 5	6	2, 4	6	7
siphonous	*Asterosiphon**		4	6		6	7
	Botrydium	1, 8	4, 5	6	2, 4	6	7
	Vaucheria	1	6, 5	6	4	6	7

Table 1. The 18 genera of Xanthophyceae of different organizational levels (first column) which have been checked for their correct phylogenetic position by DNA sequence analyses up to now. Almost all genera (except for *Excentrochloris* and *Pseudobumilleriopsis*) have repeatedly been included in several phylogenetic studies with mostly (except for the six genera marked by an asterisk) several strains/species per genus. Most genera have been studied twice in single-gene phylogenies of the nuclear-encoded 18S rRNA or plastid-encoded *rbc*L genes. Citations where the phylogenetic analyses have been published are abbreviated as follows: 1, Potter et al. (1997); 2, Bailey and Andersen (1998); 3, Andreoli et al. (1999); 4, Negrisolo et al. (2004); 5, Zuccarello and Lokhorst (2005); 6, Maistro et al. 2007; 7, Maistro et al. (2009); 8, Brown and Sorhannus (2010); 9, Hofbauer et al. (2011).

flagellates were accommodated in the Heterochloridales (syn. Chloramoebales, Silva (1979), capsoid forms (non-motile cells without wall, but embedded in mucilage) in the Heterogloeales, rhizopodial forms (vegetative cells amoeboid with rhizopods and without walls) in the Rhizochloridales, coccoid forms in the Mischococcales, filamentous (branched and unbranched forms) in the Tribonematales, and siphonous forms in the Botrydiales and Vaucheriales (Ettl, 1978; Matvienko and Dogadina, 1978). However, molecular phylogenetic studies show that most of these traditional orders do not form monophyletic groups (Potter et al., 1997; Bailey and Andersen, 1998; Maistro et al., 2009). In contrast to other algal groups (e.g. the green algae or diatoms) and despite the Xanthophyceae often attracted the attention of scientists, only rarely it became the main object of molecular studies which entirely focused on this class. A few strains of Xanthophyceae have been sequenced for nuclear- and plastid-

encoded genes and phylogenetically analyzed in studies targeting to clarify of the origins of plastids within the Stramenopiles (e.g., Scherer et al., 1991; Daugbjerg and Andersen, 1997; Bailey et al., 1998; Cho et al., 2004; Kai et al., 2008; Riisberg et al., 2009; Brown and Sorhannus, 2010; Yang et al., 2012). Another molecular study which involved strains of Xanthophyceae to better represent the stramenopile algae was about the distribution and self-splicing ability of plastid tRNA-Leu Group I introns by Simon et al. (2003). The only so far complete chloroplast genome sequence for Xanthophyceae has been determined for *Vaucheria litorea* C.Agardh which donates its chloroplasts for the symbiosis with the sea slug *Elysia chlorotica* Gould (Rumpho et al., 2008). The mechanisms that allow the long-term photosynthetic ability of the chloroplasts in the heterotrophic host have been studied in detail (for review see Pelletreau et al., 2011). Since molecular methods have been introduced into the phylogenetics and classification of algae, relationships within the Xanthophyceae have been studied several times. In general, these studies recovered conflicts between the traditional classification of the Xanthophyceae into orders as based on organizational levels and hypotheses derived from molecular phylogenetic analyses of 18S rRNA, *rbc*L and *psa*A gene sequences (Daugbjerg and Andersen, 1997; Potter et al., 1997; Bailey and Andersen, 1998; Negrisolo et al., 2004; Maistro et al., 2007; Maistro et al., 2009). Several of these studies also revealed morphologically defined genera as para- or polyphyletic within the Xanthophyceae. It has been shown that the orders Mischococcales and Tribonematales as defined by Ettl (1978) are not monophyletic (Potter et al., 1997; Maistro et al., 2009). With regard to filamentous xanthophytes there is some evidence that the filamentous species with H-shaped cell walls, the Tribonemataceae (see Chapter 2), constitute one lineage of and those with entire cell walls (e.g. *Heterococcus*) a separated second lineage, but this finding has not yet been put forward in a classification yet (Bailey and Andersen, 1998; Adl et al., 2005). Also the monophyly of Vaucheriales is questionable as well as the appropriateness of Botrydiales (Negrisolo et al., 2004; Maistro et al., 2009). Also many of morphologically defined genera, e.g. *Bumilleriopsis, Botrydiopsis, Chlorellidium, Xanthonema*, and *Ophiocytium*, have been found not to be monophyletic in molecular phylogenetic studies (Negrisolo et al., 2004; Maistro et al., 2007; Maistro et al., 2009). It follows that the criteria for species delimitation of these (and most likely several other) genera of Xanthophyceae are in need of evaluation in light of molecular analyses (Zuccarello and Lokhorst, 2005; Rybalka et al., 2009).

Modern classifications within the Xanthophyceae are in contrast to the traditional classification, but have not been put forward in formal taxonomic proposals of orders. Adl et al. (2005) reduced the classification to two groups: (1) the Tribonematales with filamentous as well as coccoid forms, sometimes becoming parenchymatous or multinucleate with age and elaborate reproductive structures lacking, and (2) the Vaucheriales comprising siphonous filaments with elaborate sexual reproductive structures (antheridia, oogonia). Based on analyses of 18S rDNA, *rbc*L and *psa*A genes

robust evolutionary relationships within the Xanthophyceae have been resolved, i.e. four major clades within the Xanthophyceae received strong statistical support and led to the distinction of major newly defined xanthophycean clades, i.e. the Botrydiopsalean, Chlorellidialean, Tribonematalean and Vaucherialean clade which may be regarded as phylogenetically supported orders of the class (Maistro et al., 2009). Interestingly, none of the three organizational levels of Xanthophyceae received support, e.g. the coccoid as well as siphonous forms were distributed on at least three clades. Filamentous Xanthophyceae, the particular subject of the current study, were recovered in two lineages by the molecular studies (Bailey and Andersen, 1998; Adl et al., 2005). Those filamentous algae with H-shaped cell walls (Tribonemataceae, see Rybalka et al. (2009) = Chapter 1, and Chapter 2) were within the Tribonematalean clade, whereas those with entire cell walls (*Heterococcus*, see Rybalka et al. (2013) were within the Chlorellidialean clade (Maistro et al., 2009).

3. Ecology, distribution, age and economic use of the Xanthophyceae

Ecology. Xanthophytes are predominately freshwater and terrestrial organisms, although species of *Vaucheria* occur also in marine and brackish water habitats. While most of terrestrial and freshwater algae and plants have “green” plastids which are derived from primary endosymbiosis, xanthophytes represent together with some soil-dwelling diatoms and Eustigmatophytes a relatively small group of terrestrial organisms which obtained their plastids from an ancestral red alga by secondary endosymbiosis. Interestingly, due to their terrestrial habitats, members of the Xanthophyceae appear somehow as exceptions among the photosynthetic Stramenopiles. Most other Stramenopile algae and other photosynthetic lineages containing plastids that originated as red algal symbionts (e.g. the Haptophyta) dominate primary production in many parts of the oceans (Falkowski and Knoll, 2007).

Many Xanthophyceae are common terrestrial organisms, but their actual role in soils is still not known. Despite xanthophytes are rarely dominant in their habitats, they may be an essential part of natural food chains and play important roles to fuel soils with energy and organic matter. Recently, it was shown that the role of stramenopile algae in general in the biogeochemical cycling of soil carbon had been underestimated and that microbial autotrophy (including facultative autotrophic bacteria) could account for up to 4% of the total CO_2 fixed by terrestrial ecosystems each year (Yuan et al., 2012). Despite many Xanthophyceae may preferentially live in soils, a number of species have also been reported to occur at both, freshwater and terrestrial habitats (Muralidhar et al., 2013, and own observations). Some Xanthophyceae are known to tolerate freezing/thawing cycles and desiccation for extended periods of time (Nagao et al., 1999; Machová et al., 2008; Wang et al., 2013). Interestingly, a coccoid xanthophycean alga, *Pseudopleurochloris antarctica* Andreoli, Moro, La Rocca, Rigoni, Dalla Valle et Bargelloni, has been described from pack-ice in the Ross Sea, Antarctica (Andreoli et al., 1999).

Another coccoid aerophytic species, *Excentrochloris fraunhoferiana* Hofbauer, Gärtner, Rennebarth, Sedlbauer, Mayer et Breuer ,has recently been described from the surface of plaster on a modern building (Hofbauer et al., 2011). The genus *Tribonema* Derbès & Solier has also been reported from seasonal pools on grasslands which were seasonally flooded in the temperate zone (Machová et al., 2008; Cunha Pereira et al., 2011), and own observations).

There are also examples of Xanthophycean algae living in close associations with other organisms, i.e. in symbiosis, kleptoplasty or parasitism. *Heterococcus* Chodat is known from lichen symbiosis, i.e. the fungal family Verrucariaceae (Thüs et al., 2011); it is the only xanthophyte proved to be a lichen photobiont. A case of kleptoplasty is presented by the sea slug *Elysia chlorotica* which has an obligate relationship with species of *Vaucheria*, feeding only on *V. litorea* or *V. compacta* (F.S.Collins) F.S.Collins ex W.R.Taylor. The settlement and metamorphosis of the veliger larvae into adult sea slugs require the presence of *Vaucheria*. *E. chlorotica* acquires plastids by ingestion of its algal food, i.e. *Vaucheria* (Pelletreau et al., 2011). The organelles are sequestered in the mollusc's digestive epithelium where they photosynthesize for months in the absence of an algal nucleus (Rumpho et al., 2008). *V. litorea* nuclear-encoded genes were found in the genomic DNA of *E. chlorotica* supporting chloroplast endosymbiosis in the slug. These horizontally transferred genes are translated and transcribed in the host cell, and vertically transmitted to subsequent generations of the host species (Schwartz et al., 2010). A parasitic animal/xanthophyte assemblage is represented by the rotifer *Proales werneckii* Ehrenberg which lives in parasitic association with *Vaucheria* (Wallace et al., 2001). A young female rotifer colonizes a *Vaucheria* filament, it disrupts development of either a *Vaucheria* gametophore or an apical tip by inducing cell hypertrophy and formation of an excrescent gall. Remaining within the vacuole of the gall for the rest of her life, the female rotifer deposits her eggs after feeding on the rich food supply furnished by the alga's organelles which continue to move within the cytoplasm.

Distribution. Xanthophyceaen algae have been reported from all six continents (Pitschmann, 1963; Ettl, 1978; Ettl and Gärtner, 1995; Lokhorst, 2003). However, the geographical distribution of genera and species of Xanthophyceae, especially of coccoid and filamentous forms, still remains unclear due to the inappropriateness of morphological features to trace species distribution. DNA sequence comparisons using high variable molecular markers (e.g. the *psb*A/*rbc*L spacer or ITS2 rDNA) are well suited to test for geographical distribution of xanthophytes, but so far have been used only for a rather limited number of cultured strains, i.e. to test for differences between isolates from Antarctica and corresponding strains from temperate regions (Rybalka et al., 2009; Rybalka et al., 2013). Studies using molecular markers providing high resolution need to be expanded to a larger number of strains and should also include cloning and sequencing or new generation sequencing (NGS) from environmental samples. Because no data for Xanthophyceae from many geographical regions, e.g. Africa, Asia, and

South America, are available, the question of species and genera distribution still remains open. Also data about the autecology of xanthophycean algae remain rather fragmentary, because of an unclear species assignment of the investigated strains which would require a molecular assessment. Because most Xanthophyceae are microscopically small as well as resistant to drought and freezing their long-distance dispersal by air currents could be easily imagined, but has not been studied so far. Interestingly, it was pointed out that a larger soil xanthophyte, *Botrydium*, yet has not been reported from Antarctica in spite of its distinctive appearance making it hard to be overlooked in the sample material (Broady, 1996).

Age of the Xanthophyceae. The earliest putative xanthophyte fossil remains may date back to ~1000 million years ago (Ma). They belong to the late Mesoproterozoic *Palaeovaucheria* T.N. Hermann which likewise shows a one-to-one morphological comparison with the extant siphonous *Vaucheria* (Butterfield, 2007). This would be in line with a putative siphonous origin as it has been assumed by early 18S rRNA and *rbc*L sequence analyses (Bailey and Andersen, 1998). It may also be plausible because multinucleate stages often occur also in non-siphonous xanthophytes, e.g. the coccoid *Ophiocytium* (Hibberd and Leedale (1971a) and the filamentous *Xanthonema* Silva (Massalski et al., 2009). However, the idea of a putative siphonous origin of the Xanthophyceae has been rejected by later molecular phylogenetic studies which included the *psa*A gene as an additional molecular marker and an increased number of xanthophycean taxa (Negrisolo et al., 2004; Maistro et al., 2007; Maistro et al., 2009).

Other putative early fossils have been attributed to the Xanthophyceae providing further support for a rather early evolution of the group, are *Jacutianema* B.V. Timofeev & T.N. Hermann (ca. 750 Ma) and *Germinosphaera* N.S. Mikhailova (750-700 Ma); they are considered as form-taxa of *Palaeovaucheria* (see references in Brown and Sorhannus (2010). However, a much younger age of the Xanthophyceae has been assumed on the basis of molecular time-scale estimations using 18S rRNA gene sequence analyses. These analyses suggest the separation of the Xanthophyceae from its putative sister-group, the Phaeophyceae, in Early Jurassic about 187 Ma (range 119–275 Ma) (Berney and Pawlowski, 2006) or an origin of the Xanthophyceae most likely in the Middle Triassic about 233-223 Ma (range 314-151 Ma) from now (Brown and Sorhannus, 2010). The latter authors consider their results from molecular clock calculations as incompatible with putative Mesoproterozoic/Neoproterozoic xanthophyte fossils, i.e. *Palaeovaucheria, Jacutianema* and *Germinosphaera*, casting doubt on the taxonomic validity of these fossils. However, the molecular analyses did not include DNA sequences of extant *Vaucheria* making it unclear which ages would have been estimated when *Vaucheria* were included.

Economic use. No member of Xanthophyceae has been exploited for biotechnological purposes so far, or was found economically important (Friedl et al., 2012). However, only recently representatives of

the filamentous xanthophycean genera *Tribonema* and *Heterococcus* received attention as a possible feedstock for carbon-neutral biodiesel production (Nelson et al., 2013; Wang et al., 2013; Wang et al., 2014)

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Aims of the study

The goals of this thesis were to test boundaries of species of Tribonemataceae and *Heterococcus* as inferred from morphological features and to develop a reliable method for the unambiguous identification of species of filamentous Xanthophyceae. Morphological and molecular data were compared for these taxa, in an effort to provide accurate identifications and more stable scientific names. The filamentous genera of Xanthophyceae are common and widespread in soils of cold regions such as the Alps or Antarctica. Therefore, we aimed at establishing a reference data base of strains unambiguously distinguished with DNA sequence data. This should facilitate the appropriate assessment of soil microorganism diversity and changes in soil micro-organism communities, especially in soils of higher altitudes and the Polar Regions. The work has also been planned as a case study on how to define species of microalgae using molecular features and a phylogenetic species concept when the strains of microalgae exhibit extensive phenotypic plasticity which was particularly pronounced in *Heterococcus*.

Two highly variable molecular markers were chosen, i.e. the chloroplast-encoded *psb*A/*rbc*L spacer region (for the Tribonemataceae and *Heterococcus*) and the nuclear-encoded internal transcribed spacer 2 of the nuclear rDNA cistron (for *Heterococcus* only) to examine species boundaries. Also full plastid-encoded *rbc*L gene sequences should be determined to infer the phylogenetic position of species. These markers were then further tested for their suitability to identify new isolates at the species level using a range of newly established isolates

Finally, the study should contribute to the discussion on whether microorganisms display biogeography or not as our study includes strains from Antarctica as well as temperate regions. Therefore, a more thorough study of Antarctic strains of Tribonemataceae and *Heterococcus* was conducted using highly variable molecular markers in order to test for endemism or cosmopolitanism by comparing the Antarctic strains to those from temperate regions on other continents.

Environmental Microbiology (2009) **11**(3), 554–565 doi:10.1111/j.1462-2920.2008.01787.x

Testing for endemism, genotypic diversity and species concepts in Antarctic terrestrial microalgae of the *Tribonemataceae* (*Stramenopiles*, *Xanthophyceae*)

Nataliya Rybalka,[1] Robert A. Andersen,[2] Igor Kostikov,[1] Kathrin I. Mohr,[3] Andrzej Massalski,[4] Maria Olech[5,6] and Thomas Friedl[3]*

[1]*Taras Shevchenko National University, Department of Botany, Volodymyrska 64, 01017 Kiev, Ukraine.*
[2]*Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, Maine 04575, USA.*
[3]*Experimentelle Phykologie und Sammlung von Algenkulturen, Georg-August-Universität, Untere Karspüle 2, 37073 Göttingen, Germany.*
[4]*Jan Kochanowski University of Art and Natural Sciences, Biology Institute, Department of Botany, 15 Swietokrzyska, 25-406 Kielce, Poland.*
[5]*Jagiellonian University, Institute od Botany, Zdzisław Czeppe Department of Polar Research and Documentation, Kopernika 27, 31-501 Kraków, Poland.*
[6]*Polish Academy of Sciences, Department of Antarctic Biology, Ustrzycka 10/12, 02-141 Warszawa, Poland.*

Summary

The genetic diversity of all available culture strains of the *Tribonemataceae* (*Stramenopiles*, *Xanthophyceae*) from Antarctica was assessed using the chloroplast-encoded *psbA/rbcL* spacer region sequences, a highly variable molecular marker, to test for endemism when compared with their closest temperate relatives. There was no species endemic for Antarctica, and no phylogenetic clade corresponded to a limited geographical region. However, species of the *Tribonemataceae* may have Antarctic populations that are distinct from those of other regions because the Antarctic strain spacer sequences were not identical to sequences from temperate regions. Spacer sequences from five new Antarctic isolates were identical to one or more previously available Antarctic strains, indicating that the *Tribonemataceae* diversity in Antarctic may be rather limited. Direct comparisons of the spacer sequences and phylogenetic analyses of the more conserved rbcL gene revealed that current morphospecies were inadequate to describe the actual biodiversity of the group. For example, the genus *Xanthonema*, as currently circumscribed, was paraphyletic. Fortunately, the presence of distinctive sequence regions within the psbA/rbcL spacer, together with differences in the rbcL phylogeny, provided significant autoapomorphic criteria to re-define the *Tribonemataceae* species.

Received 16 July, 2008; accepted 28 August, 2008. *For correspondence. E-mail tfriedl@uni-goettingen.de; Tel. (+49) 551 397868; Fax (+49) 551 397871.

Introduction

Microalgae dominate the terrestrial habitats of Antarctica in terms of biomass and species diversity (Elster and Benson, 2004; Broady, 2005). These algae play a key role in cycling soil nutrients in the isolated Antarctic ecosystem, and they provide a source of organic matter through their photosynthesis (Tibbles and Harris, 1996; Tscherko *et al*., 2003). Long periods of snow cover, low temperatures and low water availability are major controlling factors for Antarctic microbial life (Tscherko *et al*., 2003), but not all terrestrial habitats are hostile. These terrestrial algae may be continually dispersed to Antarctica from other continents or they may be endemic to Antarctica. The goal of our study was to investigate the diversity and endemicity of yellow-green microalgae belonging to the family *Tribonemataceae* (*Xanthophyceae*, *Heterokontophyta*).

The composition and diversity of Antarctic microalgal communities of terrestrial and freshwater localities have been studied frequently (e.g. Seaburg *et al*., 1979; Broady, 1986; Pankow *et al*., 1991; Broady and Smith, 1994; Mataloni *et al*., 2000). Surveys frequently list the occurrence of the yellow-green alga *Xanthonema* (homonym *Heterothrix* Pascher; Silva, 1979), which forms unbranched filaments lacking obvious H-shaped cell wall segments (Pascher, 1932; Ettl, 1978; Ettl and Gärtner, 1995). Antarctic *Xanthonema* have been isolated into culture from water-flushed soils near glaciers, stone surfaces (hypolithic) and moss, where they are epiphytes (Broady *et al*., 1997; Broady, 2005). *Xanthonema* usually occurs in small amounts and it grows intermixed with green algae and cyanobacteria; often it is detectable only using a culturing approach. Most Antarctic culture strains belonging to the *Tribonemataceae* have been assigned to

the genus *Xanthonema* and they have been assigned to six species using a traditional morphology-based approach. A few strains could not be assigned to a species due to phenotypic plasticity (Broady *et al*., 1997). Apart from *Xanthonema*, other *Xanthophyceae* isolated from terrestrial locations in Antarctica include the genera *Tribonema*, *Bumilleriopsis* and *Heterococcus* (all *Tribonemataceae*), the filamentous *Heterotrichella* (*Heteropediaceae*) and a few non-filamentous genera (Andreoli *et al*., 1999; Mataloni *et al*., 2000; Broady, 2005).

Several studies have used SSU rRNA gene sequences to investigate the diversity of cyanobacteria in Antarctica (see Komárek *et al*., 2008 for review), and a few molecular environmental studies, using SSU rRNA, have investigated the eukaryotic algal diversity (Lawley *et al*., 2004; Fell *et al*., 2006). The latter detected xanthophycean algae only at the class or generic levels. So far, there has been only one study for inferring phylogenetic relationships of Antarctic *Xanthophyceae*, and it was based on the chloroplast-encoded rbcL and psaA genes and culture strains (Maistro *et al*., 2007). The nuclear-encoded SSU rRNA gene, widely used for prokaryotes and some eukaryotes, has been too conservative for xanthophycean species level phylogenies (Bailey and Andersen, 1998; Negrisolo *et al*., 2004). Because of its limited use, only six 18S rDNA sequences for *Tribonemataceae* are in GenBank, whereas 26 rbcL sequences available (Maistro *et al*., 2007). The rbcL data showed that *Xanthonema* strains were distributed on two or three deeply diverging clades (Maistro *et al*., 2007; Rybalka *et al*., 2007), and preliminary results for Antarctic strains showed that they were intermixed with strains from other geographical regions. This finding makes the *Tribonemataceae* an appropriate group to investigate endemicity among different culture strains. We conducted a more thorough study of Antarctic strains using a highly variable molecular marker for *Xanthophyceae*, the chloroplast-encoded psbA/rbcL spacer region (Andersen and Bailey, 2002; Rybalka *et al*., 2007). We tested for endemism or cosmopolitanism by comparing the Antarctic strains to those from temperate regions on other continents (e.g., Lawley *et al*., 2004). We also compared morphological and molecular data for these taxa, in an effort to provide accurate identifications and more stable scientific names.

Results

The psbA/rbcL spacer region sequences could not be aligned across all taxa because they are so variable (see below), and therefore rbcL gene sequences were used in phylogenetic analyses to establish their evolutionary relationships. The phylogenetic analyses showed that the Antarctic filamentous *Xanthophyceae* were distributed in three deeply diverging clades, which we name *Xanthonema* 1, *Xanthonema* 2 and *Tribonema* 1 (Fig. 1). These algae were only distantly related to *Bumilleria sicula* and *Bumilleriopsis filiformis*, which showed that the clades were not misidentified taxa belonging to other genera in the *Tribonemataceae* (Fig. 1). Within the *Xanthonema* clade 1, the rbcL sequences for Antarctic strains B4-1 and B8-5 were virtually identical, i.e. they differed in only one position. The two strains differed by only 1–2 bp from strain CCAP 808/3 (isolated from a snow field in Alaska) and from GenBank AJ874710 (from identical Antarctic strains, Ohtani 889 and Ling 906; Maistro *et al*., 2007). Strains A19 and Broady773 from Antarctica and three non-Antarctic strains (CCAP 836/2, SAG 836-1 and UTEX 353) had identical rbcL sequences. Other closest relatives to the Antarctic strains were non-Antarctic strains (UTEX 155, CCAP 808/2, CCALA 516) and Antarctic strain PAB 421 (Fig. 1). Interestingly, UTEX 155 was a duplicate strain of SAG 836-1, i.e. both represented the same isolate that had been kept at two different culture collections. However, the SAG 836-1 rbcL sequence differed by 15 and 14 positions from an existing GenBank sequence of strain UTEX 155 (AF084612) and our sequence (EF455920). Finally, Antarctic strains Broady 395 and Broady 601 and the temperate strain CCALA 517 had nearly identical sequences (8 and 9 rbcL bp separated the Antarctic strains from the temperate strain).

In *Xanthonema* clade 2, the Antarctic strains A16-5 and Turner 907 shared almost identical rbcL sequences (1 bp difference). They were related to two temperate freshwater strains, CCALA 518 and SAG 60.94 (Fig. 1). The Antarctic strain Broady 759 was more distantly related (Fig. 1). Its rbcL sequence was identical with that from the authentic strain of *Xanthonema sessile* (ASIB V98; AJ874329), and Broady and colleagues (1997) found that this Antarctic strain was identical to temperate strain ASIB V98 at the morphological level. Therefore, we assigned strain Broady 759 to *X. sessile*.

The *Xanthonema* clade 3 was formed by strain Broady 735, isolated from soil attached to vegetables imported from New Zealand into Antarctica (Broady *et al*., 1997), and two temperate Ukrainian strains (SAG 2181 and SAG 2182). The three strains varied by one position. Total pairwise rbcL sequence differences among the three *Xanthonema* clades were 63–112 bp, which was approximately the same magnitude as between representatives of three different genera. That is, there were 69–103 bp differences among *Xanthonema debile* CCALA517, *B. filiformis* SAG 809-2 and *Tribonema* sp. SAG 21.94.

The *Tribonema* clade 1 contained Antarctic strains A21 and Ohtani 887 (identical sequences) as well as Antarctic strain SAG 2165 (4 bp difference). They were most closely related to two temperate freshwater strains (SAG 21.94, SAG 23.94), which were assigned to two distinct species of *Tribonema* (Table 1). There were no more than

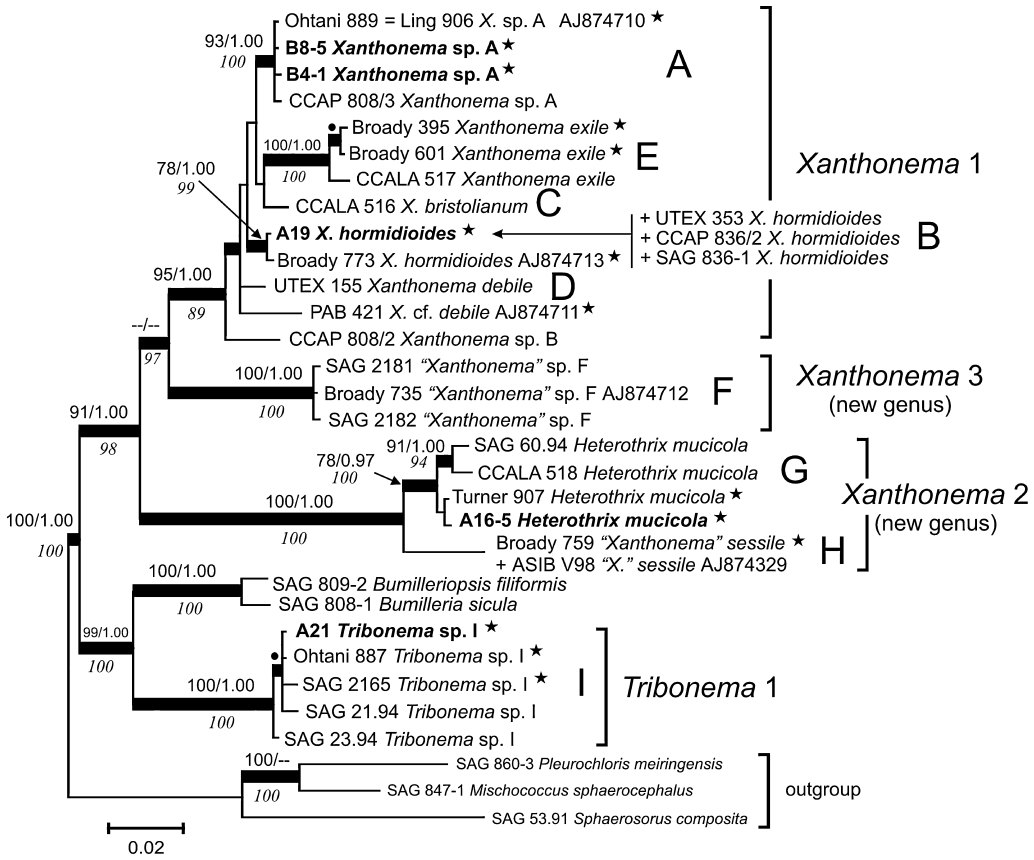

Fig. 1. Phylogenetic analysis of Antarctic strains of *Tribonemataceae* using a data set of complete rbcL sequences (1467 bp long; 295/236 variable/parsimony informative sites). Sequences in bold are the five new Antarctic isolates. Strains isolated from Antarctica are denoted by an asterisk. Sequences except those where accession numbers are given are reported for the first time in this study. Capital letters next to groups of sequences indicate psbA/rbcL spacer sequence similarity groups (see Table 1). Strains that have simply been added to the figure because their sequences were identical with those that were used in the phylogenetic analyses are marked by '+'. The phylogeny shows a Bayesian consensus tree. Thick lines indicate those internal branches that were resolved by maximum likelihood, maximum parsimony, minimum evolution distance and Bayesian analyses. High support for internal branches that connect almost identical sequences is indicated by a filled circle. Numbers at branches are bootstrap values for minimum evolution distance (> 70%; upper left), posterior probabilities from Bayesian analyses (> 0.95; upper right) and weighted maximum parsimony (below). Three unicellular *Xanthophyceae* served as an outgroup.

four and eight positions different between the rbcL sequences of these Antarctic and temperate strains.

psbA/rbcL spacer

The psbA/rbcL spacer sequences were determined to investigate the close relationships of strains within a single species or among closely related species. The spacer contained large regions of hypervariable nucleotides that were unalignable in entirety over the four major clades. However, a short region of about 30 positions at the 5′-end (pos. 93–122 of reference sequence EF455930) and a second region of about 212 bp at the 3′-end (pos. 319–531 of reference sequence EF455930) could be aligned (Fig. 2). The *Xanthonema* clade 1 psbA/rbcL spacer sequences were also aligned about 45 bp further downstream (pos. 169 of EF455930) into the hypervariable region, and five groups (A–E) were revealed (Table 1; Fig. 2). Group A strains (from Antarctica and an Alaskan snow field) were almost identical except for a single position and a single 1 bp indel. Group A did not correspond to any morphological species, and therefore it was designated *Xanthonema* sp. A.

Five strains comprised group B (Table 1; Fig. 2). Two strains were from Antarctica (A19 and Broady 773) and their sequences were almost identical, i.e. they differed in a single sequence position (Fig. 2) and by two indels of 1 bp each. Both Antarctic strains were distinct from the three temperate strains (which had identical spacer regions) by the presence/absence of a short indel (7–9 bp) at the 5′-end of the psbA/rbcL spacer as well as at two other sequence positions. Two of the three temperate strains with identical spacer sequences (CCAP 836/2, UTEX 353) were duplicate authentic strains for *Xanthonema hormidioides*. The third strain (SAG 836-1) was an authentic strain for *X. debile*. The authentic strains were

Table 1. Strains of *Tribonemataceae* grouped according to similarities of their psbA/rbcL spacers and clades as inferred from the rbcL phylogeny shown in Fig. 1.

rbcL clade	psbA/rbcL spacer similarity group/ new species designation	Strain and previous species designation	Location	Length of psbA/rbcL spacer
Xanthonema 1	A/*X.* sp. A	**B4-1** *Xanthonema* or *Bumilleria* (= SAG 2183)	King George Island (A)	439
		B8-5 *Xanthonema* sp. (= SAG 2184)	King George Island (A)	440
		Ling 906 *Xanthonema debile* (= SAG 2190)	King George Island (A)	440
		Ohtani 889 *X. debile* (= SAG 2191)	King George Island (A)	440
		CCAP 808/3 *Bumilleria* sp.	USA, cold-tolerant	439
	B/*X. hormidioides*	**A19** *Xanthonema* sp. (= SAG 2179)	King George Island (A)	431
		Broady 773 *Xanthonema pascheri* (= SAG 2188)	Ross Island (A)	429
		CCAP 836/2 *Heterothrix hormidioides* (= SAG 2288[a,b])	Europe, temperate	422
		SAG 836-1 *X. debile*	Europe, temperate	422
		UTEX 353[b] *H. hormidioides*	Europe, temperate	n.a.[c]
	C/*X. bristolianum*	CCALA 516 *Xanthonema bristolianum* (= SAG 2285[a])	Europe, cold-tolerant	449
	D/*X. debile*	UTEX 155 *Heterothrix debilis* (= SAG 2289[a])	Europe, temperate	430
	E/*X. exile*	Broady 395 *X. debile* (= SAG 2185)	Vestfold Hills (A)	451
		Broady 601 *X. debile* (= SAG 2186)	Ross Island (A)	451
		CCALA 517 *Xanthonema exile* (= SAG 2286[a])	Europe, temperate	385
Xanthonema 3 (new genus)	F/'*X.*' sp. F	Broady 735 *Xanthonema* cf. *bristolianum* (= SAG 2187)	New Zealand, cold-tolerant	451
		SAG 2182 *Xanthonema* sp.	Europe, temperate	451
		SAG 2181 *Xanthonema* cf. *exile*	Europe, temperate	451
Xanthonema 2 (new genus)	G/*Heterothrix mucicola*	**A16-5** *Xanthonema* or *Tribonema* (= SAG 2192)	King George Island (A)	476
		Turner 907 *Xanthonema hormidioides* (= SAG 2194)	Southern Victoria Land (A)	476
		CCALA 518 *H. mucicola*	Europe, temperate	475
		SAG 60.94 *Xanthonema* cf. *bristolianum*	Europe, temperate	492
	H/'*X.*' *sessile*	Broady 759 *Xanthonema sessile* (= SAG 2193[a])	Ross Island (A)	418
Tribonema 1	I/*T.* sp. I	**A21** *Xanthonema tribonematoides* (= SAG 2172)	King George Island (A)	534
		Ohtani 887 *X. tribonematoides*	King George Island (A)	n.a.[c]
		SAG 2165 *Tribonema* sp.	Adelaide Island (A)	536
		SAG 21.94 *Tribonema ulotrichoides*	Europe, temperate	536
		SAG 23.94 *Tribonema viride*	Europe, temperate	536

a. Epitype strain.
b. Duplicate strains.
c. Only a 5′ partial psbA/rbcL spacer sequences was obtained.
New isolates are in bold. (A) Strains isolated from Antarctica.

(putatively) established by Vischer when he described both *X. hormidioides* and *X. debile* (Vischer, 1936; 1945). However, a duplicate authentic strain of *X. debile*, UTEX 155, was distinctly different (group D), indicating that SAG 836-1 became mislabelled during its history. Therefore, all five group B strains were named *X. hormidioides*.

Group E (Antarctic strains Broady 395 and 601), whose rbcL sequences were identical, also had identical psbA/rbcL spacer sequences (Fig. 2). They differed from their closest relative (CCALA 517) by 15 sequence positions and an insertion of 65 bp. The CCALA 517 strain was identified as *X. exile*; it was isolated from a temperate locality, as was the culture used by Klebs (1896) when he described that species, and therefore we used that name for group E. *Xanthonema exile* was the type species for the genus *Xanthonema*, and therefore species in clade 1 must be named *Xanthonema.*

Group C and D sequences from strains CCALA 516 and UTEX 155 were rather distinct as well as strain CCAP 808/2, i.e. they could not be meaningfully aligned (Table 1; Fig. 2). Group C (CCALA 516) was identified as *Xanthonema bristolianum* and we used that name. Group D contained the authentic strain (UTEX 155) for *X. debile* and thus the group was assigned this name. Strain CCAP 808/2, which was identified as *Bumilleria exilis*, was not closely related to strain CCALA 517 (Fig. 2). Because we designated CCALA 517 as the epitype for *X. exile* (see below), CCAP 808/2 had to be assigned a different species that we designated *Xanthonema* sp. B. Note that strain PAB 421, used in the rbcL phylogenetic analyses, was not included because the strain became extinct before the psbA/rbcL spacer sequence could be determined.

Xanthonema clade 2 was deeply divergent from *Xanthonema* clade 1, and the clade represented a new genus distinct from *Xanthonema*. Clade 2 had two groups, G and H (Table 1; Fig. 2). Within group G, the Antarctic strains A16-5 and Turner 907 had identical spacer sequences. They differed from the temperate strain of that group, CCALA 518, by 18 sequence positions, an indel of one nucleotide and another indel of two nucleotides. The other temperate strain of group G, SAG 60.94, appeared to be even more distant from the two Antarctic strains based upon the spacer region, i.e. with differences in 15

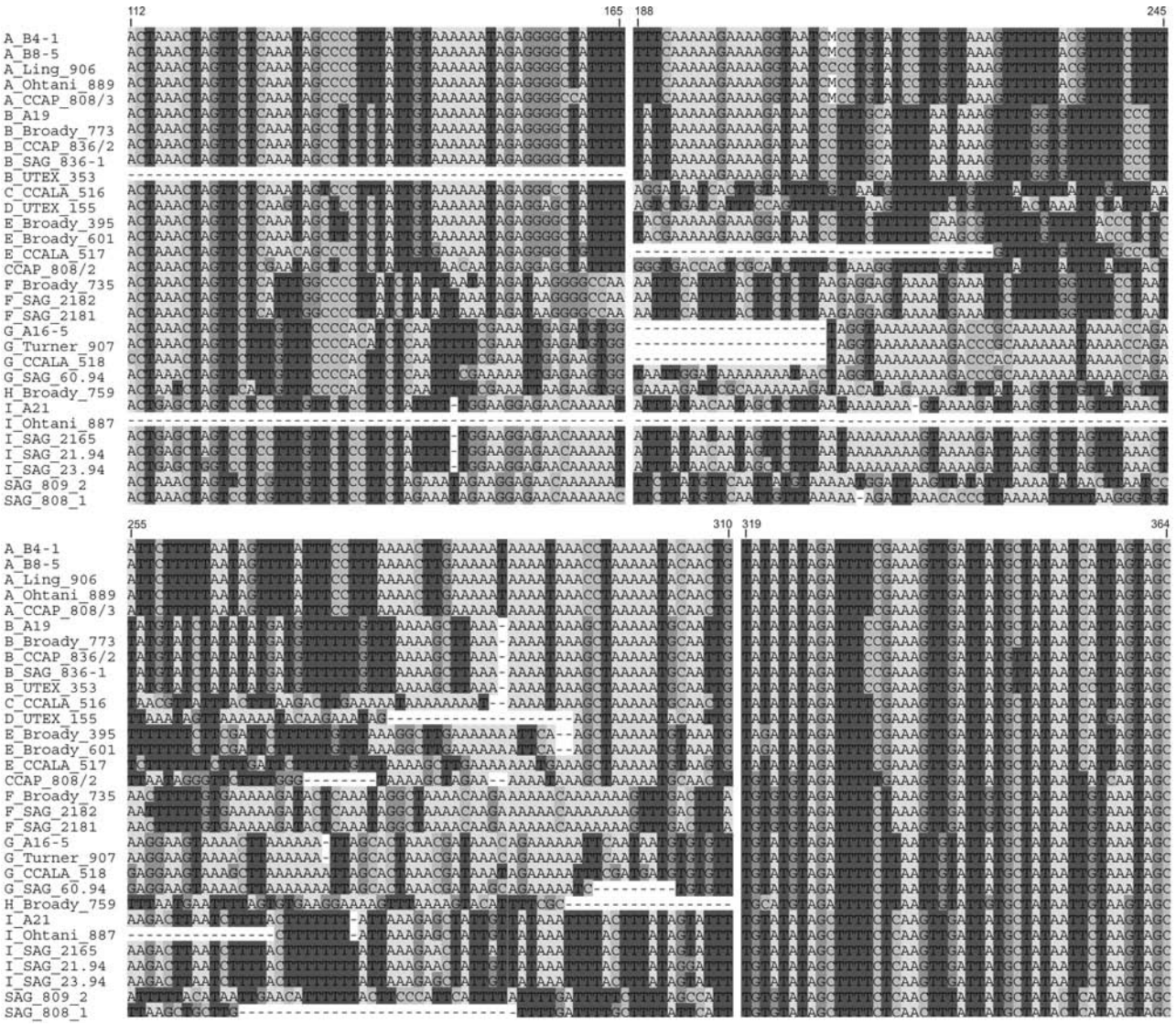

Fig. 2. Alignment of psbA/rbcL spacer sequences of studied strains of *Tribonemataceae* grouped according to sequence similarity (groups A–I, see Table 1). Parts of the more conserved 5′- and 3′-ends are shown as well as a large part of the hypervariable region in between. Numbers refer to sequence positions of strain B4-1 (Accession No. EF455930).

sequence positions (Fig. 2) and five indels of 1–17 bp long. However, the hypervariable regions of all group G strains were still easily aligned (Fig. 2). Group G contained strain CCALA 518 that represented *Heterothrix mucicola*; therefore, we temporarily assigned the group G strains to this species until a new genus can be described (see taxonomic discussion below). In group H, Antarctic strain Broady 759 (assigned *X. sessile*; see above) was more distantly related because its psbA/rbcL spacer was shorter and the spacer could not be aligned with those from group G (Fig. 2).

Xanthonema clade 3 (group F) sequences aligned well (Fig. 2) but the clade was deeply divergent, and strains appeared to belonging to a second new genus (Fig. 1). Broady 735, from New Zealand soil material, had no more than three and eight sequence differences when compared with the two Ukrainian temperate strains (SAG 2181 and SAG 2182; Fig. 2). The strains in group F did not correspond to any described morphological species, and therefore we temporarily named the strains '*Xanthonema'* sp. F until a new genus and species is described.

Tribonema clade 1 had a total of 16 variable spacer sequence positions. Isolate A21 was identical with strain Ohtani 887; however, only a 5′ partial sequence, 356 bp, could be obtained for the latter strain (Fig. 2). Antarctic strain SAG 2165 differed from strain A21 by nine sequence positions and two indels of 1 bp each. Both had only 9/12 and 6/5 sequence differences with their next

closest temperate relatives, strains SAG 21.94 and SAG 23.94. Strains in group I could not be assigned a single species name because group I contained two *Tribonema* species and therefore we temporarily named the group *Tribonema* sp. I until an unambiguous species name can be found.

Discussion

Endemism

The *Tribonemataceae* (*Stramenopiles*, *Xanthophyceae*) diversity, based upon all available Antarctic culture strains plus five new isolates, was assessed using the conserved rbcL gene and the highly variable non-coding psbA/rbcL spacer. The spacer region allows a detailed comparison of the Antarctic strains with those from temperate regions at or even below the level of species and therefore the spacer can be used to test for endemism. The term endemism refers to a group of organisms that is confined to a single geographical region, or with respect to Antarctic microorganisms, endemics have genotypes found only in Antarctica (Vincent, 2000). However, endemism applied to microorganisms is a controversial topic (Lawley *et al*., 2004). It has long been suspected that microbial dispersal is not restricted by geographic boundaries (Baas-Becking, 1934; Staley and Gosink, 1999; Finlay, 2002). Currently, there is limited evidence for microbial endemism in Antarctic terrestrial environments (e.g. Roser *et al*., 1993; Broady, 1996; 2005), but most previous studies have been based solely on traditional morphological and/or culture approaches. In a pioneering study of eukaryotic diversity in Antarctic soils, Lawley and colleagues (2004) used the rather conserved 18S rRNA gene sequences, and they found a high degree of similarity for Antarctic isolates with non-Antarctic organisms. However, they found it impossible to confirm that their taxa were conspecific with isolates from other geographic locations because 18S rRNA is so highly conserved. In our study, the Antarctic strains were distributed among six psbA/rbcL spacer sequence similarity groups, but each group also included strains from other geographic regions. The *Xanthonema* group A strains shared almost identical psbA/rbcL spacers despite they were from quite distant locations, but of similar ecology, i.e. from Alaska (strain CCAP 808/3) and from Antarctica (Table 1). Group A strains may represent a new species of *Xanthonema* that is cold-adapted because it is known only from cold regions. Also within *X. hormidioides* (group B) and group F, there were pairs of strains from distant locations within Antarctica (and thus of similar ecology; strains A19/Broady 773 and strains A16-5/Turner 907) that shared identical hyper variable spacer regions with each other. The high similarity in the psbA/rbcL spacer regions clearly indicates conspecificity, i.e. the strains from Antarctica cannot be distinguished from temperate strains at the species level. We conclude that there is no *Tribonemataceae* species endemism in Antarctica and that the high genetic similarity reflects a similar ecology rather than a geographical closeness. Furthermore, no clades in the rbcL phylogeny were restricted to a geographical region, indicating that the *Tribonemataceae* species are generally widespread where suitable habitats exist.

Despite the close similarity of Antarctic and temperate strains, we did not find a single case where an Antarctic strain was identical in its psbA/rbcL spacer region to that of an isolate from a temperate location. The fewest differences between Antarctic and temperate strains were within *X. hormidioides* (group B) and *Tribonema* sp. 1 (group I). *Xanthonema exile* (group E) and '*Xanthonema'* sp. F (group F) also had Antarctic and temperate strains with psbA/rbcL spacer regions distinct from each other. The differences between the Antarctic and temperate strains were greater than those within either the Antarctic or the temperate strains. These findings imply that the Antarctic and temperate strains represent two different populations of a single species. It further implies that the Antarctic strains for a given species share a common evolutionary history. That is, there was just one colonization event in Antarctica for each group of strains with nearly identical spacer regions or, if multiple colonization events occurred, then the invasions were too recent to produce significant divergence (Whitaker *et al*., 2003; Prosser *et al*., 2007). It is not possible to calculate divergence times because there are no reliable fossil records for the *Tribonemataceae* or the *Xanthophyceae*. It would be intriguing to investigate ancient soil samples for the presence of *Tribonemataceae* DNA because the old soils may have been isolated for long time. Also, it would be interesting to test for phenotypic differences between the Antarctic and temperate populations, e.g. with respect to cold tolerance.

Tribonemataceae *diversity in Antarctica*

Only six different psbA/rbcL spacer groups were recovered for the Antarctic *Tribonemataceae*. Although the number of culture strains currently available (15) is rather limited, it is remarkable that the psbA/rbcL spacer sequences of the five newly established Antarctic isolates were identical (or nearly so) to corresponding sequences of previously available strains. Furthermore, only two of the six known groups were not recovered by the new isolates (groups E and H). Remarkably, the diversity represented by the new isolates was found in a relatively small but ecologically diverse area, the Admiralty Bay region on King George Island. The material was collected from valleys with soils of different moisture content, i.e. slopes or tops of moraines at various distances from a glacier and the seacoast. Guano and bird excrements

further influenced at least two sites (Tscherko *et al.*, 2003). We conclude that *Tribonemataceae* diversity is rather limited in Antarctica, but once a species initially colonizes the continent, the species spreads by adapting to various habitats. Certainly, a more extensive field sampling is needed to provide more conclusive data and to confirm this hypothesis. Further studies should also include a culture-independent approach to recover *Tribonemataceae* taxa that are difficult or impossible to culture. The psbA/rbcL spacer PCR amplification approach used here appears to be selective for *Xanthophyceae* (Andersen and Bailey, 2002) and would facilitate an environmental molecular study.

Limits of current morphospecies

Species of the *Tribonemataceae* have been defined by morphological data alone even though the family is morphology-poor (Ettl, 1978; Ettl and Gärtner, 1995; Lokhorst, 2003; Zuccarello and Lokhorst, 2005). We conclude that the morphological features traditionally used to define taxa are inadequate to describe the actual biodiversity in this group. This is clearly exemplified with the five newly established Antarctic isolates. Strains A19 and A16-5 were distantly related in the rbcL phylogeny (Fig. 1), but they had only minor morphological differences (Fig. 3A and B). Conversely, strains B4-1 and B8-5 shared almost identical hypervariable spacer sequences. However, strain B4-1 exhibited an internal thick cap-like structure between two adjacent cells (Fig. 3C), a morphological character that is used to define *Bumilleria* (Ettl, 1978). Both strains fragment readily into short filaments with two to four cells (Fig. 2C and D), which is typical for *Bumilleria*, but strains B4-1 and B8-5 were rather distinct from the clade representing true *Bumilleria* and *Bumilleriopsis* (Fig. 1). The presence of H-shaped wall fragments

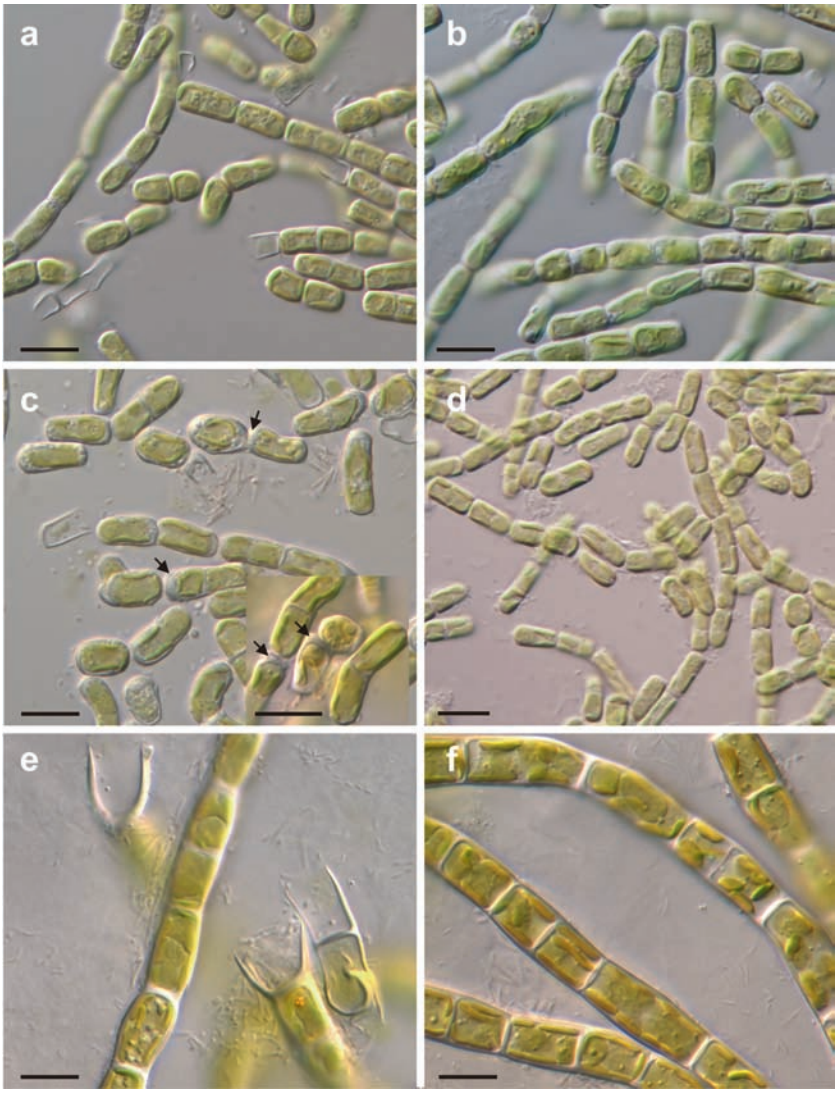

Fig. 3. Morphological features of five new strains of *Tribonemataceae* isolated from soils of King George Island, Antarctica. Terminal H-pieces at fragmented filaments obvious in strain A19 (A). Short and thin filaments with no terminal H-pieces visible at fragment ends in strain A16-5 (B). Internal cap-like thick structure (arrows) present at readily fragmented filaments of strain B4-1 (C). Strain B8-5 also exhibits fragmentation into short filaments of two to four cells, but lacks the cap-like structure (D). Strain A21 exhibits prominent H-pieces at ends of filament fragments (E) and has large cells that are somehow rounded and thickened in their middle (F). Scale 10 μm (A–F).

(Fig. 3A and E) has been the classical diagnostic feature for *Tribonema* (Ettl, 1978). However, two strains with obvious such H-pieces, A19 and A21, are not closely related to each other and only one (A21; Fig. 3E and F) is related to true representatives of *Tribonema* (Fig. 1). We conclude that the morphological features are more likely to be homoplasious than the nucleotides of the spacer region.

Molecular phylogenetic species concept

The *Tribonemataceae* are rarely (if ever) sexual and therefore the biological species concept is not applicable (e.g. Mayr and Ashlock, 1991). Johansen and Casamatta (2005) proposed the 'monophyletic species concept' (autoapomorphic species concept *sensu* Mishler and Theriot, 2000a) for asexual taxa because they consider it the most theoretically sound and well suited. The monophyletic species concept recognizes species as the smallest monophyletic groups worthy of taxonomic recognition. Monophyly within a species is recognized by the presence of autapomorphies (i.e. unique derived characters present only in those species; Mishler and Theriot, 2000a,b; Johansen and Casamatta, 2005). Johansen and Casamatta (2005) propose, in cases where morphological data are insufficient, that a monophyletic species can be identified by a maximum likelihood analysis using molecular sequence data. They proposed that in cyanobacteria distinctive 16S-23S rRNA ITS secondary structures and genetic distances in 16S rRNA gene sequences were molecular evidences for genetic separation of species. We propose that the psbA/rbcL spacer, in combination with the rbcL gene analysed with maximum likelihood, represent autoapomorphic criteria for distinguishing taxa within the *Tribonemataceae*. Within one of our species, the psbA/rbcL spacer sequences are readily aligned over their entire lengths, and differences are limited to single base pairs and short indels. For each species, the rbcL sequences have few (< 10 bp) or no differences, and the strains of each species form a monophyletic clade (Fig. 1). Between two tribonematacean species, the entire psbA/rbcL spacer sequences cannot be aligned (Fig. 2). We also note that for species within the same genus, the spacer sequences can be aligned further downstream at the 5′-end than is possible between genera. We recognize genera when the species form a deeply diverging clade in the rbcL phylogeny. For example, groups A–E form the *Xanthonema* clade 1, which corresponds to the genus *Xanthonema sensu strictu* (see below).

Xanthonema strains from Antarctica, which were originally identified based upon morphological characters, were distributed in three deeply diverging clades of the rbcL phylogeny (*Xanthonema* clades 1 and 2, *Tribonema* 1; Fig. 1). The same patterns were found using direct comparisons of the psbA/rbcL spacer sequences, i.e. the rbcL and spacer molecular data sets were congruent. Using the molecular definition for species and genera, the Antarctic *Tribonemataceae* comprises six species in three genera (*Xanthonema*, *Tribonema* and one genus listed here simply as *Xanthonema* 2). This contrasts with previous morphological studies where the same taxa were placed in approximately 10 species and three genera, *Bumilleria*, *Tribonema* and *Xanthonema*. As shown in the rbcL phylogeny (Fig. 1), strains representing *Bumilleria* are distinct from the *Xanthonema* clade 2, providing molecular evidence that clade 2 represents an undescribed new genus. Therefore, we conclude that *Xanthonema*, as defined by only morphological characters, is paraphyletic within the *Tribonemataceae*. Maistro and colleagues (2007) obtained similar results in a study using the rbcL and psaA genes (but no psbA/rbcL spacer region). Thus, *Xanthonema* clade 1 represents the genus *Xanthonema* because it includes the type species, *X. exile* (Fig. 1). *Xanthonema* clade 2 (i.e. *Xanthonema mucicola* and *X. sessile*, see below) and clade 3 represent new genera. Preliminary data showed that the clade 2 may even be identified using new morphological characters. That is, in clade 2, zoospores develop into germlings with a holdfast (attachment disk) and stalk (Broady *et al*., 1997; N. Rybalka, unpublished), whereas clade 1 and clade 3 develop elongated symmetric germlings from zoospores.

Taxonomic conclusions

We have used the molecular data to establish unambiguous identifications for the study organisms from Antarctica, and consequently we establish epitypes for four species names to better anchor the species concept (see below). The type material for *Tribonemataceae* species is generally an iconotype (ink drawing, light micrograph), and it is impossible to obtain DNA from iconotypes (Hoef-Emden *et al*., 2007; Williamson *et al*., 2007). An authentic strain, i.e. the culture strain used during the description of the species, is a valuable resource, but it lacks acceptability by the International Code of Botanical Nomenclature if it is not cryopreserved (Art. 8.4, McNeill *et al*., 2006). For many Xanthophycean species, their taxonomy has undergone a paradigm shift from morphology to gene sequences. Ideally, one would examine type material for DNA so that molecular data can be directly tied to the type specimen upon which the name is anchored. When type material is ambiguous, the International Code of Botanical Nomenclature allows that epitypes may be established as a means for clarifying and anchoring names (Art. 9.7, McNeil *et al*., 2006). Therefore, we will attempt to stabilize some *Tribonemataceae* names by formally establishing

Table 2. List of cryopreserved *Xanthonema* culture strains formally proposed as epitype material to stabilize *Tribonemataceae* names and their holotypes (iconotypes).

Epitype	Holotype (Iconotype)
X. bristolianum (Pascher) Silva, strain SAG 2285[a] (= CCALA 516)	Rabenhorst's Krypt.-Fl. Deutschl., 2. Aufl., 11: 924, fig. 778 (1939)
X. debile (Vischer) Silva strain SAG 2289[a,b] (= UTEX 155)	Ber. Schweiz. Bot. Ges. 45:379, figs 2 and 3 (1936)
X. exile (Klebs) Silva[c], strain SAG 2286[a] (= CCALA 517)	Beding. Fortpflanz. Alg. u. Pilz. p. 389, pl. II, figs. 15–20 (1896)
X. hormidioides (Vischer) Silva, strain SAG 2288[a,b] (= CCAP 836/2)	Ergeb. Wiss. Unters. Schweiz. Nationalparkes, N.F.1: 499, plate 1, figs. 6 and 14 (1945)
X. sessile (Vinatzer) Ettl et Gärtner, strain SAG 2193[a] (= Broady 759)	Plant Syst. Evol. 123:214, fig. 1 (1975)

a. Permanently preserved in a metabolically inactive state (cells stored in liquid nitrogen vapours at *c.* –165°C) (Sammlung von Algenkulturen der Universität Göttingen: Albrecht-von-Haller-Institut, Universität Göttingen, Nikolausberger Weg 18, 37073 Göttingen, Germany).
b. Authentic strain.
c. Type species for the genus.

epitypes for four species that occur in Antarctica, i.e. *X. exile*, *X. hormidioides*, *X. sessile* and *X. mucicola* as well as two other species that are closely related to the former, *X. bristolianum*, and *X. debile*, in Table 2.

Finally, we summarize some additional taxomic and culturing information. *Xanthonema hormidioides* was described by Vischer (1945) (as *Heterothrix hormidiodes*) and duplicate authentic strains CCAP 836/2 and UTEX 353 were derived from a culture established by Vischer when he described the species. Strain SAG 836-1 shared an identical psbA/rbcL spacer sequence with the duplicate strains, UTEX 353 and CCAP 836/2. However, SAG 836-1 was previously named *X. debile*, and apparently this strain/name was mixed with another strain/name in the past. Subsequently, a second strain was identified as *X. hormidioides* (Turner 907), but in our analyses, it is distinctly different and belongs to a separate species. *Xanthonema sessile* was described by Vinatzer (1975) (as *Heterothrix sessilis*) and it was transferred to *Xanthonema* by Ettl and Gärtner (1995). An authentic strain (ASIB V98; Vinatzer, 1975) was established, and that strain was previously used in DNA sequence analyses (Maistro *et al*., 2007). The strain is now extinct (G. Gärtner, pers. comm.) and it was unavailable for this study. However, strain Broady 759 has an identical rbcL sequence and we conclude that both strains represent the same species (Table 2).

Experimental procedures

Field sites, isolation of strains and microscopy

Five new strains were isolated from soil material collected by M.O. during the Antarctic summer (20 February 2002) (Table 1). Soils were not covered by snow, and came from two transects along the seashore, Admiralty Bay region, King George Island, maritime Antarctica (A), and near Ecology Glacier (B). The transects were established for botanical study (Olech, 2002). Transect A was 10–20 m inland from the seashore, whereas transect B was located about 370 m farther inland. The sampling sites for the three strains were along transect A, separated by approximately 20–30 m, i.e. strains A16-5 (62°09.993′S, 58°27.582′W; from sandr), A19 (62°10.138′S, 58°27.816′W; at plain near a lake), and A21 (62°10.175′S, 58°27.893 W; close to a lagoon). Strain A21 was collected closest to the glacier (30 m) and A16-5 was farthest from the glacier. Transect B sampling sites were about 20 m apart and yielded strain B4-1 (62°09.942′S, 58°28.073′W; at a small valley between two moraines and farthest from the glacier) and strain B8-5 [62°10.024′S, 58°28.064′W; at bottom of a moraine and closest (200 m) from the glacier]. The sea level soil surface temperatures can be considerably higher than the overlying air temperatures when the soils are not covered by snow. The soils may not even experience freeze–thaw cycles during several months, and air temperatures may reach 15°C during Antarctic summer. Therefore, the newly isolated strains may be represented by mesophilic rather than strictly psychrophilic organisms. Further details about the study area, climate, soil properties and transects are given in Olech (2002) and Tscherko and colleagues (2003). After collection, the soil material was kept frozen in plastic bags at –20°C to avoid possible contamination during transportation and storage prior to the isolation of organisms into culture. Strain A21 was isolated soon after collection by I.K., the other strains were isolated in 2006 by N.R. (A16-5, B4-1 and B8-5) and A.M. (A19). For isolating the algae, a small amount of soil was thawed at room temperature and then sprinkled onto a Petri dish with 1.5% agar enriched with modified Bold's Basal Medium containing triple nitrate (Bischoff and Bold, 1963). Petri dishes were maintained at 18°C and illuminated with white fluorescent bulbs under a light/dark regime of 14 h:10 h and a photon fluence rate of about 25 μmol photons m^{-2} s^{-1}. Colonies were further isolated and purified using standard microbiological techniques (Guillard, 2005) and the unialgal culture strains were then maintained on agar slants under the same conditions as used for isolation. The five new strains are publicly available from the SAG culture collection under the strain numbers listed in Table 1. Microscopic observations were accomplished using an Olympus BX60 microscope with Nomarski DIC optics and cultures 3–4 weeks old. Micrographs were taken with a ColorView III camera (Soft

Imaging System GmbH, Münster, Germany) and processed with the Cell^D image program (Soft Imaging System GmbH, Münster, Germany).

Other Antarctic and reference strains for molecular analyses

Cultures of additional available Antarctic strains were kindly provided by Drs C. Andreoli and P.A. Broady and these strains were accessioned by the SAG culture collection (Table 1). *Tribonema* strain SAG 2165 was kindly provided by Dr. A. Lukešova. For comparison with the Antarctic strains, closely related reference strains, isolated elsewhere than in Antarctica, were selected from a large rbcL gene sequence phylogeny (N. Rybalka, unpublished). The reference strains were from temperate regions of Europe (Germany, Switzerland, Ukraine) with three exceptions: strain Broady 735 was isolated from soil adherent to plant material that was imported into Antarctica from New Zealand (Broady *et al*., 1997), strain CCAP 808/3 was isolated from a snow field in Alaska (USA) and CCALA 516 from snow detritus in the Tatra mountains (Slovakia) (Table 1).

DNA extraction, PCR amplification and sequencing

DNA was extracted from fresh cultures after breaking the cells with glass beads in a Minibeadbeater cell homogenizer (Biospec, Bartlesvilles, OK, USA) and then using an Invisorb Spin Plant Mini DNA extraction kit (Invitek, Berlin, Germany). The spacer region that lies upstream of the rbcL gene, i.e. between the psbA and rbcL genes, and full-length sequences of the rbcL gene (1469 bp) with adjacent rbcL/rbcS spacers were amplified using the PCR approach of Andersen and Bailey (2002), which was modified to amplify the target sequence in one piece. It was noted that the target sequence was easier to amplify with DNA extracted from actively growing cultures than from cultures older than 4 weeks. The 5′ primer (psbA5, Andersen and Bailey, 2002), or Xan1F (5′-CCCATTAGATTTAGCAGCT-3′) or Xan2F (5′-TCCCATTAGATTTAGCAGCTG-3′), was anchored in the psbA gene and the 3′ primer (RS3; Andersen and Bailey, 2002) was placed in rbcS (downstream of rbcL), thereby amplifying the full-length rbcL gene and the spacer region between the psbA and rbcL genes. The PCR was performed with an initial 'hot start' for 5 min at 95°C, proceeded by 35 cycles at 94°C for 1 min, 51°C for 1 min and 72°C for 2 min 30 s. Positive PCR products were pooled, purified using Invisorb Spin PCRapid Kit (Invitek, Berlin, Germany) or NucleoSpin Extract II (Macherey-Nagel, Düren, Germany) and sequenced using the two PCR primers and a set of primers internal to the PCR product, i.e. those of Andersen and Bailey (2002) and the following newly determined ones: 4FA, the reverse complement of 4RA (Andersen and Bailey, 2002) close to the 5′-end, Xan3R (5′-TCAGGTAAAAA CTACGGTCGT-3′), Xan3F (5′-ACGACCGTAGTTTTTACCT GA-3′), X5FG (5′-ATGCGTTGGAGAG-3′, pos. 1177–1190) and 8R (5′-GACCTTGTAATCGGTTACACAG-3′) at middle positions, and X7Fm (5′-CTTCAATTTGGTGGTGGTACAA-3′), 9Fma (5′-GGTGGTGGTACA/TATTGGT-3′), 9Rma (5′-GGTGGTGGTACAATTGGT-3′) and X7Rm (5′-CTTCAA TTTGGTGGTGGTACAA-3′) close to 3′-end of the PCR product. The sequences were assembled using the program SeqAssem (Hepperle, 2004). The sequences were aligned using ClustalW in BioEdit v.6.0.7 (Hall, 1999) and then manually refined by eye.

Phylogenetic analyses

Model selection, number of rate categories, proportion of invariable sites and the gamma distribution parameter were determined with MODELTEST version 3.7 (Posada and Crandall, 1998). Phylogenetic analyses of the rbcL sequence data set was performed using maximum likelihood, maximum parsimony, minimum evolution distance (Rzhetsky and Nei, 1992) and Bayesian analyses, with the program PAUP* version 4.0b10 (Swofford, 2001) or MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003). For minimum evolution distance and maximum likelihood analyses, the GTR + I + G model (Tavaré, 1986) was used with estimations of nucleotide frequencies (A = 0.3033, C = 0.1746, G = 0.2112, T = 0.3109), a rate matrix with six different substitution types, assuming a heterogeneous rate of substitutions with a gamma distribution of variable sites (number of rate categories = 4, shape parameter $\alpha = 0.5425$) and a proportion of invariable sites (pinvar) of 0.5190. Bootstrap resamplings (1000 replications) were performed on minimum evolution distance and maximum parsimony trees. For the Bayesian analysis, the GTR + I + G model (rate matrix with six different substitution types, number of rate categories = 4, and with the nucleotide frequencies, shape parameter α and pinvar estimated from the data) was used; four Markov chains and 2 000 000 generations sampling every 100 generations were used with the first 25% of the sampled trees discarded, leaving 15 000 trees. Posterior probabilities were then calculated from two independent runs using the 50% majority rule consensus of the kept trees.

Nucleotide sequence accession numbers

The sequence alignment is available from EMBL-Align (http://www.ebi.ac.uk), Accession Number ALIGN_001299. The newly determined sequences have been submitted to the DDBJ/EMBL/GenBank databases under following accession numbers: EF455930, EF455931, EF426794, EF426796, EF426797 (new isolates); EF455932, EF455933, EF455935, EF455936, EF455947, EF455951-EF455953 (Antarctic reference strains); EF455922, EF455925, EF455934, EF455937, EF455938, EF455939, EF455948, EF431851, EF455955-EF455957, EF455962, EF455964, EF455966, EF455970, EF455972, EF455975, EF455977, EF455982 (other reference strains); EF455962, EF455970 and EF455972 (outgroup taxa).

Acknowledgements

This study was supported by a research scholarship of the DAAD extended to N.R. We appreciate the support extended to N.R. by Dr. G. Berthold, which was made possible through his generous donation to the SAG Culture Collection at the University of Göttingen. Parts of this work were financed by

the German Federal Ministry of Education and Research, BMBF (AlgaTerra Project, Grant 01 LC 0026) within the BIOLOG programme. I.K. and N.R. are thankful to National Antarctic Scientific Center of Ukraine for provision of equipment for isolation of Antarctic strains. R.A.A. acknowledges support from USA NSF Grant 0629564. N.R. and T.F. are thankful to Opayi Mudimu, Elke Zufall-Roth, Jessica Schäckermann, Ilse Kunkel and Marlis Heinemann for provision of assistance in molecular methodologies, purification and maintenance of culture strains. We thank Paul Silva for his advice on taxonomic questions.

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Chapter 2

Assessing genus and species boundaries of Tribonemataceae algae (filamentous Xanthophyceae, Stramenopiles) in culture collections using chloroplast-encoded molecular markers

ABSTRACT

To test genus and species boundaries within filamentous Xanthophyceae exhibiting bipartite cell walls (Tribonemataceae), the plastid-encoded *psb*A/*rbc*L spacer region and full *rbc*L sequences were determined for 34 strains (incl. one environmental clone), previously assigned to the four genera *Bumilleria* Borzì*, Bumilleriopsis* Printz*, Pseudobumilleriopsis* Deason & Bold and *Tribonema* Derbès & Solier. About half of this sample were new isolates which we attempted to identify at the species level using sequence comparisons with reference strains from public culture collections. Visual comparisons of the *psb*A/*rbc*L spacer regions revealed that strains previously assigned to a single genus based on morphology were split into seven distinct sequence similarity groups which we propose to represent genera. The same groups were resolved, with high statistical support, in phylogenetic analyses of complete *rbc*L sequences. The strains assigned to *Bumilleria, Bumilleriopsis* and *Pseudobumilleriopsis* were separated into three *psb*A/*rbc*L spacer similarity groups and the strains of *Tribonema* were separated into four different groups. No distinction between *Bumilleria*, *Bumilleriopsis* and *Pseudobumilleriopsis* was seen. Correspondingly, strains assigned to *Bumilleriopsis* as based on morphology were distributed on three distinct groups. Between groups the *psb*A/*rbc*L spacers differed in length and no alignment was possible and, therefore, discriminate genera. Interestingly, each group (genus) had a characteristic length of the *psb*A/*rbc*L spacer, i.e. there was no overlap in length between the groups. Strains of *Tribonema* group 2 were particularly distinct because their spacers were almost three times as long as the other Xanthophyceae. Considerable spacer sequence differences within a similarity group, in particular at the more variable 5'-end of the *psb*A-*rbc*L spacer region, were found valuable for the unambiguous identification of species. The considerable spacer differences detected within groups *Bumilleriopsis* 1 and *Bumilleriopsis* 3 (which we propose to represent the genera *Bumilleria* and *Bumilleriopsis*, respectively) imply that both consist of several species each. The absence of significant *psb*A/*rbc*L spacer differences within groups *Tribonema* 1 and *Tribonema* 2, each containing multiple strains, is interpreted that both consist of only a single species each. In the latter case molecular data are more conservative than morphology, because *Tribonema* 1 and *Tribonema* 2 strains have previously been assigned to six and three different species based on morphology.

INTRODUCTION

This chapter is based on a published extended abstract of an oral presentation held at the *11th International Conference on Culture Collections, ICCC11, Goslar, Germany, 7-11 October 2007* (Rybalka et al., 2007); it is added as appendix at the end of this thesis. The analyses of the three genera*, Bumilleria, Bumilleriopsis* and *Tribonema* were refined and are discussed in greater detail than in the extended abstract. The analyses with respect to *Xanthonema s.l.* and *Heterococcus* were excluded because they have already been published elsewhere (Rybalka et al., 2009; Rybalka et al., 2013; see chapters 1 and 3).

Filamentous microalgae of the class Xanthophyceae (Stramenopiles) are important primary producers in freshwater and soil (Andersen, 2004). However, their correct identification is often difficult due to a paucity of morphological characters and the presence of extensive phenotypic plasticity (Zuccarello and Lokhorst, 2005). Growth forms surprisingly similar to the Xanthophyceae evolved in parallel in the

green algae (Chlorophyta) and the Eustigmatophyceae, which further complicates accurate taxonomic identities (Ott et al., 2015). Species descriptions of many xanthophytes are incomplete because they were based on natural samples. In culture, however, variation of morphological features can be observed and additional distinctive features are discovered. Many public culture collections maintain filamentous xanthophycean algae and these strains could be important references for identification. However, only a few strains have been examined to verify that they were correctly identified.

Previous molecular phylogenetic studies have resolved multiple origins for the filamentous Xanthophyceae and, therefore, revealed the traditional order Tribonematales (Ettl, 1978; Silva, 1979) which encompasses all filamentous xanthophytes, not corresponding to a natural phylogenetic group. The unbranched forms with bipartite cell walls (e.g. *Tribonema*), i.e. the Tribonematacae Pascher (Silva, 1979), are not closely related to the branched forms with cell walls consisting of one piece, i.e. *Heterococcus* (Bailey and Andersen, 1998; Negrisolo et al., 2004). This divergence between Tribonemataceae and *Heterococcus* is also supported by differences in flagellar apparatus ultrastructure of both genera, *Tribonema* and *Heterococcus* (Lokhorst and Star, 2003; O´Kelly, 1989). Likewise, strains assigned to *Xanthonema* have been found be of multiple origins (Negrisolo et al., 2004; Maistro et al., 2007; Maistro et al., 2009; Rybalka et al., 2009) and also the monophyly of *Tribonema* has been questioned previously (Zuccarello and Lokhorst, 2005). For *Bumilleria* no more than just two strains have been studied at the molecular level so far (Maistro et al., 2007; Maistro et al., 2009). Therefore, we attempted with an extended sample of new isolates provisionally assigned by morphology to *Tribonema* and *Bumilleria* to further investigate the phylogenetic relationship of both genera.

The Tribonemataceae Pascher is a family of the traditional xanthophyte order Tribonematales; it comprises the unbranched filamentous genera with bipartite cell walls, i.e. *Bumilleria* Borzì, *Xanthonema* P.C. Silva and *Tribonema* Derbès & Solier (Ettl, 1978; Ott and Oldham-Ott, 2003). We use "Tribonemataceae" here for a group of genera which includes two additional genera, i.e. *Bumilleriopsis* Printz and *Pseudobumilleriopsis* Deason & Bold. For the latter genera also unbranched filamentous stages are known, e.g. *B.opsis filiformis* Vischer (strain SAG 809-2; Fig. 3), and bipartite cell walls are also characteristic features for both genera. In traditional classification, however, both *Bumilleriopsis* and *Pseudobumilleriopsis* are assigned to the order Mischococcales which includes all coccoid xanthophytes (Ettl, 1978). Recent multigene phylogenetic analyses resolved the Tribonemataceae as paraphyletic with the coccoid *Ophiocytium* Nägeli and the siphonous *Botrydium* Wallroth within the Tribonematalean clade of Xanthophyceae (Maistro et al., 2009; Fig. 2). The latter describes a group well supported in molecular phylogenetics which comprises the Tribonemataceae as well as strains of coccoid (*Bumilleriopsis*, *Ophiocytium*) and siphonous (*Botrydium*) organizations. It was concluded that

"there are no clear boundaries between siphonous *vs*. uninucleate and coccoid *vs.* filamentous taxa" in the Tribonematalean clade (Maistro et al., 2009, p. 423).

The main goal of the current study was to test genus and species boundaries within the filamentous Xanthophyceae exhibiting bipartite cell walls, i.e. the Tribonemataceae (see below). We were using a highly variable gene sequence region, i.e. the plastid-encoded *psb*A/*rbc*L spacer region which previously has successfully been employed for resolving species phylogenetic relationships with the xanthophyte *Vaucheria* (Andersen and Bailey, 2002). In the current study the groupings of strains based on *psb*A/*rbc*L spacer sequence similarities were compared to groups and clades as revealed in gene phylogenies using full *rbc*L sequences. The latter has been found rather useful in phylogenetic studies of the Xanthophyceae (Bailey and Andersen, 1998; Negrisolo et al., 2004; Maistro et al., 2007; Maistro et al., 2009). The highly variable *psb*A/*rbc*L spacer region sequences were established for a range of reference strains for the Tribonemataceae incl. *Bumilleriopsis* as available from public culture collections (Table 1). These sequences were then further tested for their suitability to identify new isolates at the species level using a sample of 17 newly established isolates (Table 1).

MATERIALS AND METHODS

In this study, we examined 34 Xanthophyceae strains (incl. one environmental clone, EF431847) and we focused on the taxonomically difficult filamentous genera (Tribonemataceae), i.e. *Tribonema* (21 strains), *Bumilleria* (5 strains), *Bumilleriopsis* (7 strains) and *Pseudobumilleriopsis* (1 strain). A total of 17 new isolates of Tribonemataceae (Table 1) was compared with the same number of reference strains (including three authentic strains) from the *Sammlung von Algenkulturen der Universität Göttingen*, Germany (SAG*;* www.epsag.uni-goettingen.de; Friedl and Lorenz, 2012), and the Culture Collection of Algae at the University of Texas at Austin, USA (UTEX; www.utex.org). We included many strains whose species identification was not determined yet. We used DNA sequence comparisons of the chloroplast-encoded *rbc*L gene and the *psb*A/*rbc*L spacer region. Only the Xanthophyceae have the *psb*A gene consistently upstream of the *rbc*L gene and this allows for a PCR approach selective for Xanthophyceae (Andersen and Bailey, 2002). The 5' primer was anchored in the *psb*A gene and the 3' primer was placed in *rbc*S (downstream of *rbc*L), thereby amplifying the full-length *rbc*L gene and the spacer region between the *psb*A and *rbc*L genes. For details about DNA extraction, PCR, sequencing and phylogenetic analyses see chapter 1. For four studied strains of *Tribonema* (SAG 880-1, SAG 200.80, SAG 2177, SAG 2178; the group *Tribonema* 2, see below) which exhibited extraordinarily long *psb*A/*rbc*L spacers the primers spacer1R (5'-TAGACATGTATTCCTCC-3', pos. 1420-1404 of reference sequence EF431848 from strain SAG 2178), spacer2R (5'-ATTCGAGTACGCTCTTGTAC-3', pos. 1445-1426 of EF431848) and trispacR (5'-TTCACCTACTAAAGCTGATATAGG-3', pos. 383-360 of EF431848)

were used for sequencing of their long spacers. Seven strains previously assigned to *Bumilleria* or *Bumilleriopsis*, i.e. SAG 2158, SAG 2159, SAG 34.93, SAG 57.94, SAG 58.94, SAG 809-2, and SAG 809-3, were also studied for their ultrastructure, in particular for the presence or absence of a girdle lamella in the chloroplasts. Standard methods for Transmission Electron Microscopy (TEM) according to Massalski et al. (2009) were applied and the studies carried out the laboratory of Prof. A. Massalski at Jan Kochanowski University of Art and Natural Sciences, Kielce, Poland.

RESULTS AND DISCUSSION

Analyses of *psb*A/rbcL spacer sequences and *rbc*L phylogeny

The 5'-end of the *psb*A/*rbc*L spacer region was highly variable and it was valuable for the unambiguous identification of species (Fig. 1). Visual comparisons of *psb*A/*rbc*L spacer sequences revealed that strains previously assigned to a single genus based on morphology were split into several distinct groups (Fig. 1). The same groups were resolved, with high statistical support, in phylogenetic analyses of complete *rbc*L sequences (1467 base pairs long); each group represented monophyletic subclades of the Tribonematalean clade of Maistro et al. (2009) (Fig. 2). The strains assigned to *Bumilleria, Bumilleriopsis* and *Pseudobumilleriopsis* were separated into three *psb*A/*rbc*L spacer similarity groups, i.e. *Bumilleriopsis* 1, *Bumilleriopsis* 2 and *Bumilleriopsis* 3 of Rybalka et al. (2007), which was in disagreement with a distinction of the former genera. No distinction between *Bumilleria*, *Bumilleriopsis* and *Pseudobumilleriopsis* was seen, i.e. group *Bumilleriopsis* 1 included strains assigned to all three genera (Table 1; Figs. 1, 2). Correspondingly, strains assigned to the genus *Bumilleriopsis* as based on morphology were distributed on three distinct groups or clades, *Bumilleriopsis* 1-3. The strains of *Tribonema* were separated into four different groups, i.e. *Tribonema* 1, *Tribonema* 2, *Tribonema* 3 and *Tribonema* 4 (Table 1; Figs. 1, 2).

The *rbc*L phylogeny showed evolutionary relationships among the groups, e.g., *Bumilleriopsis* group 2 and *Xanthonema* group 2 were closely related with each other. Also, *Tribonema* groups 1, 2 and 3 were closely neighboring, but without statistical support (Fig. 2). There was also no statistical support for the closer relatedness of *Tribonema* 1 and *Tribonema* 3. *Tribonema* group 2 was a well-supported sister taxon to *Tribonema* group 3, even though it had a much longer *psb*A/*rbc*L spacer (Fig. 2). Most intra-group relationships within those groups which contained more than just two strains, i.e. *Tribonema* 1 and *Bumilleriopsis* 1, had weak statistical support.

Between groups the *psb*A/*rbc*L spacers differed in length and no alignment was possible. Interestingly, each spacer similarity group of Tribonemataceae had a characteristic length of the *psb*A/*rbc*L spacer, i.e. there was no overlap in length between the groups (Table 1). Strains of *Tribonema* group 2 were

particularly distinct because their spacers were with 1325 nts almost three times as long as the other Xanthophyceae, however, the sequences within that group were almost invariant. Therefore, assignment to *Tribonema* group 2 was already possible after PCR amplification because the amplicon was larger than for other Xanthophyceae (3.0 kB compared to about 2.2 kB). Spacers in the *Tribonema* 1 group had only minor length variation (534-537 nts, but for five strains no full sequences of the spacer could be obtained), whereas in *Bumilleriopsis* group 1 they varied considerably, i.e. 461-539 nts.

The *psb*A/*rbc*L spacer regions and *rbc*L gene phylogenies help define genera and species. Strains with spacer sequences that exhibit significant differences, but are still easily aligned, may represent different species within a single genus. Similarly, strains with nearly identical spacer sequences may represent a single species. For example, spacer sequences between *Tribonema* groups 2 and 3 cannot be aligned, and they are apparently two distinctly different genera, despite their close relationship in the *rbc*L gene phylogeny. There was also a considerable spacer sequence difference within a spacer similarity group, despite within each group at least the less variable region at the 3'-end of the *psb*A/*rbc*L spacer region could be well aligned (Fig. 1). This was found within groups *Bumilleriopsis* 1 and *Bumilleriopsis* 3, implying that both consist of several species each.

Group *Bumilleriopsis* 1: genus *Bumilleria* with six species (Fig. 3A-E)

Bumilleria. The *Bumilleriopsis* 1 group comprises strains which clearly exhibit filaments or filamentous stages that become easily fragmented. Both authentic strains in the group, *B.opsis filiformis* SAG 809-2 and *Pseudobumilleriopsis pyrenoidosa* SAG 69.90, have been described as exhibiting clearly filamentous growth forms (Vischer, 1945; Deason and Bold, 1960); Fig. 3A, B). Filamentous growth was also observed in *Bumilleria sicula* SAG 808-1 (Fig. 3 C) and our isolates SAG 2157, SAG 2158, SAG 2159 and SAG 2160 (Fig. 3D, E). Strain *B. sicula* SAG 808-1 is so far the only available *Bumilleria* strain which has been identified to species level; it has also been used in other molecular phylogenetic studies to represent the genus (Maistro et al., 2007; Maistro et al., 2009). Because there is no authentic strain available to represent *Bumilleria*, we suggest strain SAG 808-1 to be used as a reference and epitype strain to represent the genus. We assign group *Bumilleriopsis* 1 to the genus *Bumilleria* because it contains filamentous xanthophytes closely related with the reference strain, SAG 808-1.

B. filiformis, B. klebsiana* and *B.* sp. A.** Among the 10 strains which formed group *Bumilleriopsis* 1, there were only three pairs of strains with identical or almost identical *psb*A/*rbc*L spacer sequences (Table 1). Each pair may represent a distinct species, i.e. *Bumilleria filiformis , B. klebsiana* Pascher and unnamed species A. ***B. filiformis (Table 1)**.** Strain SAG 2161, a new isolate from soil in Ukraine, shared an identical *psb*A/*rbc*L spacer (and *rbc*L sequence*)* with the authentic strain of *Bumilleriopsis filiformis*, SAG 809-2 (Vischer, 1945), and, therefore, was readily identified as the same species. We propose a new combination for the authentic strain, *Bumilleria filiformis*, and to transfer the species to the genus

Bumilleria. ***B. klebsiana*** (Table 1; Figs 3D, E)**.** Two isolates from distant localities in Ukraine, SAG 2157 and SAG 2158, had identical *psb*A/*rbc*L spacers and, therefore, represent the same species. Using morphological features observed in culture, strain SAG 2158 was assigned to *Bumilleria klebsiana.* We suggest the latter strain to be used as an epitype to link the name to a culture strain (Table 1). **Species A** (Table 1)**.** Strains SAG 2159 and SAG 2160, two new isolates from soils of distant localities in Ukraine, had *psb*A/*rbc*L spacers identical except for 6 positions and three indels of 1, 6 and 13 nucleotides in lengths; three positions differed among their *rbc*L gene sequences. Both strains were left unidentified because they were distant from any named reference strain. The rather variable regions at the 5' end of the *psb*A/*rbc*L spacers of both strains were still well alignable and, therefore, we regard both strains as single independent species, unnamed species A.

B. pyrenoidosa, B. sicula, B. **sp. B and** ***B.*** **sp. C**. Four more strains had distinct spacer sequences within the clade and, therefore, may be regarded as four more independent species, i.e. *B. pyrenoidosa, B. sicula* and unnamed species B and C. ***B. pyrenoidosa.*** Strain SAG 69.90 is the authentic strain of the type of the genus *Pseudobumilleriopsis* Deason and Bold (1960). Its very close relationship with other strains of *Bumilleria* (*Bumilleriopsis* group 1) is revealed by the *psb*A/*rbc*L spacer sequence comparisons and it is placed within the same clade, *Bumilleriopsis* 1, in the *rbc*L gene phylogeny (Fig. 2). Consequently, we suggest the new combination *Bumilleria pyrenoidosa* here. **Species B.** Unidentified strain *Bumilleria* SAG 57.94 was most closely related to *B. pyrenoidosa* SAG 69.90 , i.e. the spacer sequences of both strains were readily alignable except for an indel at the 3'-end of the *psb*A/*rbc*L spacer, 73 (SAG 69.90) and 19 (SAG 57.94) nucleotides long, and they differed at 14 sites. We regard these differences as sufficient to keep strain SAG 57.94 as another independent, yet unnamed species of *Bumilleria*. ***B. sicula*** (Fig. 2 C). Strain SAG 808-1 was another independent species within the *psb*A/*rbc*L spacer similarity group of *Bumilleria* and we suggest to establish strain SAG 808-1 as a reference and an epitype to link the name *Bumilleria sicula* Borzì to a culture strain. **Species C.** Unidentified strain SAG 58.94 exhibited a *psb*A/*rbc*L spacer sequence distinct from that of any other strain of the group and, therefore, it may represent another independent species of *Bumilleria*, i.e. yet unnamed species C (Table 1).

Group *Bumilleriopsis* 1 has already been resolved previously, i.e. the monophyletic origin of strains SAG 808-1, SAG 809-2, SAG 69.90, SAG 57.94 and SAG 58.94 has been well supported using plastid-encoded *rbc*L, *psa*A and nuclear 18S rRNA genes (Maistro et al., 2007; Maistro et al., 2009). However, both previous molecular-phylogenetic studies made only few suggestions regarding the distinction of species within the group. Maistro et al. (2009) kept *Pseudobumilleriopsis pyrenoidosa* as name for strain SAG 69.90 because this species is the type of *Pseudobumilleriopsis*, despite the species had already been assigned *Bumilleriopsis pyrenoidosa* (Deason and Bold) Ettl ealier (Ettl, 1978; Ettl and

Gärtner, 1995). They even assigned authentic strain *Bumilleriopsis filiformis* SAG 809-2 together with SAG 69.90 to *Pseudobumilleriopsis*. That Maistro et al. (2009) regarded the three strains as closer relatives and distinct from *Bumilleria* may be due to that they used molecular markers with less resolution than the *psb*A/*rbc*L spacer sequences and a reduced strain selection compared to our study here.

The distinctness of group *Bumilleriopsis* 1 may also be seen in unique ultrastructural features that define the group, i.e. the genus *Bumilleria.* Strain SAG 808-1 *Bumilleria sicula* and all other strains representing distinct species within the *Bumilleriopsis* 1 group have unique ultrastructural features in common which may serve as synapomorphies to distinguish the group as an independent genus. The strains are characterized by the absence of a girdle lamella in the chloroplast (Fig. 4A, C, D), a feature which is quite exceptional because a girdle lamella is found in all other Xanthophyceae examined so far (Hibberd, 1990), and it is a widespread feature that characterizes most photosynthetic stramenopiles (Andersen, 2004). In addition, in those strains also a pyrenoid-like structure was found, i.e. a region in the plastid where the spaces between thylakoids are somehow widened (Fig. 4C, D). These features have already been observed in strains SAG 808-1 and SAG 69.90 previously (Massalski and Leedale, 1969; Deason, 1971) and are reported for five additional strains for the first time in this study, i.e. SAG 809-2, SAG 57.94, SAG 58.94, SAG 2158 and SAG 2159. In contrast, both the strains SAG 34.93 and SAG 809-3 assigned to *Bumilleriopsis* and studied for their ultrastructure here, exhibited a typical girdle lamella (Fig. 4B). The latter strains were not members of group *Bumilleriopsis* 1 (*Bumilleria*), but group *Bumilleriopsis* 2 and *Bumilleriopsis* 3 (see below).

Group *Bumilleriopsis* 3: *Bumilleriopsis* with two species (Fig. 3F, G)

This group included only two strains, *Bumilleriopsis peterseniana* SAG 809-3 which is the authentic strain of that species (Vischer, 1936) and unidentified strain SAG 33.93. For both strains only partial sequences at the 3'-ends of the *psb*A/*rbc*L spacers could be obtained (Table 1). Here both strains were readily alignable except for two unalignable segments of different lengths, i.e. 17/16 nucleotides long between positions 15 and 33 of the SAG 809-3 sequence (acc. no. EF455942) and 80/72 nucleotides long between positions 63 and 144 of the same reference sequence. Consequently, we regard strain SAG 33.93 to represent a species distinct from *B.opsis Peterseniana* Vischer & Pascher, but closely related to it within the same group (genus). The monophyletic origin of both strains has already been revealed by the multi gene analyses of Maistro et al. (2007, 2009) and found distinct from "*Pseudobumilleriopsis*" and *Bumilleria* strains. The strain *B.opsis peterseniana* SAG 809-3 is authentic and we regard this strain to represent well the features of the genus *Bumilleriopsis*. Because no strain of the type species, *B.opsis brevis* (Gerneck) Printz (Silva, 1979), is available, we use strain SAG 809-3 instead to assign group *Bumilleriopsis* 3 to the genus *Bumilleriopsis.*

Group *Bumilleriopsis* 2: new genus with one species (Fig. 3 H)

A single strain previously assigned to *Bumilleriopsis*, SAG 34.93, represented an independent lineage within the Tribonematalean clade (Fig. 2); it had a sister group-relationship with group *Xanthonema* 2 of Rybalka et al. (2009) in the *rbc*L phylogeny. Also, its *psb*A/*rbc*L spacer was unalignable with any other Tribonemataceae studied here. We conclude strain SAG 34.93 represents a still unnamend species of an independent (new) genus.

Group *Tribonema* 1: one genus with one species

The *psb*A/*rbc*L spacers were well alignable among 11 strains assigned to the genus *Tribonema* and two strains assigned to *Xanthonema tribonematoides* (Pascher) P.C. Silva (SAG 2172, SAG 2173) based on morphology as well as one environmental clone (Helgoland 7; EF431847). They formed a distinct well supported monophyletic clade in the *rbc*L phylogeny, group *Tribonema* 1 (Fig. 2). Their *psb*A/*rbc*L spacers were not alignable with any other studied strains assigned to *Tribonema* or *Xanthonema*. The group *Tribonema* 1 strains had a characteristic *psb*A/*rbc*L spacer length of 534-537 nucleotides (Table 1). The length variation was due to a total of 4 indels of one nucleotide in length each and varied at no more than 41 positions within the group. There were two pairs of strains with identical *psb*A/*rbc*L spacer as well as identical *rbc*L sequences, i.e. SAG 2165/SAG 2168, SAG 2172/SAG 2173. The *psb*A/*rbc*L spacers were also identical in two more groups, i.e. SAG 21.94/SAG 2166 and SAG 2176/SAG2174/SAG2175, which, however, had one and two pairwise differences in their *rbc*L sequences. According to the molecular species definition of Rybalka et al. (2009) strains which belong to the same species have identical *psb*A/*rbc*L spacer sequences or their spacer sequences can be readily aligned over their entire lengths in conjunction with no or few (< 10 bp) differences in the full *rbc*L sequences. Between two different species of the same genus, the entire *psb*A/*rbc*L spacer cannot be aligned, but only a more conserved region at the 3'-end of the spacer. There was no strain within group *Tribonema* 1 which would fulfill the latter condition, i.e. they were rather few spacer sequence differences within the group. Therefore, all strains in group *Tribonema* 1 may represent just a single species. However, there was a particular group of eight strains (i.e. SAG 21.94, SAG 2166, SAG 2174, SAG 2175, SAG 2176, SAG 23.94, SAG 2172, and SAG 2173) among which no more than 4 pairwise *psb*A/*rbc*L spacer sequence differences were observed and one to 6 pairwise differences in their *rbc*L sequences. This group of strains had slightly larger differences with other *Tribonema* group 1 strains, i.e. 5 to 8 spacer sequence and 3 to 10 *rbc*L differences with the other 5 strains of the same group. Interestingly, the molecular data are more conservative than morphology. *Tribonema* 1 strains have previously been assigned to six different species based on morphology, i.e. *T. ulotrichoides, T. intermixum, T. tribonematoides, T. viride, T. regulare,* and *T. minus* (Table 1).

Group *Tribonema* 2: one genus with one species

The group *Tribonema* 2 was formed by five strains previously assigned to *Tribonema* which exhibited extraordinarily long *psb*A/*rbc*L spacers, i.e. 1325 nucleotides in length. Therefore, members of this group could already be identified by the size of their PCR products when using the same primers as for other tribonematacean strains. Interestingly, the group *Tribonema* 2 was supported as a close sister group with group *Tribonema* 3 in the *rbc*L phylogeny (Fig. 2). Therefore, without comparisons of the highly variable *psb*A/*rbc*L spacer sequences the *Tribonema* 2 group would probably not have been recognized. In previous molecular phylogenetic studies of the Xanthophyceae using less variable gene sequences just a low support in bootstrap tests for the common origin of *Tribonema* strains was noticed, but no distinction into groups was made (Maistro et al., 2007; Maistro et al., 2009). Among *Tribonema* 2 strains there was only little sequence variation in their *psb*A/*rbc*L spacers, i.e. among strains SAG 200.80, SAG 808-1 and SAG 2178 there were no more than 4 sequence positions different and the spacers of strains SAG 200.80 and SAG 808-1 were even identical with each other. Only strain SAG 2177 had 40/43 *psb*A/*rbc*L spacer positions different with other strains of *Tribonema* 2, but its spacer was readily alignable with the corresponding sequences of other members of the group. The partial sequence of the *psb*A/*rbc*L spacer we could obtain for strain *T. missouriense* UTEX 2549 had no more than two positions different with corresponding sequences of other *Tribonema* 2 strains. Therefore, only a single strain, SAG 880-1, was used in the *rbc*L phylogenetic analysis (Fig. 2). We conclude the five strains studied here for *Tribonema* 2 group represent just a single species of another distinct genus due to their very few sequence differences. Therefore, group *Tribonema* 2 is another case where molecular data were more conservative than morphology. Based on morphology the *Tribonema* 2 strains previously have been assigned to three different species (Table 1).

Group *Tribonema* 3: one genus with one species

Group *Tribonema* 3 included only two strains, *T. utriculosum* SAG 22.94 and *T. vulgare* SAG 24.94. With their *psb*A/*rbc*L spacer sequences they were distinct from all other studied strains and they formed a monophyletic lineage in the *rbc*L phylogeny (Fig. 2). The *rbc*L gene sequences of both strains had 14 pairwise differences. Their *psb*A/*rbc*L spacer sequences were readily alignable, except for three indels of 9/9, 21/11 and 65/117 (SAG 22.94/SAG 24.94) nucleotides which were unalignable and accounted for the spacer length differences between both strains, i.e. 459 nts in SAG 22.94 and 502 nts in SAG 24.94. The spacer sequences had just 5 differences at single positions at both ends and there was an indel of a single nucleotide at the 3'-end of the spacer between both strains. Consequently, because of their rather similar *psb*A/*rbc*L spacers and just very few differences in their *rbc*L gene sequences, we conclude both strains represent a single species.

Group *Tribonema* 4: one genus with one species

Strain UTEX 639 *Tribonema* sp. exhibited a *psb*A/*rbc*L spacer sequence of 417 nts in length, distinct from all other studied strains. It also had an independent position within the *rbc*L phylogeny, i.e. there was no significant statistical support for a grouping of the strain with any other group of the studied Tribonemataceae (Fig. 2). Therefore, strain UTEX 639 is assigned a genus of its own where it represents a still unnamed species.

CONCLUSIONS

Using our *psb*A/*rbc*L spacer and *rbc*L gene sequence data as references, we attempted to unambiguously identify new isolates (12 our strains, one environmental clone and four strains of other scientists) from terrestrial and freshwater habitats in the Ukraine and from soils in Antarctica (Table 1). We found that all the new isolates could be unambiguously assigned to our groups. All these examples demonstrate that a single sequence of the *psb*A/*rbc*L spacer, when compared to reference strains, can unambiguously identify new Xanthophyceae culture strains. Those strains assigned to *Bumilleria* on the basis of morphology were confirmed to represent *Bumilleria* (group *Bumilleriopsis* 1) by the molecular data. Identification for the new isolate, SAG 2161, tentatively assigned to *Bumilleriopsis filiformis* on the basis of morphology, was confirmed at the species level, i.e. the new isolate even shared identical *psb*A/*rbc*L spacer and *rbc*L gene sequences with authentic strain SAG 809-2 *B.opsis filiformis*. This means that the molecular distinctions were corroborated by morphological differences observed in culture, e.g. cell shapes and dimensions, formation of filamentous stages and germination of zoospores (N. Rybalka, unpubl. data). The 12 new isolates assigned to *Tribonema* and the environmental clone were distributed on two groups (genera), *Tribonema* 1 and *Tribonema* 2. Interestingly, 10 of these strains and the environmental clone were members of the *Tribonema* 1 group which previously was represented by only two SAG strains (SAG 21.94 and SAG 23.94). The latter group obviously represents a rather common genus widespread at geographically distant localities, it has even been recorded from Antarctic soils (Table 1; Rybalka et al., 2009). An Antarctic strain, SAG 2165, shared an identical *psb*A/*rbc*L spacer with strain SAG 2168 from Europe (Ukraine). This finding supports widespread distribution rather than specialization for temperate or cold habitats in the Tribonemataceae (Rybalka et al., 2009). To find morphological characters that corroborate the molecular distinction between the four genera on which the strains previously assigned to a single genus *Tribonema* are distributed a detailed re-investigation of morphological, including ultrastructural, features is needed. The morphological features used to describe the three genera *Bumilleria, Bumilleriopsis* and *Tribonema* are not congruent with the molecular data and, therefore, are not phylogenetically informative at the generic level. *Bumilleriopsis* group 1, the genus *Bumilleria*, lacks a

girdle lamella in their chloroplasts, whereas the strains of both, group *Bumilleriopsis* 2 and *Bumilleriopsis* 3 (i.e. *Bumilleriopsis*) possess a girdle lamella. Thus, it appears that some morphological features will corroborate the molecular data while other morphological features (e.g. those currently used to differentiate species of *Tribonema*) appear to have little taxonomic value for distinguishing species or even genera of Tribonemataceae (Rybalka et al., 2009).

The 17 studied new Tribonemataceae isolates were assigned to five species out of which only two could be named (Table 1). Our study recovered six species of *Bumilleria*, out of which three were left unnamed, and two species of *Bumilleriopsis* of which was one left unnamed. One species of *Bumilleriopsis* (*B.opsis. filiformis*) and *Pseudobumilleriopsis pyrenoidosa* were transferred to *Bumilleria* here. One additional genus (*psb*A/*rbc*L spacer similarity group) for a strain with *Bumilleria/ Bumilleriopsis*-like morphology was revealed. The still unnamed species may correspond to already described species of either genera, *Bumilleria, Bumilleriopsis* or *Tribonema*, for which, however, no cultures are available. For example, a total of four and five other species for *Bumilleria* and *Bumilleriopsis* are listed as taxonomically accepted in (Guiry and Guiry, 2015). Because no cultures are available for them, they first need to be rediscovered and isolated into culture and epitypes based on the cryopreserved cultured strains need to be proposed for these species in order to stabilize the names. Then sequence comparisons with our strains might reveal names for our still unnamed species. For *Xanthonema* studied culture strains which were cryopreserved have been proposed as epitypes to stabilize six species names within the genus (Rybalka et al., 2009). Similarly, epitypes for *Bumilleria borziana* N. Wille and *Bumilleriopsis brevis* (Gerneck) Printz, the type species for both genera, need to be established (after determining their *psb*A/*rbc*L spacer sequences) in order to clarify whether our *psb*A/*rbc*L spacer similarity groups *Bumilleriopsis* 1 and *Bumilleriopsis* 3 in fact correspond to *Bumilleria* and *Bumilleriopsis*. None of the strains studied here correspond to the descriptions of either species.

The 21 studied strains previously assigned to *Tribonema* (as nine named species and six strains unidentified at the species level) were found to represent only four species which, however, were distributed on four different genera. An epitype of *Tribonema bombycina* (C.Agardh) Derbès & Solier, the type species of *Tribonema*, needs to be established in order to fix *Tribonema* to one of our groups *Tribonema* 1-4. For *Tribonema* strains species assignments may be even more complicated because of the large number of species that have been described (Lokhorst, 2003); currently there are 16 species of *Tribonema* listed as taxonomically accepted in Guiry and Guiry (2015). There is also some confusion about the status of the type *T. bombycina*. It is stated in Zuccarello and Lokhorst (2005) that *T. bombycina* were "usually ascribed to the more commonly used name, *T. viride*" (p. 384) whereas Guiry and Guiry (2015) list *T. bombycina* as a taxonomic synonym of *T. utriculosum* (Kützing) Hazen. Strains

which could represent *T. viride* and *T. utriculosum* were found in two distinct *psb*A/*rbc*L spacer similarity groups, i.e. *Tribonema* 1 and *Tribonema* 3 (Table 1).

We hope that our study will encourage researchers to establish further new isolates of Tribonemataceae in order to rediscover more of the previously described Tribonemataceae species and to subject them to DNA sequence comparisons. This will be crucial to finally clarify the taxonomy at both generic and species levels of this common and widely distributed group of yellow green algae.

ACKNOWLEDGEMENTS

Parts of this work were supported by the German Science Foundation (DFG) by a grant extended to T.F. (Fr 905 ⁄20-1). An earlier version of this work was supported by travel grants of the DAAD and ICCC11 (2007) conference organizing committee extended to N.R.

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Table 1. The 34 xanthophycean strains (incl. one environmental clone) previously assigned to three genera, *Bumilleria, Bumilleriopsis*, *Pseudobumilleriopsis* and *Tribonema* studied here for their *psb*A/*rbc*L spacer and *rbc*L gene sequences, their distribution on seven *psb*A/*rbc*L spacer groups (genera), their new genus/species assignments, their origins, corresponding DNA sequence accession numbers for their *psb*A/*rbc*L spacer (with their lengths in brackets) and full *rbc*L sequences and the sources where these molecular data have been analyzed for the first time.

Boxed are those groups of strains which are considered to represent a single species (see text). Asterisks mark the 17 new isolates used in this study; [1,2]strains studied for their chloroplast ultrastructure, [2]results reported here for the first time.

*psb*A/*rbc*L spacer group	new genus/species designation	previous genus/species designation	strain/isolate	origin (isolated by)	*psb*A/*rbc*L spacer (length)	
Bumilleriopsis 1	*Bumilleria filiformis*	*Bumilleriopsis filiformis*	[2]SAG 809-2 (authentic strain)	Switzerland, soil (Vischer)	EF431851 (520)	this study
		Bumilleriopsis cf. *filiformis*	SAG 2161*	Ukraine, soil (Rybalka)	EF426793 (520)	this study
	Bumilleria klebsiana	*Bumilleria klebsiana*	[2]SAG 2158* (suggested epitype and reference strain)	Ukraine, freshwater (Rybalka)	EF460492 (461)	this study
		Bumilleria sp.	SAG 2157*	Ukraine, soil (Rybalka)	EF426792 (461)	this study
	Bumilleria pyrenoidosa	*Pseudobumilleriopsis pyrenoidosa*	[1]SAG 69.90 (authentic strain)	USA Texas, soil (Deason)	EF455978 (534)	this study
	Bumilleria sicula	*Bumilleria sicula*	[1]SAG 808-1 (suggested epitype and reference strain)	UK, soil (George)	EF455982 (491)	this study
	Bumilleria sp. A	*Bumilleria* sp.	[2]SAG 2159*	Ukraine, soil (Rybalka)	EF455927 (521)	this study
		Bumilleria sp.	SAG 2160*	Ukraine, soil (Demchenko)	EF460494 (539)	this study
	Bumilleria sp. B	*Bumilleriopsis* sp.	[2]SAG 57.94	Germany, soil (Neuhaus)	EF431850 (479)	this study
	Bumilleria sp. C	*Bumilleriopsis* sp.	[2]SAG 58.94	Germany, soil (Neuhaus)	EF455963 (482)	this study
Bumilleriopsis 2	new genus and species	*Bumilleriopsis* sp.	[2]SAG 34.93	Germany, soil (Oesterreicher)	EF455960 (369)	this study
Bumilleriopsis 3	*Bumilleriopsis peterseniana*	*Bumilleriopsis peterseniana*	[2]SAG 809-3 (authentic strain)	Switzerland, freshwater (Vischer)	EF455942 (5' partial)	this study
	Bumilleriopsis sp.	*Bumilleriopsis* sp.	SAG 33.93	Germany, soil (Oesterreicher)	EF431849 (5' partial)	this study
Tribonema 1	new genus and one species	*Tribonema intermixum*	SAG 2166*	Ukraine, freshwater (Rybalka)	EF460491 (536)	this study
		Tribonema minus	SAG 2168*	Ukraine, soil (Rybalka)	EF455924 (5' partial)	this study
		Tribonema regulare	SAG 2164*	Ukraine, freshwater (Rybalka)	EF455928 (537)	this study
		Tribonema sp.	SAG 2165*	Antarctica, soil (Lukeshova)	EF460489 (536)	Rybalka et al. (2009)
		Tribonema sp.	SAG 2174*	Ukraine, freshwater (Rybalka)	EF460490 (5' partial)	this study
		Tribonema sp.	SAG 2175*	Ukraine, freshwater (Rybalka)	EF455944 (536)	this study
		Tribonema sp.	SAG 2176*	Ukraine, freshwater (Rybalka)	EF455950 (5' partial)	this study
		Tribonema ulotrichoides	SAG 21.94	Germany, freshwater (Hübel)	EF455964 (536)	Rybalka et al. (2009)
		Tribonema viride	SAG 2167*	Ukraine, freshwater (Rybalka)	EF460493 (5' partial)	this study
		Tribonema viride	SAG 23.94	Germany, freshwater (Hübel)	EF455966 (536)	Rybalka et al. (2009)
		"Xanthonema" cf. *tribonematoides*	SAG 2172*	Antarctica, soil (Kostikov)	EF426797 (534)	Rybalka et al. (2009)
		"Xanthonema" tribonematoides	SAG 2173*	Antarctica, soil (Ohtani)	EF455951 (5' partial)	Rybalka et al. (2009)
		"Xanthonema" sp.	environmental clone Helgoland 7	Germany, freshwater (Mohr)	EF431847 (536)	this study
Tribonema 2	new genus and one species	*Tribonema aequale*	SAG 200.80	UK (?), freshwater (Fogg)	EF455968 (1325)	this study
		Tribonema aequale	SAG 880-1	Slovakia, soil (Pringsheim)	EF455969 (1325)	this study
		Tribonema cf. *minus*	SAG 2177*	Ukraine, soil (Rybalka)	unpubl. (1325)	this study
		Tribonema cf. *minus*	SAG 2178*	Ukraine, freshwater (Rybalka)	EF431848 (1325)	this study
		Tribonema missouriense	UTEX 2549	USA (?), unclear (Nichols)	EF455923 (5' partial)	this study
Tribonema 3	new genus and one species	*Tribonema utriculosum*	SAG 22.94	Germany, freshwater (Hübel)	EF455965 (459)	this study
		Tribonema vulgare	SAG 24.94	Germany, freshwater (Hübel)	EF455967 (502)	this study
Tribonema 4	new genus and one species	*Tribonema* sp.	UTEX 639	UK, freshwater (Christensen)	EF455921 (417)	

FIGURES AND FIGURE LEGENDS

Figure 1. Illustration of the seven *psb*A/*rbc*L spacer sequence similarity groups (genera) on which the studied Tribonemataceae strains (Table 1) were distributed. Upper half, 5'-end of the sequences with alignment of the *psb*A gene 3'-ends (pos. 41-67 of references sequence EF455969), followed by a short conserved region and start of the highly variable spacer region (pos. 68-220 of references sequence EF455969). The latter region was used to discriminate species within a spacer sequence similarity group (see text). Lower half, more conserved 3'-end of the spacer (pos. 1181-1411 of references sequence EF455969) providing a clear distinction of sequence similarity groups (genera) across a selection of studied Tribonemataceae strains.

B1 – B3, groups *Bumilleriopsis* 1-3; T1-T4, groups *Tribonema* 1-4

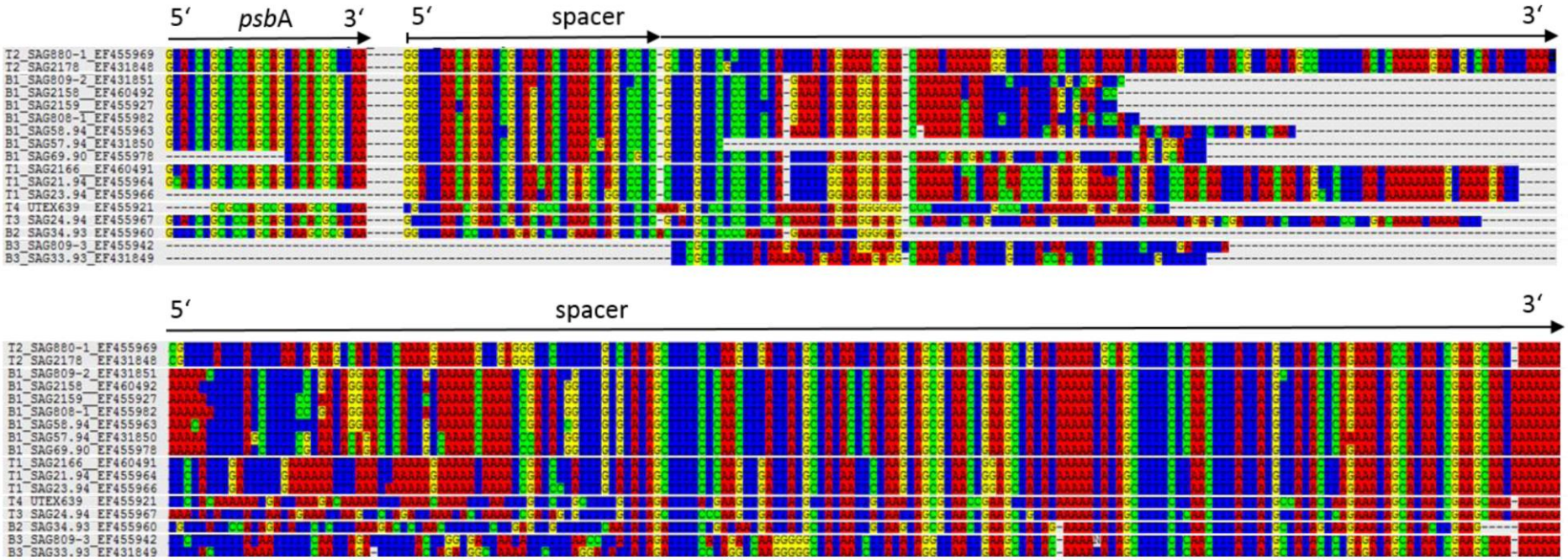


Figure 2. Phylogenetic analysis of 34 strains of filamentous Xanthophyceae (Tribonemataceae, incl. one environmental clone) using a data set of 54 complete *rbc*L sequences (1467 bp long; 565/445 variable/parsimony informative sites). Sequences in bold are authentic strains. New isolates of Tribonemataceae (except *Xanthonema s.l.*) are denoted by an asterisk. Indicated or highlighted are groups of sequences from Tribonemataceae corresponding to *psb*A/*rbc*L spacer sequence similarity groups and the filamentous *Heterococcus*. Highlighted in boxes are the seven sequence similarity groups of Tribonemataceae treated here (see Table 1), the other groups and *Heterococcus* have been treated previously (Rybalka et al., 2009; Rybalka et al., 2013). Sequences above the dotted line are from members of the Tribonematalean clade (Maistro et al., 2009). The taxon sampling for non-filamentous Xanthophyceae is approximately as in Rybalka et al. (2013). Two brown algae (Phaeophyceae) served as outgroup. The phylogeny shows a Maximum Likelihood tree obtained with the program Treefinder (Jobb et al., 2004; Jobb, 2011). The optimal model of sequence substitution was selected using the AIC criterion in Treefinder. The GTR model (Rodríguez et al., 1990) was selected with the rate parameters set to optimal and frequency parameter estimated empirically assuming a discrete Gamma model for the heterogeneity of rate of substitutions with the number of rate categories = 5. Confidence values for the obtained groups (edge support) were inferred from expected-likelihood weights (Strimmer and Rambaut, 2002) applied to local rearrangements (1000x, search depth = 2) of the tree topology as provided in Treefinder.

Figure 2

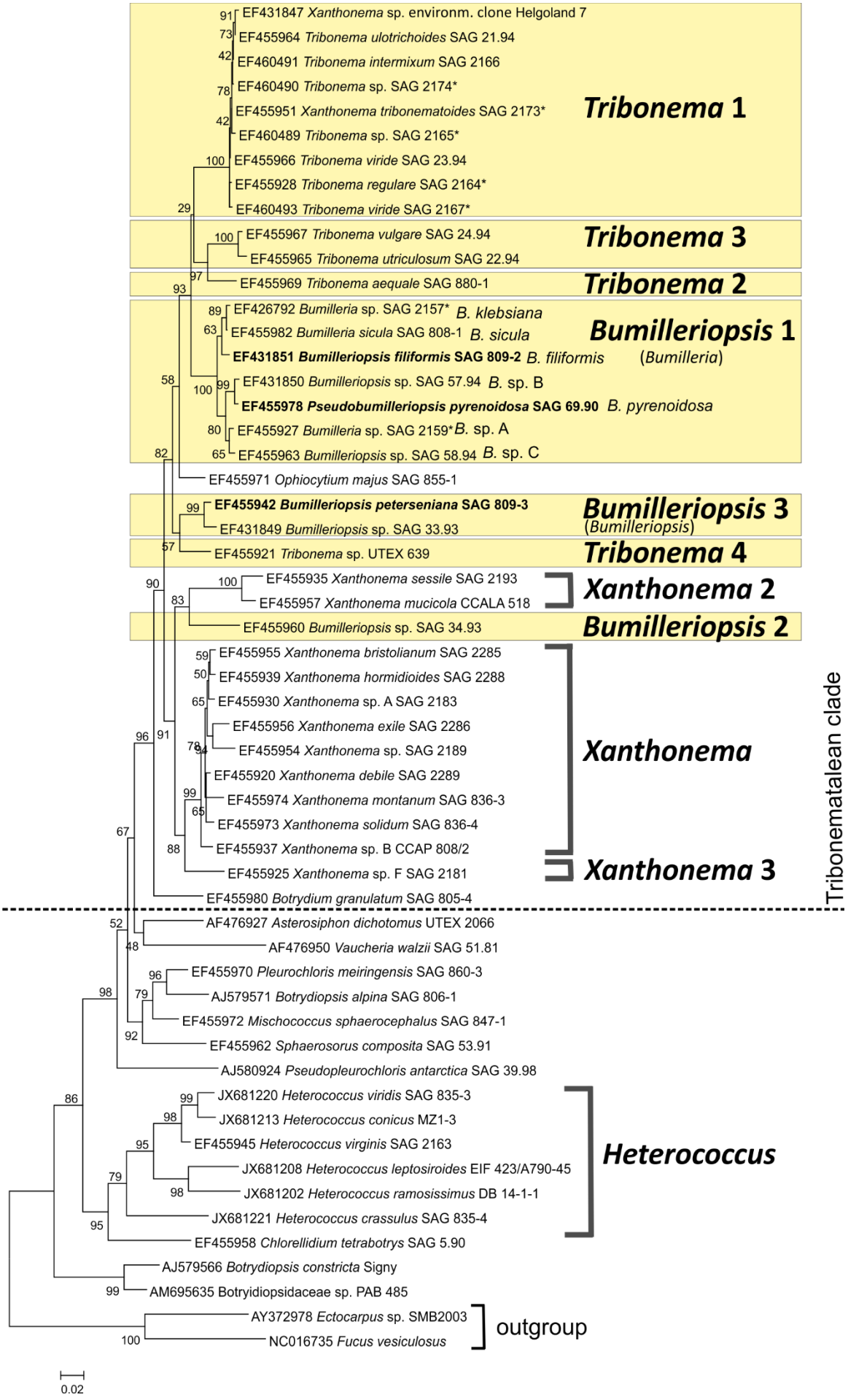

EF431847 Xanthonema sp. environm. clone Helgoland 7
EF455964 Tribonema ulotrichoides SAG 21.94
EF460491 Tribonema intermixum SAG 2166
EF460490 Tribonema sp. SAG 2174*
EF455951 Xanthonema tribonematoides SAG 2173*
EF460489 Tribonema sp. SAG 2165*
EF455966 Tribonema viride SAG 23.94
EF455928 Tribonema regulare SAG 2164*
EF460493 Tribonema viride SAG 2167*
Tribonema 1
EF455967 Tribonema vulgare SAG 24.94
EF455965 Tribonema utriculosum SAG 22.94
Tribonema 3
EF455969 Tribonema aequale SAG 880-1
Tribonema 2
EF426792 Bumilleria sp. SAG 2157* B. klebsiana
EF455982 Bumilleria sicula SAG 808-1 B. sicula
EF431851 Bumilleriopsis filiformis SAG 809-2 B. filiformis (Bumilleria)
EF431850 Bumilleriopsis sp. SAG 57.94 B. sp. B
EF455978 Pseudobumilleriopsis pyrenoidosa SAG 69.90 B. pyrenoidosa
EF455927 Bumilleria sp. SAG 2159* B. sp. A
EF455963 Bumilleriopsis sp. SAG 58.94 B. sp. C
Bumilleriopsis 1
EF455971 Ophiocytium majus SAG 855-1
EF455942 Bumilleriopsis peterseniana SAG 809-3
EF431849 Bumilleriopsis sp. SAG 33.93
Bumilleriopsis 3 (Bumilleriopsis)
EF455921 Tribonema sp. UTEX 639
Tribonema 4
EF455935 Xanthonema sessile SAG 2193
EF455957 Xanthonema mucicola CCALA 518
Xanthonema 2
EF455960 Bumilleriopsis sp. SAG 34.93
Bumilleriopsis 2
EF455955 Xanthonema bristolianum SAG 2285
EF455939 Xanthonema hormidioides SAG 2288
EF455930 Xanthonema sp. A SAG 2183
EF455956 Xanthonema exile SAG 2286
EF455954 Xanthonema sp. SAG 2189
EF455920 Xanthonema debile SAG 2289
EF455974 Xanthonema montanum SAG 836-3
EF455973 Xanthonema solidum SAG 836-4
EF455937 Xanthonema sp. B CCAP 808/2
Xanthonema
EF455925 Xanthonema sp. F SAG 2181
Xanthonema 3
EF455980 Botrydium granulatum SAG 805-4
Tribonematalean clade
AF476927 Asterosiphon dichotomus UTEX 2066
AF476950 Vaucheria walzii SAG 51.81
EF455970 Pleurochloris meiringensis SAG 860-3
AJ579571 Botrydiopsis alpina SAG 806-1
EF455972 Mischococcus sphaerocephalus SAG 847-1
EF455962 Sphaerosorus composita SAG 53.91
AJ580924 Pseudopleurochloris antarctica SAG 39.98
JX681220 Heterococcus viridis SAG 835-3
JX681213 Heterococcus conicus MZ1-3
EF455945 Heterococcus virginis SAG 2163
JX681208 Heterococcus leptosiroides EIF 423/A790-45
JX681202 Heterococcus ramosissimus DB 14-1-1
JX681221 Heterococcus crassulus SAG 835-4
Heterococcus
EF455958 Chlorellidium tetrabotrys SAG 5.90
AJ579566 Botrydiopsis constricta Signy
AM695635 Botryidiopsidaceae sp. PAB 485
AY372978 Ectocarpus sp. SMB2003
NC016735 Fucus vesiculosus
outgroup
0.02

Figure 3. Microscopy of studied Tribonemataceae strains previously assigned to *Bumilleria* and *Bumilleriopsis.* (A-E) Filamentous growth of representatives of group *Bumilleriopsis* 1 (*Bumilleria*, see text). (A, B) *Bumilleriopsis* (*Bumilleria*) *filiformis* SAG 809-2. (C). *Bumilleria sicula* SAG 808-1. (D, E) *Bumilleria klebsiana.* (D) SAG 2158. (E) SAG 2157. (F-H) Coccoid vegetative stages. (F, G) Group *Bumilleriopsis* 3 (*Bumilleriopsis*). (F) *Bumilleriopsis peterseniana* SAG 809-3. (G) *Bumilleriopsis* sp. SAG 33.93. (H) Group *Bumilleriopsis* 2, *Bumilleriopsis* sp. SAG 34.93. Scale bar, 10 µm.

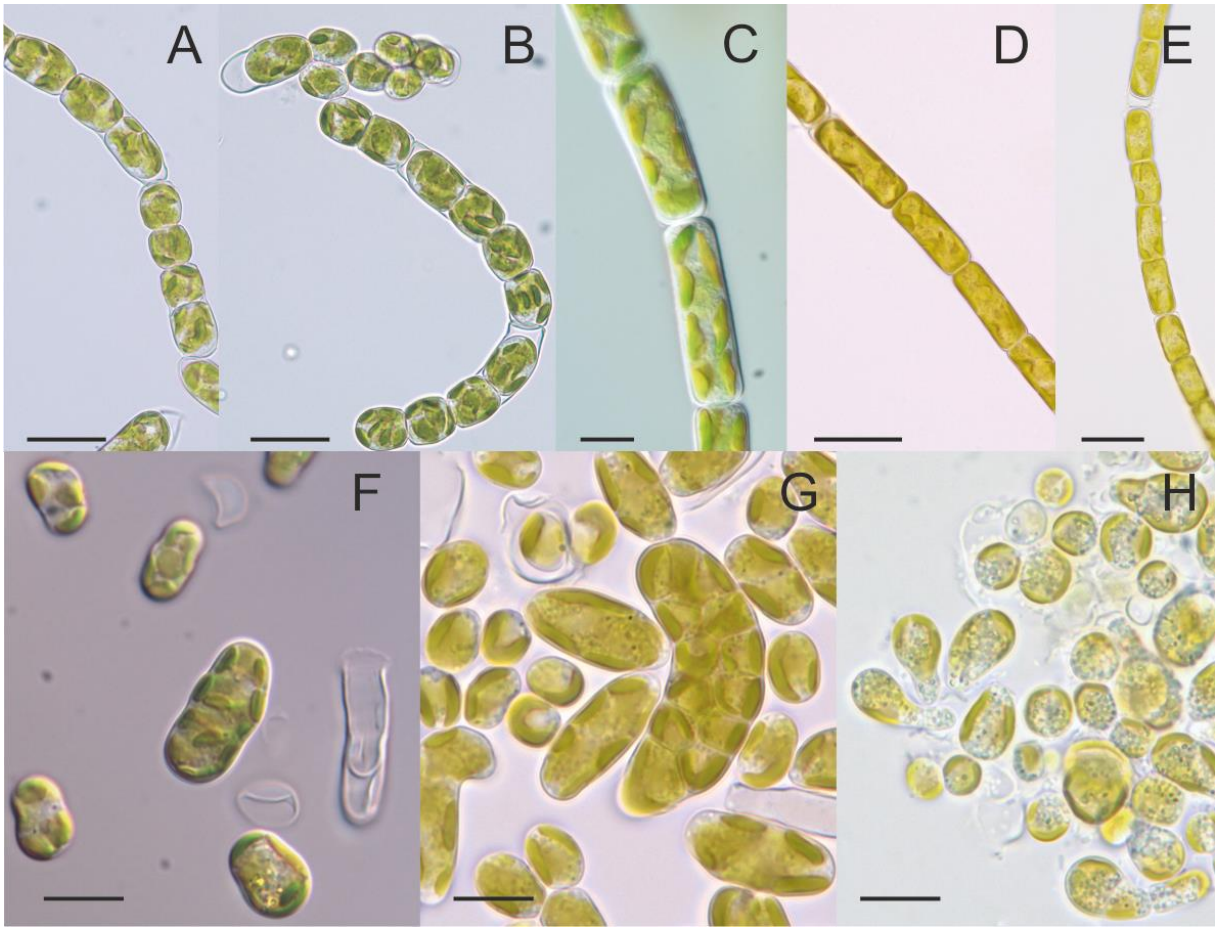
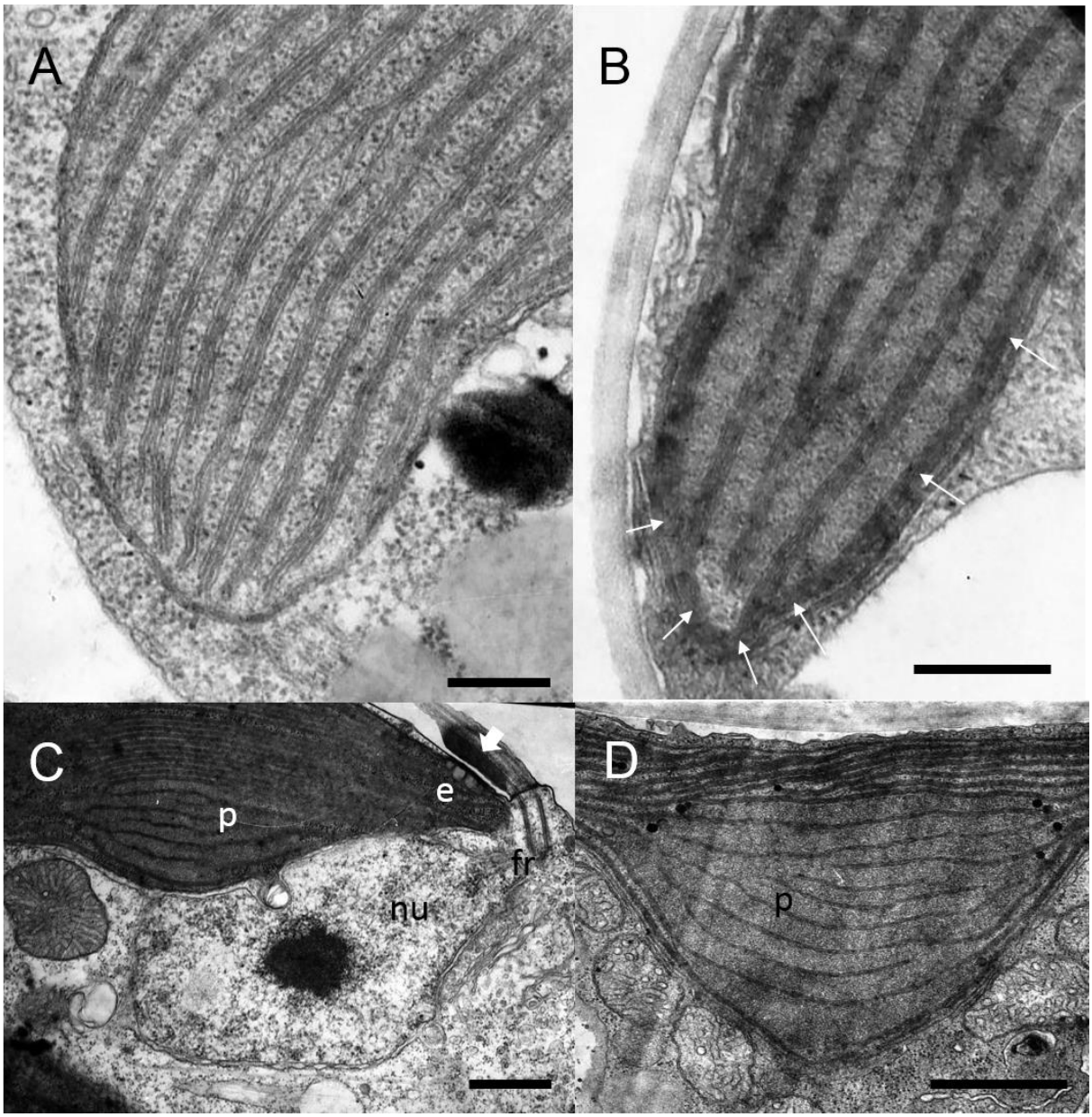


Figure 4. Chloroplast ultrastructure of strains of *Bumilleriopsis* groups 1 (*Bumilleria*) and 3 (*Bumilleriopsis*). (A) *Bumilleria klebsiana* SAG 2157, cross section of chloroplast with girdle lamella absent. Note lamellae of three stacked thylakoids. (B) *Bumilleriopsis peterseniana* SAG 809-3, cross section of chloroplast and cell wall of. Arrows mark the girdle lamella surrounding all the other lamellae in the plastid. (C) Zoospore of *B. klebsiana* SAG 2158. Cross section of chloroplast containing a pyrenoid-like structure (p). A chloroplast endoplasmatic reticulum with nucleus (nu) is adjacent to the chloroplast. The arrow indicates the swelling on the short, smooth flagellum which is opposite of the eyespot (e) within the chloroplast, a characteristic feature of Xanthophyceae. fr, flagellar microtubular root. (D) *Bumilleria* sp. SAG 58.94, cross section of chloroplast with pyrenoid-like structure (p) in a vegetative cell. Scale bars: (A, B) 0.1 µm; (C, D) 0.25 µm



Rybalka *et al. BMC Evolutionary Biology* 2013, **13**:39
http://www.biomedcentral.com/1471-2148/13/39

BMC Evolutionary Biology

RESEARCH ARTICLE Open Access

Congruence of chloroplast- and nuclear-encoded DNA sequence variations used to assess species boundaries in the soil microalga *Heterococcus* (Stramenopiles, Xanthophyceae)

Nataliya Rybalka[1,2†], Matthias Wolf[3†], Robert A Andersen[4†] and Thomas Friedl[1*†]

Abstract

Background: *Heterococcus* is a microalgal genus of Xanthophyceae (Stramenopiles) that is common and widespread in soils, especially from cold regions. Species are characterized by extensively branched filaments produced when grown on agarized culture medium. Despite the large number of species described exclusively using light microscopic morphology, the assessment of species diversity is hampered by extensive morphological plasticity.

Results: Two independent types of molecular data, the chloroplast-encoded *psb*A/*rbc*L spacer complemented by *rbc*L gene and the internal transcribed spacer 2 of the nuclear rDNA cistron (ITS2), congruently recovered a robust phylogenetic structure. With ITS2 considerable sequence and secondary structure divergence existed among the eight species, but a combined sequence and secondary structure phylogenetic analysis confined to helix II of ITS2 corroborated relationships as inferred from the *rbc*L gene phylogeny. Intra-genomic divergence of ITS2 sequences was revealed in many strains. The 'monophyletic species concept', appropriate for microalgae without known sexual reproduction, revealed eight different species. Species boundaries established using the molecular-based monophyletic species concept were more conservative than the traditional morphological species concept. Within a species, almost identical chloroplast marker sequences (genotypes) were repeatedly recovered from strains of different origins. At least two species had widespread geographical distributions; however, within a given species, genotypes recovered from Antarctic strains were distinct from those in temperate habitats. Furthermore, the sequence diversity may correspond to adaptation to different types of habitats or climates.

Conclusions: We established a method and a reference data base for the unambiguous identification of species of the common soil microalgal genus *Heterococcus* which uses DNA sequence variation in markers from plastid and nuclear genomes. The molecular data were more reliable and more conservative than morphological data.

Keywords: Soil algae, *Heterococcus*, Xanthophyceae, *psb*A/*rbc*L spacer, ITS2, Systematics, Molecular phylogeny, Species concept

* Correspondence: tfriedl@uni-goettingen.de
†Equal contributors
[1]Experimental Phycology and Culture Collection of Algae (SAG), Georg August University Göttingen, Untere Karspüle 2a, Göttingen 37073, Germany
Full list of author information is available at the end of the article

Background

Heterococcus is a genus of yellow-green microalgae (Xanthophyceae, Stramenopiles) that is common and widespread in soils of cold regions such as the Alps or Antarctica [1,2]. In addition to soils, three species have been reported from freshwater [3-6], and *Heterococcus* is the only xanthophyte known from lichen symbiosis [7,8]. *Heterococcus* produces extensively branched filaments when grown on agarized culture medium (Figure 1); however, in field samples it produces unicellular coccoid cells that are weakly connected. Perhaps uniquely for microalgal genera, all species have been described based upon isolates grown in culture and observed with a light microscope [1,2,6]. Without culturing, *Heterococcus* is often mistaken for other coccoid xanthophytes, eustigmatophytes or green algae. Sixty-one *Heterococcus* species have been described [9], and 51 species are recognized [10]. Extensive ultrastructural observations were undertaken by Lokhorst [2], but he reluctantly concluded that ultrastructural features were not sufficient to distinguish species.

Sexual reproduction is unknown for *Heterococcus*, and therefore the biological species concept cannot be employed (e.g. [11]); only the morphological (typological) species concept has been used. That is, *Heterococcus* species identity is limited to light microscopic morphological characters interpreted within the extensive plasticity that is exhibited during culture studies [1,2,6]. For example, branching patterns are not present in very young or old cultures, and filament formation is suppressed (coccoid cells are produced) when cultures are grown at suboptimal temperature ranges [1] (Figure 1). Cladistic analysis of these morphological features would be extremely difficult because cell sizes, branching patterns, colony growth, chloroplast number and other features overlap extensively among the species, even when grown under optimum conditions.

Molecular phylogenetic analysis is often a reliable alternative for identification of species; however, species diversity of *Heterococcus* using molecular markers was unstudied and no molecular reference data base existed. From only seven *Heterococcus* species DNA sequences had previously been reported, and all these sequences were from conserved molecular markers. The sequences revealed the probable monophyletic origin of the genus and its basal position within the Xanthophyceae, which was distinct from other filamentous members (e.g. *Tribonema, Vaucheria*) [12-15]. We used molecular phylogenetics, especially within the framework of the monophyletic species concept [16-18], to evaluate 33 culture strains identified as *Heterococcus* (Figure 2). Fourteen strains were originally identified to species level using morphology, and ten of those strains were authentic culture strains, i.e. the culture strains used to describe the species [1,3-5,19]. Unfortunately, the cultures used to describe all other species have been lost. For nine authentic strains, there are extended morphological descriptions with numerous illustrations produced by two independent authors [2-5]. We added 19 unidentified culture isolates, including twelve cultures recently isolated. Our goals were (1) to test boundaries of *Heterococcus* species as inferred from morphological features and (2) to establish a reference data base of strains unambiguously distinguished with DNA sequence data. We chose two highly variable molecular markers, i.e. the chloroplast-encoded *psb*A/*rbc*L spacer region [20,21] and the nuclear-encoded internal transcribed spacer 2 of the nuclear rDNA cistron [22-24], to examine species boundaries. We also determined full plastid-encoded *rbc*L gene sequences to infer the phylogenetic position of species.

Results

Four of the strains, identified as *Heterococcus*, were green algae (Figure 2). These were not included in the rest of the study. The *rbc*L gene sequences were used to assess the phylogenetic relationships of the remaining 29 strains (Figure 3, Additional file 1). For 25 strains, PCR amplification was successful for the whole region from *psb*A (downstream), through the *rbc*L, through the *rbc*L/

Figure 1 Morphology of three strains of *Heterococcus viridis* in culture. (**A**) Prostrate colonies produced by branched filaments on the surface of an agarized culture, 16 weeks old (strain B10). (**B**) Enlarged view of a young (4 weeks old) colony, liquid culture, strain SAG 835–7. (**C**) Enlarged filament, 6 week old agarized culture (strain MZ3-7). (**D**) Coccoid cells in a 4 weeks old liquid culture (strain SAG 835–7). Scale bar in (**A**) 500 μm, in (**B**) – (**D**) 20 μm.

*rbc*L clade or lineage	*psb*A/*rbc*L spacer and ITS2 group	Strain	New species designation	Origin
H. viridis	A	**SAG 835-3 *H. viridis* ***	*H. viridis*	Switzerland, freshwater
		SAG 835-6 *H. mainxii* *		Czech Republic, freshwater
		SAG 835-7 *H. marietanii**		Switzerland, freshwater
		SAG 835-1 *H. brevicellularis* *		Switzerland, soil
		SAG 835-8 *H. moniliformis* *		Switzerland, soil
		SAG 56.94 *H.* sp.		Germany, soil
		EIF 398 *H.* sp.		Antarctica, soil
		EIF PAB 398/473 *H. pleurococcoides*		Antarctica, soil
		EIF 430/A801-2 *H.* sp.		Antarctica, soil
		EIF PAB 397/380 *H. pleurococcoides*		Antarctica, soil
		MZ2-4 *H.* sp.[a]		Antarctica, soil
		MZ2-5 *H.* sp.[a]		Antarctica, soil
		B10 *H.* sp.[a]		Antarctica, soil
		SAG 2162 *H.* sp.[a]		Antarctica, soil
		MZ3-7 *H.* sp.[a]		Antarctica, soil
	B	**MZ1-3 *H.* sp.**[a]	*H. conicus*	Antarctica, soil
		MZ1-6 *H.* sp.[a]		Antarctica, soil
		DB14-15 *H.* sp.[a]		Germany, freshwater
		DB15-5 *H.* sp.[a]		Germany, freshwater
	C	**SAG 2163 *H.* sp.**[a]	*H. virginis*	Antarctica, soil
H. caespitosus	D	**EIF 423/A790-45 *H.* sp.**	*H. leptosiroides*	Antarctica, soil
		EIF PAB 399/372 *H. caespitosus*		Antarctica, soil
		EIF 128/A788-70 *H. protonematoides*		Antarctica, rock surface
	E	**SAG 835-2a *H. caespitosus* ***	*H. caespitosus*	Germany, soil
		SAG 835-9 *H. protonematoides* *		Switzerland, rock surface
H. sp.	F	**DB14-1-1 *H.* sp.**[a]	*H. ramosissimus*	Germany, freshwater
		DB14-5-1 *H.* sp.[a]		Germany, freshwater
H. crassulus	G	**SAG 835-4 *H. crassulus* ***	*H. crassulus*	Switzerland, rock surface
H. fuornensis	n.a.[1]	**SAG 835-5 *H. fuornensis* ***	*H. fuornensis*	Switzerland, soil
n.a.[2]	n.a.[2]	**SAG 63.90 *H. endolithicus* ***	*Desmococcus antarctica*	Antarctica, soil
		EIF434/A801-133 *H.* sp.	*Demococcus*-like green algae	Antarctica, soil
		EIF446/A812-63a *H.* sp.		Antarctica, soil
		EIF447/A834-545 *H.* sp.		Antarctica, soil

Figure 2 The 33 strains identified as *Heterococcus* used in this study. The *Heterococcus* strains are listed with their species names (where provided) from previous morphological analyses, their assignment to clades and lineages in the *rbc*L phylogeny (boxed with thick lines; see Figure 3), their assignments to a certain species recognized in this study (boxed with thin lines), their new species designations (see Discussion) and their geographic origin. Highlighted in green are genotypes, i.e. groups of strains exhibiting high sequence similarities (see text). Strains in bold letters represent cryopreserved epitypes (reference strains) designated for each species (see Discussion). An asterisk marks an authentic reference strain (see text). [a] marks those strains that have recently been isolated by us or were provided to us for this study; n.a.[1], not applicable because the *psb*A/*rbc*L spacer sequence could not be determined (see text); n.a.[2], not applicable because strains were identified as green algae (see text).

*rbc*S spacer and to the *rbc*S gene; therefore the full *rbc*L gene, 1467 base pairs long, was determined (Additional file 2). We failed to obtain full *rbc*L sequences for three authentic strains, *Heterococcus fuornensis* Vischer strain SAG 835–5, *H. caespitosus* Vischer strain SAG 835-2a, and *H. protonematoides* Vischer strain SAG 835–9, but we used available sequences (AM421004, AM421002 and AJ579575) for these three strains. Also, for strains DB14-15 and MZ1-6 the full *rbc*L failed to amplify. Fifteen different *rbc*L sequences were recovered among the 29 strains, which implies that the *rbc*L gene was identical among many strains (Additional file 3). Only the 15 different *rbc*L sequences were used for phylogenetic analyses (Figure 3, Additional file 1). Monophyly of *Heterococcus* was highly supported with all methods except maximum likelihood, and this confirmed the generic identity of the 29 strains. The analyses resolved two well supported clades, named "*H. caespitosus* clade" and "*H. viridis* clade". In addition, there were three independent lineages representing *H. crassulus* Vischer, *H. fuornensis* and an unidentified strain ("*H.* sp."). Relationships among the clades and lineages remained ambiguous (Figure 3, Additional file 1).

*psb*A/*rbc*L spacer

To further examine the relationships, the *psb*A/*rbc*L spacer sequences were determined for 28 strains (*H. fuornensis* strain SAG 835–5 failed to amplify). The spacers varied greatly in length and primary sequences; the sequences could not be aligned across all strains. Nevertheless, two short sequence stretches were aligned across all strains. The first was 23 nucleotides at the 5'-end (pos. 78–99 of reference sequence *H. viridis* Chodat strain SAG 835–3, JX681220) and the second was 36 nucleotides at the 3'-end (pos. 312 – 347, same reference sequence).

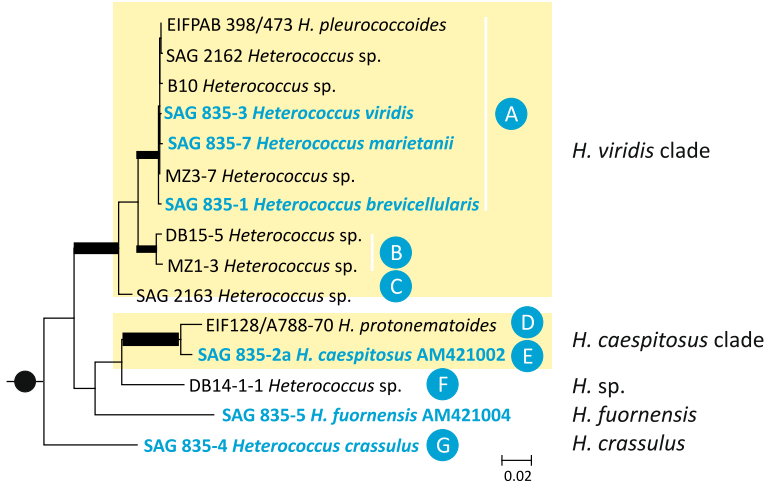


Figure 3 Maximum likelihood (ML) phylogeny of *rbc*L gene sequences for 15 *Heterococcus* strains. Twelve other strains had sequences identical to one of the 15 shown (Additional file 3). Sequences without accession numbers are reported for the first time. Sequence names highlighted in blue indicate authentic reference strains; names used in the tree are those used to identify the original cultures (see text). Capital letters in filled blue circles indicate the seven species apart from *H. fuornensis* resolved by the *psb*A/*rbc*L spacer and ITS2 sequence analyses (see text). The names next to the tree represent clades and lineages recovered in the phylogenetic analyses. Thick lines indicate internal branches resolved by maximum likelihood, maximum parsimony, minimum evolution distance and Bayesian analyses and with significant statistical support (bootstrap >95%, posterior probability = 1.0). Black filled circle marks the branch indicating the monophyletic origin of *Heterococcus* that was significantly supported (bootstrap >95%, posterior probability = 1.0) except for the maximum likelihood analyses. The phylogeny shown is part of a larger ML phylogeny (calculated with GARLI v0.96 [25,26]) based on a *rbc*L data set (1325 bp long, 517/418 variable/parsimony informative sites) consisting of 15 *Heterococcus* sequences and 32 other Xanthophyceae sequences corresponding to clades C, B, T, and V as defined in [14] (see Additional file 1) as well as two outgroup taxa. Scale bar, substitutions per site.

In most *Heterococcus* strains the nucleotide length of the *psb*A/*rbc*L spacer ranged from 275 nucleotides (*H. caespitosus* strain SAG 835-2a,) to 289 nucleotides (*H.* sp. strain DB14-15). The sequence for *H. crassulus* strain SAG 835–4 was 1762 nucleotides, and the identical sequences for two strains, DB14-1-1 and DB14-5-1, were 2143 nucleotides. Sequence similarities further downstream grouped the strains into seven "spacer groups", A – G, within which the *psb*A/*rbc*L spacers were identical or displayed only very few differences (Figure 2, Additional file 3). When mapped on the *rbc*L phylogeny, the strains of spacer groups A, B and C were included in the *H. viridis* clade, strains of spacer groups D and E fell in the *H. caespitosus* clade, and spacer groups F and G represented the lineages "*H.* sp." and *H. crassulus* (Figure 3).

Between closely related groups or within a group, also other regions of the *psb*A/*rbc*L spacer sequences could be aligned. For example, strains of the *Heterococcus viridis* clade (groups A-C) had sequence regions that aligned well, but there were up to 28 nucleotide differences among them. In addition, there was a hypervariable region of different lengths (20–31 nucleotides, between pos. 172 and 193 of the reference sequence *H. viridis* SAG 835–3, JX681220) that was not alignable among the three groups, but clearly distinguished them from each other. In the *H. caespitosus* clade, i.e. between groups D and E, the *psb*A/*rbc*L spacers also aligned well over the entire lengths, but differed at 14 sequence positions and a single indel. Similarly, there was a maximum of 13 *psb*A/*rbc*L spacer sequence differences between strains of group A. In group A there were nine strains isolated from Antarctica (Figure 2). There were no more than two nucleotides difference among them when Antarctic strain MZ3-7 was not considered and the previously unidentified strain SAG 56.94, isolated from Germany, had just one to three sequence differences with the eight Antarctic isolates. Conversely, strain MZ3-7 was with seven to nine spacer differences more distant to the other eight Antarctic strains. Strain *H. brevicellularis* Vischer SAG 835–1 was the closest neighboring strain of strain MZ3-7; there were just 4 sequence positions different between both strains. Group B contained two Antarctic strains (MZ1-3, MZ1-6) that had identical spacers; Group B also contained two German strains (DB14-15, DB15-5) with identical spacers; however, the Antarctic strains differed at 4 positions when compared to the German strains. Finally, group D had three strains that had only one nucleotide difference, while two strains in group F had only two sequence differences.

ITS2

Nuclear-encoded ITS2 sequences were determined for 28 strains as an independent assessment of the plastid-encoded sequences. *Heterococcus fuornensis* strain SAG

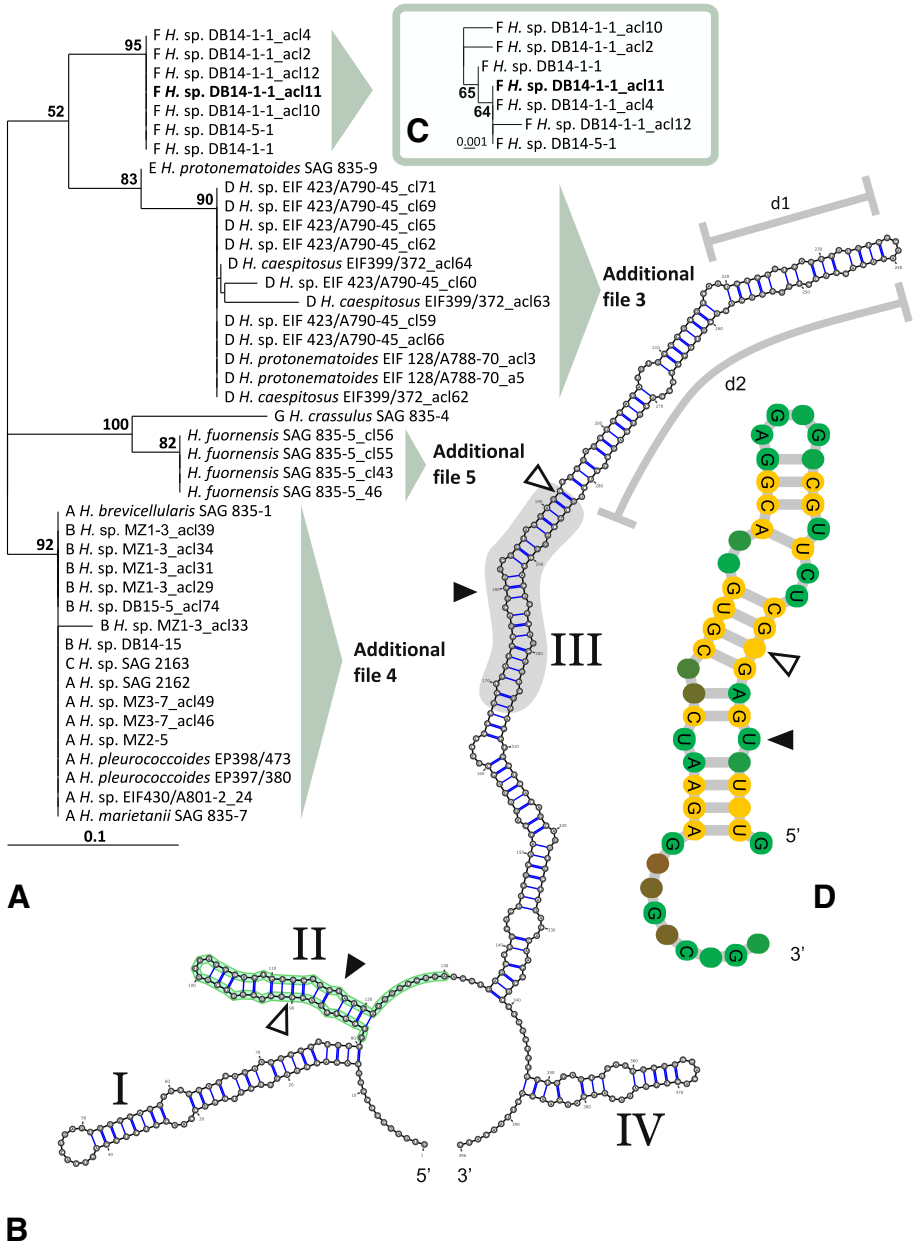


Figure 4 ITS2 sequence and secondary structure phylogenetic analyses of 28 strains of *Heterococcus*. (**A**) ProfDistS [27] sequence-structure NJ tree (unrooted) as derived from the multiple sequence-structure alignment of ITS2 helix II. Bootstrap values (100 pseudo-replicates) are mapped to the appropriate internodes. Branch lengths are drawn proportional to inferred changes. The template ITS2 variant used in B) is highlighted in bold. (**B**) ITS2 secondary structure of ITS2 variant DB14-1-1_acl11 (group F, *H. ramosissimus*) used for homology modeling of helix II (shaded) for all strains of *Heterococcus*. The secondary structure was visualized with VARNA [28]. Helices are numbered I–IV. Typical ITS2 motifs are highlighted by filled arrowheads. Open arrowheads mark positions of two CBCs that distinguish groups D (= *H. leptosiroides*) and E (= *H. caespitosus*). An additional conserved region throughout all strains of *Heterococcus* is indicated by a cloud (see text). In contrast to the template structure the region d1 is deleted in four strains (group D, see Additional file 4). The region d2 is deleted in all other strains not classified in group F. (**C**) Subtree as obtained by using the complete sequence-structure information from helices I-IV (template highlighted in bold). Further subtrees as derived using clade specific structural templates (helices I-IV) are provided as Additional files 4, 5, 6. (**D**) Visualization of the complete sequence-structure alignment used to generate the tree as shown in A). Consensus structure (51%) of helix II for all ITS2-sequences obtained from the complete multiple sequence-structure alignment without gaps. Sequence conservation is indicated from red (not conserved) to green (conserved). Nucleotides which are 100% conserved in all sequences are written as A, U, G or C. Nucleotide bonds which are 100% conserved throughout the alignment are marked in yellow. Note the U-U mismatch. The figure was generated with 4SALE [29].

835–5 was successfully amplified and included; however, amplification failed for strain MZ1-6 and this strain was not included in the ITS2 analyses. Based upon alignment similarity, the ITS2 sequences formed the same groups that were recovered in the *psb*A/*rbc*L spacer analysis; therefore, we used the same group notation for both datasets. Within a spacer group, the ITS2 sequences and their secondary structures were easily aligned and rather similar; conversely, between spacer groups, the sequences and secondary structures were highly variable, i.e. they could be aligned with confidence only for a few short segments. The ITS2 sequences exhibited a considerable length variation of up to about 130 nucleotides between spacer groups. The shortest ITS2 had 285 nucleotides (strain SAG 2163 from group C; strain EIF 399/372 from group D); the longest sequence had 416 nucleotides (strains DB14-1-1, DB 14-5-1 from group F). Within each spacer group, the ITS2 sequences were relatively constant in length (variation < 10 nucleotides), except for group D where sequences were either short (285–287 nucleotides) or long (315–319 nucleotides), and the difference was due to an indel at the terminal end of helix III in the secondary structure model (see below; Additional file 4). The ITS2 sequence from *Heterococcus fuornensis*, which had a distinctive *rbc*L gene but could not be amplified for the *psb*A/*rbc*L spacer, showed little similarity to other spacer groups.

The inferred RNA secondary structures folded into the common core structure known for eukaryotes [23] which consisted of four helices with the third being the longest and helix IV the shortest (Figure 4, Additional files 4, 5, 6). Because of the high sequence length variation there was not a single ITS2 secondary structure from which the secondary structure models of the remaining sequences could be deduced using homology modeling. Only helix II could be modeled throughout the set of sequences independent of the used sequence-structure pair. However, within each group complete secondary structures could be obtained by homology modeling (Figure 4, Additional files 4, 5, 6). Throughout the set of sequences, conserved regions were restricted to the entire helix II (pos. 86–125 of reference sequence *H. viridis* SAG 835–3, JX681147), which had a constant length of 40 nucleotides, and a segment of about 50 nucleotides (pos. 165–189 and 205–228 of the same reference sequence) located at or close to the distal end of helix III (Figure 4, Additional files 4, 5, 6). It was followed by an extended terminal end of the helix III of 45 and 133 nucleotides in spacer groups D and F, whereas the corresponding sequence region in other spacer groups comprised of six (*H. fuornensis* strain SAG 835–5, no assigned group) to 18 nucleotides (spacer group E). That means there was a continuous lengthening/shortening of the ITS2 helix III within *Heterococcus* (Figure 4, Additional files 4, 5, 6). Another conserved ITS2 region useful to distinguish groups among *Heterococcus* strains was an unpaired sequence segment (~12 nucleotides) adjacent to helix II (Figure 4; pos. 126–137 of reference sequence *H. viridis* SAG 835–3, JX681147). It separated *H. crassulus* SAG 835–4, *H. fuornensis* SAG 835–5, and two clusters of strains from each other. The one cluster comprised the strains from groups A-C, the other the strains from groups D-F. Within each cluster the sequence segments were invariant.

Multiple copies of ITS2 were recovered in eight strains (from groups A, B, D, F and G; Additional file 2), i.e. there were no clear sequence reads possible without cloning. Four to 12 clones per strain were sequenced and this revealed up to seven ITS2 variants per strain (Additional file 2). Differences between ITS2 variants consisted of one to seven sequence positions and a few small indels (< 5 nucleotides); they were mostly located in helices I, IV and the basal part of helix III preceding the conserved segment. In groups B and D differences between ITS2 variants were also located in the conserved helix II. In group D three out of the ten detected ITS2 variants were lacking the extended 45 nucleotides long terminal end of helix III. These shorter variants were present in all three strains of group D or in about half (10) out of the sequenced 21 clones, while the longer ITS2 variants were retrieved only from two strains, EIF 423/A790-45 and EIF 128/A788-70.

The ITS2 phylogenetic analyses were confined to helix II (with adjacent unpaired conserved region, pos. 85–134 of reference sequence *H. viridis* SAG 835–3, JX681147) for assessing relationships among all studied strains. The sequence alignment was with 50 positions relatively short; it contained no more than 14/9 variable/parsimony informative sites and just nine sequences were not identical with others. However, a well-resolved phylogeny was obtained when secondary structure was considered in addition to primary structure information (Figure 4). The resolved helix II sequence groups were congruent with the groups recovered in the *rbc*L phylogeny (see spacer group letters on Figure 4). A common origin of *H. crassulus* with *H. fuornensis* was well supported in the unrooted ITS2 (helix II) phylogeny, and this contrasted with the *rbc*L phylogeny where the relationships of both were unresolved (Figure 3). Also an unrooted (maximum likelihood) phylogeny of only *Heterococcus rbc*L gene sequences did not support the common origin of both species (not shown). The helix II phylogenetic tree resolved a close relationship of groups D and E (as the *rbc*L phylogeny, *H. caespitosus* clade in Figure 3), but at the same time both groups were clearly separated species because there were two CBCs [23,24] in helices II and III (Figure 4, Additional file 4); also their helices I and IV could not be aligned. No resolution

was provided within the *H. viridis* clade, i.e. among spacer groups A, B and C (Figure 4). The complete ITS2 sequence was used to produce phylogenetic trees for individual spacer groups or *rbc*L clades. For example, then within the *rbc*L *H. viridis* clade the spacer groups A-C were resolved (Additional file 5). Within group A, both variants of strain MZ3-7 shared a common origin and were separated from other strains of the group. The three authentic strains, *Heterococcus viridis* SAG 835–3, *H. mainxii* SAG 835–6, and *H. marietanii* SAG 835–7, shared identical ITS2 sequences with each other (Additional file 7). Similarly, the ITS2 sequences of the two Antarctic strains EIF 398 and EIF PAB 398/473 were identical (Figure 2, Additional file 3). Two authentic strains, *H. brevicellularis* SAG 835–1 and *H. moniliformis* SAG 835–8, and one unidentified strain (SAG 56.94) shared identical ITS2 sequences except for a short indel (4 nucleotides) in helix IV. Another congruence with the chloroplast-encoded data was within group E where the ITS2 sequences of two authentic strains, *H. caespitosus* SAG 835-2a and *H. protonematoides* SAG 835–9, were identical. Conversely, within group B no differentiation among strains was possible due to the extensive radiation of multiple ITS2 variants of strain MZ1-3 (Additional file 5). Similarly, group D had extensive radiation of ITS2 variants and no relationships among strains were resolved (Additional file 4). Here the shorter variants of both strains EIF 423/A790-45 and EIF PAB 399/372 were intermixed among each other; they formed two independent lineages distinct from a clade comprising the variants with extended terminal end of helix III. Within group F no clear distinction of the two strains DB14-1-1 (with multiple variants) and DB14-5-1 was provided (Figure 4).

Discussion

Monophyletic species concept

Our results show that morphological features do not characterize species; for example, we found that five authentic culture strains – used in the original descriptions for the five species – had nearly identical DNA sequences and ITS2 secondary structures. Furthermore, we found other examples where authentic strains or identified strains were synonymous with another species (see below). Almost all *Heterococcus* species have been described using the same morphological approach, we have examined all existing authentic culture strains, and we find that morphological species descriptions are inadequate for this asexual genus. We conclude that morphological features characterize only individuals, not species. Therefore, we must apply a different species concept for *Heterococcus*.

The 'monophyletic species concept' of Johansen and Casamatta [18], which is derived from the 'phylogenetic (autapomorphic) species concept' of Mishler and Theriot [16,17], is easily applied to asexual species when molecular data are available. In our study, the DNA sequences and ITS2 secondary structure comparisons recovered a clear and robust phylogenetic structure for the 29 *Heterococcus* strains. Eight groups of sequences were repeatedly recovered using three different molecular markers; sequences within each group were very similar or identical while those between groups were highly variable. Using the monophyletic species concept, we recognize these groups as eight distinct species, and we identify previously unidentified strains and environmental clones to species level.

In a previous study, the *rbc*L gene and *psb*A/*rbc*L spacer were used, in conjunction with the monophyletic species concept, to define species in the Tribonemataceae, another asexual lineage of filamentous Xanthophyceae [21]. In that study, strains of the same species formed a monophyletic clade in the maximum likelihood *rbc*L gene phylogeny, and strains within the same species differed by less than 10 nucleotides. Within each species, the *psb*A/*rbc*L spacer was easily aligned, and within species variation was limited to single nucleotide differences and short indels. As with our study, the entire spacer could not be aligned between species. Therefore, the molecular-based monophyletic species concept identifies species in the same way for both studies.

The original iconotypes used to nomenclaturally anchor all *Heterococcus* names consist of ink drawings of various morphological features. We have shown that these morphological features are not reliable for species identity, and ink drawings are very limited for reference. In some cases, neotype material was dried and deposited in a herbarium [2], but this too is ambiguous because in at least one case, the wrong culture was used (see below) and because the material does not clearly separate species (still based upon morphology). Therefore, the names are herein further anchored with epitypes to avoid all ambiguity. The epitypes here designated are cryopreserved culture strains that can be re-investigated. The nomenclatural details are summarized below.

Taxonomy and nomenclature

Group A strains differed by no more than five sequence positions (one nonsynonymous substitution) in their *rbc*L genes, and the *psb*A/*rbc*L spacer regions aligned well over their entire lengths, with no more than 11 sequence differences. Their ITS2 sequences also aligned well over their entire lengths and there were no more than eight ITS2 sequence positions different. Therefore, we regard group A as a single species, *Heterococcus viridis*, which is the type species for the genus. It is

noteworthy that we used Chodat's [19] authentic strain, SAG 835–3 [3,30]. Group A also contained four additional species that were based upon authentic strains, *H. brevicellularis, H. mainxii, H. marietanii* and *H. moniliformis* [4,5]. We conclude that interpretations of largely overlapping morphological features, which were used to establish these as separate species, are not taxonomically sound; therefore, we consider these to be heterotypic synonyms of *H. viridis* (Figure 2, Additional files 3 and 7). Previously, Lokhorst [2] found that three of these strains were morphologically almost indistinguishable and he considered them as varieties. Group A also includes two strains previously identified as *H. pleurococcoides* Pitschmann [1]. However, the two strains were not authentic strains, and we cannot completely conclude that *H. pleurococcoides* is a heterotypic synonym of *H. viridis*. In addition, eight unidentified strains are now identified as *H. viridis* based on our study (Figure 2).

Heterococcus viridis **Chodat in *Bull. Herb. Boissier*, ser. 2, 8: p. 81 (1907).**

NEOTYPE: Material (authentic culture strain SAG 835-3) deposited in Nationaal Herbarium Nederland, Leiden University (L) by G.M. Lokhorst in *Taxonomic Studies in the Genus* Heterococcus. *Cryptogamie Studies* Vol. 3, p. 40. (1992).

EPITYPE DESIGNATED HERE: Cryopreserved culture strain SAG 835-3, deposited in the Sammlung von Algenkulturen (SAG), Universität Göttingen, Germany.

Heterotypic synonyms:

Heterococcus brevicellularis Vischer in *Ergeb. Wiss. Unters. Schweiz. Nationalparkes, N.F.* 1: p. 504; pl. 4, figures 1–3; figure 17A, d-f; figure 18. (1945).

Heterococcus mainxii Vischer in *Ber. Schweiz. Bot. Ges*. 47: p. 233; figures 4–6 (1937).

Heterococcus marietanii Vischer in *Ber. Schweiz. Bot. Ges*. 47: p. 235; figure 7 (1937).

Heterococcus moniliformis Vischer in *Ber. Schweiz. Bot. Ges*. 47: p. 238; figures 8–9 (1937).

Heterococcus marietanii Vischer var. *moniliformis* Lokhorst in *Taxonomic Studies in the Genus* Heterococcus. *Cryptogamie Studies* Vol. 3, p. 39. (1992).

Two authentic strains in group E, *H. caespitosus* SAG 835-2a and *H. protonematoides* SAG 835–9, were identical when considering the three markers. We recognize group E as a single species. *H. caespitosus* was described first [3], and therefore has nomenclatorial priority over *H. protonematoides* [5], which becomes a heterotypic synonym.

Heterococcus caespitosus **Vischer in *Ber. Schweiz. Bot. Ges.* 45: p. 391, figures 4–10 (1936).**

ICONOTYPE: Figures 4-10 in Vischer, W. *Ber. Schweiz. Bot. Ges.* 45: 372-410 (1936).

NEOTYPE: Material (authentic culture strain SAG 835-9) deposited in Nationaal Herbarium Nederland, Leiden University (L) by G.M. Lokhorst in *Taxonomic Studies in the Genus* Heterococcus. *Cryptogamie Studies* Vol. 3, p. 12. (1992). Note: The culture strain used to designate the neotype material belonged to *Heterococcus protonematoides*, not *H. caespitosus*; see Lokhorst (1992, p. 12).

EPITYPE DESIGNATED HERE: Cryopreserved culture strain SAG 835-2a, deposited in the Sammlung von Algenkulturen (SAG), Universität Göttingen, Germany.

Heterotypic synonyms:

Heterococcus protonematoides Vischer in *Ergeb. Wiss. Unters. Schweiz. Nationalparkes, N.F.* 1: p. 502, pl. 2, figures 1–3; figures 15,16. (1945).

For group G, *H. crassulus* was represented by an authentic strain, and we accept this as a recognized species. Similarly, for an unnamed group (see Figure 2), *H. fuornensis* was represented by an authentic strain, and therefore we recognize this as a distinct species.

Heterococcus crassulus **Vischer in *Ergeb. Wiss. Unters. Schweiz. Nationalparkes, N.F.* 1: p. 503, pl. 3, figures 1–3; figures 17, 17A, l-o (1945).**

ICONOTYPE: Figure 17 in Vischer, W. *Ergeb. Wiss. Unters. Schweiz. Nationalparkes, N.F.* 1: 479-512 (1945).

NEOTYPE: Material (authentic culture strain SAG 835-4) deposited in Nationaal Herbarium Nederland, Leiden University (L) by G.M. Lokhorst in *Taxonomic Studies in the Genus* Heterococcus. *Cryptogamie Studies* Vol. 3, p. 12. (1992).

EPITYPE DESIGNATED HERE: Cryopreserved culture strain SAG 835-4, deposited in the Sammlung von Algenkulturen (SAG), Universität Göttingen, Germany.

Heterococcus fuornensis **Vischer in *Ergeb. Wiss. Unters. Schweiz. Nationalparkes, N.F.* 1: p. 506, pl. 5, figures 1–3; figure 17A, a-c; figure 19 (1945).**

ICONOTYPE: Figure 19 in Vischer, W. *Ergeb. Wiss. Unters. Schweiz. Nationalparkes, N.F.* 1: 479-512 (1945).

NEOTYPE: Material (authentic culture strain SAG 835-5) deposited in Nationaal Herbarium Nederland, Leiden University (L) by G.M. Lokhorst in *Taxonomic Studies in the Genus* Heterococcus. *Cryptogamie Studies* Vol. 3, p. 12. (1992).

EPITYPE DESIGNATED HERE: Cryopreserved culture strain SAG 835-5, deposited in the Sammlung von Algenkulturen (SAG), Universität Göttingen, Germany.

In group B, there were no more than nine different sequence positions (one nonsynonymous substitution) among the complete *rbc*L sequences and only four nucleotide differences among the *psb*A/*rbc*L spacers. Strains of group B formed a well-supported monophyletic clade independent of other groups/species in the *rbc*L phylogeny (Figure 3, Additional file 1) as well as phylogenetic analysis of the whole ITS2 region

(Additional file 5). Therefore, we recognize group B as a distinct species. Placing a scientific name on group B (species B) is problematic because our study included all existing authentic cultures. Our molecular data, which were rigorously analyzed with phylogenetic methods, contradict species distinctions based upon non-rigorous intuition using highly variable morphological features, and we conclude that our rigorous analyses are more scientifically sound. Nonetheless, there are 61 named species, and perhaps group (species) B belongs to one of those species. If we simply propose a new name, then we are defying the intent of the International Code of Botanical Nomenclature (or any other Code). Therefore, we simply apply four of the oldest names used in [6] for group (species) B and the three other groups (C, D, F) which contained no authentic strains. We assume that none of these names is in contradiction with the morphology of the strains we designate to represent the four species. We argue that establishing axenic cultures and examining filaments at a certain age of a culture time (as it has been done to define species of *Heterococcus* previously [2-6]) is a poor way to identify species and this does not allow field samples to be identified to species. With *Heterococcus* growth in culture is a measure of meaningless differences and there is no hope that morphology will ever be useful when trying to put a name on these four groups (species). We suggest that close phylogenetic relationship with defined reference (epitype) strains as well as genetic distance from corresponding strains of other species, evidenced by *rbc*L gene phylogenies and differences in the *psb*A/*rbc*L spacers are appropriate to identify the species. Secondary structure of ITS2 constitutes an additional autapomorphic feature to define species of *Heterococcus*. We use *Heterococcus conicus* Pitschmann as name for group (species) B.

***Heterococcus conicus* Pitschmann *in* Pitschmann, H. *Nov. Hed.* 5 (3/4), p. 498, plate 96, Figures 11-16, (1963)**

ICONOTYPES: Plate 96, Figures 11-16, in Pitschmann, H. *Nov. Hed.* 5 (3/4), (1963)

NEOTYPE: Material (culture V 111) deposited in Nationaal Herbarium Nederland, Leiden University (L) by G.M. Lokhorst in *Taxonomic Studies in the Genus* Heterococcus*. Cryptogamie Studies* Vol. 3, p. 12. (1992).

EPITYPE DESIGNATED HERE: Cryopreserved culture strain MZ1-3, deposited in the Sammlung von Algenkulturen (SAG), Universität Göttingen, Germany.

Group C consisted of a single strain, SAG 2163 (Figure 2), which formed a distinct lineage in the *rbc*L and full ITS2 phylogenies (Figure 3, Additional files 1 and 4). It was also distinct in its *psb*A/*rbc*L spacer from *H. viridis* and *H. conicus* which were the closest relatives with SAG 2163. Therefore, we recognize group C as a distinct species and we use *Heterococcus virginis* Pitschmann as name. Two unidentified lichen photobionts share identical partial *rbc*L sequences (JN573801 and JN573802; [8]) and these differed by only one nucleotide from SAG 2163. Therefore, we assign these lichen photobionts to *H. virginis* as well.

***Heterococcus virginis* Pitschmann *in* Pitschmann, H. *Nov. Hed.* 5 (3/4), p. 497, plate 96, Figures 1-5, (1963)**

ICONOTYPES: Plate 96, Figures 1-5, in Pitschmann, H. *Nov. Hed.* 5 (3/4), (1963).

EPITYPE DESIGNATED HERE: Cryopreserved culture strain SAG 2163 deposited in the Sammlung von Algenkulturen (SAG), Universität Göttingen, Germany.

Group D comprised three strains with no nucleotide difference in the *rbc*L and a single in the *psb*A/*rbc*L spacer. In the ITS2 phylogeny, the three strains could not be distinguished due to different ITS2 variants that are intermixed (Additional file 4). Strains of group D exhibit a unique ITS2 secondary structure with a rather long helix III with considerable length variation at its terminal end (Additional file 4). Despite being closely related to *H. caespitosus* (group E) in the *rbc*L phylogeny (Figure 3 and Additional file 1) there are two CBCs in ITS2 that separate group D strains from the latter species. Consequently, we recognize group D as a distinct species, *Heterococcus leptosiroides* Pitschmann. One environmental clone sequence from Antarctic soils (AJ580925) shared full sequence identity in *rbc*L gene with group D strains, and therefore, we conclude that the environmental clone belongs to *H. leptosiroides*. Group D included strains identified as *H. caespitosus* and *H. protonematoides* based on morphology; however, neither was an authentic culture and again we consider identification based on

***Heterococcus leptosiroides* Pitschmann *in* Pitschmann, H. *Nov. Hed.* 5 (3/4), p. 497, plate 96, Figures 6–10, (1963).**

ICONOTYPES: Plate 96, Figures 6-10, in Pitschmann, H. *Nov. Hed.* 5 (3/4), (1963).

EPITYPE DESIGNATED HERE: Cryopreserved culture strain EIF 423/A790-45 deposited in the Sammlung von Algenkulturen (SAG), Universität Göttingen, Germany.

Finally, group F contained two strains with fully identical complete *rbc*L sequences and two differences in their *psb*A/*rbc*L spacers. In the ITS2 phylogeny the two strains could not be distinguished due to the variation of multiple copies (Figure 4C). The group F strains had a unique ITS2 secondary structure with a particularly long helix III (Figure 4B). Group F forms an independent lineage within the *Heterococcus* clade in the *rbc*L phylogeny (Figure 3, Additional file 1). Consequently, we recognize group F as a distinct species and use *Heterococcus ramosissimus* Pitschmann as name.

***Heterococcus ramosissimus* Pitschmann *in* Pitschmann, H. *Nov. Hed.* 5 (3/4), p. 499, plate 97, Figures 1–4, (1963)**

ICONOTYPES: Plate 97, Figures 1-4, in Pitschmann, H. *Nov. Hed.* 5 (3/4), (1963).

EPITYPE DESIGNATED HERE: Cryopreserved culture strain DB14-1-1 deposited in the Sammlung von Algenkulturen (SAG), Universität Göttingen, Germany.

The authentic strain of *H. endolithicus* was described by Darling and coworkers [1], 195/A790-35 (accessioned as strain SAG 63.90 by the SAG culture collection), but our study revealed that it represents a green alga, i.e. a close relative of *Desmococcus* species (Trebouxiophyceae) (Figure 2). Our microscopic investigation of SAG 63.90 revealed the same morphology as described previously [1]. Significantly, this morphology is somewhat similar to the morphology of *Desmococcus* [10], and this makes us confident that SAG 63.90 still represents the original isolate. Despite Darling and coworkers [1] having reported a "typical xanthophycean plastid structure" based on electron microscopy, they already considered *H. endolithicus* distinct from all other *Heterococcus* species because it did not form long filaments. In addition, three more strains from Antarctic soils were also identified as *Desmococcus*-like green algae (Figure 2). Therefore, we exclude *H. endolithicus* from the genus *Heterococcus* and propose a new nomenclatural combination for this authentic strain, but unfortunately we cannot apply the specific epithet (*endolithicus*) because the name *Desmococcus endolithicus* Broady & Ingerfeld already exists [31]. Therefore, we propose an avowed substitute name:

Desmococcus antarctica* (Darling & Friedmann) Rybalka, Wolf, Andersen & Friedl *comb. nov.

Basionym: *Heterococcus endolithicus* Darling & Friedmann In Darling et al. *J Phycol* **23:** 599, Figures 2a-c, 3. (1987).

EPITYPE DESIGNATED HERE: Cryopreserved culture strain SAG 63.90 deposited in the Sammlung von Algenkulturen (SAG), Universität Göttingen, Germany.

Infraspecific Variation and Geographical Distribution

Our relatively small sample of 29 *Heterococcus* strains already showed eight groups (= eight species). Within the five species for which multiple strains were available, the *psb*A/*rbc*L spacer sequences even resolved groups of strains with nearly identical sequences (genotypes; Figure 2). Strains with identical, or nearly identical, sequences were repeatedly found in our relatively small sample of *Heterococcus* strains and, importantly, established at different times from geographically distant localities. This implies that the number of species within *Heterococcus* might be rather limited. The same genotypes were confined to certain habitats (soil or freshwater) and geographical regions (Europe or Antarctica). For example, *H. viridis* strains SAG 835–3, SAG 835–6 and SAG835-7 were collected from freshwater habitats in Europe while all other strains of the species were from soil in Europe or Antarctica (Figure 2); they represent a distinct subgroup (genotype) within the species. Similarly, two strains of *H. conicus* were collected from freshwater in Europe (DB14-15, DB15-5) whereas the other two *H. conicus* strains were collected from Antarctic soil (MZ1-3, MZ1-6; Figure 2). We draw two conclusions. First, the two species are geographically widespread and will grow where suitable habitats exist. Second, genotypes of those growing in freshwater are distinct from those growing in soil. The sample size is exceedingly small, but there is a suggestion that our molecular data are separating populations within both species that have distinctly different habitats.

We also note that half of the *Heterococcus* genotypes in our sample originated from Antarctica but not a single genotype was shared between Antarctic and European strains, i.e. none of the Antarctic *Heterococcus* strains shared identical *psb*A/*rbc*L spacer sequences with the European strains. A previous study showed that Antarctic strains within a single species of the xanthophyte *Xanthonema* were distinguished from their temperate counterparts by only few nucleotides for the highly variable *psb*A/*rbc*L spacers [21]. Therefore, our findings for *Heterococcus* support the view that the Antarctic and temperate strains of xanthophyte species represent different populations of a single species. That is, the Antarctic strains of a given species share their own common evolutionary histories, implying that there was only one (relatively recent) colonization event in Antarctica for each species. Alternatively, if multiple colonization events occurred, then the invasions were too recent to produce significant divergence [32,33].

ITS2 sequence features

Our ITS2 sequences are, to our knowledge, the first ITS2 sequences available for Xanthophyceae. Given that available ITS2 sequence information for stramenopile algae is still limited, two aspects of the *Heterococcus* ITS2 sequences appear unusual, but might be useful for taxonomy. First, in *Heterococcus* ITS2 lengths were approximately 300 nucleotides long in most strains; group F (*H. ramosissimus*) sequences were almost 400 bps. The average length of ITS2 across all eukaryotes is about 210 bps as inferred from the ITS2 database IV [34]. In group D, two size classes occurred, i.e. either ~250 or ~300 bps, due to a large indel at the terminal end of helix III. Other stramenopile algal groups, the Bacillariophyceae and Phaeophyceae, show a bimodal distribution of their ITS2 sequence lengths, i.e. around 250/290 bps and around 250/350 bps, respectively. Second, the ITS2 sequences were rather variable, i.e. only few and rather

short sequence segments were alignable with confidence across the eight *Heterococcus* species. Such a high sequence variation among species of a single genus is unusual, at least as compared to genera and species of green algae where ITS2 has been revealed as a reliable molecular marker already many times, e.g. [35-38]. Finally, because the ITS2 rDNA sequences were so variable in *Heterococcus*, it is not possible to safely define compensatory base changes (CBCs), which can be deduced only from well aligned sequences. CBCs in conserved regions of the helices of ITS2 have been proposed for distinguishing microalgal species when sexual reproduction is unknown [23,24]. However, the concept of CBCs does not imply that two strains lacking CBCs must belong to the same species. That is, there may be other criteria that define microalgal species.

The high ITS2 sequence variability is in line with our maximum likelihood (GARLI and RAxML) analyses that had weak support for the monophyletic origin of the genus (Additional file 1). The monophyletic origin of *Heterococcus* was also weakly supported by a multiple gene phylogenetic analyses of photosynthetic stramenopiles that included three of our *Heterococcus* species [15]. Therefore, our results may suggest that more data (and better taxon sampling) are required to firmly demonstrate the monophyly of *Heterococcus*, or they may suggest that some of the species defined in our study belong to a separate, and sister, genus.

Conclusions

Application of the monophyletic species concept using the highly variable chloroplast-encoded *psb*A/*rbc*L spacer, the more conserved plastid *rbc*L gene, and the nuclear-encoded ITS2 provided a reference data base for unambiguous identification of the common cold soil microalga *Heterococcus*. Eight species were recognized and characterized at the molecular level. Previous taxonomic studies relied entirely on morphological features produced in cultures; our data will facilitate diversity assessments that are independent of culturing. In addition, the PCR amplification approach for the *psb*A/*rbc*L spacer is specific for Xanthophyceae. Using the new reference data base, partial sequences of the *psb*A/*rbc*L spacer and/or ITS2 may already be sufficient for the assignment of a new strain to a certain species. There are some difficulties; amplification of the *psb*A/*rbc*L spacer may be hampered by length variations, and sequence analyses of ITS2 may be complicated by multiple variants per strain. Using the monophyletic species concept, our species are mostly in contrast to those defined by the morphological (typological) species concept. We conclude that the extensive morphological plasticity displayed in culture cannot be interpreted without rigorous methods (e.g. cladistics), and the largely overlapping morphological characteristics make cladistic analysis very difficult or impossible. The identical, but highly variable, sequences that were repeatedly recovered among the species, suggest that the species diversity of *Heterococcus* is not extensive, especially considering the repetition that occurred in our small sampling from Europe and Antarctica. The observed sequence changes within a species may reflect adaptations to different types of habitats or climates and distinguish geographically widely separated strains.

Methods

Culture strains

Twenty three culture strains were received from the SAG culture collection [39,40]; five strains were provided by other workers in the field. Another five isolates (strains MZ1-3, MZ1-6, MZ2-4, MZ2-5, MZ3-7) were newly established using methods described previously [21] from Antarctic soil samples, i.e. the forefield of Baranowski Glacier, King George Island (collected December 12 2008 by M. Olech). Strains MZ1-3 and MZ1-6 were from the same sample, about 5 m from the glacier (62°12′34,9″S, 58°26′55,7″W) at 10 m a.s.l. Strains MZ2-4 and MZ2-5 also were from a single sample, a frontal moraine (62°12′34.4″S- 58°26′50.2″W) at 16 m a.s.l. Strain MZ3-7 was from a basal moraine (62°12′33.4″S – 58°26′41.1″W) at 6 m a.s.l. Antarctic strain B10 (provided by A. Massalski) was isolated also from King George Island, but from transect B near Ecology Glacier about 370 m farther inland; [41]). Four isolates (DB14-1-1, DB14-5-1, DB14-15 and DB15-5; provided by K. M. Mohr) were from cyanobacteria-dominated biofilms covering rocks at two neighboring locations of the main spring of the tufa-forming karst-water creek, Deinschwanger Bach, located at the western margin of the Franconian Alb, approximately 30 km ESE of Nürnberg, Germany (49°23'N, 11°28'E) [42]. The ten new isolates have been accessioned by the SAG culture collection under strain numbers as given in Additional file 2.

DNA extraction, PCR amplification and sequencing of strains

DNA was isolated from fresh cultures as in [21]. For determining sequences of the plastid-encoded *psb*A/*rbc*L spacer which lies upstream of the *rbc*L gene, i.e. between the *psb*A and *rbc*L genes, and full-length sequences of the *rbc*L gene the PCR approach of Andersen and Bailey [20] modified to amplify the target sequence in one piece [21] was used. The 5' primer *psb*A5 [20] or Xan2F [21], anchored in the *psb*A gene, and the 3' primer RS3 [20] placed in *rbc*S (downstream of *rbc*L) were used. However, for strains with extremely long *psb*A/*rbc*L spacers, PCR amplification was in two overlapping fragments, i.e. with primer pairs *psb*A5 and X5RG (the

reverse complement of primer X5FG [21]) and Xan3F [21] and RS3 [20]. For amplification of ITS2, PCR primers Xits2F (5' –GCTACACTCTGACACCTG -3'; which binds at the 5'-end of the 18S rRNA gene, i.e. pos. 1462–1477 of reference sequence AM490822 *H. viridis* SAG 835–3, and LR1850 [43] were used to amplify a rDNA fragment that expanded from 3'-end of SSU downstream to the 5'-end of the LSU rDNA. The same cycling parameters were used for all PCR reactions as described previously [21]. PCR products were purified using Invisorb Spin PCRapid Kit (Invitek, Berlin, Germany) or MSB Spin PCRapace Kit (Invitek, Berlin, Germany). Sequence determination of the *psb*A/*rbc*L spacer was as previously [21], but complemented by nine additional primers to obtain the sequences of the extremely long *psb*A/*rbc*L spacers present in some *Heterococcus* strains, i.e. hetnew_F (5'–GGTACAACTGAY CAATT-3'), het_F (5'–GGTGGTACAATTGGYCATC CAGA-3'), spacer2R (5'–ATTCGAGTACGCTCTTGTA-3'), DB_F (5'–GGCAAGCCTTTCACTCTTGAT–3'), DB_R (5'-CCACCCGGATTTAAAAGAGTT-3'), DB_F2 (5'–TTCGATACGGGAAACAACTT–3'), DB_R2 (5'–G ATCCTTTGGTTCAACTTAGAAGA–3'), SAG_F (5'–C AAGCTTCGACTGAGGCTT–3'), and SAG_R (5'–AT TGCAAGGCAAGCCTTG–3'). The latter two sequencing primers were used only for *H. crassulus* strain SAG 835–4, the "DB" primers only with the two isolates DB14-1-1 and DB14-5-1. The *rbc*L sequences were checked against the NCBI gene sequence database using nucleotide BLAST (blastn) [44,45] to confirm that they were Xanthophyceae. For four strains from the SAG culture collections no PCR products of plastid-encoded markers as described above could be obtained and then a portion of the nuclear-encoded 18S rRNA gene was sequenced with primer 895R [46] after PCR amplification with primers preferentially binding to green algal rDNA, primers 20 F [8] and CH1750R [46] and checked against the NCBI gene sequence database. For ITS2 sequence determination, the sequencing primers were 5.8SbF and 5.8SbR [47], 1800 F [43] and ITS4Xan (5'-TCCTCCGCTTAGTTATATGC-3'), which was a modification of primer ITS4 [48]. In several cases no clear sequence reads were obtained, even after repeated PCR and sequencing attempts, due to multiple copies of the ITS2 which varied in primary sequences (see Results). Then cloning of the PCR products was performed with the TOPO TA cloning kit and the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA). Ligations were transformed into competent *E. coli* TOP 10 cells as supplied by the manufacturer. In the plasmid screening, white *E. coli* colonies containing correct DNA insertions were identified by direct amplification of the inserted DNA fragment with a vector-specific primer set M13F/M13R. The ITS fragments were re-amplified from M13F/M13R PCR products with primer pair Xits2F/LR1850 as described above or the clones were cultivated overnight in LidBac reaction tubes (Qiagen, Hilden, Germany) with 1 ml LB medium containing 100 μg ampicillin and plasmid DNA was prepared from the clones with a NucleoSpin-Plasmid kit (Macherey and Nagel, Düren, Germany) following manufacturer's instructions. Sequencing reactions were performed with the Dye Terminator Cycle Sequencing v3.1 kit (Applied Biosystems, Darmstadt, Germany) and separated on an ABI Prism 3100 (Applied Biosystems, Darmstadt, Germany) sequencer. The sequences were assembled using the program SeqAssem [49]. For GenBank accession numbers of newly determined sequences for the 29 *Heterococcus* strains see Additional file 2; the accession numbers for the four green algal sequences determined in this study are JX681197 - JX681200.

Chloroplast-encoded marker analysis

The chloroplast-encoded marker sequences (from 3'-end of *psb*A downstream to 5'-end of *rbc*S) were manually aligned using Bioedit [50] and Seaview [51] editors from which the *rbc*L sequence alignment used for the phylogenetic analyses was extracted. The *rbc*L sequence alignment was constructed using 15 of the sequences newly determined for *Heterococcus* in this study to which 32 other sequences available for the Xanthophyceae clades C, B, T, and V as defined previously [14] were added (Additional file 1). The two phaeophycean sequences *Fucus vesiculosus* NC016735 and *Ectocarpus* sp. AY372978 were employed to root the phylogeny. The alignment was subjected to distance, maximum-parsimony (MP) and maximum-likelihood (ML) approaches. ModelTest 3.7 [52] used in conjunction with PAUP* 4b10 [53] determined that the GTR+I+G model [54] provided the best fit to the data according to the AIC criterion with estimations of nucleotide frequencies (A = 0.2859, C = 0.1447, G = 0.1981, T = 0.3714), a rate matrix with six different substitution types, assuming a heterogeneous rate of substitutions with a gamma distribution of variable sites, number of rate categories = 4, shape parameter $\alpha = 0.8249$ and proportion of invariable sites (*pinvar*) of 0.4977. This model was used for the minimum evolution distance (ME) approach performed with PAUP* 4b10 (DNA distances set to maximum likelihood) and the maximum likelihood ML (approach) using GARLI v0.96 [25,26]. A complementary ML phylogeny construction was done with the program RAxML [55], using the GTR+Γ+I model and with 100 bootstrap replicates. Neighbor-joining distance (NJ) phylogenies were constructed in connection with the "HKY85 model" [56] with PAUP* 4b10. For ME and maximum parsimony (MP) tree reconstruction (PAUP* 4b10) a heuristic search procedure with 10 random input orders of sequences and TBR were employed to find the best tree. Best scoring trees were held at each step. In MP analyses,

the sites were weighted (RI over an interval of 1–1000). Bootstrap resampling was performed on NJ, ME, MP with 1000 replications and 2000 replications on ML GARLI trees. For the Bayesian analysis the program MrBayes version 3.1.2 [57] was used with procedures as described earlier [58].

Nuclear-encoded ITS2 sequence-structure analysis

Using hidden Markov models (HMMs) nuclear ITS2 sequences have been annotated according to [59]. One ITS2 sequence from each group A, D, F and *H. fuornensis* strain SAG 835–5 was used for secondary structure prediction. Based on minimum free energy ITS2 secondary structures were directly folded with the help of the "RNAstructure" software [60,61] and manually corrected. The four sequence-structure pairs were used as templates for homology modeling of the remaining 39 secondary structures [62]. In accordance to [63] the phylogenetic analysis followed the procedure outlined in [23,34,64,65]: automatically, a multiple sequence-structure alignment was generated in 4SALE v1.7 [29,66], i.e. either partial (Figure 4) or full (Additional files 4, 5, 6) sequences and their secondary structures were synchronously aligned, making use of an ITS2 sequence-structure specific scoring matrix [66,67]. Based simultaneously on the primary sequence and the secondary structure information, phylogenetic relationships were reconstructed using NJ through in conjunction with an ITS2 sequence-structure specific general time reversible (GTR) substitution model as implemented in ProfDistS v0.9.9 [27,67]. Bootstrap support [68] was estimated based on 100 pseudo-replicates (Figure 4, Additional files 4, 5, 6). Trees were visualized using Treeview [69].

Additional files

Additional file 1: Maximum likelihood (ML) phylogeny of *rbc*L gene sequences for *Heterococcus* and other members of Xanthophyceae. The phylogeny was calculated with the programme GARLI v0.96 [25,26] based on a *rbc*L data set (1325 bp long, 517/418 variable/parsimony informative sites) consisting of 15 *Heterococcus* and 32 other Xanthophyceae sequences (corresponding to clades C, B, T, and V as defined in [14]) as well as two sequences from Phaeophyceae as outgroup. Scale bar, substitution per site. Numbers mapped to internodes are bootstrap values from 2000 replicates, only values >70% have been recorded. The phylogeny in this Figure includes the phylogeny of 15 *Heterococcus* strains shown in Figure 3 (highlighted). The inserted table lists bootstrap values mapped to internodes of the *Heterococcus* clade using six different analysis methods (see text). Scale bar, substitution per site.

Additional file 2: DNA sequences newly determined for 29 *Heterococcus* strains and their GenBank sequence accession numbers. For the *psb*A/*rbc*L spacer and full *rbc*L gene all determined sequences are listed, for ITS2 only those sequences that were different from each other. (p), only *psb*A/*rbc*L spacer and partial full *rbc*L gene could be determined; (a), already made available previously; n.a., not applicable.

Additional file 3: Groups of *Heterococcus* strains with fully identical *rbc*L and/or *psb*A/*rbc*L spacer sequences. Strains marked in bold were used for the *rbc*L phylogeny (Figure 3, Additional file 1). Species assignment is according to the new species designation as in Figure 2 (see Discussion).

Additional file 4: ITS2 sequence and secondary structure phylogenetic analyses of three strains of *Heterococcus* group D *(H. leptosiroides).* (**A**) ProfDistS [27] sequence-structure NJ tree (unrooted) as derived from the multiple sequence-structure alignment of ITS2 helices I-IV recovered for strains of group D, *H. leptosiroides*. Bootstrap values based on 100 pseudo-replicates are mapped to the appropriate internodes. Branch lengths are drawn proportional to inferred changes. The template ITS2 variant used in B) is highlighted in bold. Scale bar, substitutions per site. (**B**) ITS2 secondary structure of ITS2 variant EIF 423/A790-5_cl65 used for homology modeling of secondary structures for all strains of group D (*H. leptosiroides*). The secondary structure was visualized with VARNA [28]. Helices are numbered I–IV. Four strains indicated by an asterisk are devoid of the apical part of helix III. An arrowhead indicates the highly conserved GGU motif 5′ to the apex of helix III. A cloud highlights the segment of helix III conserved across all studied strains. Open arrowheads mark positions of two CBCs that distinguish groups D and E.

Additional file 5: ITS2 sequence and secondary structure phylogenetic analyses of twelve strains of *Heterococcus* groups A-C *(H. viridis, H. conicus, H. virginis).* (**A**) ProfDistS [27] sequence-structure NJ tree (unrooted) as derived from the multiple sequence-structure alignment of ITS2 helices I-IV recovered for strains of the *H. viridis* clade, i.e. groups A-C, *H. viridis, H. conicus* and *H. virginis*. Bootstrap values based on 100 pseudo-replicates are mapped to the appropriate internodes. Branch lengths are drawn proportional to inferred changes. The template ITS2 variant used in B) is highlighted in bold. Scale bar, substitutions per site. (**B**) Secondary structure of ITS2 variant *H. viridis* EIF 430/A801-2_24 used for homology modeling of secondary structures for all strains of *Heterococcus* groups A-C. The secondary structure was visualized with VARNA [28]. Helices are numbered I–IV. An arrowhead indicates the highly conserved GGU motif 5′ to the apex of helix III. A cloud highlights the segment of helix III conserved across all studied strains.

Additional file 6: ITS2 sequence and secondary structure phylogenetic analyses of *Heterococcus fuornensis* strain SAG 835–5. (**A**) ProfDistS [27] sequence-structure NJ tree (unrooted) of ITS2 variants recovered from strain *H. fuornensis* SAG 835–5 as derived from the multiple sequence-structure alignment of ITS2 helices I-IV. Bootstrap values based on 100 pseudo-replicates are mapped to the appropriate internodes. Branch lengths are drawn proportional to inferred changes. The template ITS2 variant used in B) is highlighted in bold. Scale bar, substitutions per site. (**B**) Secondary structure of ITS2 variant SAG 835-5_46 used for homology modeling of secondary structures for all ITS2 variants of the same strain. The secondary structure was visualized with VARNA [28]. Helices are numbered I–IV. An arrowhead indicates the highly conserved GGU motif 5′ to the apex of helix III. A cloud highlights the segment of helix III conserved across all studied strains.

Additional file 7: DNA sequence differences among five authentic strains of *Heterococcus* group A. Distance matrices with number of sequence position differences from the *rbc*L gene, the *psb*A/*rbc*L spacer and ITS2 between the five authentic strains of *Heterococcus* group A (assigned to *H. viridis* , see text). In brackets, the total number of differences found with a certain molecular marker among the five strains. An asterisk marks the strain that is distinct from others by the presence of a "GCAA" indel in helix IV of ITS2.

Abbreviations

SAG: Culture Collection of Algae at Göttingen University, Germany (*Sammlung von Algenkulturen der Universität Göttingen, Göttingen*, Germany); ML: Maximum likelihood; MP: Maximum parsimony; ME: Minimum evolution; NJ: Neighbor joining.

Competing interests

The authors declare that they have no competing interests.

Authors' contribution

NR conceived and designed the study, carried out the molecular genetic studies, established cultures, participated in the phylogenetic analyses including ITS2 secondary structure models and drafted the manuscript. MW performed the analyses of ITS2 secondary structure models and ITS2 phylogenetic analyses, contributed to interpretation of the results and was involved in critically revising the manuscript. RAA developed all aspects regarding taxonomy of *Heterococcus*, participated in the interpretation of the molecular data and critically revised the manuscript. TF and RAA wrote the final manuscript. TF participated in all phylogenetic analyses and interpretation of the molecular data. All authors have contributed on the manuscript drafting, read and approved the final manuscript.

Acknowledgments

NR acknowledges the various support provided by Professor Rüdiger Schulz, Kiel University, Germany. This work would not have been possible without the generous financial support of grants extended by the *Christiane Nüsslein-Volhard-Stiftung* (CNV) and the "L'Oréal-UNESCO "For Women in Science" Award to NR; RAA acknowledges National Science Foundation support for grant DEB-0949211. NR acknowledges Professors Maria Olech (Jagiellonian University, Kraków, Poland, and Polish Academy of Sciences) and Andrzej Massalski (Jan Kochanowski University, Kielce, Poland) for provision of Antarctic soil samples and strain B10. Dr. Katrin I. Mohr is acknowledged for provision of four strains she isolated from freshwater; development and analyses of these strains were made possible through a grant of the Deutsche Forschungsgemeinschaft (DFG) extended to T.F. (FR 905/13-2,3). NR and TF acknowledge the assistance in purification, and maintenance of the studied culture strains as well as provision of reference strains from the SAG culture collection, by Ilse Kunkel, Marlis Heinemann, Hella Timmermann and Dr. Maike Lorenz. Robert Rockstuhl helped in isolating strains from Antarctic soil samples.

Author details

[1]Experimental Phycology and Culture Collection of Algae (SAG), Georg August University Göttingen, Untere Karspüle 2a, Göttingen 37073, Germany. [2]Plant Cell Physiology and Biotechnology, Botanical Institute, Christian Albrechts University of Kiel, Am Botanischen Garten 1-9, Kiel 24118, Germany. [3]Department of Bioinformatics, Biocenter, University of Würzburg, Würzburg 97074, Germany. [4]Friday Harbor Laboratories, University of Washington, Friday Harbor, WA 98250, USA.

Received: 15 September 2012 Accepted: 5 February 2013
Published: 13 February 2013

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doi:10.1186/1471-2148-13-39
Cite this article as: Rybalka *et al.*: Congruence of chloroplast- and nuclear-encoded DNA sequence variations used to assess species boundaries in the soil microalga *Heterococcus* (Stramenopiles, Xanthophyceae). *BMC Evolutionary Biology* 2013 **13**:39.

Authors' contributions

All chapters of this thesis were designed and written for peer-reviewed publications. The contribution of each author, including my own, is specified in this section.

Introduction.

Nataliya Rybalka provided the Introduction regarding the two classes of stramenopile algae, Xanthophyceae and Eustigmatophyceae, to a book chapter, *Freshwater Algae of North America, 2nd Edition Ecology and Classification*, which to revise and update for a new edition NR has been invited (Ott et al., 2015; publication no. 6 in section "List of publications", p. 85). The parts of this book chapter introduction as far as it concerns the class Xanthophyceae has been used for the Introduction of this thesis. NR wrote the Introductions to the Xanthophyceae and Eustigmatophyceae for this book chapter independently by herself. It was then revised and language-edited by Thomas Friedl and the editors of the book (John Wehr).

Chapter 1. Testing for endemism, genotypic diversity and species concepts in Antarctic terrestrial microalgae of the Tribonemataceae (Stramenopiles, Xanthophyceae)

NR conceived and designed the study, carried out the molecular genetic studies, established cultures, participated in the phylogenetic analyses and wrote the manuscript independently by herself. Robert A. Andersen and Thomas Friedl then revised the text. NR, RAA and TF together wrote the final manuscript of Rybalka et al. (2009). Igor Kostikov and Andrzej Massalski assisted in designing the study until an early stage, Kathrin I. Mohr and Andrzej Massalski provided their own isolates to the study and Maria Olech contributed the Antarctic soil samples.

Chapter 2. Assessing genus and species boundaries of Tribonemataceae algae (filamentous Xanthophyceae, Stramenopiles) in culture collections using chloroplast-encoded molecular markers

NR conceived and designed the study, carried out the molecular genetic studies, established cultures, participated in the phylogenetic analyses and wrote the manuscript independently by herself. The chapter has been based on publication Rybalka et al. (2007) which is a summary of NR's studies concerning the Tribonemataceae except for the genus *Xanthonema*. For publication Rybalka et al. (2007) the manuscript text was written by NR and then revised by RAA and TF. All three together wrote the final manuscript.

Chapter 3. Congruence of chloroplast- and nuclear-encoded DNA sequence variations used to assess species boundaries in the soil microalga *Heterococcus* (Stramenopiles, Xanthophyceae)

NR conceived and designed the study, carried out the molecular genetic studies, participated in the phylogenetic analyses including ITS2 secondary structure models and wrote the manuscript independently by herself. Matthias Wolf performed the analyses of ITS2 secondary structure models and ITS2 phylogenetic analyses, contributed to interpretation of the results and was involved in critically revising the manuscript. RAA developed all aspects regarding taxonomy of *Heterococcus*, participated in the interpretation of the molecular data and critically revised the manuscript. NR, RAA and TF together wrote the final manuscript of Rybalka et al. (2013).

Appendix 1: Introduction of book chapter, Ott et al. (2015), used for the Introduction of this thesis

Ott, D.W., Oldham-Ott C.K., Rybalka, N., and Friedl, T. (2015) Xanthophyte, Eustigmatophyte, and Raphidophyte Algae. In: Wehr, J.D., Sheath, R.G., Kociolek, J.P. (Eds.). *Freshwater Algae of North America: Ecology and Classification, 2nd edition*. Academic Press, Amsterdam, pp. 483-534.

Chapter 11

Xanthophyte, Eustigmatophyte, and Raphidophyte Algae

Donald W. Ott,[1] Carla K. Oldham-Ott,[2] Nataliya Rybalka,[3,4] and Thomas Friedl[4]

[1]*Department of Biology, The University of Akron; Akron, Ohio, USA.* [2]*School for Professional Studies, Walsh University, Akron, Ohio, USA.* [3]*Institut für Mikrobiologie und Genetik, Georg-August-Universität Göttingen, Göttingen, Germany.* [4]*Experimentelle Phykologie und Sammlung von Algenkulturen, Georg-August-Universität Göttingen, Göttingen, Germany*

Chapter Contents

I GENERAL INTRODUCTION

The three classes treated in this chapter, Xanthophyceae, Eustigmatophyceae, and Raphidophyceae, represent three independent photosynthetic lineages of the Stramenopiles (heterokont algae) and are not closely related to each other. Remarkably, members of the Eustigmatophyceae appear morphologically and in coloration so similar to coccoid members of the Xanthophyceae that they often cannot be distinguished in field material. Even with pure cultures, DNA sequence data or a detailed pigment analysis is required for an unambiguous distinction between coccoid Xanthophytes and Eustigmatophytes. In contrast, freshwater members of Raphidophyceae are rather distinct, because they form flagellated vegetative stages. Probably the most particular characteristic feature, shared by the three classes, is that they represent biflagellate "heterokont" organisms, commonly referred to as Stramenopiles. Flagellated stages of most Stramenopiles possess two different flagella, an anteriorly directed one with tripartite and rigid tubular hairs (mastigonemes) in two opposite rows along the flagellum and a posteriorly directed usually smooth flagellum without hairs (Patterson, 1989; Adl et al., 2005; Yoon et al., 2009; Yang et al., 2012). Because of the presence of heterokont flagella, the Xanthophytes, Eustigmatophytes (in the past as a single class: Xanthophyceae), and Raphidophytes (as Chloromonadophyceae) were combined into the division "Heterokontae" more than hundred years ago (Luther, 1899). Later, the Heterokontophyta (van den Hoek and Jahns, 1978) was introduced for the division containing the stramenopile algae. In addition, the terms *chromophyte*, *Chromista*, *ochrophyte*, and *Ochrophyta* have been referred to stramenopile algae (Yoon et al., 2009). Sixteen classes of photosynthetic Stramenopiles have been described up to now. Most molecular phylogenetic analyses show them as a monophyletic group within the Stramenopiles that is either derived from, or sister to, a clade of entirely non-photosynthetic Stramenopiles (Riisberg et al., 2009; Yoon et al., 2009). A recent phylogeny based on multigene DNA sequences resolved three major clades within the photosynthetic Stramenopiles (Yang et al., 2012). The molecular phylogenetic studies show the Xanthophyceae and Raphidophyceae being less distant from each other than the Xanthophyceae

Freshwater Algae of North America. http://dx.doi.org/10.1016/B978-0-12-385876-4.00011-6

to the Eustigmatophyceae (Yoon et al., 2009; Yang et al., 2012). The Xanthophyceae and Raphidophyceae formed together with five other classes (among which were the Phaeophyceae, brown algae; see Chapter 19) the "SI clade" of Yang et al. (2012), whereas the Eustigmatophyceae were together with the Chrysophyceae (Chapter 12) and Synurophyceae (Chapter 14) in the "SII" clade. The Bacillariophyceae, diatoms (Chapters 15 and 16), were placed in another clade, the "SIII" (Yang et al., 2012).

All photosynthetic stramenopiles acquired their plastids through secondary endosymbiosis, which is the uptake and retention of a primary algal cell by another eukaryotic lineage (Keeling, 2009). The primary algal cell involved was a red algal cell. However, recently phylogenomic evidence emerged for a third partner that was involved in the secondary endosymbiosis. These data indicate an endosymbiosis with a green alga most likely preceded the red algal endosymbiont (Moustafa et al., 2009; Tirichine and Bowler, 2011). The plastids of all stramenopile algae are so-called complex plastids with four membranes. The outermost of them is continuous with the outer nuclear membrane, giving rise to a shared endomembrane system, the plastid endoplasmatic recticulum (PER), and resulting in an intimate connection of the plastid to the nucleus (Yoon et al., 2009).

Most other shared features of stramenopile algae refer to their pigmentation and chloroplasts (plastids). Chlorophyll *b* is absent, but Xanthophyceae and Rhaphidophyceae exhibit chlorophyll *c* instead (characteristic for most stramenopile algae). However, chlorophyll *c* is absent in the Eustigmatophyceae, the only lineage of stramenopile algae without that pigment. Another peculiarity of the three classes with respect to photosynthetic pigments is the absence of the brown carotenoid fucoxanthin in both the Xanthophyceae and Eustigmatophyceae, whereas the Raphidophyceae as well as all other 13 classes of photosynthetic Stramenopiles have a more brownish appearance due to the presence of fucoxanthin. Therefore, the color of Xanthophytes and Eustigmatophytes is bright green to yellowish green. Members of Raphidophyceae together with Bolidophyceae are the only class of stramenopile algae with naked vegetative cells (Yoon et al., 2009). Most Stramenopile plastids exhibit lamellae of three stacked or appressed more or less plate-like thylakoids with a spheroidal girdle lamella surrounding all the other lamellae in the plastid. However, Eustigmatophyceae is the only stramenopile algal lineage where such a girdle lamella is absent (which may also be true for the xanthophyte genus *Bumilleriopsis*, Massalski and Leedale, 1969). Similarly, an eyespot in flagellated cells (if present) associated with the shorter smooth flagellum is located within the plastid in the Xanthophyceae and Raphidophyceae as in most other stramenopile algae, but it is located outside of the plastid in Eustigmatophyceae.

II XANTHOPHYTES

A Introduction

The most recent summary of general characteristics of the class Xanthophyceae that distinguishes it from other photosynthetic Stramenopiles has been presented by van den Hoek et al. (1995). Although still based on data obtained before the introduction of molecular phylogenetics to examine the Xanthophyceae as well as the Stramenopiles, it is still regarded as generally valid. According to van den Hoek et al. (1995, pp. 123-130) the main distinguishing features of Xanthophyceae are as follows:

1. *Organizational level*. Most genera of Xanthophytes are unicellular or colonial coccoid algae, but there is also a considerable number of species in which the thalli are composed of multinucleate siphons, and a few that consist of multicellular filaments. Many non-flagellate species, however, produce flagellate cells (zoids) at some stage in their life cycles. In the zoids the flagella are inserted close to the apex of the cell.
2. *Eyespot*. A typical photoreceptor apparatus is present in the zoids, consisting of a swelling on the short, smooth flagellum and an eyespot lying within the chloroplast.
3. *Chloroplasts and pigments*. Chloroplasts are discoid and green or yellow-green. In additional to chlorophyll *a*, there are also small amounts of chlorophylls *c1* and *c2*. The principal accessory pigments are ß-carotene, vaucheriaxanthin, diatoxanthin, diadinoxanthin, and heteroxanthin. Fucoxanthin, the brown pigment of many other stramenopile algae, is absent.
4. *Cell wall in vegetative cells and resting cysts*. Spherical or ellipsoidal cysts are formed by some species. Each cyst is formed within a cell (it is an endogenous cyst). Its wall is impregnated with silica and consists of two unequal halves that fit tightly together. The cell wall often can be seen to consist of two halves that overlap to some extent in the middle of the cell. The cell wall, like the cyst wall, is often impregnated with silica, although it seems to consist for the most part of cellulose microfibrils.

B Diversity and Morphology

1 Classification, Phylogeny, and Morphology

Van den Hoek and Jahns (1978) and van den Hoek et al. (1995) have summarized the repeated and continuous taxonomic rearrangements to which the Xanthophyceae was subjected since its establishment in the early twentieth century. The name Xanthophyceae was proposed by Allorge in 1930 (Allorge, 1930) and first validly published by Fritsch in 1935 (see Hibberd, 1990a; Fritsch, 1935, 1956). Principles of traditional morphological classification of Xanthophytes algae were established by Pascher (1925, 1939) and with minor changes used by subsequent authors (e.g., Ettl, 1978; Matvienko and Dogadina, 1978) until today.

The yellow-green algae or Xanthophyceae are also known as the Tribophyceae (Hibberd, 1981). Their yellow-green color is not always easy to distinguish from true green algal taxa. Despite about 600 species from about 100 genera of Xanthophyceae that were described in the twentieth century mostly on the basis of morphology (van den Hoek et al., 1995), the number of true members of Xanthophyceae remains unclear. There are no summarized data about taxa that were excluded from Xanthophyceae and moved to other classes on the basis of ultrastructural or molecular investigations. Genera and species of such groups as Eustigmatophyceae, Raphidophyceae, Phaeothamniophyceae, and Chlorarachniophyta were originally described as members of Xanthophyceae (Bailey et al., 1998; Hibberd, 1990a,b). In addition, many species from the traditional xanthophycean order Chloramoebales were found to belong to other stramenopile classes, for example, Chrysophyceae, and even other phyla, for example, Haptophyta and Cryptophyta.

Traditional morphological classifications of the Xanthophyceae include six or seven orders corresponding to the level of organization of vegetative cells (Ettl, 1978; Silva, 1979): Unicellular flagellates were accommodated in the Heterochloridales (syn. Chloramoebales Silva, 1979), capsoid forms (non-motile cells without wall, but embedded in mucilage) in the Heterogloeales, rhizopodial forms (vegetative cells amoeboid with rhizopods and without walls) in the Rhizochloridales, coccoid forms in the Mischococcales, filamentous (branched and unbranched forms) in the Tribonematales, and siphonous forms in the Botrydiales and Vaucheriales (Ettl, 1978; Matvienko and Dogadina, 1978). However, molecular phylogenetic studies show that most of these traditional orders do not form monophyletic groups (Potter et al., 1997; Bailey et al., 1998; Maistro et al., 2009). Therefore, Adl et al. (2005) reduced the classification to two groups: (1) the Tribonematales with filamentous as well as coccoid forms, sometimes becoming parenchymatous or multinucleate with age and elaborate reproductive structures lacking, and (2) the Vaucheriales comprising siphonous filaments with elaborate sexual reproductive structures (antheridia, oogonia). All six genera of Heterochloridales (Chloramoebales) as treated in the previous edition of this book have been recognized as members of Chrysophyceae or Synurophyceae until now and, therefore, are no longer treated here. Similarly, four out of the six previously treated genera of Heterogloeales have been transferred to the Chrysophyceae or Synurophyceae and, therefore, are not treated here. The two Heterogloeales genera still remaining in the Xanthophyceae are *Characidiopsis* and *Pleurochloridella*, but are treated here as "doubtful" members of Xanthophyceae. DNA sequence analyses showed *Chlamydomyxa labyrinthuloides* W. Archer, characterized by amoeboid or plasmodioid structures as the main stage in the life history and placed by A. Pascher within the yellow-green algae (Pascher, 1937-1939; Ettl, 1978), formerly assigned to Rhizochloridales, has been found to be a close relative with the recently described class Synchromophyceae (Kai et al., 2008), which is also supported by morphology, pigment, and DNA sequence analyses (Wenderoth et al., 1999; Schmidt et al., 2012). *Myxochloris*, to be merged with *Chlamydomyxa* (Pascher, 1937-1939; Silva, 1979), may likely also have a similar phylogenetic position, therefore, both genera are not treated here anymore. The Xanthophyceae is one of the two major lineages of the "PX clade" (Kai et al., 2008) for which the presence of cell walls and the absence of flagellate cells in the vegetative phase are characteristic features that may be a distinct contrast to the other classes of Heterokontophyta. Also, there is no ultrastructural or molecular evidence in support of their presumed taxonomic status (i.e., being xanthophytes) for the genera of Rhizochoridales and Heterogloeales treated here. Kai et al. (2008) even suggest that because there is no naked species belonging to the Xanthophytes and the PX clade as a whole on the basis of reliable modern data, "it would be reasonable to exclude these doubtful species from consideration" (Kai et al., 2008). However, four genera of Rhizochloridales and one of Heterogloeales are mentioned here because they are eukaryotes with a lifestyle peculiar for algae that is poorly investigated and have frequently been reported from associations with vascular plants. The heterotrophic *Rhizochloris* has been reported to live within *Sphagnum* water-holding cells, similar to *Chlamydomyxa* and *Myxochloris*, which may live endophytically as well. *Rhizolekane*, *Stipitococcus*, and *Stipitoporos* have been described to live epiphytically on filamentous algae, a habitat that has been poorly studied so far. It has been shown that orders Mischococcales and Tribonematales as defined by Ettl (1978) are not monophyletic (Potter et al., 1997; Maistro et al., 2009). With regard to filamentous Xanthophytes, there is some evidence

that the filamentous species with H-shaped cell walls constitute one lineage of the Tribonematales and those with entire cell walls (e.g., *Heterococcus*) constitute a second lineage, but this finding has not yet been put forward in a classification (Bailey et al., 1998; Adl et al., 2005). Also the monophyly of Vaucheriales is questionable as well as the appropriateness of Botrydiales (Negrisolo et al., 2004; Maistro et al., 2009). Many investigated morphologically defined genera, for example, *Bumilleriopsis*, *Botrydiopsis*, *Chlorellidium*, *Xanthonema*, and *Ophiocytium*, were found not to be monophyletic (Negrisolo et al., 2004; Maistro et al., 2007, 2009). Using DNA sequence data, it has also been shown that criteria for species delimitation and species borders should be re-evaluated within many other genera of Xanthophyceae (Zuccarello and Lokhorst, 2005; Rybalka et al., 2009).

A recent phylogeny based on *rbc*L gene sequence comparisons summarizes the findings from several previous studies that none of the three organizational levels in the Xanthophyceae—coccoid, filamentous, or siphonous—define monophyletic lineages (Rybalka et al., 2013).

Today only 13 morphologically defined genera from four orders of the traditional Xanthophyceae (Ettl, 1978) may be attributed to Xanthophyceae with confidence on the basis of molecular phylogenetics ("approved" Xanthophytes, Table 1). As based on DNA sequence phylogenetic analyses, there are no species without cell walls and no species with flagellate cells in the vegetative phase belonging to the Xanthophyceae. This is also true for other members of the PX clade *sensu* Kai et al. (2008) of Stramenopiles, a clade that unites the Xanthophyceae with the Phaeophyceae, Chrysomerophyceae, Phaeothamniophyceae, and Schizocladiophyceae.

TABLE 1 Overview of Genera of Xanthophytes Treated in this Chapter and Their Organizational Level

1. Approved Xanthophytes	
The 13 genera checked by molecular phylogenetics for their correct assignment to Xanthophyceae	
coccoid (7)	*Botrydiopsis*
	Botryochloris
	Bumilleriopsis
	Chlorellidium
	Mischococcus
	Ophiocytium
	Sphaerosorus
filamentous (4)	*Bumilleria*
	Heterococcus (incl. *Aeronemum*[1], *Capitulariella*[1], *Chaetopedia*[1], *Fremya*[1], *Heteropedia*[1])
	Tribonema
	Xanthonema
siphonous (2)	*Botrydium*
	Vaucheria
2. Untested Xanthophytes	
The 44 genera of former or current Xanthophytes, mostly poorly studied, in need of being cultured and checked for their correct phylogenetic position by molceular phylogenetics. Many of these genera may in fact be Eustigmatophytes, belong to other photosynthetic Stramenopiles or Green Algae (Chlorophyta).	
coccoid (42)	*Akanthochloris*
	Arachnochloris
	Asterogloea
	Aulakochloris
	Bracchiogonium
	Centritractus
	Chlorallantus
	Chlorarkys
	Chlorellidiopsis
	Chlorogibba
	Chlorokoryne
	Chloropedia
	Chlorosaccus
	Chlorothecium
	Diachros
	Dioxys
	Ducellieria
	Endochloridion
	Excentrochloris
	Gloeopodium
	Gloeosphaeridium
	Hemisphaerella
	Heterodesmus
	Ilsteria
	Isthmochloron
	Keriosphaera
	Lutherella
	Meringosphaera
	Monallantus
	Peroniella
	Pleurogaster
	Polygoniochloris
	Prismatella
	Pseudopolyedriopsis
	Pseudotetraedron
	Raphidiella
	Rhomboidella
	Sklerochlamys
	Tetraktis
	Tetraplektron
	Trachychloron
	Trachycystis

(Continued)

TABLE 1 Overview of Genera of Xanthophytes Treated in this Chapter and Their Organizational Level—Cont'd

filamentous (2)	*Chadefaudiothrix*
	Neonema
3. Doubtful Xanthophytes	
The 12 genera of former xanthophyte genera poorly known, i.e., for each genus there is an indication that it does not belong to Xanthophyceae or Eustigmatophyceae, but to other photosynthetic Stramenopiles or the Green Algae (Chlorophyta).	
amoeboid (5)	*Rhizochloris*
	Rhizolekane
	Stipitococcus
	Stipitoporos
	Perone
capsoid[2] (1)	*Characidiopsis*
coccoid (5)	*Chlorocloster*
	Chytridiochloris
	Dichotomococcus
	Nephrodiella
	Pleurochloridella
filamentous (1)	*Heterodendron*

There are three groups of genera based on the current knowledge about their phylogenetic placement: Keys 1, 2, and 3.
Descriptions of genera in sections "Approved Xanthophytes," "Untested Xanthophytes," "Doubtful Xanthophytes," and "Approved Eustigmatophytes," and "Candidate Eustigmatophytes".

[1]*Genus with unclear distinction from Heterococcus and still not studied by molecular phylogenetics.*

[2]*With eyespots in the plastid and contractile vacuoles in the cytoplasm.*

2 Reproduction

Asexual reproduction in xanthophytes is mostly by means of flagellated zoospores and immotile autospores, as well as by fragmentation in the filamentous forms. The formation of aplanospores (similar to zoospores, but no flagella) and resting stages (akinetes, composed of two pieces of approximately equal size and with silicified walls) have also been observed. Zoospores are formed in many xanthophyte genera including coccoid, filamentous, and siphonous forms (e.g., *Heterococcus*). One or more plastids (usually two) are present in the xanthophyte zoospores (Hibberd, 1990a). The zoospores of *Vaucheria* (Fig. 11D) differ from those of other xanthophytes in producing large compound zoospores (= synzoospores) with many pairs of unequal, smooth flagella (Koch, 1951; Greenwood et al., 1957; Ott and Brown, 1974). The numerous plastids do not have eyespots (Ott and Brown, 1974). Autospore formation is common in the coccoid forms, for example, *Botryochloris*, *Chlorellidium*, and *Sphaerosorus* (Figs. 3H–L). Fragmentation frequently occurs in filamentous forms (e.g., *Bumilleria* and *Xanthonema*, Figs. 9A–D). Akinetes generally occur in filamentous genera (Lee, 2008), such as *Tribonema* (Smith, 1950) but have also been observed in *Botrydium*, possibly in *Ophiocytium* (Poulton, 1930), and in *Vaucheria* under suboptimal culture conditions (Fig. 11F).

Sexual reproduction has been observed in only three xanthophyte genera. *Tribonema* is reported to be isogamous (Smith, 1920; Ross, 1954; Bold and Wynne, 1985), while *Botrydium* may be isogamous or anisogamous (Iyengar, 1925; Moewus, 1940). *Vaucheria* has sexual reproduction and is oogamous; antheridia and oogonia are formed either directly on the filament (Figs. 11C, H, K, L) or on gametophores (Figs. 11G, I, J, M). Most species are monoecious, but some brackish water species are dioecious (Ott and Hommersand, 1974).

C Ecology and Distribution

Xanthophytes are predominately freshwater and terrestrial organisms, although representatives of genus *Vaucheria* occur also in marine and brackish water habitats. In that respect the Eustigmatophyceae, as far as currently circumscribed, are very similar. While most terrestrial and freshwater algae and plants have "green" plastids that are derived from primary endosymbiosis, Xanthophytes represent, together with some soil-dwelling diatoms and Eustigmatophytes, a relatively small group of terrestrial organisms that obtained their plastids from an ancestral red alga by secondary symbiosis. Interestingly, due to their terrestrial habit, members of the Xanthophyceae (and the Eustigmatophyceae as well) appear somehow as exceptions among the photosynthetic Stramenopiles. Most other photosynthetic clades containing plastids that originated as red algal symbionts (such as the brown algae and diatoms) that are related to xanthophytes and eustigmatophytes dominate primary production in many parts of the oceans (Falkowski and Knoll, 2007).

Xanthophytes may be euplanktonic, tychoplanktonic, neustonic (on/under the water surface), metaphytic, or epiphytic on vascular plants and filamentous algae (Lowe, 1927; Poulton, 1930; Hirsch and Palmer, 1958; Prescott and Vinyard, 1965; Schumacher et al., 1966; Meyer et al., 1970; Tarapchak, 1972; Duthie and Socha, 1976; Colt, 1974; Dillard et al., 1976; Goldstein and Manzi, 1976; Sheath and Hellebust, 1978). Less common are taxa that are epilithic (growing on rocks), epipelic (growing on mud, silt, or soil), or epizooic (growing on animals) (Poulton, 1930; Schumacher et al., 1963, 1966; Whitford and Schumacher, 1969). The unbranched filamentous genera similarly occur in many ecological conditions, even occur on or in soils of polar regions (Rybalka et al., 2009), but are rarely endophytic (Prescott and Vinyard, 1965; Whitford and Schumacher, 1969; Croasdale, 1973; Stein, 1975; R.L. Meyer personal communication). The genus *Heterococcus* recently has been proved to be a lichen photobiont of the fungal family Verrucariaceae (Thüs et al., 2011). The siphonous vesiculate *Botrydium* (Botrydiales) is nearly always epipelic and often terrestrial (Thompson, 1938; Stein, 1975; LaRivers, 1978). The coenocytic filamentous *Vaucheria* (Vaucheriales) is most often epipelic, but species are both aquatic and terrestrial; less often they are tychoplanktonic (Prescott, 1931; Tiffany, 1937; Thompson, 1938; Harris, 1964; Woodson, 1962; Colt, 1985). Only few xanthophyte algae are found in great numbers or with extensive biomass in the field, with the exceptions of *Tribonema* (Colt, 1974; Stein, 1975) (Figs. 9F–I) and *Vaucheria* (Figs. 11C–M). *Botrydium* (Figs. 11A–B) although cosmopolitan in distribution (Poulton, 1930), can form extensive carpets on soil, but is often overlooked because it is admixed with higher plants in moist habitats (Transeau, 1913; Stein, 1975; LaRivers, 1978). *Ophiocytium* (Figs. 1B–G) and *Mischococcus* (Fig. 1A) are also often collected in North America (Poulton, 1930; Prescott, 1931; Taylor, 1934; Duthie and Socha, 1976; Duthie et al., 1976; Smith, 1916; Meyer et al., 1970; Stein, 1975; Stein and Borden, 1978; Sheath and Hellebust, 1978; Sheath and Steinman, 1981). Coccoid xanthophytes are mostly ubiquitous in freshwaters with regard to their ecological habit.

Xanthophyte algae occupy a wide variety of ecosystems and habitats. They have been collected from temporary pools, swamps, ponds, lakes, ditches, streams, rivers, and on mud or soil, but little is known of the physical and chemical factors influencing the occurrence of all but a few Xanthophytes. Current velocity, light regime, specific conductance, oxygen, and nutrient concentration ranges have not been established for more than a few members of this group. In general, xanthophyte species richness and abundance may be greatest in dystrophic (Round, 1981) and mesotrophic conditions or in waters enriched with dissolved organic matter. Waters stained with organic acids and having pH values within the range from 4.5 to neutral typically have the most diverse flora. The greatest abundance and diversity of xanthophytes may occur under conditions in which the water temperatures range from 0 to 20°C (Meyer personal communication). In Minnesota, xanthophytes have been collected from under the ice and throughout the open water season. In lower latitudes, such as Arkansas, the taxa are most abundant during the spring and fall as well as in the free water under the ice. In Alaska, the taxa may be present under the ice, but snow frequently limits the amount of light available. This may give the impression that there is a tendency for Xanthophytes to be more competitive in colder habitats. However, an interesting exception is the genus *Centritractus*, which has been collected most often from warmer locations, including Jamaica (Hegewald, 1976), Puerto Rico (Tiffany and Britton, 1944), and many southern U.S. states (Camburn, 1982; Dillard, 2007). It is important to note that many of the unicellular genera included in this chapter have been rarely collected (some only once), thus few clear distribution patterns or ecological preferences can be determined for the vast majority of the xanthophyte algae.

Some data on light and nutrient requirements for culturing *Vaucheria* and a few other xanthophyte algae have been published (League and Greulach, 1955; Schneider et al., 1993; Schneider et al., 1999). *Vaucheria* species tend to grow well in culture at 10 to15°C (the authors, unpublished data) and collections in the field are often most successful between fall and late spring; collections have even been made from under ice. Some species of *Vaucheria* are habitat specific. *Vaucheria ornithocephala* (Fig. 11H) and *V. geminata* (Fig. 11J) often occur in the shallow waters of lakes (e.g., collected by the authors from an Ohio lake at a depth of 5 m) and ponds, while *V. dillwynii* (Fig. 11K, L), and *V. frigida* (Fig. 11G) usually occur on mud in seeps, ditches, and bogs. Other *Vaucheria* species are apparently less particular; *Vaucheria sessilis* (Fig. 11C) has been collected from all of the habitats described above. Oospores of *Vaucheria* are able survive at least five months of anoxia within riparian soils and recolonize damp soils when conditions improve (Schneider et al., 2008). Apparently, earthworms facilitate their dispersal among different soils (Schneider and McDevit, 2002). Several xanthophyte genera are used for the production of essential fatty acids, including *Botrydium*, *Tribonema*, and *Monodus* (Mercer et al., 1974).

III EUSTIGMATOPHYCEAE

A Introduction

The Eustigmatophytes are distinct from other members of the stramenopile algae based on several ultrastructural features and pigment composition.

1. *Organizational level*. Based on current information, only coccoid unicellular members of Eustigmatophyceae are known, and none are flagellates or multicellular or siphonous forms in the vegetative stage. Several species, however, produce flagellate cells (zoids) at some stage in their lifecycles. In the zoids the flagella are inserted close to the apex of the cell. In some genera (*Eustigmatos*, *Pseudostaurastrum*, *Vischeria*) only a single emergent flagellum is present, but two basal bodies (visible by TEM) are expressed (Hibberd and Leedale, 1971a,b; Santos and Leedale, 1995). A single plastid without a pyrenoid is present in the zoospores (Hibberd, 1990b). Other genera (e.g., *Pseudocharaciopsis*) have two emergent flagella, while for some (e.g., *Chlorobotrys* and *Nannochloropsis*), the production of zoospores is unknown.
2. *Eyespot*. The photoreceptor apparatus present in the zoids consists of a swelling on the long pleuronematic (bearing mastigonemes) flagellum in cases where only a single emergent flagellum is present and an eyespot that lies outside of the chloroplast. This peculiar feature gives the class its name: Eu = well developed; stigma = eyespot. The latter is a distinct, orange–red extraplastidial eyespot (Trzcyńska et al., 2014).
3. *Chloroplasts and pigments*. The chloroplasts are discoid but lack the girdle lamella present in all other classes of stramenopile algae. Color of the chloroplasts is green or yellow-green because fucoxanthin, the brown pigment of many other stramenopile algae, is absent (as in the Xanthophyceae). There is no chlorophyll *c* present as in all other Stramenopile algae. Accessory pigment (carotenoid) composition is similar to Xanthophytes except for the absence of heteroxanthin (Andersen, 2004; Kai et al., 2008).

Reproduction in the Eustigmatophyceae is by means of walled autospores (non-motile spores without the potential to produce flagella) or naked (non-walled) zoospores (flagellated asexual reproductive cells) (Hibberd, 1990b). Sexual reproduction has not yet been observed in the Eustigmatophytes.

B Diversity and Morphology

Several species have been transferred from the coccoid xanthophytes into new genera within the Eustigmatophyceae, including *Eustigmatos* (Hibberd, 1981, from *Pleurochloris*), *Monodopsis* (Hibberd, 1981, from *Monodus*), *Pseudocharaciopsis* (Lee and Bold, 1973, from *Monodus* and *Characiopsis*), and *Vischeria* (Hibberd, 1981, from *Polyedriella*). This has also required reassigning several type species. In case of *Eustigmatos*, the previous type of *Pleurochloris*, *P. commutata*, was moved as a type of the new genus, renamed to *E. vischeri*. In case of *Vischeria*, the type of *Polyedriella*, *P. helvetica*, was formally transferred to the eustigmatophyte genus (as *V. helvetica*; Hibberd, 1981), leaving two more species without types in the xanthophyte genus.

A particular case of taxonomic confusion has arisen because another species of *Pleurochloris*, *P. meiringensis*, has been proven to be a member of the Xanthophyceae by molecular phylogenetic analyses (Andreoli et al., 1999; Rybalka et al., 2009). In the case of *Monodopsis*, the type *M. acuminatus* formally remained in the Xanthophyceae; it is at present unstudied and therefore, the actual phylogenetic position of *Monodus* remains unresolved. Therefore, three genera previously in the Xanthophyceae that gave rise to the Eustigmatophyceae still remain. We regard them here as "candidate" Eustigmatophyte genera (Table 2), because molecular analyses of their other species (as they become available for study) may reveal still more species of Eustimatophytes or confirm their position within the Xanthophyceae. 18S rRNA gene sequence data (A. Bombosch and T. Friedl, pers. comm.) revealed two species of *Tetraedriella*, the type *T. acuta*, not yet included as members of Eustigmatophyceae and, therefore, *Tetraedriella* is also assigned a "candidate" eustigmatophyte genus here (Table 2). In addition, the three xanthophyte genera—*Gloeobotrys*, *Gloeoskene*, and *Merismogloea*— are also assigned to the "candidate" eustigmatophytes because they were already assumed to be synonymous with the Eustigmatophyte *Chlorobotrys* and even with each other (see genus descriptions). In case of *Pseudocharaciopsis*, the type *Ps. texensis* has begun its existence as a new species already described under the Eustigmatophyceae.

An exception to the moving of traditional Xanthophytes to Eustigmatophytes is the genus *Nannochloropsis*. Species of this genus (type *N. oculata*) have been transferred from the green algal genus *Nannochloris* Naumann to the Eustigmatophyceae (Hibberd, 1981). For nine other traditional genera of Xanthophyceae, their nature as eustigmatophycean algae has been revealed by using the type of each of these genera for studies: *Characiopsis* (*C. minuta*; Fawley et al., 2014), *Chloridella* (*C. neglecta*; Fawley et al., 2014), *Chlorobotrys* (*C. regularis*; Hibberd, 1974, 1981), *Goniochloris* (*G. sculpta*; Přibyl et al., 2012), *Pseudostaurastrum* (*P. enorme*; Hegewald et al., 2007; Fawley et al., 2014), *Trachydiscus* (*T. minutus*; Přibyl et al., 2012), *Vischeria* (*V. stellata*; Hibberd, 1981). *Chlorobotrys* and *Vischeria* have been transferred to the Eustigmatophyceae (Hibberd, 1981; Guiry and Guiry, 2013), the other genera were assigned to newly established families under the Eustigmatophyceae (Fawley et al., 2014) and therefore are regarded as Eustigmatophyte genera here (Table 2). The genus *Ellipsoidion* has been listed as a member of Eustigmatophyceae by Guiry and Guiry (2013), but the only species investigated by molecular phylogenetics so far, *E. parvum*, turned out to be a green alga (Eliáš et al., 2013). Therefore, *Ellipsoidion* may be a doubtful Eustigmatophyte, and other *Ellipsoidion* species are in urgent need of being checked for their correct phylogenetic position. Three more genera, *Pseudotetraedriella* (Hegewald et al., 2007), *Botryochloropsis* (Preisig and Wilhelm, 1989), and *Pseudellipsoidion* (Neustupa and Němcová, 2001), have been established under the Eustigmatophyceae, but are not treated in this chapter because they have not been reported from the United States until now. This makes a total of 14 approved Eustigmatophyte genera, which already outnumbers the total of approved coccoid Xanthophytes (7) and is about the total number of all approved Xanthophytes (13). There is a good chance that further studies will discover new Eustigmatophytes from the collection of 42 coccoid untested xanthophyte genera listed in Table 1, as they become available for study.

Despite being phylogenetically distant from the Xanthophyceae, Eustigmatophytes are virtually impossible to distinguish from coccoid Xanthophytes without molecular phylogenetic or detailed pigment analysis. Their inclusion in this chapter reflects historical groupings only. With a growing number of cultures available for those species regarded as "traditional"

TABLE 2 Overview of Genera of Eustigmatophytes Treated in this Chapter. There are Two Groups of Genera Based on the Current Knowledge About their Phylogenetic Placement

1. Eustigmatophytes (12)
Coccoid genera formerly assigned to Eustigmatophyceae and treated in this chapter.
Characiopsis
Chloridella
Chlorobotrys
Ellipsoidion (doubtful)
Eustigmatos
Goniochloris
Monodopsis
Nannochloropsis
Pseudocharaciopsis
Pseudostaurastrum
Trachydiscus
Vischeria
2. Candidate Eustigmatophytes
The 7 coccoid former genera of Xanthophyceae treated in this chapter for which there is some indication that they may be members of Eustigmatophyceae.
Gloeobotrys
Gloeoskene
Merismogloea
Monodus
Pleurochloris (contains one approved Xanthophyte species)
Polyedriella
Tetraedriella

coccoid xanthophytes, studies employing DNA sequence analyses are likely to increase the number of genera and species in the Eustigmatophyceae. These form pragmatic reasons to include both coccoid Xanthophyceae and currently known or suspected members of Eustigmatophyceae in one identification key (see below) and arrange their figures together (see Figs. 2, 4–8).

C Ecology and Distribution

Eustigmatophytes occur mostly in freshwaters or terrestrial habitats. Only a single genus of Eustigmatophytes, *Nannochloropsis*, is comprised of mostly marine species; so far, only a single *Nannochloropsis* species is known from freshwaters. The coccoid cells of eustigmatophyte genera are frequently encountered in the euplankton (true plankton community of open water) and the tychoplankton (formerly benthic or epiphytic carried into the plankton by wave action) (Tarapchak, 1972; Sheath and Hellebust, 1978). They are also often a component of the metaphyton (loosely associated among vascular plants and other algae) (Whitford and Schumacher, 1969, R.L. Meyer personal communication). Less often, eustigmatophytes may be epiphytic (growing on other plants) (Schumacher et al., 1966), epilithic (growing on rocks) (Poulton, 1930), or even as an endosymbiont in the freshwater sponge *Corvomeyenia* (Frost et al., 1997). Eustigmatophytes may also be common on soils (Hibberd, 1990b; Graham et al., 2009; Trzcyńska et al., 2014).

Aquatic habitats in which eustigmatophytes have been collected include ponds, pools, bogs, streams, and lakes. Dystrophic (nutrient-poor acidic waters) and mesotrophic (waters with a moderate amount of nutrients present) conditions, coupled with low temperatures (0-15°C) seem to be preferred by most eustigmatophytes (R.L. Meyer personal communication). However, Tarapchak (1972) has collected them in waters with temperatures from 17.5-30°C, and Transeau (1913) observed *Chlorobotrys regularis* in a small Michigan pool in August.

Eustigmatophyte genera have been collected in the Arctic (Sheath and Hellebust, 1978), Canada (Stein, 1975; Duthie and Socha, 1976; Stein and Borden, 1978), Minnesota (Tarapchak, 1972), Wisconsin (Smith, 1920), Connecticut and Massachusetts (Poulton, 1930; Andersen et al., 1998) North Carolina (Schumacher et al., 1966; Krienitz et al., 2000), South Carolina (Goldstein and Manzi, 1976), Texas (Andersen et al., 1998), and Alaska, Arkansas, and Montana (R.L. Meyer personal communication). *Trachydiscus minutus* has been found to have high levels of the essential fatty acid eicosapentaenoic acid (Rezanka et al., 2010).

IV KEYS AND DESCRIPTIONS OF GENERA OF XANTHOPHYTES AND EUSTIGMATOPHYTES

Based in part on Bourrelly (1968, 1981), Ettl (1978), Prescott (1978), Hibberd (1990b), and with the advice of R.L. Meyer.

1 Keys

Key 1. Xanthophytes and Eustigmatophytes of Coccoid Organization

Genera of coccoid organization, that is, with vegetative cells non-motile and cell wall. Vegetative cells are uninucleate, of non-filamentous organization, non-flagellated and non-motile, having a distinct cell wall, solitary or in colonies, with and without mucilage, without contractile vacuole and eyespot. The genera in this key have been assigned to the traditional order Mischococcales of the Xanthophyceae or have been described as members of Eustigmatophyceae. Due to the uncertain position of many taxa and sparse collection and culture data, genera have been categorized according to whether they have been verified using molecular analyses (Figs. 1–8).

- *Descriptions of genera in sections "Approved Xanthophytes", "Untested Xanthophytes", "Doubtful Xanthophytes", and "Approved Eustigmatophytes", and "Candidate Eustigmatophytes".*
- *Genera listed in* Table 1 *(Xanthophytes) and* Table 2 *(Eustigmatophytes).*

Approved Xanthophyte. This is a genus placed in the Xanthophyceae that has yet been checked by molecular phylogenetics for its correct assignment to this class. There are 13 approved genera (Table 1).
Untested Xanthophyte. This is a genus currently listed in the Xanthophyceae that (in most cases) is still rather poorly studied and in need of being cultured and checked for its correct phylogenetic position by molecular phylogenetics. Many of these genera may in fact be Eustigmatophytes, belong to other photosynthetic Stramenopiles, or possibly green algae (Chlorophyta). There are 42 coccoid untested genera treated in this chapter (Table 1).
Doubtful Xanthophyte. This is a genus yet still formally assigned to the Xanthophyceae although there is some indication that it does not belong to Xanthophyceae or Eustigmatophyceae, but to other photosynthetic Stramenopiles or the green algae (Chlorophyta).
Candidate Eustigmatophyte. This is a coccoid genus currently listed as a member of the Xanthophyceae, but treated in this chapter because there is an indication that it may be member of the Eustigmatophyceae (Table 2).

1. Cells united on thin, mucilaginous stipes, dendritic (forming tree-like colonies) (Fig. 1A) ..... *Mischococcus* (approved Xanthophyte).
1. Unicellular, cells in small clusters, or colonial but not forming tree-like Colonies 2
2. Cells elongate, cylindrical, with two or more nuclei, solitary or colonial (Figs. 1B–G) ..... *Ophiocytium* (approved Xanthophyte)
2. Cells either elongate and uninucleate or of other shapes, solitary or colonial 3
3. Cells prominently elongate, uninucleate, solitary 4
3. Cells of other shapes, solitary or colonial 5
4. Cells cylindrical, straight, or slightly bent with long spine at each end (Fig. 1H) ..... *Centritractus* (untested Xanthophyte)
4. Cells cylindrical, straight or curved, without spines (Figs. 1I–K) ..... *Bumilleriopsis* (approved Xanthophyte)
5. Cells in flat colonies, attached without stipe or disc (Fig. 1 L) *Chloropedia* (untested Xanthophyte) .....
5. Cells in other types of colonies or solitary 6
6. Cells in thick, stratified, mucilaginous stalks, sometimes branched (Figs. 1M–P) *Gloeopodium* (untested Xanthophyte) .....
6. Cells in other types of colonies or solitary 7
7. Older cells large, with many nuclei and plastids. Spherical or irregularly shaped. Cell wall unornamented 8
7. Cells solitary or colonial, mostly uninucleate 9
8. Cells forming amoeboid stages, epiphytic (Figs. 3A, B) *Perone* (doubtful Xanthophyte) .....
8. Not epiphytic. Cells spherical or irregularly ellipsoidal 10
9. Cells spherical (Figs. 3A, B) ..... *Botrydiopsis* (approved Xanthophyte)
9. Cells irregularly ellipsoidal (Figs. 3C) ..... *Excentrochloris* (untested Xanthophyte)
10. Cells attached to substratum directly with mucilaginous disc or by stipe-like extension of cell wall. Cells solitary or in twos or fours 11
10. Cells either attached to one another or embedded in a common mucilage. Solitary and free-living or colonial 18
11. Cells pyriform (Fig. 2A) ..... *Chlorokoryne* (untested Xanthophyte)
11. Cells either hemispherical, stipulate, with thin thread-like stipe, with basal disc, or directly attached to the substrate 12
12. Cells hemispherical (Figs. 2B) *Hemisphaerella* (untested Xanthophyte)
12. Cells either stipulate, with thin thread-like stipe, with basal disc, or directly attached to the substrate 13
13. Cells stipulate (Figs. 2C–H) 14
13. Cells attached by thin thread-like stipe, basal disc, or directly to the substrate 16
14. Stipe short; cells irregularly fusiform, often forming flattened triangles (Figs. 2C–F) ..... *Dioxys* (untested Xanthophyte)
14. Stipe long, cells spherical, ovoid, ellipsoidal, or fusiform 15
15. Cells spherical, stipe thread-like, attached to other algae (Figs. 2G, H) ..... *Peroniella* (untested Xanthophyte)
15. Cells ovoid to ellipsoidal on stipe with small basal disc (Figs. 2K, N, O) ..... *Characiopsis/Pseudocharaciopsis* (Eustigmatophyte)
16. Cells cylindrical to spindle-shaped, attached with short stalk, cell wall usually in two pieces of unequal size (Figs. 2L, ... *Chlorothecium* (untested Xanthophyte)
16. Cells attached directly to the substrate 17
17. Cells long, cylindrical to fusiform, usually on filamentous algae (Fig. 3C) ... *Chytridiochloris* (doubtful Xanthophyte)
17. Cells spherical to upright ovals, solitary or in groups of two to four (Figs. 2I, J) ..... *Lutherella* (untested Xanthophyte)
18. Cells colonial 19
18. Cells solitary, free-living 32
19. Cells attached to one another 20
19. Cells embedded in a common mucilage 27
20. Cells attached in chains of two to eight cells (Fig. 3O) ..... *Heterodesmus* (untested Xanthophyte)
20. Cells attached in other groups of two, tetrads, spherical colonies, or unorganized clusters 21
21. Cells elongate, fusiform, 18-30 µm in length in groups of twos, fours, or solitary (Fig. 3E) ..... *Raphidiella* (untested Xanthophyte)
21. Cells spherical, hemispherical, cylindrical, recurved ovals, or pyramidal 22

2: Publication Rybalka et al. (2007), used for Chapter 2

Rybalka, N., Mohr, G., Kostikov, I., Andersen, R.A., Bombosch, A., and Friedl, T. (2007) Unambiguous rapid identification of Xanthophycean algae in culture collections using chloroplast gene spacer region sequences. In *Science for Service, Service for Science - Connections between Collections: Proceedings of the 11th International Conference on Culture Collections, ICCC11, Goslar, Germany, 7-11 October 2007*. Stackebrandt, E., Wozniczka, M., Weihs, V., and Sikorski, J. (eds). Goslar: DSMZ and WFCC, pp. 36-39 (ISBN 978 – 3 – 00 – 022417 – 1)

Unambiguous rapid identification of Xanthophycean algae in culture collections using chloroplast gene spacer region sequences

N. Rybalka[1], K. Mohr[2], I. Kostikov[1], R.A. Andersen[3], A. Bombosch[2], T. Friedl[2]

[1]Department of Botany, Taras Shevchenko National University, 01017, Kyiv, Ukraine bumilleria@mail.univ.kiev.ua
[2]Experimentelle Phykologie und Sammlung von Algenkulturen, Universität Göttingen, 37073, Göttingen, Niedersachsen, Germany
[3]Bigelow Laboratory for Ocean Sciences, Maine 04575, West Boothbay Harbor, Niedersachsen, Germany
[4]Experimentelle Phykologie und Sammlung von Algenkulturen, Universität Göttingen, 37073, Göttingen, Maine, United States

Microalgae of the class Xanthophyceae (Stramenopiles) are important primary producers in freshwater and soil (Andersen 2004). However, their correct identification is often difficult due to a paucity of morphological characters and the presence of extensive phenotypic plasticity (Zuccarello & Lokhorst 2005). Growth forms surprisingly similar to the Xanthophyceae evolved in parallel in the Green Algae and the Eustigmatophyceae, which further complicates accurate taxonomic identities. Species descriptions of many xanthophytes are incomplete because they were based on natural samples. In culture, however, variation of morphological features can be observed and additional distinctive features are discovered. Many public culture collections maintain xanthophyceaen algae and these strains could be important references for identification. However, only a few strains have been examined to verify that they were correctly identified.

In our study, we examined 82 Xanthophyceae strains and we focused on the taxonomically difficult filamentous genera, i.e. *Tribonema* (27 strains), *Xanthonema* (28 strains), *Bumilleria* (7 strains), *Bumilleriopsis* (8 strains) and *Heterococcus* (2 strains). Strains were obtained from various public culture collections, especially the *Sammlung von Algenkulturen der Universität Göttingen* (SAG). Our goals were to distinguish the strains using DNA sequence comparisons in order to independently test their morphology-based taxonomic classification. We included many strains whose species identification was not determined and we also established new cultures from terrestrial habitats. We used DNA sequence comparisons of the chloroplast-encoded *rbcL* gene and the *psbA-rbcL* spacer region. Only the Xanthophyceae have the *psbA* gene consistently upstream of the *rbcL* gene and this allows for a PCR approach selective for Xanthophyceae (Andersen & Bailey 2002). The 5' primer was anchored in the *psbA* gene and the 3' primer was placed in *rbcS* (downstream of *rbcL*), thereby amplifying the full-length *rbcL* gene and the spacer region between the *psbA* and *rbcL* genes.

The 5'-end of the *psbA-rbcL* spacer region was highly variable and it was valuable for the unambiguous identification of species. Visual comparisons of *psbA-rbcL* spacer sequences revealed that strains previously assigned to a single genus were split into several distinct groups (Fig. 1). For example, strains of *Tribonema* were separated into four different groups, those of *Bumilleriopsis* into three and those of *Xanthonema* into two groups. Within each of the ten groups the 5' end of the *psbA-rbcL* spacer region could be well aligned. However, between groups this region differed in length and no alignment was possible.

Strains of *Tribonema* group 2 were distinct because their spacers were almost three times as long as the other Xanthophyceae, however, the sequences within that group were almost invariant. Therefore, assignment to *Tribonema* group 2 was already possible after PCR amplification because the amplicon was larger than for other Xanthophyceae (3.0 kB compared to about 2.2 kB).

The same groups were resolved, with high statistical support, in phylogenetic analyses of complete *rbcL* sequences (1466 bp). Furthermore, the *rbcL* phylogeny showed evolutionary relationships among the groups, e.g., *Bumilleriopsis* group 2 and *Xanthonema* group 2 were closely related with each other. Also, *Tribonema* groups 1, 2 and 3 were closely related. Even though *Tribonema* group 2 had a much longer spacer, it was a well-supported sister taxon to *Tribonema* group 3 (Fig. 1). However, most intra-group relationships had weak statistical support (not shown).

The *psbA-rbcL* spacer regions and *rbcL* gene phylogenies help define genera and species. Strains with spacer sequences that exhibit significant differences, but are still easily aligned, may represent a single genus. Similarly, strains with nearly identical spacer sequences may represent a single species. For example, spacer sequences between *Xanthonema* groups 1 and 2 cannot be aligned, and they are apparently two distinctly different genera. Furthermore, there is considerable spacer sequence difference within *Xanthonema* groups 1 and 2, implying that each group consists of several species. These patterns are clearly supported by the *rbcL* phylogeny that shows that both *Xanthonema* groups are far removed from each other (Fig. 1). In a second example, *Tribonema* groups 1 and 2 have almost identical spacer sequences within their group but the between-group sequences cannot be aligned. Therefore, the two groups appear to represent two distinct, monotypic genera. The *rbcL* phylogenetic analysis also supports the recognition of two distinct clades (Fig. 1).

The generic and specific assignments using DNA sequences differed from current morphology-based taxonomy. Strains from *Tribonema* group 1 were previously assigned to five different species based on morphology and those from *Tribonema* group 2 were assigned to three species. Perhaps surprisingly, the *Tribonema* DNA sequences gave a more conservative taxonomic classification than that based on morphology. Likewise, strains placed in our *Bumilleriopsis* group 1 had almost identical spacer sequences, but based upon morphology, the strains had been placed into three different genera (*Bumilleria*, *Bumilleriopsis* and *Pseudobumilleriopsis*). The morphological features used to describe these three genera are not congruent with the molecular data and, therefore, are not phylogenetically informative at the generic level.

The different xanthophycean groups revealed in our DNA analyses were often corroborated by morphological differences observed in culture, e.g. cell shapes and dimensions, formation of filamentous stages and germination of zoospores (N. Rybalka, unpubl. data). However, detailed re-investigation of morphological, including ultrastructural, features is still needed. We recently discovered that strains of *Bumilleriopsis* group 1 lack a girdle lamella in their chloroplasts, whereas the strains of *Bumilleriopsis* group 2 and 3 possess a girdle lamella (N. Rybalka & A. Massalski, unpubl. data). A girdle lamella is found in all other examined Xanthophyceae (Hibberd 1990), and it is a widespread feature that characterizes most photosynthetic stramenopiles (Andersen 2004). Thus, it appears that some morphological features will corroborate the molecular data while other morphological features appear to have little taxonomic value for distinguishing genera and species.

Using our *psbA-rbcL* spacer and *rbcL* gene sequence data as references, we attempted to unambiguously identify new isolates (our strains and those of other scientists) from terrestrial and freshwater habitats in the Ukraine and from soils in Antarctica. We found that all the new isolates could be unambiguously assigned to our groups. Furthermore, many of the new strains had identical sequences to the reference strains from the SAG culture collection. *Tribonema* group 1 was originally represented by only two SAG strains, but it was the most common of new *Tribonema* strains, i.e., it included 14 new isolates from geographically distant regions. All the new Antarctic isolates were identified as *Xanthonema* using traditional morphology-based taxonomy. However, visual sequence comparisons of their *psbA-rbcL* spacer sequences suggested that they belonged two four different lineages, i.e. two lineages within the *Xanthonema* group 1 and one each in *Xanthonema* group 2 and *Tribonema* group 1. Interestingly, except for two isolates, the spacer sequences of the Antarctic strains were virtually identical with strains from temperate regions. This result supports widespread distribution rather than specialization for temperate or cold habitats. All these examples demonstrate that a single sequence of the *psbA-rbcL* spacer, when compared to reference strains, can unambiguously identify new Xanthophyceae culture strains.

Duplicate cultures of the same strain (representing the same algal isolate) are often held in different collections. The duplicate cultures, therefore, should be genetically identical. However, our comparisons of the *psbA-rbcL* spacer often showed clear differences between duplicate cultures, and conversely, cultures of unrelated strains had identical spacers. This clearly indicates that cultures had been mislabelled and mixed up by one of the culture collections during serial transfer. Therefore, strains used as taxonomic references should be held in several collections, and if possible they should be cryopreserved. In other cases where spacer amplification was not possible, the nuclear-encoded 18S rRNA gene was sequenced and then the strain was identified either as a green alga or eustigmatophycean alga. Thus, the *psbA-rbcL* spacer region can assist culture collections in providing correctly identified strains and to prevent mislabelling of Xanthophyceae cultures.

This work was supported by travel grants of the DAAD and ICCC11 conference organizing committee extended to NR.

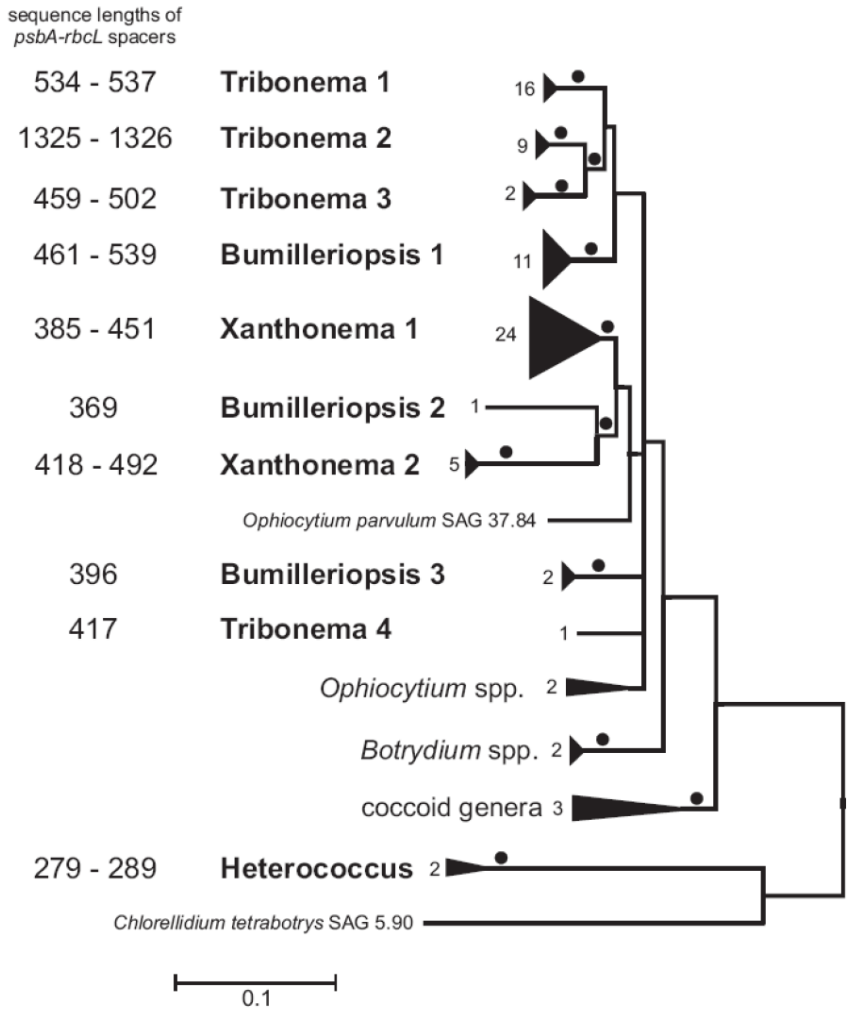

Fig. 1. Groups of strains of filamentous Xanthophyceae as revealed from visual comparisons of their *psbA-rbcL* spacer region sequences opposed to a phylogeny of complete *rbcL* sequences for the same strains. Numbers in the left column are lengths (bp) of the spacer regions. Names for the groups are in the middle. The phylogenetic tree on the right side was produced by a Bayesian analysis of 41 sequences (421/333 variable/phylogenetic informative sites). Groups of sequences were collapsed into triangles, and the number of strains is next to each clade. Filled circles indicate significant statistical support (> 0.95 posterior probability).

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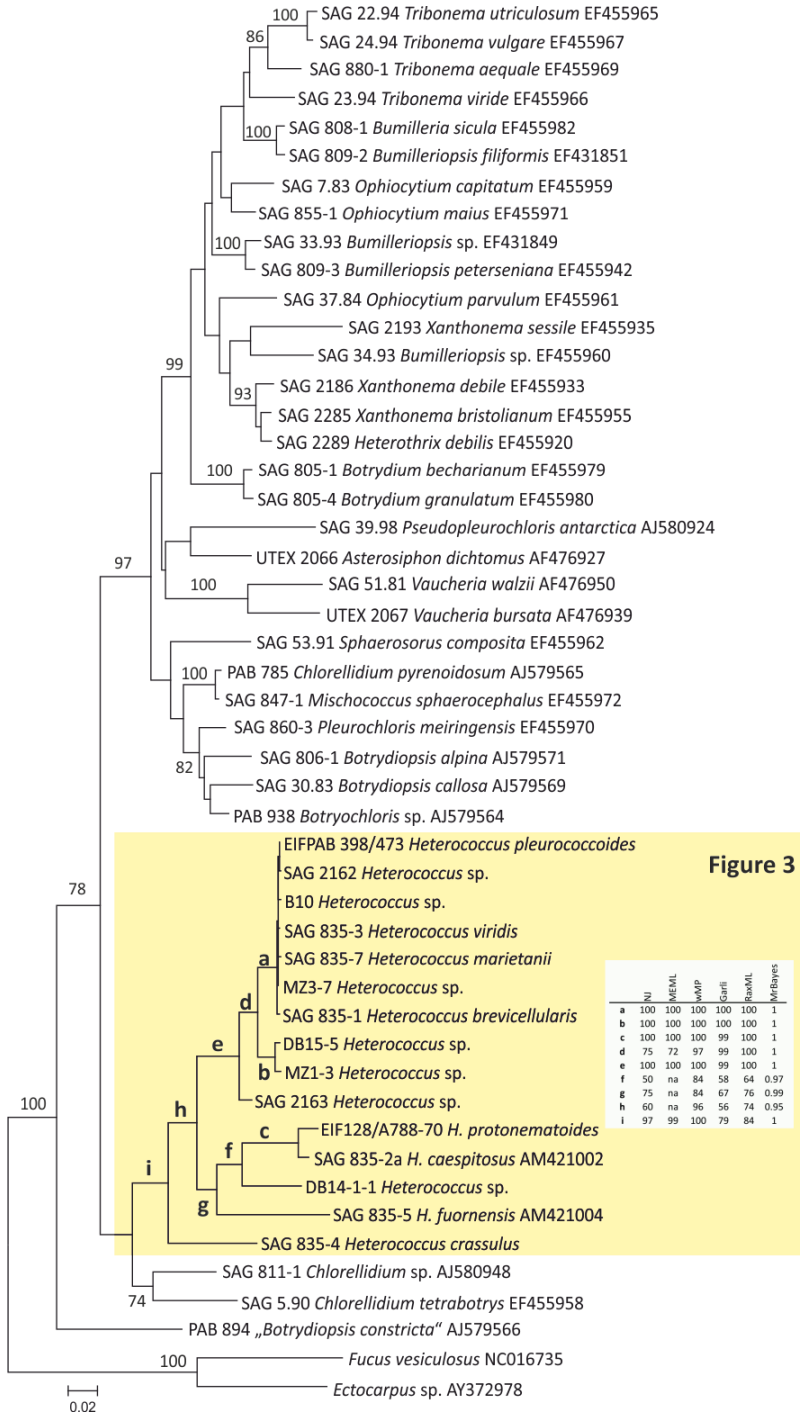
Appendix 3: Additional files of publication Rybalka et al. (2013) (= Chapter 3)

Additional file 1. Maximum likelihood (ML) phylogeny of rbcL gene sequences for *Heterococcus* and other members of Xanthophyceae. The phylogeny was calculated with the programme GARLI v0.96 (Zwickl, 2006) based on a *rbc*L data set (1325 bp long, 517/418 variable/parsimony informative sites) consisting of 15 *Heterococcus* and 32 other Xanthophyceae sequences (corresponding to clades C, B, T, and V as defined in Maistro (Maistro et al., 2009) as well as two sequences from Phaeophyceae as outgroup. Scale bar, substitution per site. Numbers mapped to internodes are bootstrap values from 2000 replicates, only values >70% have been recorded. The phylogeny in this Figure includes the phylogeny of 15 *Heterococcus* strains shown in Figure 3 of Rybalka et al. (2013; highlighted). The inserted table lists bootstrap values mapped to internodes of the *Heterococcus* clade using six different analysis methods (see text of Rybalka et al., 2013). Scale bar, substitution per site.

GARLI. Phylogenetic analysis of molecular sequence data using the maximum likelihood criterion. https://code.google.com/p/garli/.



Additional file 2. DNA sequences newly determined for 29 *Heterococcus* strains and their GenBank sequence accession numbers. For the *psb*A/*rbc*L spacer and full *rbc*L gene all determined sequences are listed, for ITS2 only those sequences that were different from each other. (p), only *psb*A/*rbc*L spacer and partial full rbcL gene could be determined; (a), already made available previously; n.a., not applicable.

Strain	psbA/rbcLspacer and rbcL	ITS2 variant	ITS2
SAG 835-3	JX681220		JX681147
SAG 835-6	JX681222		JX681148
SAG 835-7	JX681223		JX681149
SAG 835-1	JX681218		JX681150
SAG 835-8	JX681224		JX681151
SAG 56.94	JX681226		JX681152
EIF 398	JX681207		JX681153
EIF 430/A801-2	JX681209		JX681154
EIF PAB 398/473	JX681211		JX681155
EIF PAB 397/380	JX681210		JX681156
MZ2-4 (= SAG MZ2-4)	JX681215		JX681157
MZ2-5 (= SAG MZ2-5)	JX681216		JX681158
B10 (= SAG B10)	JX681201		JX681159
SAG 2162	EF426795 (a)		JX681160
MZ3-7 (= SAG MZ3-7)	JX681217	MZ3-7_acl46	JX681161
		MZ3-7_acl49	JX681162
MZ1-6	JX681214 (p)		n.a.
MZ1-3 (= SAG 3334)	JX681213	MZ1-3_acl29	JX681163
		MZ1-3_acl31	JX681164
		MZ1-3_acl33	JX681165
		MZ1-3_acl34	JX681166
		MZ1-3_acl39	JX681167
DB14-15 (= SAG 3335)	JX681204 (p)		JX681168
DB15-5 (= SAG 3336)	JX681205	DB15-5_acl74	JX681169
SAG 2163	EF455945 (a)		JX681170
EIF PAB 399/372	JX681212	EIF PAB 399/372_acl62	JX681171
		EIF PAB 399/372_acl63	JX681172
		EIF PAB 399/372_acl64	JX681173
EIF 423/A790-45	JX681208	EIF 423/A790-45_cl59	JX681174
		EIF 423/A790-45_cl60	JX681175
		EIF 423/A790_45_cl62	JX681176
		EIF 423/A790-45_cl65	JX681177
		EIF 423/A790-45_cl66	JX681178
		EIF 423/A790-45_cl69	JX681179
		EIF 423/A790-45_cl71	JX681180
EIF 128/A788-70	JX681206	EIF 128/A788-70_a5	JX681181
		EIF 128/A788-70_acl3	JX681182
SAG 835-2a	JX681219 (p)		JX681183
SAG 835-9	JX681225 (p)		JX681184
DB14-1-1 (= SAG 3337)	JX681202	DB14-1-1	JX681185
		DB14-1-1_acl2	JX681186
		DB14-1-1_acl4	JX681187
		DB14-1-1_acl10	JX681188
		DB14-1-1_acl11	JX681189
		DB14-1-1_acl12	JX681190
DB14-5-1 (= SAG 3338)	JX681203		JX681191
SAG 835-4	JX681221		JX681192
SAG 835-5	n.a.	SAG 835-5_cl43	JX681193
		SAG 835-5_cl43	JX681194
		SAG 835-5_cl55	JX681195
		SAG 835-5_cl56	JX681196

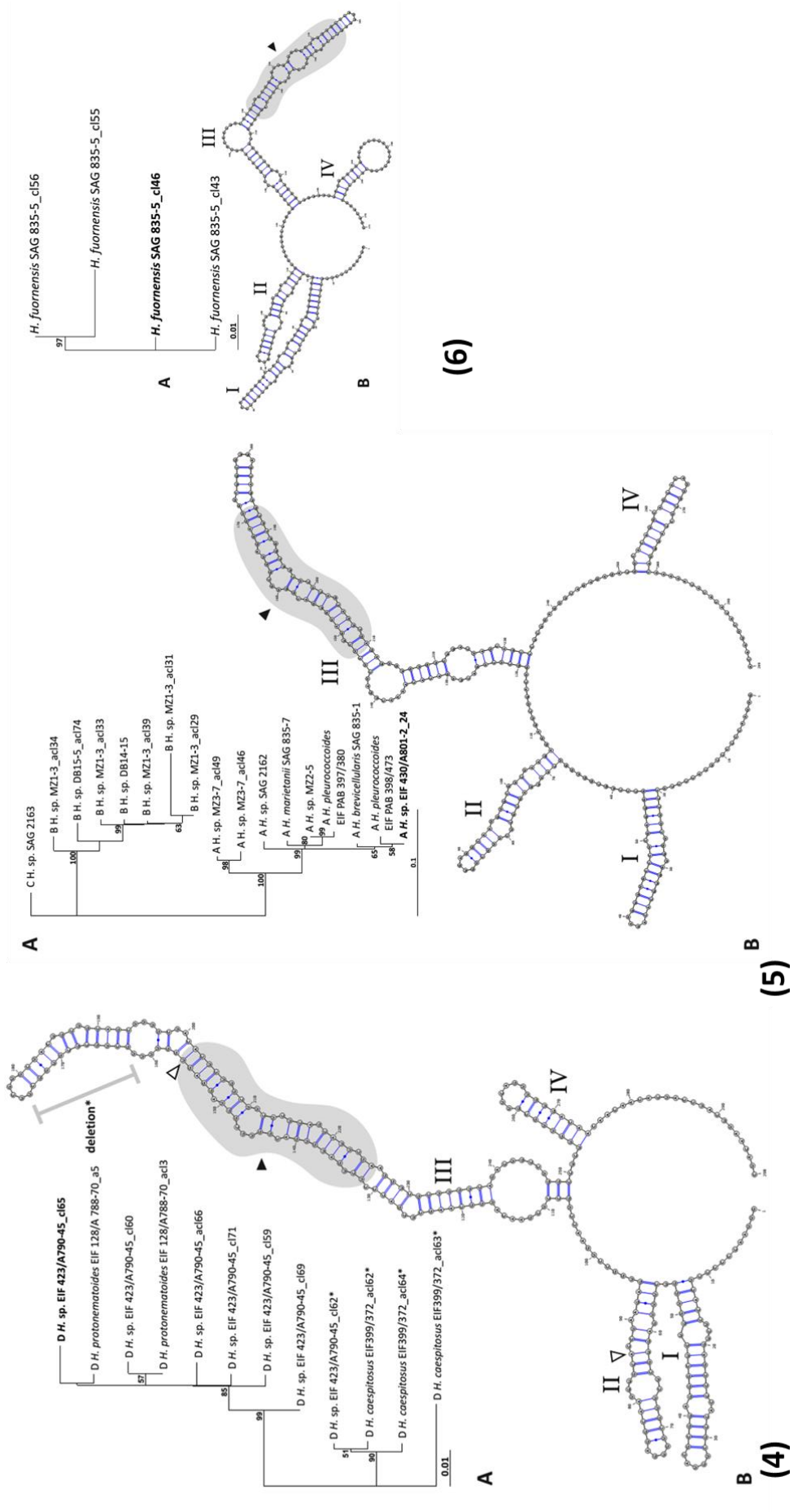
Additional file 3. Groups of *Heterococcus* strains with fully identical rbcL and/or psbA/rbcL spacer sequences. Strains marked in bold were used for the rbcL phylogeny (Figure 3, Additional file 1). Species assignment is according to the new species designation as in Figure 2 (see Discussion).

	rbcL identical	psbA/rbcL spacer identical
H. viridis	**SAG 835-7 *H. marietanii***	SAG 835-7 *H. marietanii*
	SAG 835-6 *H. mainxii*	SAG 835-6 *H. mainxii*
	SAG 835-1 *H. brevicellularis*	
	SAG 835-8 *H. moniliformis*	
	SAG 56.94	
		EIF 398
	EIF 398	EIF PAB 398/473
	EIF PAB 398/473	
	EIF PAB 397/380	EIF PAB 397/380
	EIF 430/A801-2	EIF 430/A801-2
	MZ2-4	MZ2-4
	MZ2-5	MZ2-5
	MZ3-7	B10
species B	**MZ1-3**	
	DB14-15	
species D	**EIF 128/A788-70**	EIF 128/A788-70
	EIF 423/A790-45	EIF 423/A790-45
	EIF PAB 399/372	
H. caespitosus	**SAG 835-2a *H. caespitosus***	SAG 835-2a *H. caespitosus*
	SAG 835-9 *H. protonematoides*	SAG 835-9 *H. protonematoides*
species F	**DB 14-1-1**	
	DB 14-5-1	

Additional file 4. (A) ProfDistS (Wolf et al., 2008) sequence-structure NJ tree (unrooted) as derived from the multiple sequence-structure alignment of ITS2 helices I-IV recovered for strains of group D, H. leptosiroides. Bootstrap values based on 100 pseudo-replicates are mapped to the appropriate internodes. Branch lengths are drawn proportional to inferred changes. The template ITS2 variant used in B) is highlighted in bold. Scale bar, substitutions per site. (B) ITS2 secondary structure of ITS2 variant EIF 423/A790-5_cl65 used for homology modeling of secondary structures for all strains of group D (*H. leptosiroides*). The secondary structure was visualized with VARNA (Darty et al., 2009). Helices are numbered I–IV. Four strains indicated by an asterisk are devoid of the apical part of helix III. An arrowhead indicates the highly conserved GGU motif 5' to the apex of helix III. A cloud highlights the segment of helix III conserved across all studied strains. Open arrowheads mark positions of two CBCs that distinguish groups D and E.

Additional file 5. ITS2 sequence and secondary structure phylogenetic analyses of twelve strains of *Heterococcus* groups A-C (*H. viridis, H. conicus, H. virginis*). (A) ProfDistS (Wolf et al., 2008) sequence-structure NJ tree (unrooted) as derived from the multiple sequence-structure alignment of ITS2 helices I-IV recovered for strains of the *H. viridis* clade, i.e. groups A-C, *H. viridis*, *H. conicus* and *H. virginis*. Bootstrap values based on 100 pseudo-replicates are mapped to the appropriate internodes. Branch lengths are drawn proportional to inferred changes. The template ITS2 variant used in B) is highlighted in bold. Scale bar, substitutions per site. (B) Secondary structure of ITS2 variant *H. viridis* EIF 430/A801-2_24 used for homology modeling of secondary structures for all strains of *Heterococcus* groups A-C. The secondary structure was visualized with VARNA (Darty et al., 2009). Helices are numbered I–IV. An arrowhead indicates the highly conserved GGU motif 5' to the apex of helix III. A cloud highlights the segment of helix III conserved across all studied strains.

Additional file 6. (A) ProfDistS (Wolf et al., 2008) sequence-structure NJ tree (unrooted) of ITS2 variants recovered from strain *H. fuornensis* SAG 835–5 as derived from the multiple sequence-structure alignment of ITS2 helices I-IV. Bootstrap values based on 100 pseudo-replicates are mapped to the appropriate internodes. Branch lengths are drawn proportional to inferred changes. The template ITS2 variant used in B) is highlighted in bold. Scale bar, substitutions per site. (B) Secondary structure of ITS2 variant SAG 835-5_46 used for homology modeling of secondary structures for all ITS2 variants of the same strain. The secondary structure was visualized with VARNA (Darty et al., 2009). Helices are numbered I–IV. An arrowhead indicates the highly conserved GGU motif 5' to the apex of helix III. A cloud highlights the segment of helix III conserved across all studied strains.


H. fuornensis SAG 835-5_cl56
H. fuornensis SAG 835-5_cl55
H. fuornensis SAG 835-5_cl46
H. fuornensis SAG 835-5_cl43
A
B
I
II
III
IV
(6)
C H. sp. SAG 2163
B H. sp. MZ1-3_acl34
B H. sp. DB15-5_acl74
B H. sp. MZ1-3_acl33
B H. sp. DB14-15
B H. sp. MZ1-3_acl39
B H. sp. MZ1-3_acl31
B H. sp. MZ1-3_acl29
A H. sp. MZ3-7_acl49
A H. sp. MZ3-7_acl46
A H. sp. SAG 2162
A H. marietanii SAG 835-7
A H. sp. MZ2-5
A H. pleurococcoides EIF PAB 397/380
A H. brevicellularis SAG 835-1
A H. pleurococcoides EIF PAB 398/473
A H. sp. EIF 430/A801-2_24
(5)
D H. sp. EIF 423/A790-45_cl65
D H. protonematoides EIF 128/A 788-70_a5
deletion*
D H. sp. EIF 423/A790-45_cl60
D H. protonematoides EIF 128/A788-70_acl3
D H. sp. EIF 423/A790-45_acl66
D H. sp. EIF 423/A790-45_cl71
D H. sp. EIF 423/A790-45_cl59
D H. sp. EIF 423/A790-45_cl69
D H. sp. EIF 423/A790-45_cl62*
D H. caespitosus EIF399/372_acl62*
D H. caespitosus EIF399/372_acl64*
D H. caespitosus EIF399/372_acl63*
(4)

Additional file 7. DNA sequence differences among five authentic strains of *Heterococcus* group A. Distance matrices with number of sequence position differences from the rbcL gene, the *psb*A/*rbc*L spacer and ITS2 between the five authentic strains of *Heterococcus* group A (assigned to *H. viridis* , see text). In brackets, the total number of differences found with a certain molecular marker among the five strains. An asterisk marks the strain that is distinct from others by the presence of a "GCAA" indel in helix IV of ITS2.

		rbcL (6)				**psbA/rbcL (8)**				**ITS2 (2 + indel)**			
		1	2	3	4	1	2	3	4	1	2	3	4
1	*H. viridis* SAG 835-3												
2	*H. mainxii* SAG835-6	3				1				0			
3	*H. marietanii* SAG 835-7	3	0			1	0			0	0		
4	*H. brevicellularis* SAG 835-1*	4	5	5		7	6	6		2	2	2	
5	*H. moniliformis* SAG 835-8	4	5	5	0	4	3	3	5	2	2	2	0

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Maistro, S., Broady, P.A., Andreoli, C., and Negrisolo, E. (2009) Phylogeny and Taxonomy of Xanthophyceae (Stramenopiles, Chromalveolata). *Protist* **160**: 412-426.

Wolf, M., Ruderisch, B., Dandekar, T., Schultz, J., and Müller, T. (2008) ProfDistS: (profile-) distance based phylogeny on sequence—structure alignments. *Bioinformatics* **24**: 2401-2402.

Zwickl, D.J. (2006) Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. In: The University of Texas at Austin, Ph.D. thesis.

Curriculum Vitae

PERSONAL DETAILS

Name and Surname:	Nataliya Rybalka
Date of Birth:	20 January 1981
Place of Birth:	Vinnitsa (Ukraine)
Nationality:	Ukrainian
Additional information:	married, two children (born in 2009 and 2013)

EDUCATION

since summer term 2011:	PhD studies at Kiel University
2004 – 2006:	PhD studies at Taras Schevchenko National University, Kyiv, Ukraine
2002 – 2004:	Studies of Biology with final exam MSc (with honor) at Taras Schevchenko National University, Kyiv, Ukraine
1998 – 2002:	Studies of Biology with final exam BSc (with honor) at Taras Schevchenko National University, Kyiv, Ukraine
1998:	School leaving exam (with honor and gold medal for excellence) at M.I: Pirogow grammar school No. 1, Vinnitsa, Ukraine

WORK EXPERIENCE

since 10/2014:	scientific collaborator (full position) in DFG project “Diversity of microalgae and cyanobacteria communities in Antarctic soils: changes along soil developmental stages and abiotic variables and testing for endemism/geographical distribution”, employed at Georg-August-University, Institute of Microbiology and Genetics, Dept. Genome Analysis (Prof. Dr. Rolf Daniel)
10/2013 – 03/2014:	scientific collaborator (half position) at Georg-August-University, Institute of Microbiology and Genetics, Dept. Genome Analysis (Prof. Dr. Rolf Daniel)
02/2013 – 04/2014:	maternity leave and “Elternzeit” according to German “BEEG”
05/2009 – 04/2012:	scientific collaborator (half position) at Christian-Albrechts-University of Kiel, Dept. Plantphysiology and Biotechnology at Botanical Institute(Prof. Dr. Rüdiger Schulz), working space at the Culture Collection of Algae at Göttingen University (SAG)
12/2008 – 04/2009:	maternity leave
02 – 09/2008:	trained assistant for biotechnology at company “CJSC Promsvyaz”, Kyiv, Ukraine
2007 – 2008:	employment as assistant at “Speko Ukraina” (Spanish-Ukrainian joint venture company) division of pharmacy, Vinnitsa, Ukraine

STAYS ABROAD

02 - 04/2006	Jan Kochanowski Universität, Kielce, Polen, Prof. Dr. A. Massalski: "Ultrastructure of selected taxa of filamentous Xanthophyceae"
05 - 09/2006	Georg-August-Universität Göttingen, Abt. Experimentelle Phykologie und Sammlung von Algenkulturen, Prof. Dr. T. Friedl: "Molecular phylogeny of Xanthophyceae" (DAAD-Stipendium)

TRAVEL GRANTS

ICCC 11	Conference attendance, organizing committee of the conference "11th International Conference on Culture Collections"
EPC 5	Conference attendance, "5th European Phycological Congress", Rhodos, Greece, 2011. Sektion Phykologie in der Deutschen Botanischen Gesellschaft
EPC 6	Conference attendance, "6th European Phycological Congress", London, 2015. Awarded the cost of registration for the congress by the Young Scientists Meeting Grant of FEMS.

AWARDS

2010	Awards "For Women in Science" of the Christiane Nüsslein-Volhard-Stiftung (CNV), the German UNESCO commission and L'Oréal, Germany

CONFERENCE ATTENDENCES (oral presentations)

2015:	"Microalgae communities in Antarctic soils: changes along soil developmental stages and testing for geographical distribution". 6th European Phycological Congress, London, UK (August 23-28, 2015)
2014:	- "Changes in Antarctic soil microalgal communities along a gradient of soil developmental stages and testing for geographical distribution of Antarctic soil algae" Gesellschaft für Ökologie (GfOe), the Ecological Society of Germany, Austria and Switzerland, 44th Annual meeting, Hildesheim, Germany (September 8-14, 2014) - "Molecular markers for species taxonomy and DNA-barcoding of Xanthophyceae (Stramenopiles)" 15. Wissenschaftliche Tagung der Sektion Phykologie in der Deutschen Botanischen Gesellschaft,Stralsund, Germany (February 23-26, 2014)
2011:	- "Testing for species and genus concepts and endemism in filamentous Xanthophyceae (Stramenopiles)". 5th European Phycological Congress, Rhodos, Greece (September 4-9, 2011) - "Hidden diversity in microalgae: assessing taxa boundaries in Filamentous yellow-green algae (Xanthophyceae)". International Botanical meeting at Charkhov University, Charkhov, Ukraine, (February 2-4, 2011)
2010:	"Assessing phylogenetic delineations of taxa of filamentous Xanthophyceae (Stramenopiles) using chloroplast gene spacer region sequence and morphology". 13. Wissenschaftliche Tagung der Sektion Phykologie in der Deutschen Botanischen Gesellschaft, Insel Reichenau, Germany (March 14-17 2010)
2007:	"Unambiguous rapid identification of Xanthophycean algae in Culture Collections using chloroplast gene spacer region sequences". 11th International Conference on Culture Collections, Goslar, Germany (October 7 – 11, 2007)
2006:	"Testing genus- and species level assignments in filamentous Xanthophyceae (heterokont algae)". 11. Wissenschaftliche Tagung der Sektion Phykologie in der Deutschen Botanischen Gesellschaft,Helgoland, Germany (August 28-31, 2006)

List of Publications

Nataliya Rybalka has co-authored the following publications until submission of this thesis. The four publications 6-9 are considered for this thesis. The publications 7 and 9 form the thesis chapters 1 and 3 and have been published in peer-reviewed international journals. Thesis chapter 2 is based on publication no. 8 which has been published in the form of conference proceedings and parts of the Introduction of this thesis have been published with paper no. 6 in the form of a book chapter. The latter two publications have not undergone the regular peer-review of scientific journals.

1) Abdel-Basset, R., Friedl, T., Mohr, K.I., **Rybalka, N.**, and Martin, W. (2011) High growth rate, photosynthesis rate and increased hydrogen(ases) in manganese deprived cells of a newly isolated Nostoc-like cyanobacterium (SAG 2306). *International Journal of Hydrogen Energy* **36**: 12200-12210.

2) Friedl, T., and **Rybalka, N.** (2012) Systematics of the Green Algae: a brief introduction to the current status. In: Lüttge, U., Beyschlag, W., Büdel, B., and Francis, D. (Eds.). *Progress in Botany 73*. Springer, Berlin, Heidelberg, pp. 259-280.

3) Friedl, T., **Rybalka, N.**, and Kryvenda, A. (2012) Phylogeny and systematics of microalgae: An overview. In *Microalgal Biotechnology: Potential and Production*. Posten, C., and Walter, C. (eds): De Gruyter., pp. 11-38.

4) Mudimu, O., **Rybalka, N.**, Bauersachs, T., Friedl, T., and Schulz, R. (2015) Influence of different CO_2 concentrations on microalgae: growth rate, α-tocopherol content and fatty acid composition. *Geomicrobiology Journal* **32**: 291-303 (DOI: 10.1080/01490451.01492014.01889784).

5) Mudimu, O., **Rybalka, N.**, Bauersachs, T., Born, J., Friedl, T., and Schulz, R. (2014) Biotechnological screening of microalgal and cyanobacterial strains for biogas production and antibacterial and antifungal effects. *Metabolites* **4**: 373-393.

6) Ott, D.W., Oldham-Ott C.K., **Rybalka, N.**, and Friedl, T. (2015) Xanthophyte, Eustigmatophyte, and Raphidophyte Algae. In: Wehr, J.D., Sheath, R.G., Kociolek, J.P. (Eds.). *Freshwater Algae of North America: Ecology and Classification, 2nd edition* Academic Press, Amsterdam, pp. 483-534.

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Acknowledgments

I am very thankful to Professor Rüdiger Schulz, Kiel University, for his various support of my thesis study. In particular, the work would not have been possible without his financial support which was possible through a funding by the Centre of Excellence of Biomass in Schleswig Holstein.

This work would not have been possible without the generous financial support of grants extended by the Christiane-Nüsslein-Volhard-Stiftung (CNV) and the "L'Oréal-UNESCO "For Women in Science" Award to Nataliya Rybalka (N.R.).

This study was supported by a research scholarship of the DAAD extended to N.R. Also, N.R. appreciates the support by Dr. G. Berthold, which was made possible through his generous donation to the SAG Culture Collection at the University of Göttingen. This thesis work was also supported by travel grants of the ICCC11 (2007) conference organizing committee and the "Sektion Phykologie in der Deutschen Botanischen Gesellschaft" (2011) extended to N.R..

Parts of this work were financed by the German Federal Ministry of Education and Research, BMBF (AlgaTerra Project, Grant 01 LC 0026) within the BIOLOG programme or supported by the German Science Foundation (DFG) through a grant extended to T.F. (Fr 905/20-1). Dr. Katrin I. Mohr is acknowledged for provision of four strains she isolated from freshwater; development and analyses of these strains were made possible through a grant of the DFG extended to T.F. (Fr 905/13-2,3).

N.R. acknowledges Professors Maria Olech (Jagiellonian University, Kraków, Poland, and Polish Academy of Sciences) and Andrzej Massalski (Jan Kochanowski University, Kielce, Poland) for provision of Antarctic soil samples and several strains of filamentous Xanthophyceae from Antarctica.

N.R. acknowledges the assistance in purification, and maintenance of the studied culture strains as well as provision of reference strains from the SAG culture collection, by Ilse Kunkel, Marlis Heinemann, Hella Timmermann and Dr. Maike Lorenz. Robert Rockstuhl helped in isolating strains from Antarctic soil samples.

Lastly, I am thankful to my parents, my two little daughters and my husband, for their patience and support.

Declaration

Hereby I declare that,

- apart from my supervisor's guidance, the content and design of this dissertation is product of my own work. Co-author's contributions to specific paragraphs are listed in the section "Authors' contributions" (p. 67).
- this thesis has not been submitted either partially or wholly as part of a doctoral degree to another examination body, and no other materials are published or submitted for publication than indicated in the thesis.
- the preparation of the thesis has been subjected to the Rules of Good Scientific Practice of the German Research Foundation.

Kiel, August 18 2015