Molecular Systematics of the Green Algal Order Trentepohliales (Chlorophyta).

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MOLECULAR SYSTEMATICS OF THE GREEN ALGAL ORDER
TRENTEPOHLIALES (CHLOROPHYTA)

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Plant Biology

by

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ABSTRACT

Molecular, karyological, and nuclear genome quantification data from representatives of the order Trentepohliales (Chlorophyta) were used to infer evolutionary relationships with other green algal classes and orders. Phylogenetic analyses of the nuclear-encoded small subunit ribosomal DNA (18S rDNA) sequences from taxa representing all of the major lineages of green algae consistently indicated that the subaerial Trentepohliales are closely related to Ulvophycean marine green algae, particularly to the siphonous and hemisiphonous orders.

The phylogenetic distribution of continuous and discontinuous types of mitochondrial large subunit ribosomal RNAs (mtLSU rRNA) in green algae has been shown to be consistent with phylogenetic relationships previously suggested by both ultrastructural data from the flagellar apparatus and nuclear rRNA sequence analysis. Our studies indicated the presence of a continuous mtLSU rRNA in Cephaleuros parasiticus; continuous mtLSU rRNA have been reported in all the investigated zoosporic green algae with a counterclockwise orientation of the flagellar apparatus and their autosporic descendants; a result that is consistent with an ulvophycean affinity.

Microspectrophotometry with the DNA-localizing fluorochrome DAPI was used to quantify nuclear DNA content in eight species representing three genera of the subaerial green algal order Trentepohliales (Chlorophyta). Comparisons of mean fluorescence intensity (I/) values of algal nuclear genomes to those of chicken erythrocytes (RBC) resulted in an estimate of 1.1-4.1 pg for the algae. DNA levels in Cephaleuros parasiticus Karsten for 2 C nuclei in gametophytic phase closely approximate 50% of the 4 C values in the sporophytic phase, confirming previous
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Amplification and sequencing of the chloroplast-encoded large subunit ribulose-1,5-bisphosphate carboxylase/oxygenase (rbcL) gene was used to assess its reliability as a phylogenetic marker of the order Trentepohliales and the green algal classes. Our present results suggest using the rbcL gene sequences is more useful within green algal classes than for the analysis of phylogenetic analysis among major groups of algae.
CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW
SYSTEMATICS OF THE GREEN ALGAE

Introduction. The green algae appeared on earth 500 to 900 million years ago as revealed by fossils (Waters and Chapman 1996; Bold and Wynne 1985). Such antiquity has provided this group of organisms with an opportunity to develop an amazing diversity in habitat, morphology, and life history. Despite the array of morphologies, reproductive strategies, and ecological roles shown by the green algae, the group is surprisingly uniform in some biochemical and ultrastructural features (Silva 1982). The concept of what comprises the green algae has varied. Generally, the green algae contains eukaryotic algae possessing chlorophylls $a$ and $b$, starch as storage products in plastids, in which thylakoids are stacked up to two to six per granulum (McCourt 1995). As currently circumscribed the green algae, with approximately 500 genera and 16,000 species (Melkonian 1990b), are considered one of the most diverse plant groups on earth.

The following is a brief account of the history of our knowledge of the green algae. Additional historical discussion of phycology and green algal systematics can be found in Prescott (1951), Papenfuss (1955), and Round (1984).

History. Algae were recognized in the literature as far back as the Greek writers such as Theophrastus and Dioscorides as phykos (root of phycology) or seaweed, which became fucus with the Romans (Prescott 1951). The algae, like most of the plant groups, were first cited in Linnaeus' (1753) Species Plantarum, in which were recognized four genera in the modern sense: Conferva, Ulva, Fucus, and Chara. The green algae were recognized as a distinct group early by Lamouroux (1813) with the name of Ulvacées, an order, to receive the genera Ulva, Bryopsis, and Caulerpa. Harvey
(1836) recognized the “green” algae as the Chlorospermeae (green algae). Lamouroux and Harvey had, without knowing it at the time, created a classification that would be discovered to be based on biochemical features, their major groups remain as valid entities to this day. Kutzing (1845) proposed the term Chlorophyceae to replace Chlorospermeae and it is the name we use now.

By the nineteenth century, five assemblages of green algae were recognized: protococcoid, palmelloid, confervoid (Chlorophycean), conjugate (Zygnematophycean), and macroscopic-multicellular (Charophycean) (Round 1984). Zooid forms such as chlamydomonads were grouped later into the Coccophyceae by Rabenhorst (1863). Stitzenberger (1860) recognized the Siphonophyceae, which included the Valoniaceae, Caulerpaceae, and Dasycladaceae. Rabenhorst (1868) in his “Flora europaea algarum...” recognized four basic groups of green algae: Coccophyceae, Zygophyceae, Siphophyceae and Nematophyceae; the last group includes Chroolepidaceae, better known today as Trentepohliaceae. During this period, 1800-1900, phycology, like the study of other plant groups, was a field of intense, almost feverish, activity, a time that has been called the “Golden age of plant taxonomy” (Prescott 1951).

By the turn of the century, the green algae were viewed from an evolutionary approach; it was Blackman (1900) who pointed out that the Volvocine, Tetrasporene, and Chlorococcine pathways were the result of evolutionary developments from primitive forms of green flagellates such as a Chlamydomonas-like organism. An important contribution was made by Pascher (1914, 1931) who classified the green algae in six classes: Volvocineae, Tetrasporeneae, Protococcineae, Ultorichineae, Siphonineae, and Siphonocladineae, and with a modern view he proposed that the
charophytes be set apart from other green algae. The classic and extensive work of Fritsch (1935) on the structure and reproduction of the algae was a landmark in phycology. His textbook was recognized throughout the world. In addition to his classification of the green algae in nine orders: Volvocales, Chlorococcales, Ulotrichales, Cladophorales, Chaetophorales, Oedogoniales, Conjugales, Siphonales, and Charales, he also recognized the green algae as the descendants of the ancestors that gave rise to the embryophytes. Fritsch’s monumental work is considered as the end of an era in which morphology was the predominant feature of the classification of the algae.

**The Modern Era.** After 1950, phycology was invigorated by biochemical, molecular, and ultrastructure approaches. New technological advances along with theoretical input from Margulis and her popularization of the endosymbiosis theory created an exciting time of new ideas, new data, and new analyses.

**Ultrastructure.** With the advent in the 1960s of powerful electron microscopes, it became possible to analyze the fine structure of algal cells. Details of organelles such as chloroplasts, nuclei, flagellar roots, and some important biological processes such as mitosis and cytokinesis, were found to be highly diverse and this diversity was the basis for new concepts, especially for green algal phylogeny and systematics. Therefore new approaches in the classification of the green algae started to proliferate. A new generation of researchers was soon studying the ultrastructure of the green algae with a keen eye to the phylogeny of this group. An important and prolific leader in this new field, Jeremy D. Pickett-Heaps, showed that the ultrastructural features of algal cells can be used for systematic purposes. Two main areas of ultrastructural cytology were
exploited with great success; the morphological features of cell division and the flagellar apparatus of the green algal motile cells. Ultrastructural details in the cell division of green algae provided important information for the classification, not only of the green algae, but also higher plants. It was shown that cell division in green algae is by no means uniform. In some green algae, such as *Chlamydomonas*, the mitotic apparatus during the final phase of cell division (telophase) allows the two daughter nuclei to approach each other closely, because the mitotic spindle is not persistent. In this case, a system of microtubules arranged in a plane parallel to the plane of cell division separate the daughter nuclei; this system of microtubules is called a phycoplast. In other groups of green algae (e.g. *Chara*) the daughter nuclei are kept farther apart, with a persistent mitotic spindle, and the microtubules of the spindle are oriented in a plane perpendicular to the plane of cell division. This system is termed phragmoplast and is the system that is found in all embryophytes.

The distribution of these patterns of cytokinesis among the green algae led Pickett-Heaps (1969, 1972) to propose two major green algal lineages: green algae exhibiting phycoplast-type cytokinesis formed the class Chlorophyceae whilst green algae with phragmoplast-type cytokinesis were grouped in the class Charophyceae. The Charophyceae were hypothesized to have given rise to the land plants. This distinction was received with great excitement, especially because ultrastructural data were used as evidence to classify some heretofore apparently unrelated orders such as Chlorokybales, Klebsormidiales, Zygnematales, and Coleochaetales with the “higher” order Charales, which earlier had been considered by phycologists to be a distinct lineage clearly separate from the other green algae (e.g. Pascher 1914, 1931). The dichotomy of green
algae also underscored a close relationship between the green algae of the Charophyceae and the tracheophytes, lending support to the theory that the green algae had given rise to the land plants.

The ultrastructural features of the flagellar apparatus in green algae provided reliable taxonomic characters for the green algae. Several features were studied from the flagellar apparatus, specially from the flagellar root system. Several phycologists, especially Chapman (1981), Graham (1982), Mattox and Stewart (1984), Melkonian (1984), O’Kelly and Floyd (1984), J. D. Pickett-Heaps (1975), and Sluiman (1989), produced a considerable amount of information that provided the basis for arranging the green algae in groups based on ultrastructural features of the flagellar roots. Two groups were evident: one group shows a cruciate root system (clockwise, counterclockwise, or directly opposed; fig. 1.1), associated with 180° rotational symmetry (or reversed bilateral symmetry) and the presence of an eyespot, such as Chlamydomonas. The flagellates in the second group (fig. 1.2) have the flagella inserted on one side and never have an eyespot (e.g. Chara). Other groups are more restricted in occurrence, and have an unusual combination of features, for example in the Trentepohliales with a CCW cruciate flagellar apparatus, and two multilayered structures associated with two roots. In addition, although less obvious in micrographs of the green algal cells, the presence or absence of scales on the flagella and/or cell surface was observed. The importance of these features for inferring relationships among green algal groups and between green algae and land plants was soon noted by phycologists using electron microscopy.

**Classification.** The systematic arrangement of such a highly diverse group by phylogeny has been a major challenge to every taxonomic phycologist. Former
Fig. 1.1. Diagram of the cruciate arrangement of flagellar basal bodies and microtubular roots, anterior view, Ulvophyceae (left) and Chlorophyceae (right) (from Mattox and Stewart 1984).

Fig. 1.2. Diagram of the lateral arrangement in Charophyceae in side view, scaly cell with one large root (the MLS), one smaller root, and flagella (from Mattox and Stewart 1984).
classification schemes emphasized morphological and biochemical characteristics (pigments, cell walls, storage product) as main features to distinguish groups in the green algae. Recognition of classes and orders of green algae was commonly based on comparative morphology to delineate major evolutionary lines of Chlorophyta; some examples can be found in the classic books on algae such as Fritsch (1935), Smith (1955), and Bold and Wynne (1985).

As mentioned earlier, the advent of electron microscopy made it possible to study representatives of the major groups of green algae in a detailed comparative manner. Additional studies during the 1970’s and 1980’s produced an array of new characters to be used as phylogenetic indicators in the Chlorophyta. Several systems of classification making use of the new ultrastructural features in green algae appeared. Mattox and Stewart (1984), based on two decades of research on comparative cytology, proposed an alternative scheme that modified the two classes system of J. D. Pickett-Heaps. The Mattox and Stewart (1984) system has become the most widely cited system of chlorophytan classification. Stewart and Mattox (1975) had originally erected 3 classes of green algae; however, this system was based only on a few genera and was designed to explain progress in the field and not intended as a general-use classification. Later Mattox and Stewart (1984) erected five classes of green algae:

Micromonadophyceae, Charophyceae (including the orders Chlorokybales, Klebsormidiales, Zygmematales, Coleochaetales, and Charales), Ulvophyceae, Pleurastrophyceae (with the orders Tetraselmidales and Pleurastrales), and Chlorophyceae (including Chlamydomonadales, Volvocales, Chlorococcales, Sphaeropleales, Chlorosarcinales, Chateophorales, and Oedogoniales). Van den Hoek et
al. (1995) proposed a new system based upon a more "holistic" approach, including architecture of the zooids, type of cell division, place of meiosis in the life history, thallus morphology, habitat type, and chloroplast type. This systematic arrangement includes eleven classes of green algae: Prasinophyceae, Chlorophyceae sensu stricto, Ulvophyceae, Cladophorophyceae, Bryopsidophyceae, Dasycladophyceae, Trentepohliophyceae, Pleurastrophyceae, Klebsormidiophyceae, Zygmatophyceae, and Charophyceae. Although the classification of the green algae is still in flux, as is evident from the various systematic treatments published recently on the green algae (see Margulis et al. 1990), the system proposed by Mattox and Stewart (1984) is often the starting point used for modern discussions of green algal systematics and evolution (McCourt 1995). The importance of the Mattox and Stewart (1984) system lies in the fact that it is based on correlated characters on which phylogenetic inferences can be made (Waters and Chapman 1996).

Molecular Systematics. Recently, new technological advances in DNA sequencing and phylogenetic analysis have produced a revolutionary approach to phylogenetic studies in general and for the algal systematics in particular. The impact of the new technology in providing new characters is similar to the advent of electron microscopy. Determining the nucleotide sequence of genes, using the polymerase chain reaction (PCR) even for algae that cannot be cultured, provided the opportunity to analyze hundreds or thousands of characters (nucleotides) for each taxon. For the first time, phycologists found themselves with not just a large number of characters to be analyzed, but also with independent sets of features which, if from homologous sites represented valuable phylogenetic information. Data analyses of gene sequences by
cladistic, phenetic, and maximum likelihood methods, have been used routinely in the search for a true phylogeny of the green algae. The advantage of these approaches resides in the fact that they work as independent data sets to test hypotheses of relationships based on other source such as morphological, biochemical, and ultrastructural data.

First attempts in the new field of the molecular systematics of the green algae were not without pitfalls. The first gene sequences used were the 5.8 S rDNA (Hori et al. 1985; Hori and Osawa 1987), which are only 120 base pairs in length and rapidly evolving, and thus potentially misleading phylogenetically (Steele et al. 1991). Other, longer, genes were tested, and found to be more phylogenetically informative. Among these useful genes, the chloroplast-encoded large subunit ribulose-1,5-bisphosphate carboxylase/oxygenase (rbcL) (Manhart 1994, McCourt et al. 1995), and the nuclear-encoded ribosomal small subunit RNA or 18S rDNA (SSU rDNA) (see Chapman and Buchheim 1991 for a review) are most widely used in testing for phylogenetic inference. The mitochondrial genome, specifically the large subunit ribosomal DNA, has been used only recently (Nedelcu et al. 1996, Nedelcu 1997a, b).

Gene sequences from SSU rDNA are commonly used in molecular systematics of the green algae. Analysis of these sequences consistently supports Pickett-Heaps' idea (Pickett-Heaps and Marchant 1972) of two lineages of green algae: the chlorophycean lineage and the charophycean lineage. The charophycean lineage gave rise to the land plants, thus forming a monophyletic group named Streptophyta. The monophyletic chlorophycean group includes the Micromonadophyceae, Pleurastrophyceae, Chlorophyceae, and Ulvophyceae (sensu Mattox and Stewart 1984).
More recently, Friedl (1995) using molecular studies, revised this group to contain the Prasinophyceae, Trebouxiophyceae, Chlorophyceae, and Ulvophyceae. There is consistent support from various molecular analyses for the basal phylogenetic splitting of the Viridiplantae into the chlorophycean and charophycean groups. Relationships amongst the taxa comprising these groups is still under study, but some agreements are clear (fig. 1.3); the Prasinophyceae is not monophyletic, but probably a paraphyletic assemblage of basal clades in the chlorophycean lineage with at least one clade among the streptophytes. The monophyly of the Trebouxiophyceae, Chlorophyceae, and Ulvophyceae is supported by various studies. The charophycean group appears to be the sister group to the land plants (Embryophyta). The charophycean group, as defined by Mattox and Stewart (1984), is supported by molecular analysis and comprises the orders Charales, Coleochaetales, Klebsormidiales, Chlorokybales, and Zygnematales. More recently the class Chaetosphaeridiophyceae was added as a basal clade within this group (Sluiman and Guihal 1999) along with the "prasinophyte" *Mesostigma viride* (Melkonian et al. 1995, Bhattacharya et al. 1998).

**BIOLOGY AND SYSTEMATICS OF THE TRENTEPOHLIALES**

**Introduction.** The Trentepohliales consists of subaerial green algae growing on humid soil, rocks, tree bark, and leaves. Some are endophytic or parasitic in leaves, stems and fruits while others grow in close association with fungi forming lichens. Representatives of Trentepohliales develop a filamentous structure which forms uniseriated, branched, erect tufts or laterally coherent, prostrate discs. Other forms are highly reduced and produce only a short vegetative filament a few cells in length. The cells of the Trentepohliales are uninucleate or multinucleate, with several parietal
Fig. 1.3. Summary results of molecular analyses of the evolutionary relationships among the green plants.

The main characteristics of the order Trentepohliales are as follows:

a) Differentiated reproductive cells which differ from the vegetative cells by a unique zoosporangial abscission process.

b) The presence of β-carotene, haematochrome (i.e., astaxanthin), coloring the algal thallus yellow, orange, or red;

c) The absence of a pyrenoid in the parietal reticulate chloroplasts.

d) Unique flagellar apparatus.

f) Transverse cell walls with centrally located plasmodesmata.

Reproduction and Life Histories. The life cycle of the Trentepohliales is still terra incognita. Early taxonomic treatments, such as Printz’s (1939), made no observations on this topic, and modern reviews (O’Kelly and Floyd 1990) acknowledge limited data on this subject. The only modern reports on the life cycle of Cephaleuros and Stomatochroon are from Thompson (1961) and Thompson and Wujek (1997). The life cycle as reported includes an alternation of heteromorphic generations. The haploid gametophyte produces stalked zoosporangia which release quadriflagellated zoospores which repeat the gametophytic phase; in addition on the same plant or thallus biflagellated isogametes are produced within sessile gametangia. Fertilization may take place within the gametangia or outside, and the mating system is homothallic. The zygote germinates to produce a dwarf sporophyte that develops small zoosporangia, and which, in turn, produce four quadriflagellated zoospores (microzoospores or
meiozoospores). The site of meiosis was tentatively placed in the dwarf zoosporangia (Thompson 1961), and later confirmed by Chapman and Henk (1982) who discovered the synaptonemal complexes in the dwarf sporangia of *Cephaleuros virescens*. The life cycle of both *Trentepohlia* and *Phycopeltis* has been described as an alternation of isomorphic generations (Chapman 1984, Thompson and Wujek 1997). The haploid gametophyte bears gametangia (in some species also zoosporangia) and produces biflagellated gametes. The zygote develops into a diploid sporophyte bearing only meiozoosporangia and quadriflagellated zoospores which germinate to form haploid gametophytes. Again, the putative site of meiosis is the meiozoosporangium. Chapman (1984) observed that isomorphic alternation of generations occurs in *Trentepohlia* and *Phycopeltis* when the vegetative morphology is simpler, whereas a more complicated heteromorphic alternation occurs in the genera *Cephaleuros* and *Stomatochroon*, both
of which have a complex vegetative morphology and possibly secondarily reduced morphology. Thus, *Trentepohlia* and *Phycopeltis* could be considered to be basal taxa.

The structure of the sporangium is unique in this order of algae because of the suffultory cell (or “stalk cell”) forming the “sporangiate-lateral”, considered to be an important taxonomic character for the circumscription of the order Trentepohliales (fig. 1.4). The suffultory cell was defined by Thompson and Wujek (1997) as “the retrorsely bent cell that immediately subtends a sporangium;” the area of contact between the suffultory cell and the sporangium has been the object of ultrastructural studies in *Cephaleuros* (Chapman 1976) and *Phycopeltis* (Good and Chapman 1978b). The head cell, which can be lateral or terminal on a sporangiophore (erect filament) bears the sporangiate-laterals. The process of abscission was described by Good and Chapman (1978b) involving a central area rich in plasmodesmata surrounded by a thickened area of an “internal” ring. In the periphery of this abscission septum there is a second “external” ring of thickened wall material, the region between the internal and external ring lacks plasmodesmata. During the development of the sporangium, the external thickening undergoes a circumsessile tearing, and the suffultory cell and the sporangium remain attached only at the central region. During favorable conditions of high humidity for the swimming zoospores, the suffultory cell expands, holding the sporangium farther out from the head cell. It is believed that turgor causes the final separation (abscission) of the sporangia.

**Ultrastructure.** The ultrastructural details of reproductive structures, quadriflagellate zoospores, and biflagellated gametes in the Trentepohliales have been reported by several phycologists. From the genera studied, *Trentepohlia* (Graham and
McBride 1975, Roberts 1984)) and *Cephaleuros* (Chapman 1976, 1980, 1981, Chapman and Henk 1982, 1983) and *Phycopeltis* and *Stomatochroon* (Good 1978), a pattern has emerged. The flagellated cells are compressed in a dorsiventral fashion with either two (gametes) or four (zoospores) flagella, and four microtubular roots in a cruciate arrangement. The counter-clockwise flagellar apparatus in Trentepohliaceae has been cited as evidence for an affinity to the Ulvophyceae (Roberts 1984). The overlapping configuration of the basal bodies is termed 11 o’clock-5 o’clock. Flagellar apparatus in the Trentepohliaceae also shows distinct and unique features. One is the pair of columnar structures which resemble, and may be homologous to, the multilayered structures (MLS) typical of the unilateral flagellated cells such as in Charales. Another unusual feature for the motile cells in Trentepohliaceae is listed in Van den Hoek et al. (1995): the four microtubular roots do not follow the usual arrangement of the x-2-x-2 pattern which is typical of the green algae with a cruciate flagellar root system. Instead, in *Trentepohlia* the arrangement is 6-4-6-4, and in other Trentepohliaceae the arrangements also vary, due the compressed nature of the cell, the microtubular roots are appressed to the basal bodies and each flagellum bears bilateral wing-like structures.

Developmental stages in mitosis have been reported for *Trentepohlia* sporangia (Graham and McBride 1978) and *Cephaleuros parasiticus* (Chapman and Good 1977). A closed and centric mitosis has been reported during mitosis in vegetative cells, the interzonal spindle is present and at telophase is a distinct massive bundle of microtubules associated with membrane vesicles at the plane of cell division, forming a phragmoplast. The importance of the phragmoplast in this type of cytokinesis has been cited as a way to assess the phylogenetic affinity in green algae (Mattox and Stewart 1975, 1980, 2016).
1984). It is found only in a few charophycean algae (i.e. *Nitella missouriensis* [Turner 1968], *Chara fibrosa* [Pickett-Heaps 1967], *Coleochaete scutata* [Marchant and Pickett-Heaps 1973], and *Spirogyra* sp. [Fowke and Pickett-Heaps 1969]), in *Cephaleuros parasiticus* (Chapman and Henk 1986) and *Trentepohlia* (Waters et al. 1998). The well developed phragmoplast-type cell division in the Trentepohliales could link the group to the charophycean green algae (characterized by this cytokinetic apparatus) and directly contradict the ulvophycean affiliation inferred from the CCW cruciate flagellar arrangement.

Plasmodesmata are a common feature for the Trentepohliaceae (Chapman and Good 1978; Chappell et al. 1978; Good 1978). They occur in the cross walls between the cells, a central area which has been termed “pit field” by Chappell et al. (1978). Plasmodesmata also occur in the central abscission zone of the zoosporangium.

**Biochemistry.** Some biochemical by-products in Trentepohliaceae have been found to be specific for this group of green algae. Feige and Kremer (1980) reported an unusual carbohydrate pattern in *Trentepohlia* species. Patterson and VanValkenburg (1991) mentioned the presence and accumulation of unusual carbohydrates in *Cephaleuros* and *Trentepohlia*, as well as polyhydroxy alcohols (polyols and alditols), considered rare among the green algae. Among the sterols discovered in *Cephaleuros*, cholesterol made up 19% of the total sterol. The novel sterol 4, 24-dimethylcholest-7-enol is a new algal sterol and this is the first report of this sterol being the principal sterol of any organism. Kremer and Kirst (1982) demonstrated that species of *Trentepohlia* showed the widest spectrum of accumulated alditols reported for any algal group. They suggested that this sterol complement was related to the aerophytic habit
(subaerial). The same observation has been advocated by Chapman and Good (1983), although these last authors pointed out the need for more studies in this field.

**Fossils.** Eocene fossils identifiable as belonging to Trentepohliaceae have been reported by Tappan (1980). Thompson and Wujek (1997) mentioned the report of Dilcher (1965) and Reynolds and Dilcher (1984) of excellent preserved thalli, sporangiophores and hairs of *Cephaleuros*, from leaf compressions from the Eocene 40,000 years ago (sic, the period should be 40 million years ago). O'Kelly and Floyd (1990), based on cytological and ultrastructural features, suggested that the Trentepohliales are a derived group that has arisen relatively recently. The presence of sporopollenin-like substances in the cell wall (Good and Chapman 1978a) would help preserve the algae as fossils, and since trentepohlialean fossils are found only as far back as the Eocene, one can suggest that they must be of recent origin vis-a-vis the origin of the green algae some 500 to 900 mya.

**Ecology and Economic Importance.** The geographic distribution of Trentepohliaceae is basically pantropical (Bourrelly 1966). However some taxa of *Trentepohlia* and *Phycopeltis* have been reported from colder regions such as northern Europe (Chapman 1984, O'Kelly and Floyd 1990). All Trentepohliaceae are subaerial, none having found in aquatic habitats, freshwater or marine. The presence of sporopollenin-like substances in the cell walls (Good and Chapman 1978b) as well as a special pattern of carbohydrates and alcohols (Feige and Kremer, 1980; Patterson and VanValkenburg 1991), probably provide adaptative features against dessication in the subaerial habitat.
The ecological distribution of Trentepohliaceae is also remarkable. *Trentepohlia* commonly occurs upon rocks, pilings, walls, bark of trees wherever a solid substrate, light, and favorable humid conditions are found (Bold and Wynne 1985). *Phycopeltis* is generally described as a epiphyllous alga, growing on the surface of leaves but able to grow potentially on any object (Thompson and Wujek 1997). *Cephaleuros* is more restricted in habitat requirements. It is considered a strict epiphyte, living in the subcuticle or, deeper, in the tissue of leaves, twigs, and fruits of vascular plants (Chapman and Good 1983). *Cephaleuros virescens* seems to be the most common of the trentepohlialean community inhabiting tropical hosts and it has been reported from numerous host species, representing 218 genera and 62 families of vascular plants just in the Gulf of Mexico coast and numerous hosts (119 genera) from Brazil (Holcomb 1986). *Stomatochroon* has an even more specialized habitat; it is found in the air chambers and stomates of leaves of tropical vascular plants (Bourrelly 1966).

The economic importance of the Trentepohliales lies in species of the genus *Cephaleuros*. Species of this genus are very common on the leaves of tropical trees and shrubs with economic importance such as tea (*Camellia sinensis*), pepper (*Piper nigrum*), coffea (*Coffea arabica*), oil palm (*Elaeis guinmeensis*), avocado (*Persea americana*), vanilla (*Vanilla planifolia*), guava (*Psidium guajava*), and cacao (*Theobroma cacao*), as well some citrus (*Citrus* spp.) cultivars, causing death (necrosis) of the cells just beneath the algal thallus (Thompson and Wujek 1997) and perhaps injuring the host plants. Infection on tea and coffea were erroneously called “red rust.” Thompson and Wujek (1997) suggested that it is a fungus (which sometimes forms an association with the alga to form a lichen) not the algal growth, that is responsible for
the disease. A different scenario is portrayed by *Cephaleuros parasiticus* and allied species, which develop intramatrically within the leaf tissue, causing necrosis in the lower epidermis. The new genus and species of fungus, *Veralucia brasiliensis*, was erroneously described from the Amazon Basin in Brazil (Reynolds and Dunn 1982), based on samples of a "fungus-like" alga, later recognized as *Cephaleuros parasiticus* Karsten (Reynolds and Dunn 1984). This species is a serious pest on *Magnolia grandiflora* in Florida. However, it has been noted that the parasitic species of *Cephaleuros* are not as widespread in a variety of hosts as is *C. virescens* (Thompson and Wujek 1997). Salleh and Kamsari (1994) reported an ecological study on *Cephaleuros virescens* from the rubber tree (*Hevea brasiliensis*) from Malaysia. They found some factors associated with the infection, with rain and high temperatures as the limiting factors. Marche-Marchad (1981) reported from Senegal another ecological study on the same species. Her report concluded that the limiting factor on this trentepohlialean community was evapotranspiration or ETP and that decreasing ETP adds to the biocenosis richness, density and diversity; with foliicolous populations as ETP indicators.

Trentepohliales are well known to form lichenic associations with fungi (Alexopoulous et al. 1996; Chapman and Good 1983; Matthews et al. 1989). The phycobionts usually are representatives of the genera *Cephaleuros* and *Trentepohlia*. *Cephaleuros* has been described as the phycobiont in 14 species of obligately foliicolous lichens (Santesson 1952) in the genera *Strigula* and *Raciborskiella* (Chapman 1976). *Racodium* and *Coenogonium* are other genera of lichens with trentepohliaceous phycobionts (Davis and Rands 1993). *Phycopeltis* has been reported
as not lichenized but in close association with a fungus (Chapman and Good 1976). *Trentepohlia* has been found associated with eight families of loculascomycetes and discomycetes just in one city in Louisiana (Tucker et al. 1991). Chapman (1976) has shown in *Strigula elegans* that fungal penetration of haustoria occurs in the phycobiont cells; the lichen and the nonlichenized phycobiont occur in the same habitat. Since some phycobiont cells are destroyed by the mycobiont, the author concluded that the phycobiont does not benefit from this association and the mycobiont is, in fact, parasitizing the trentepohlialean alga. In another study, Davis and Rands (1993) found that *Physolinum monile* is a common trentepohliaceous phycobiont which is almost identical, except for larger cells than in the free-living filaments of the phycobiont.

**Systematics of the Trentepohliales.** The order Trentepohliales is represented by one family, Trentepohliaceae. The family name has also been cited as “Chroolepidaceae” or “Chroolepaceae.” Pappenfuss (1962) noted the precedence of Chroolepidaceae Rabenhorst over Trentepohliaceae Hansgirg; although Silva (Chapman 1984) has suggested the use of Chroolepidaceae is erroneous. In response Chapman (1984) recommended the appropriate use of Trentepohliaceae over the other names.

The Trentepohliales has received few taxonomic revisions. Early treatments were made by Karsten (1891) and Hariot (1889, 1890, 1893).

The major taxonomic accounts consist of the publications of Printz (1921, 1927, 1939). In his major revision Printz (1939) recognized only subaerial genera within the Trentepohliales: *Physolinum, Trentepohlia, Phycopeltis, Cephalaeuros,* and *Stomatochroon.* He placed Trentepohliales into the order Chaetophorales (Printz 1964). A summary of his classification follows (Printz 1939):
<table>
<thead>
<tr>
<th>Genus</th>
<th>Number of Species</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Trentepohlia</em></td>
<td>36</td>
</tr>
<tr>
<td><em>Phycopeltis</em></td>
<td>12</td>
</tr>
<tr>
<td><em>Cephaleuros</em></td>
<td>13</td>
</tr>
<tr>
<td><em>Stomatochroon</em></td>
<td>1</td>
</tr>
<tr>
<td><em>Physolinum</em></td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>63</strong></td>
</tr>
</tbody>
</table>

*Trentepohlia* Martius 1817. (Synonyms: *Chroolepus* Agardh 1824; *Amphiconium* Nees, *Dematium* Rebent; *Mycinema* Hooker et Arnott, *Phytoconis* Bory; *Cystocoleus* Thwaites; *Coenogonium* Nylander; *Nylandera* Hariot.

Section I. *Chroolepus* (C. Agardh 1824) Wille 1909 (*Eutrentepohlia* Hariot 1889)

1. *T. dialepta*
2. *T. calamicola*
3. *T. abietina*
4. *T. treubiana*
5. *T. jucunda*
6. *T. cucullata*
7. *T. annulata*
8. *T. bossei*
9. *T. lueto-fusca*
10. *T. elongata*
11. *T. arborum*
12. *T. negeri*
13. *T. uncinata*
14. *T. aurea*
15. *T. villosa*
16. *T. jolithus*
17. *T. lagenifera*
18. *T. santurcensis*
19. *T. umbrina*
20. *T. odorata*
21. *T. diffracta*
22. *T. rigidula*

Section II. *Heterothallus* Hariot 1890

23. *T. leprieurii*
24. *T. depressa*
25. *T. ellipsiocarpa*
26. *T. cyanea*
27. *T. minima*
28. *T. effusa*
<table>
<thead>
<tr>
<th>Number</th>
<th>Species</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>26.</td>
<td>T. dusenii</td>
<td>T. diffusa</td>
</tr>
<tr>
<td>31.</td>
<td>T. peruana</td>
<td>T. tentaculata</td>
</tr>
<tr>
<td>32.</td>
<td>T. bogoriensis</td>
<td>T. willei</td>
</tr>
<tr>
<td>33.</td>
<td>T. Lagerheimii</td>
<td>T. prolifera</td>
</tr>
</tbody>
</table>

**Phycopeltis** Millardet 1870. **Phyllactidium** Kutzing 1849; **Chromopeltis** Reinsch 1875;

**Hansgirgia** de Toni 1888.

**Section I. Euphycopeltis** Wille 1909

<table>
<thead>
<tr>
<th>Number</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>P. epiphyton</td>
</tr>
<tr>
<td>2.</td>
<td>P. microcystis</td>
</tr>
<tr>
<td>3.</td>
<td>P. arundinaceae</td>
</tr>
<tr>
<td>4.</td>
<td>P. expansa</td>
</tr>
</tbody>
</table>

**Section II. Hansgirgia** (de Toni 1888) Wille 1905

<table>
<thead>
<tr>
<th>Number</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.</td>
<td>P. treubii</td>
</tr>
<tr>
<td>6.</td>
<td>P. aurea</td>
</tr>
<tr>
<td>7.</td>
<td>P. maritrina</td>
</tr>
<tr>
<td>8.</td>
<td>P. flabelligera</td>
</tr>
<tr>
<td>9.</td>
<td>P. irregularis</td>
</tr>
<tr>
<td>10.</td>
<td>P. prostrata</td>
</tr>
<tr>
<td>11.</td>
<td>P. amboinensis</td>
</tr>
<tr>
<td>12.</td>
<td>P. nigra</td>
</tr>
</tbody>
</table>

**Cephaleuros** Kunze in Weigelt 1827

<table>
<thead>
<tr>
<th>Number</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>C. solutus</td>
</tr>
<tr>
<td>2.</td>
<td>C. laevis</td>
</tr>
<tr>
<td>3.</td>
<td>C. purpureus</td>
</tr>
<tr>
<td>4.</td>
<td>C. karstenii</td>
</tr>
<tr>
<td>5.</td>
<td>C. pulvinatus</td>
</tr>
<tr>
<td>6.</td>
<td>C. henningsii</td>
</tr>
<tr>
<td>7.</td>
<td>C. candelabrum</td>
</tr>
<tr>
<td>9.</td>
<td>C. virescens Kunze in Fries 1829</td>
</tr>
<tr>
<td>10.</td>
<td>C. albidus</td>
</tr>
<tr>
<td>11.</td>
<td>C. minimus</td>
</tr>
<tr>
<td>12.</td>
<td>C. parasiticus</td>
</tr>
<tr>
<td>13.</td>
<td>C. mycoidea Karsten 1891, Phyllacticium tropicum Mobius 1888</td>
</tr>
</tbody>
</table>
Another major publication concerning the Trentepohliales was the well known textbook of Fritsch (1935). Fritsch envisioned the Trentepohliaceae as a wide ensemble of genera of green algae possessing a filamentous morphology and various differentiated reproductive structures (O'Kelly and Floyd 1990). Genera included in the Trentepohliaceae by Fritsch (1935)

Section Gongrosireae  
- *Chloroclonium*  
- *Chlorotylium*  
- *Endophyton*  
- *Grongrosira*  
- *Leptosira*  

Section Gomontieae  
- *Gomontia*  

Section Trentepohliaceae  
- *Trentepohlia*  
- *Cephaleuros*  
- *Phycopeltis*  

Section Trentepohliaceae  
- *Trentepohlia*  
- *Cephaleuros*  
- *Phycopeltis*  

Smith (1950) classified the Trentepohliaceae into the Order Ulotrichales, together with aquatic and subaerial forms. The genera included by Smith are as follows:

Genera included in the Trentepohliaceae by Smith (1950)

- *Leptosira*  
- *Gomontia*
Flint (1959) merged the monotypic genus Physolinum into Trentepohlia, a treatment widely accepted by many authors (Chapman 1984, Bourrelly 1966); however some authors advocate the use of Physolinum (Davis and Rands 1993, Davis 1994) as a valid genus.

More recently, Thompson and Wujek (1992), based on the morphological characteristics of the sporangia, published a new genus, Printzina (Typus speciei: Printzina lagenifera) by describing a new species and transferring nine species of Trentepohlia, many of them from Hariot’s (1890) section Heterothallus. The new species is P. ampla; the transferred species are:

- P. lagenifera var. africana (= T. lagenifera var. africana)
- P. lagenifera var. rugulosa (= T. lagenifera var. rugulosa)
- P. bossei (= T. bossei; T. bossei f. major)
- P. diffusa (= T. diffusa; T. pinnata)
- P. dusenii (= T. dusenii)
- P. effusa (= T. effusa; T. setifera; T. effusa var. subtropica)
- P. lagerheimii (= T. lagerheimii)
- P. luteo-fusca (= T. luteo-fusca)
- P. santurcensis (= T. santurcensis)
Thompson and Wujek (1997) prepared a monograph of the Trentepohliales (except the genus *Trentepohlia*) recognizing only subaerial genera within the Trentepohliales as follow:

**Cephaleuros** Kunze in Fries 1829

1. *C. solutus*  
2. *C. druetii*  
3. *C. tumidae-setae*  
4. *C. karstenii*  

**Phycopeltis** Millardet 1870

1. *amboinensis*  
2. *arundinacea*  
3. *aurea*  
4. *costaricensis*  
5. *dorsopapillosa*  
6. *epiphyton*  

**Stomatochroon** Palm 1934

1. *S. lagerheimii*  
2. *S. coalitus*  
3. *S. consociatus*  
4. *S. reniformis*  

The Order Trentepohliales, as currently circumscribed (Thompson and Wujek 1997), includes one family Trentepohliaceae and 6 genera which can be separated by the following key:
Key To The Genera Of Trentepohliales

1. Thallus reduced to few cells, endophytic
   Stomatochroon

1. Thallus well developed, with filaments free or coalesced to form discs
   2. Aplanospores present, filaments free and moniliform
      Physolinum
   2. Aplanospores absent, filaments with cylindrical or inflated cells
      3. Filaments free; epiphytic or not; papilla-pore always basal, adjacent to
         the sporangium attachment
      Printzina
      4. Sporangia globular-reniform; prostrate filaments well
         developed, scanty erect system
      Trentepohlia
      4. Sporangia ovoid; scanty prostrate system, profuse erect system
      Trentepohlia

3. Filaments regularly coalesced to form discs; sometimes free; always
   associated with a host; papilla-pore basal or terminal
   5. Supracuticular; papilla-pore terminal, opposite to the
      attachment of the sporangium
      Phycopeltis
   5. Subcuticular; papilla-pore basal, adjacent to the attachment of
      the sporangium
      Cephaleuros

Trentepohlia Martius 1817

The genus Trentepohlia is the most diversified of the described
Trentepohliacean algae, consisting of branched heterotrichous filaments growing
epilithic or epiphytic on the bark of trees, or in lichenic associations at exposed habitats
forming conspicuous masses, usually yellow to orange in color. As currently redefined
by Thompson and Wujek (1992), Trentepohlia shows a profused erect system, with a
scarce or absent prostrate system. Sporangia are ovoid, sporangiate-laterals, solitary or grouped, borne terminally or on an enlarged terminal head-cell of a branched sporangiophore. Gametangia are terminal only. An alternation of isomorphic generations is indicated for *Trentepohlia* (Thompson and Wujek 1997). In the most recent taxonomic account of *Trentepohlia* species (Printz (1939), twenty-seven species of *Trentepohlia* are retained after transferring some taxa to the genus *Printzina* by Thompson and Wujek (1997).

*T. dialepta* (Nylander) Hariot; *T. calamicola* (Zeller) de Toni et Levi; *T. abietina* (Flotow) Hansgirg; *T. jucunda* (Cesati) Hariot; *T. cucullata* De Wildeman; *T. annulata* Brand; *T. elongata* (Felies) de Toni; *T. arborum* (C. A. Agardh) Hariot; *T. negeri* Brand; *T. uncinata* Gobi) Hansgirg; *T. aurea* (Linnaeus) Martius; *T. villosa* (Kützing) Hariot; *T. jolithus* (Linnaeus) Wallroth; *T. umbrina* (Kützing) Bornet; *T. odorata* (Wiggers) Wittrock; *T. diffracta* (Krempelhuber) Hariot; *T. rigidula* (Muller) Hariot; *T. lepreiurii* Hariot; *T. depressa* (Muller) Hariot; *T. ellipsiocarpa* Schmidle; *T. cyanea* Karsten; *T. minina* Schmidle; *T. tentaculata* (Hariot) de Wildeman; *T. peruana* (Kützing) Printz; *T. willei* (Tiffany) Printz; *T. bogoriensis* de Wildeman; *T. prolifer* de Wildeman.

*Printzina* Thompson et Wujek 1992

*Printzina*, in honor of Prof. H. Printz, is remarkably similar to *Trentepohlia* (see above), the major basis for its erection are a) the presence of globular to reniform sporangia, and b) an almost pseudoparenchymatous thallus with few or any upright filaments. Sporangiate-laterals are solitary and sessile on prostrate filaments or erect filaments. An alternation of isomorphic generations has been described for *Printzina*.
(Thompson and Wujek 1997). The only taxonomic treatment of this genus is Thompson and Wujek (1992), which recognized nine species

\[ P. \ \text{lagenifera} \ (\text{Hildebrand}) \ \text{Thompson} \ \text{and Wujek}; \ P. \ \text{ampla} \ \text{Thompson} \ \text{and Wujek}; \ P. \ \text{bossei} \ (\text{De Wildeman}) \ \text{Thompson} \ \text{and Wujek}; \ P. \ \text{diffusa} \ (\text{de Wildeman}) \ \text{Thompson} \ \text{and Wujek}; \ P. \ \text{dusenii} \ (\text{Hariot, Wittrock et Nordst}) \ \text{Thompson} \ \text{and Wujek}; \ P. \ \text{effusa} \ (\text{Krempehüber}) \ \text{Thompson} \ \text{and Wujek}; \ P. \ \text{lagerheimii} \ (\text{de Wildeman}) \ \text{Thompson} \ \text{and Wujek}; \ P. \ \text{santurcensis} \ (\text{Tiffany}) \ \text{Thompson} \ \text{and Wujek}. \]

**Physolinum** H. Printz 1921

The species type for the genus *Physolinum* was originally described as *Trentepohlia monile* De Wildeman, later the specific epithet was changed to *T. moniliformis* Karsten. Printz (1921) erected the genus *Physolinum* based on his discovery of aplanospores as the sole reproductive structures found in this alga, and hence concluding that his material was markedly different from *Trentepohlia*. More recently, Davis and Rands (1993) reestablished this genus when studying samples, both free-living and lichenized, of an alga from central Missouri and concluding that the presence of aplanospores, absence of plasmodesmata in the cross walls and absence of flagellated cells, supported the assignment of the alga to the genus *Physolinum*. Only one species is known. *Physolinum monile* (De Wildeman) Printz.

**Phycopeltis** Millardet 1870

The filaments of *Phycopeltis* grow openly or form a pseudoparenchymatous thallus, but always superficial upon the plant host or other surfaces. Sporangiate-laterals are solitary, sessile to terminal on erect filaments. Another feature distinguishing
Phycopeltis from other superficial trentepohlialean genera (Trentepohlia, Physolinum, Printzina, and Cephaleurus) is the papilla-pore terminal on the sporangium, which is opposite to the end of attachment (Thompson and Wujek 1997) whereas, in the other genera it is basal and adjacent to the area of attachment. An alternation of isomorphic generations is described for Phycopeltis (Thompson and Wujek 1997). The modern taxonomic treatment of this genus (Thompson and Wujek 1997) reports 18 species

P. amboinensis (Karsten) Printz; P. arundinacea (Mont.) de Toni; P. aurea Karsten; P. costaricensis Thompson and Wujek; P. dorsopapillosa Thompson and Wujek; P. epiphyton Millardet; P. flabellata Thompson and Wujek; P. irregularis (Schmidle) Wille; P. minuta Thompson and Wujek; P. novae-zealandiae Thompson and Wujek; P. parva Thompson; P. pilosa Thompson and Wujek; P. pseudotreubii Thompson and Wujek; P. terminopapillosa Thompson and Wujek; P. treubii Karsten; P. treubioides Thompson and Wujek; P. umbrina (Kützing) Thompson and Wujek; P. vaga Thompson and Wujek.

Cephaleuros Kunze in Fries 1829

This genus is usually reported as an obligate epiphyte and subcuticular, that may be parasitic. The prostrate portion can be open-filamentous to pseudoparenchymatous. Sporangiophores bear one or more head cells subtending sporangiate-laterals. Cephaleuros is one of the most studied genera among the Trentepohliales, in part for its worldwide distribution, in part for the obvious presence and sometimes economic damage to their host. An alternation of heteromorphic generations is describe for Cephaleuros (Thompson and Wujek 1997) with the sporophyte reduced to a dwarf plant
(the stalk cell, head cell, and one or more suffultory cells, and the meiosporangia). The modern treatment of this genus by Thompson and Wujek (1997) described 13 species.

*C. biolophus* Thompson and Wujek; *C. diffusus* Thompson and Wujek; *C. druetii* Thompson; *C. expansa* Thompson and Wujek; *C. henningsii* Schmidle; *C. karstenii* Schmidle; *C. lagerheimii* Schmidle; *C. minimus* Karsten; *C. parasiticus* Karsten; *C. pilosa* Thompson and Wujek; *C. solutus* Karsten; *C. tumidae-setae* Thompson and Wujek; *C. virescens* Kunze in Fries.

*Stomatochroon* Palm emend. Thompson and Wujek 1997

This trentepohlialean alga grows as a branching system of filaments endophytically in the substomatal chamber and through the intercellular spaces of the host, protruding its sporangiophores through the stomata. The reduced morphology of this alga is extraordinary, one species has become reduced to a single massive and lobed anchoring cell. An alternation of heteromorphic generations is described for *Stomatochroon* (Thompson and Wujek 1997), with the sporophyte consisting of either a sporangiate lateral alone, or accompanied by a stalk cell. Since this taxon was originally described by Palm (1934) from a single species, the addition of three more new species to this genus warranted Thompson and Wujek (1997) to perform an amendment of the original generic description.

*S. lagerhaimii* Palm; *S. consociatus* Thompson and Wujek; *S. coalitus* Thompson and Wujek; *S. reniformis* Thompson.

**Hypotheses on Molecular Systematics and Phylogeny.** Based on ultrastructural and cytological characters, conflicting views have been hypothesized about the systematic position of the Trentepohliales among the several classes of green
algae. Taxonomic features such as the counter-clockwise flagellar apparatus components (Roberts 1984) can be cited as evidence for an affinity to the Ulvophyceae sensu Mattox and Stewart (1984). The presence of multilayered structures (MLSs) in the flagellar apparatus and the demonstration of a phragmoplast-type cytokinesis in Cephaleuros parasiticus (Chapman and Henk 1986) suggest affinity with the Charophyceae. There is also evidence that a similar phragmoplast-type cell division occurs in Trentepohlia sp. (Boullion 1985, unpubl. observations; Waters et al. 1998). Raven (1987) classified the Trentepohliales among the Pleurastrophyceae based on biochemical, biophysical, and physiological features. Moreover, the Trentepohliales share an ultrastructural feature (presumptive mating structures or PMSs in the gametes) with members of Chlorophyceae (Chapman and Henk 1983, 1985). Consequently, the Trentepohliales have been placed in three out of the five major classes of green algae according to the system proposed by Mattox and Stewart (1984), and the order still remains incompletely characterized for phylogenetic purposes (O’Kelly and Floyd 1990).
CHAPTER 2
PHYLOGENETIC AFFINITIES OF THE TRENETEPOHLIALES INFERRED FROM SMALL SUBUNIT RIBOSOMAL DNA
INTRODUCTION

The green plants are an assemblage of highly diverse organisms, including the green algae and land plants. Recently, molecular studies have challenged the concept of the green algae as a natural group (for a review see Chapman et al. 1999; Waters and Chapman 1996). Molecular evidence consistently supports the monophyly of the green algae plus land plants, forming the group Viridiplantae (Cavalier-Smith 1981) or Chlorobionta (Bremer 1985). It is important to note that although Euglenophyta and Chlorarachniophyta have chlorophylls a and b, they are not considered green algae (Gilson and McFadden 1997, 1999; Ishida et al. 1999, Lipscomb et al. 1998).

Current reviews (e.g. Chapman et al. 1999; Waters and Chapman 1996) of the analyses of the nuclear-encoded small subunit ribosomal DNA (SSU rDNA) as well as the chloroplast-encoded large subunit rubisco gene ($rbcL$) provide support for the original suggestion, based on ultrastructural data, of two main lineages among the green plants (Pickett-Heaps and Marchand 1972). One of the lineages comprises the charophycean algae and their descendents, the land plants, forming a monophyletic group named Streptophyta. The charophycean algae include at least five orders: the Chlorokybales, Klebsormidiales, Zygnematales, Coleochaetales, and Charales. The second lineage consists exclusively of the remaining green algae, forming the monophyletic group Chlorophyta (Friedl 1997; Melkonian et al. 1995). The term "chlorophyte" has often been used to denote all green algae; however, it should now be used only as an informal designation for green algae in the Chlorophyta.

Assignment of orders and classes of green algae in a classification system reflecting evolutionary relationships has been one of the goals of phycologists;
however, the high degree of morphological diversity in such a group challenged earlier attempts at inferring an evolutionary hierarchical classification.

Mattox and Stewart (1984) proposed one of the most widely accepted systems of classification of green algae. These authors analyzed the ultrastructural data for flagellated cells as well as for types of cell division accumulated in the previous 20 years. They proposed a system with five classes: Micromonadophyceae, Pleurastrophyceae, Ulvophyceae, Chlorophyceae, and Charophyceae. The class Micromonadophyceae included, among others, *Micromonas*, *Pedinomonas*, *Pyramimonas* and *Scourfieldia*. This classification was described as "more natural than any previous system" by Mattox and Stewart (1984). However, the same authors were inclined to consider the Micromonadophyceae as an unnatural group because it was defined by primitive features. Charophycean taxa were represented by the orders Chlorokybales, Klebsormidiales, Coleochaetales, Zygnematales, and Charales. The class Ulvophyceae included all of the coenocytic or siphonous forms (except coenocytic members of the Chlorococcales and genera of the Sphaeropleaceae), marine species of *Ulothrix* and those freshwater species of *Ulothrix* that have codiolum stages or other ulvophycean features, any other genera that produce a codiolum stage, all marine branched filaments that were formerly classified in the Chaetophorales, genera usually included in the Ulvales (except *Schizomeris*), the Trentepohliaceae, *Pseudendoclonium*, *Trichosarcina*, *Pseudendocloniopsis*, *Ctenocladus*, and *Smithsoniella*. The Pleurastrophyceae of Mattox and Stewart (1984) consisted of Tetraselmidales and Pleurastrales, and the Chlorophyceae included Chlamydomonadales, Volvocales, Chlorococcales, Sphaeropleales, Chlorosarcinales, Chaetophorales, and Oedogoniales.
The system proposed by Mattox and Stewart (1984) is often the starting point used for modern discussions of green algal systematics and evolution (McCourt 1995), and its importance lies in the fact that it is based on correlated characters on which phylogenetic predictions can be made (Waters and Chapman 1996).

In the original paper by Mattox and Stewart (1984) the order Trentepohliales was included in the class Ulvophyceae, but was not discussed by the authors. Members of the Trentepohliales (Chlorophyta) are most abundant in tropical and subtropical regions worldwide. A distinct assemblage of green algae characterized by special adaptations to subaerial habitats, the group includes vascular plant epiphytes, some of which have been thought to be economically important, such as *Cephaleuros virescens* which is a parasite on tea leaves (Chapman 1984).

Conflicting views have been hypothesized about the systematic position of the Trentepohliales among the several classes of green algae. The presence of multilayered structures (MLSs) in the flagellar apparatus and the demonstration of a phragmoplast-type cytokinesis in *Cephaleuros parasiticus* (Chapman and Henk 1986) and *Trentepohlia odorata* (Waters et al. 1998) suggest an affinity with the class Charophyceae. However, taxonomic features of the Trentepohliales such as the counterclockwise flagellar apparatus components can be cited as evidence for an affinity with the Ulvophyceae (Roberts 1984). Based on biochemical, biophysical, and physiological features, Raven (1987) classified the Trentepohliales among a third class, the Pleurastrophyceae. Moreover, it was noted that the Trentepohliales even share a rare ultrastructural feature (presumptive mating structures or PMSs in the gametes) with members of a fourth class, the Chlorophyceae (Chapman and Henk 1983, 1985).
Therefore, the Trentepohliales exhibit some features associated with four of the five major classes of green algae in the system proposed by Mattox and Stewart (1984); but the major discussions have been focused on ulvophycean versus charophycean affinities of this enigmatic order. The order remains incompletely characterized for phylogenetic purposes (O’Kelly and Floyd 1990).

Where does the Trentepohliales belong in the various proposed classification schemes of green algal classes? What are the closest relatives of the Trentepohliales? In this study we addressed these questions by using phylogenetic analysis of the SSU rDNA gene sequences from several genera of Trentepohliales and representatives of the major classes and orders of Chlorophyta.

MATERIALS AND METHODS

Taxa. Unialgal cultures of Trentepohliales were grown in liquid and/or agarized MWH media (Nichols 1973) under continuous cool white fluorescent light. Trentepohlialean taxa included in this study were Cephaleuros parasiticus Karsten, Cephaleuros virescens Kunze in Fries, Trentepohlia sp., Trentepohlia dialepta (Nylander) Hariot, and Physolinum monile (De Wildeman) Printz. Species included for comparison and their GenBank accession numbers or reference are shown in Table 2.1.

DNA Extraction. Total cellular DNA was extracted from trentepohlialean samples as follows. Aliquots of unialgal cultures were rinsed with Tris-HCl 0.5M pH 7.2. Cells were disrupted by grinding in liquid nitrogen with a chilled mortar and pestle. Samples were treated as described in the Dneasy plant mini handbook for DNA isolation from plant tissue, Dneasy Plant Mini Kit (QIAGEN, Inc. Valencia, CA). The nucleic acid pellet was dissolved in sterile distilled water. DNA samples were stored at 37°C.
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(Zechman et al. 1990)
-20°C. The integrity of DNA preparations were checked by electrophoresis in agarose gels stained with ethidium bromide.

**PCR Amplification and Sequencing.** DNA amplification via polymerase chain reaction of the nuclear-encoded SSU rDNA gene was performed by using the primers designed originally for both green algae and land plants (Hamby et al. 1988). These primers were designed based on the conserved region from the SSU rRNA and have been shown to amplify rRNA genes from other green algae, bryophytes, and lower vascular plants (Chapman and Buchheim 1991). Template DNA was amplified in 0.2 mL thin-wall PCR tubes (DOT Scientific Inc., Burton, MI) in a total reaction volume of 50 µL. Each amplification reaction consisted of 2 µL of template DNA, 2 µL of two flanking primers, 5 µL of MgCl₂ (25 mM), 5 µL of Thermophilic DNA Polymerase 10X buffer (Promega Corp., Madison, WI), 4 µL of 1 mM deoxynucleotide mix (containing dATP, dTTP, dCTP, and dGTP), 0.4 µL of *Taq* DNA polymerase in storage buffer B (Promega Corp., Madison, WI), and sterile water. Negative controls without DNA template were included. Amplification cycles were controlled in a GeneAmp PCR System 2400 (Perkin-Elmer) thermocycler, with an initial denaturation step at 95°C for 3 min, followed by 30 cycles of 1 min at 95°C, primer annealing at 52°C for 1 min, and extension at 72°C for 2 min. Amplified products and a standard 1Kb DNA ladder (Life Technologies, Inc. Gaithersburg, MD) were visualized by electrophoresis on 0.8%
agarose gels stained with ethidium bromide for correct length, yield, and purity. DNA products were purified with Millipore UFC3 TTK 00 (30,000 NMWL) filter units (Millipore Corporation, Bedford, MA) with an Eppendorf 5415 C table-top microcentrifuge. Purified DNA amplification products were sequenced with internal SSU primers using the protocol of the DNA Sequencing Kit, Big-Dye Terminator Cycle Sequencing Ready Reaction ABI PRISM (Perkin Elmer Applied Biosystems, Foster City, CA) in an ABI 377 Prism Automated Sequencer. DNA sequences were captured in text as well as color-coded electropherograms.

**Sequence Alignment and Data Analysis.** Sequence data were converted using ReadSeq with the Pearson/Fasta output format available at http://dot.imgen bcm.tmc.edu:9331/seq-util/readseq.html (Baylor College of Medicine, Houston, TX). Converted sequences were submitted for automatic multiple alignment to MultiAlin, multiple sequence alignment program (Corpet 1988) available at the internet site: http://W3.toulouse.inra.fr/lgc/multalin/multalin.html (Laboratoire du Génétique Cellulaire, Institut National de la Recherche Agronomique, Toulouse, France). The SSU sequences were manually adjusted by using SeqPup v. 0.6, available at ftp://iubio.bio.indiana.edu/molbio/seqpup/. All analyses were performed using a G3 Macintosh computer (Apple Computer Inc., Cupertino, CA). Regions in the data matrix that could not be unambiguously aligned were excluded from the analyses. A total of 106 green plant and 3 outgroup taxa (glaucocystophytes) were used to construct a general data matrix. A subset with 1733 positions was used as input for distance and maximum parsimony analyses of fifty-one taxa of Viridiplantae with three glaucocystophyte taxa as the outgroups to position the Trentepohliales among the
Viridiplantae. A more restricted subset from the large matrix containing twenty-three taxa of ulvophycean algae, including representatives of all the orders, was used as input of distance, maximum parsimony, and maximum likelihood analyses to evaluate the position of the Trentepohliales within the ulvophycean algae. Distance analyses were performed with the PAUP* 4.0 package (Swofford, 1999). The two-parameter model of Kimura (1980) was used to generate distance matrices, which were converted to phylogenetic trees with the neighbor joining method (Saitou and Nei 1987). Maximum parsimony analyses were performed in PAUP* 4.0 with the heuristic search option with a branch-swapping algorithm (tree bisection-reconnection) and random sequence addition (100 replicates). For Maximum Likelihood analyses, PAUP* 4.0 program was used. Bootstrap analyses were performed in PAUP* 4.0 to assess the stability/support of nodes with 100-500 replications for maximum parsimony and neighbor-joining analyses.

RESULTS AND DISCUSSION

To elucidate the overall position of the Trentepohliales in a nuclear-encoded SSU rDNA phylogeny of the Viridiplantae, a global analysis was performed with a subset of 53 taxa (Fig. 1) and 1733 (equally weighted) aligned characters. Two glaucocystophytes (*Cyanophyceae gloeocystis* and *Glaucocystis nostochinearum*) were included as the outgroup. Fifty-one green plants were included as representatives of the Viridiplantae, viz., from the charophycean lineage (Charophyceae and Embryophyta), as well as from the chlorophycean lineage. Both the distance and maximum parsimony analyses positioned the trentepohlialean taxa unequivocally within the chlorophycean lineage, the Chlorophyta. The Chlorophyta lineage forms a monophyletic group
(bootstrap support >82%). Present results based on nuclear-encoded SSU rDNA confirm the previous reports from both SSU data and chloroplast-encoded LSU rubisco data on the existence of two lineages of Viridiplantae. The land plants within the Streptophyte clade, diverged from the Charophyceae as expected from previous reports. As predicted by Mattox and Stewart (1984) the micromonadophycean taxa (*Mantoniella* spp., *Micromonas pusilla*, *Pycnococcus provasoli*, and *Pseudoscourfieldia marina* and *Mamiella* sp.) are not a natural or monophyletic group but rather a series of basal divergences forming a grade. This paraphyletic group is also known as Prasinophyceae, and the use of this term has been recommended by Sym and Pienaar (1993). Similar results supporting the paraphyletic nature of this group have been reported (Nakayama et al. 1998, Fawley et al. 1999). Our results in the clade formed by *Pseudoscourfieldia marina* and *Pycnococcus provasolii* (bootstrap support 100%) confirm the recent study by Fawler et al. (1999) using 18S rDNA sequence data in grouping these two taxa in one family, Pycnococcaceae. Furthermore, recent rDNA and actin analysis of *Mesostigma* (not shown on fig. 2.1) has placed this taxon in the charophycean lineage (Melkonian et al. 1995, Bhattacharya et al. 1998), thus indicating polyphyly for the algae we call the prasinophytes. Therefore it may be time to end the use of "Prasinophyceae" or "Micromonadophyceae," and maybe even the very convenient term "prasinophytes." In the absence of new names for the seven distinct lineages, the term (prasinophytes) will be used in this report. In general, the prasinophytes are considered as the modern representatives of the earliest green algae (Graham and Wilcox 2000). Despite the fact they are not a monophyletic group, their basal position is well supported.
Fig. 2.1. Phylogeny of the Viridiplantae based on nuclear-encoded SSU rDNA gene sequences comparisons. Tree inferred from neighbor-joining and maximum parsimony analysis by using a total of 1733 aligned positions. This phylogenetic tree corresponds to the bootstrap of the neighbor-joining analysis. Maximum parsimony tree with a length of 3682 and a Cl 0.427. The phylogeny is rooted with the glaucocystophyte taxa *Glaucocystis nostochinearum* and *Cyanopythyc glaucoctyis*. * different topology in the parsimony. Bootstrap values of distance (neighbor-joining number above the nodes) and maximum parsimony analysis (number below the nodes) using an identical data set are indicated (only values >50% were recorded). A = Chlorophyceae, B = Prasinophyceae, C = Trebouxiophyceae, D = Trentepohliales, E = Ulvophyceae, F = Streptophyta, and G = Outgroup.
The representatives of the ulvophycean taxa in this study consistently form a monophyletic group (bootstrap support >72%) that includes the taxa of the order Trentepohliales. This clade is the sister group of the remaining green algae, the Chlorophyceae and the Pleurastrophyceae. At present there is no strong support for the recognition of the monophyletic nature of the Chlorophyceae and the Pleurastrophyceae sensu Mattox and Stewart. The Pleurastrophyceae of Mattox and Stewart has been shown to be a polyphyletic group (Friedl and Zeltner 1994). Friedl (1995) erected a new class name, the Trebouxiophyceae, to include many coccoid green algae that completely lack a motile stage (autosporic coccoids) and members of the Microthamniales (sensu Melkonian [1982, 1990a] or Pleurastrales sensu Mattox and Stewart [1984]) based on rDNA sequence comparisons (Friedl 1995, 1997).

The Chlorophyceae analyzed in the present study formed a well-supported clade and comprise two distinct monophyletic lineages defined by ultrastructural details of the flagellar apparatus: one group (Chlamydomonas reinhardtii, Volvox carterii, Gongrosira papuasica, Hydrodictyon reticulatum, Botryococcus braunii, Protosiphon botryoides, and C. moewusii) with a clockwise basal body configuration (CW group) and the other group (Scenedesmus obliquus, Neochloris aquatica, and Pedistrum duplex) with directly opposed basal bodies (DO group) (Lewis et al. 1992, Nakayama et al. 1996).

In the present study, ulvophycean taxa formed a monophyletic group in all analyses. Distance methods and maximum parsimony analysis consistently positioned the taxa of Trentepohliales within this ulvophycean clade. The monophyly of the Trentepohliales is not surprising since some features such as the sporangium-associated
apparatus and the flagellar apparatus are unique for this order. An affinity with the Pleurastrophyceae (Trebouxiophyceae) as suggested by Raven (1987) is not supported. An ulvophycean affinity for the Trentepohliales has been expressed before (Roberts 1984) based on ultrastructural features of the flagellar apparatus. Both ulvophycean and Trentepohliales taxa share a counterclockwise basal body configuration as well as an alternation of generations. Based on preliminary partial nuclear-encoded SSU rDNA sequence data Zechman et al. (1990) also related the Trentepohliales to the Ulvophycean clade. In the 18S rDNA trees the genus Cephaleuros appears nonmonophyletic, however in the analysis of the internal transcribed spacer regions (ITS) of the same gene (not shown) this genus forms a well supported clade.

One question arising from these results is how to explain the presence of the phragmoplast in Cephaleuros (Chapman and Henk 1986). The presence of a phragmoplast-type cytokinesis is well documented in some charophycean algae and is the typical mode of cytokinesis in land plants. The presence of a phragmoplast-type cytokinesis in the chlorophycean lineage raises a question on the "homology" of this process: Is it possible that the phragmoplast evolved more than once? It is difficult to understand how a highly sophisticated cytological process probably involving more than one gene evolved in two different lineages. However, a recent immunofluorescence study in Trentepohlia odorata (Waters et al. 1998) has confirmed the ultrastructural evidence (Chapman and Henk 1986) of the phragmoplast. The authors believe that the answers for the enigmatic presence of a phragmoplast in the Trentepohliales in the ulvophycean clade will come from a thorough study of the
ultrastructural and immunological analyses of the evolution of the phragmoplast in the basal lineages of the streptophytes as well as in the Trentepohliales.

To elucidate the position of the Trentepohliales within the nuclear-encoded SSU rDNA phylogeny of the ulvophycean green algae, a subset of 27 taxa from the complete data matrix was analyzed with 1729 (equally weighted) characters (Fig. 2). Two trebouxiophycean algae (Myrmecia israelensis and Trebouxia impressa) and two chlorophycean algae (Volvox carterii and Chlamydomonas reinhardtii) were included as the outgroup taxa. Twenty-three algal taxa were included in the ingroup representing most of the major orders of the Ulvophytes.

In all phylogenetic analyses, including neighbor-joining, parsimony, and likelihood methods, the Trentepohliales formed a monophyletic group. This Trentepohliales clade was well supported by bootstrap analysis in the neighbor-joining method (100%), maximum parsimony method (97%) as well as the maximum likelihood approach. The Trentepohliales emerged as a sister group to the clade containing the Siphonocladales/Cladophorales complex and Dasycladales, both of which contain representatives mainly from the marine environment.

In the Mattox and Stewart scheme (1984) the Ulvophyceae were defined in terms of ultrastructural features that make them a group distinct from the chlorophycean and charophycean algae. In the same paper Ulvophycean algae were considered more "advanced" since their vegetative state is non-motile and non-flagellated, and presumably derived from scaly green flagellates. A more detailed study of the Ulvophyceae was presented by O'Kelly and Floyd (1984), defining the Ulvophyceae in terms of ultrastructural, reproductive, and biochemical features. The scheme recognized
Fig. 2.2. Phylogeny of the Ulvophyceae based on nuclear-encoded SSU rDNA analyses by distance, maximum parsimony, and maximum likelihood methods. This phylogenetic tree corresponds to the bootstrap analysis (neighbor-joining) of 29 taxa and 1729 aligned positions. Maximum parsimony tree with a length = 2137 and a CI = 0.647. Maximum likelihood tree with a Ln likelihood = -12660.44857. Tree rooted with two outgroups sets: two chlorophycean taxa (Volvox carterii and Chlamydomonas reinhardtii) and two trebouxiiophycean algae (Myrmecia israelensis and Trebouxia impressa). * different topology in the parsimony/likelihood analysis. Bootstrap values of distance (neighbor-joining; number above the nodes) and maximum parsimony analysis (number below the nodes) using an identical data set are indicated (only values >50% were recorded). A = Outgroup, B = Ulotrichales, C = Ulvales, D = Trentepholiales, E = Siphonocladales/Cladophorales Complex, and F = Dasycladales.
five orders: Ulotrichales, Ulvales, Siphonocladales (including Cladophorales), Dasycladales, and Caulerpales. In this study, the Ulvales and Ulotrichales were considered primitive orders of the Ulvophyceae, and the Siphonocladales, Dasycladales, and Caulerpales, more advanced orders. The authors also implied that the Siphonocladales and Dasycladales are sister groups. This arrangement of orders, as well the relationships of the orders in the Ulvophyceae, were based on a) the absence of quadriflagellated motile cells or modification of the flagellar apparatus, b) development of basal body orientation perpendicular to the long axis of the cell during forward swimming, c) loss of the isomorphic life history, d) an increasing complexity in the zoosporangial and gametangial structure and development, and e) structural and chemical composition of the cell walls. O'Kelly and Floyd (1984), although recognizing that the trentepohlialean motile cells are consistent with their definition of the class Ulvophyceae, decided to exclude the order Trentepohliales from the Ulvophyceae because the presence of multilayered structures (MLS) associated with two of the four rootlets, and plasmodesmata in the cross walls between vegetative cells.

Sluiman (1989) considered the Ulvophyceae to be represented by eight orders: Ulotrichales, Ctenocladiales, Trentepohliales, Pleurastrales, Acrosiphoniales, Cladophorales, Bryopsidales, and Dasycladales. His system was based mainly on the ultrastructural features of the flagellar apparatus, de-emphasizing the importance of the cell division at the ordinal level. It is important to mention that this was the first paper to formally assign the order Trentepohliales to the Ulvophyceae. Mattox and Stewart (1984) mentioned the order Trentepohliales in the Ulvophyceae by referring to the ultrastructural studies of Roberts (1984), and O'Kelly and Floyd (1984) did not mention
the order Trentepohliales in their Ulvophycean scheme. However, the Sluiman's concept of the orders in the class Ulvophyceae, acknowledging the ulvophycean nature of the order Trentepohliales, implied that some features such as the cell division may be homoplasious (independently derived), particularly the phragmoplast-type cytokinesis in the overall scheme of the Chlorophytes.

Results from the present study supports two groups within the Ulvophycean algae. The first group is represented by the orders Ulotrichales (Gloeotilopsis planctonica, Pseudendoclonium basiliense, Ulothrix zonata, Monostroma grevillei, and Acrosiphonia sp.) and Ulvales (Ulva rigida and Enteromorpha intestinalis). The second group contains the order Trentepohliales forming a clade which is a sister group of the remaining genera of ulvophycean algae. This latter group comprises the siphonous and semisiphonous ulvophycean algae and forms a monophyletic group. Within this group the Siphonocladales/Cladophorales complex (represented by Siphonocladus tropicus, Valonia utricularis, Ernodesmis verticillata, Cladophora vagabunda, and Chatomorpha sp.) is a sister group to the Dasycladales (Acetabularia major, Polyphysa peniculus, Cymopolia vanbossea, Neomeris dumentosa, and Batophora occidentalis). Our results confirm previous analyses of partial nuclear-encoded SSU rDNA sequences (Zechman et al. 1990) suggesting a close relationship between the orders Ulvales and Ulotrichales, as well as the relationships among the Siphonocladales/Cladophorales complex, Dasycladales and Trentepohliales. However, Zechman et al. (1990) reported nonmonophyly for Ulvophyceae. The two groups of ulvophytes, that is, the Ulvales + Ulotrichales and Siphonocladales/Cladophorales + Dasycladales ("primitive" versus "advanced" orders sensu O'Kelly and Floyd 1984) were separated by the
Pleurastrophyceae and Chlorophyceae. However, Zechman et al. (1990) also noted that these "intermediate clades" (chlorophycean and pleurastrophycean algae) were resolved by just a few evolutionary steps and were susceptible to rearrangement by changes in taxon and/or character sampling. Our results do not support the concept of Sluiman (1989) that includes representatives of the Pleurastrales (pleurastrophycean algae sensu Mattox and Stewart 1984) within the Ulvophyceae. Similar results based on cladistic analysis of nuclear rDNA sequence data have been reported by Kantz et al. (1990) and reviewed by Chapman et al. (1999).

In conclusion, our study provides robust support to position the order Trentepohliales within the ulvophycean algae in the chlorophycean lineage. Our results clearly indicate the "advanced" marine taxa (orders Siphonocladales/Cladophorales complex and Dasycladales) are the most closely related to the trentepohlialean algae. Our analyses strongly support the monophyly of the class Ulvophyceae. The evolutionary implications of these results for the origin of the Trentepohliales indicate that they presumably diverged from a ulvophycean-like macroscopic filamentous marine ancestor. The presence of the phragmoplast-type cytokinesis in the order Trentepohliales remains enigmatic, but if such a system evolved in the freshwater Streptophyta lineage, there is no reason why an almost identical system could not have evolved in the marine portion of the Chlorophyta lineage. Finally, it is interesting to mention that in both lineages, the phragmoplast-mediated cytokinesis is associated with terrestrial (subaerial) habits.
CHAPTER 3
CONTINUOUS MITOCHONDRIAL LARGE SUBUNIT RIBOSOMAL RNA IN
CEPHALEUROS PARASITICUS KARSTEN
INTRODUCTION

The order Trentepohliales consists of subaerial green algae growing on humid soil, rocks, tree bark and leaves; others are endophytic and/or parasitic in leaves, stems and fruits; and still others form lichens. Some representatives of Trentepohliales develop a filamentous structure that forms uniseriated, branched, erect tufts, or laterally coherent, prostrate discs. Some other forms are highly reduced and show a short vegetative filament only a few cells in length. The cells of trentepohlialean taxa are uninucleated or multinucleated, with several parietal, discoid, or band-shaped chloroplasts. Reproduction occurs by asexual, quadriflagellated zoospores or sexual, biflagellated gametes. An incompletely documented life history has been proposed, consisting of an alternation of isomorphic or heteromorphic generations (Thompson 1961, Bourrelly 1966, Silva 1982, Sluiman 1989, O’Kelly and Floyd 1990, Chapman and Buchheim 1991).

The main characteristics of the order Trentepohliales include: a) reproductive cells which differ from the vegetative cells morphologically and in having a unique, zoosporangial abscission process, b) presence of a β-carotene, hematohrome, coloring the algal thallus yellow, orange, or red, c) absence of both pyrenoid and starch in the parietal chloroplasts, d) a unique flagellar apparatus, and f) transverse cell walls with centrally located plasmodesmata.

Taxonomically, the order Trentepohliales is represented by one family, Trentepohliaceae. The family name has been cited also as “Chroolepidaceae” or “Chroolepaceae;” however, Chapman (1984) documented the appropriate use of Trentepohliaceae over the other names.
The phylogenetic position of this enigmatic group remains questionable. On the basis of the counterclockwise flagellar apparatus (Chapman and Henk 1983, Roberts 1984), as well as on the phylogenetic analysis of gene sequences of the nuclear-encoded rDNA (López-Bautista and Chapman in preparation), this group was assigned to the class Ulvophyceae. However, the phragmoplast-type cell division reported for *Cephaleuros parasiticus* (Chapman and Henk 1986) as well as for *Trentepohlia odorata* (Waters et al. 1998) relates this order to the charophycean algae which, along with the land plants, possess this elaborate type of cytokinesis. In the present investigation, the mitochondrial large subunit ribosomal RNA (mtLSU rRNA) from a representative of the order Trentepohliales is compared with previous reports of other green algae to elucidate its phylogenetic relationship.

Ribosomal RNAs (rRNAs) are essential components for the structure and function of ribosomes in all prokaryotes and in the cytosol and cell compartments of all eukaryotes. The ribosome is a compact particle (ribonucleoprotein) consisting of two subunits, one small and one large subunit (Lewin 1997). Most rRNAs are continuous molecules, but some examples are known wherein the polyribonucleotide chain is split (discontinuous) rather than continuous. Discontinuous large subunit (LSU) rRNAs have been documented for mitochondrial, chloroplast, and nucleocytosolic compartments of eukaryotes and in eubacteria (Nedelcu et al. 1996, Nedelcu 1997a, b).

Both continuous and discontinuous mitochondrial LSU rRNAs (mtLSU rRNAs) have been reported in green algae. The phylogenetic distribution of these two types of mtLSU rRNA in green algae has been shown to be consistent with phylogenetic relationships previously suggested by both ultrastructural data and nuclear rRNA
sequences (Nedelcu et al. 1996). Discontinuous mtLSU RNAs have been found in zoosporic chlorophycean lineages (*Chlamydomonas reinhardtii, Polytomella agilis, and Carteria crucifera*) with a clockwise (CW) flagellar apparatus configuration, chlorophycean taxa whose quadriflagellated zoospores exhibit a directly opposed (DO) configuration in their flagellar apparatus (*Neochloris aquatica, Hormotilopsis gelatinosa, Planophila terrestris*), as well as an autosporic chlorococcalean relative (*Scenedesmus obliquus*). In contrast, continuous mtLSU RNAs have been reported in all the investigated zoosporic green algae with a counterclockwise (CCW) orientation (*Hafniomonas montana, Pyramimonas parkae, and Pleurastrum terrestre*) and in related autosporic chlorococcalean species (*Chlorella vulgaris, Prototheca wickerhamii*). Continuous mtLSU rRNA was also reported in *Uronema belkae*, a chlorophycean lineage the zoospores of which have a flagellar apparatus including DO upper basal bodies and lower basal bodies in the CW orientation (Nedelcu et al. 1996). Therefore, mtLSU rRNA type could be an useful phylogenetic character for resolving conflicting inferences of relationships such as the placement of the Trentepohliales among green algal lineages. If the Trentepohliales are ulvophycean algae, one would predict that they would have continuous mtLSU rRNA. Given the unique features of the Trentepohliales and unique mixture of phylogenetically useful features in this order, characterization of the mitochondrial rRNA was undertaken to further document the features of this enigmatic order.

**MATERIALS AND METHODS**

**Source and Growing Conditions.** *Cephaeleuros parasiticus* Karsten was originally collected on the campus of Louisiana State University, Baton Rouge, LA,
USA. Specific identifications are based on previously published descriptions and diagnostic criteria (Thompson and Wujek 1997). Specimens of C. parasiticus were maintained in culture in MBV-medium (Friedl 1989) and illuminated by 75W cool-white fluorescent tubes on a continuous light regimen at room temperature. Axenic cultures were treated with the antibiotics ampicillin and kanamycin at 200 µg/mL and 20 µg/mL, respectively.

**Total RNA Extraction and Fractionation.** Algal filaments were pulverized with a mortar and pestle in liquid N₂ and resuspended in saline-EDTA. Total RNA was extracted as described in Nedelcu et al. (1996). Ten µg of RNA was fractionated by gel electrophoresis in 1.5% agarose, then transferred to a Hybond-N nylon membrane (Amersham).

**Northern blot hybridization.** RNA blots were prehybridized at 37°C with the buffer: 5X SSPE (20X SSPE=3.6 M NaCl, 200 mM NaH₂PO₄, 20 mM EDTA, pH 7.4), 1X BLOTTO (10X BLOTTO = 5% Skim Milk powder, 10% sodium dodecyl sulfate [SDS], pH 7.8) and 50% formamide. To detect putative mitochondrial rRNAs, four synthetic probes were used that are complementary to highly conserved regions within the LSU rRNA. Probe 4 is a 32-mer (5’-CCGAACTTGATTGCTTTCCACCCCTAGCCAC-3’) complementary to the 5’-half LSU rRNA of *Chlamydomonas reinhardtii* mtLSU rRNA. Probe 6 is a 27-mer (5’-GCTGATAAACCTGTTATCCCTAGCGTA-3’) complementary to the 3’-half mtLSU rRNA of *Scenedesmus obliquus*. Probe 7 is a 29-mer (5’-AGGACGCGATGATCCACATCGAGGTGCC-3’) complementary to the 3’-half mtLSU rRNA of *C. reinhardtii*. Probe 8 is a 26-mer (5’-
GGGTCTCTAATCCGGTTCGCTACCCA-3') complementary to the mSSU rRNA of C. reinhardtii. The oligonucleotide probes were 5'-end-labeled using [γ-32P]ATP and polynucleotide kinase (Pharmacia). Hybridization was carried out at 37 °C for 21 h. The blots were washed twice, 15 min each time at room temperature, first in 2X SSPE, 0.1% SDS, then in 0.5X SSPE, 0.1% SDS.

RESULTS AND DISCUSSION

In this study, one species of the order Trentepohliales, Cephalleuros parasiticus, was investigated and compared with previous reports for representatives of three classes of green algae. Figure 3.1 shows the electrophoretic pattern of total RNA fractionated in 1.5% agarose gel and stained with ethidium bromide (EtBr). The ethidium bromide (EtBr) stained gel was loaded with two RNA ladders (first two columns, L1 and L2) in order to compare fragment sizes. The third column represents C. reinhardtii (Cr) and the last column, C. parasiticus (Cp). The most abundant fragments were the cytosolic LSU (3500 nt) and SSU (1500 nt) rRNAs as well as the chloroplast SSU (1500 nt) and LSU rRNA types (δ=1700 nt and γ=810 nt) in Cr, which served both as a reference and as a basis for the identification of its rRNA counterpart, Cp. Although the fragment signals from Cp were less abundant than from Cr, it was possible to detect, in the gel, some of the counterparts for the cytosolic SSU (1800 nt) and LSU (3500 nt) rRNAs in Cp. In the EtBr stained gel it was not possible to observe mitochondrial rRNA due to its low concentration compared with cytosolic and chloroplast rRNAs.

Under the hybridization conditions carried out in this experiment, probes annealed not only with the mitochondrial rRNAs, but also with cytosolic and
Fig. 3.1. Ethidium bromide staining of RNA fractionated on a 1.5% agarose gel. L1 = 0.16-1.77 Kb RNA Ladder; L2 = 0.24-9.5 Kb RNA Ladder; Cr = Chlamydomonas reinhardtii; Cp = Cephaleuros parasiticus.
chloroplast rRNAs. Hybridizing RNAs with no visible counterparts in the ethidium bromide stained gel and no hybridizing counterparts with the cytosolic and chloroplast rRNAs of C. reinhardtii were considered of mitochondrial origin. Only oligonucleotide probes 4 and 8 produced detectable hybridization signals in all of the northern blots analyzed. They are shown in Fig. 2. Probes 6 and 7 were originally designed to target the 3'-half of LSU rRNA of Cr and Scenedesmus obliquus. The successful probes, 4 and 8, were originally designed to target the 5'-half of LSU and SSU rRNA, respectively, of Cr. Figure 3.2 shows the northern blot analysis of total rRNA fractionated in 1.5% agarose gel and hybridized with the oligonucleotide probes. The oligonucleotide probe 8 directed to a region within the SSU rRNA identified a small fragment of 410 nt for Cr that was not detectable in the EtBr gel, corresponding to the mitochondrial rRNA, as well as two abundant rRNA fragments of chloroplast (1700 nt) and cytosolic (3500 nt) origin. The cytosolic fragment was also evident in the lane of Cp. The fragment of mtSSU rRNA with a size of 410 nt corresponds with the fragment S3 of the SSU rRNA reported previously (Nedelcu 1997a, b). The oligonucleotide probe 4 directed to a 5'-half LSU rRNA identified two fragments in the Cr lane corresponding to the LSU rRNA (3500 nt) and the SSU rRNA, both of cytosolic origin. A small fragment of 190 nt was also present, but it was not recorded by the photograph because of its poor signal. It corresponds to the fragmented mitochondrial LSU rRNA reported on previously (Nedelcu et al. 1996, Nedelcu 1997a, b). In C. parasiticus sample the oligonucleotide probe 4 identified clearly a 3 Kb fragment with no visible counterpart on the EtBr stained gel compatible with the expected size of a continuous mitochondrial
Fig. 3.2. Northern blot analysis of total RNA fractionated in 1.5% agarose gels and hybridized with oligonucleotide probes. Cr = Chlamydomonas reinhardtii, Cp = Cephaleuros parasiticus. Only informative bands, taken from several gels, are shown. RNA molecular sizes are expressed in nucleotides.
LSU rRNA. Similar results of a continuous mitochondrial LSU rRNA for other green algae have been reported. A similar band in size (3000 nt) has been reported for *Prototheca wickerhamii* (fig. 1-A6 in Nedelcu 1997a) using northern blots protocols. The detection of a continuous mtLSU rRNA of about 3100-3200 nt in *Hafniomonas montana, Chlorella vulgaris, Pleurastrum terrestre,* and *Pyramimonas parkae* identified the presence of a continuous mitochondrial LSU rRNA in these taxa (Nedelcu et al. 1996). In *Uronema belkae,* the 2900 nt rRNA detected probably represents a smaller continuous mitochondrial LSU rRNA in this species. Taking into account previous analysis (Nedelcu et al. 1996), the mitochondrial LSU rRNA is discontinuous in *Polytomella agilis, Carteria crucifera, Scenedesmus obliquus, Planophila terrestris* and *Hormotilopsis gelatinosa.*

In an evolutionary perspective it has been suggested that the mitochondrial fragmentation pattern can be used as a phylogenetic indicator of relationships among the green algae. Taxa showing a continuous mitochondrial LSU rRNA such as *Pyramimonas parkae* (Prasinophycean or Micromonadophycean [sensu Mattox and Stewart 1984]), the autosporic chlorococcalean species such as *Chlorella vulgaris* and *Prototheca wickerhamii* (Trebouxiophycean algae [sensu Friedl 1995]) also share a counterclockwise (CCW) orientation in their flagellar apparatus (*Uronema belkae* with a continuous mitochondrial LSU rRNA shows a combination of DO upper basal bodies and CW lower basal bodies, and according to O'Kelly et al. [1994] forming a separate chlorophycean lineage).

Taxa showing discontinuous mitochondrial LSU rRNAs are the chlorophycean algae *Chlamydomonas reinhardtii, Polytomella agilis,* and *Carteria crucifera* sharing a
clockwise (CW) configuration of their flagellar apparatus, *Hormotilopsis gleatinosa* and *Planophila terrestris* sharing a directly opposed (DO) configuration in their flagellar apparatus as well as autosporic chlorococcacean species phylogenetically related to this group such as *Scenedesmus obliquus*. Taxa in the class Chlorophyceae are grouped not only by the configuration of the flagellar apparatus (CW and DO), but also by the fragmentation pattern of the mitochondrial LSU rRNA (Nedelcu 1997a, b, 1998) and the phylogenetic analysis of the nuclear-encoded SSU rRNA sequences (Lewis et al. 1992).

In the particular case of *Cephaleuros parasiticus*, a trentepohlialean taxon, a CCW flagellar configuration has been well-documented (Chapman and Henk 1983, Roberts 1984). The presence of a continuous mitochondrial LSU rRNA in *C. parasiticus* supports the previous hypothesis of Nedelcu et al. (1996) that relates all green algal taxa studied with a CCW flagellar configuration to having a continuous mtLSU rRNA. The CCW in the trentepohlialean algae (Chapman and Henk 1983, Roberts 1984) has been cited as an indicator of an affinity with the class Ulvophyceae.

Previous indications of a possible, albeit remote chlorophycean affinity based on the presence of a presumptive mating structures (PMSs) in the gametes of *Chlamydomonas* and the Trentepohliales (Chapman and Henk 1983, 1985) is not supported by the mitochondrial LSU rRNA data.

The presence of a phragmoplast-type cytokinesis reported in the Trentepohliales (Chapman and Henk 1986) suggests an affinity with the charophycean algae. However, a phylogenetic analysis of the nuclear-encoded SSU rRNA (López-Bautista and Chapman, in preparation) of representatives of the Trentepohliales compared with the
major lineages of the green algae (Charophyceae, Prasinophyceae
[ Micromonadophyceae sensu Mattox and Stewart 1984 ], Ulvophyceae,
Treouxiophyceae [ Pleurastrophyceae sensu Mattox and Stewart 1984 ] and
Chlorophyceae ) consistently positioned the Trentepohliales within the class
Ulvophyceae and never in the charophycean clade.

Our results suggest that the basal body configuration of the flagellar apparatus,
as well as the fragmentation pattern of the mitochondrial LSU rRNA in the green algae,
are reliable phylogenetic markers and can be used in assessing phylogenetic affiliations
among the green algae at the class level. Characterization of the mitochondrial LSU
rRNA in charophycean and other ulvophycean algae would be a very appropriate
subject for further examination of the phylogenetic distribution of continuous versus
discontinuous mitochondrial LSU rRNA.
INTRODUCTION

Members of the Trentepohliales, a subaerial green algal order, are basically filamentous in construction, ranging from free branching thalli, to coalesced filaments forming discs, to highly reduced forms. It has been reported (Thompson 1961) that life cycles in this order are diplobiontic, with genera Trentepohlia and Phycopeltis representing an alternation of isomorphic generations, and Cephaleuros and Stomatochroon representing an alternation of heteromorphic generations with a diploid dwarf sporophyte. Meiosis presumably occurs in the zoosporangium of the diploid dwarf thallus during the formation of quadriflagellate “meiozoospores” (Chapman 1984). However, direct support for the presence of an alternation of gametophyte/sporophyte generations has not been confirmed, and except for the discovery of synaptonemal complexes in sporangia of Cephaleuros virescens Kunze in Fries (Chapman and Henk 1981), a sexual life cycle in Trentepohliales remains a matter of speculation.

Approximately 60 species are currently recognized in the Trentepohliales, and warmer climates are generally considered to be the most species-rich. The taxonomy of the Trentepohliales is confused by the high degree of morphological and anatomical variation exhibited within the species. In floristic studies, specimens are often overlooked, resulting in misrepresentation of their geographic distribution. Modern concepts of these taxa are often a matter of speculation, even though some Trentepohlialean species (Cephaleuros virescens and C. parasiticus) adversely affect plants that are of economic value, such as tea, Citrus, Cacao, Inga, and coffee.

An early account of karyology in Trentepohliales (Chowdary 1959) reported on the extremely small dot-like appearance of chromosomes in several unnamed species of
Trentepohlia and in Cephaleuros virescens. Chowdary (1963) reported twenty-two chromosomes for Physolinum monilia (De Wildeman) Printz (for a taxonomical discussion of this species see Chowdary [1963] and Davis and Rands [1993]). Abbas et al. (1964) reported a chromosome count of 18 for Trentepohlia aurea (Linnaeus) Martius. Other karyological studies on Trentepohlialean algae (Jose and Chowdary, 1977 and 1978) reported chromosome numbers for 14 isolates of Cephaleuros solutus Karsten and C. virescens as well as several isolates from nine species of the genus Trentepohlia. The most current and comprehensive list of chromosome numbers in algae summarized the research of the previous 30 years (Sarma 1982). In that review, Trentepohlialean algae (under the order Chaetophorales) were reported as having minute chromosomes in most of the taxa and chromosomal races in species of Trentepohlia and Cephaleuros.

Since karyotypes evolve as chromosomes undergo translocations, inversions, fusions, deletions, and non-disjunction, they are likely to provide information on evolutionary processes and trends (Cole 1990). To take advantage of this information, modern approaches to the karyology of taxonomic groups of green algae, including the use of DNA localizing fluorochromes and microspectrophotometry (e. g., Kapraun 1993), are needed. Almost two decades after Sarma’s summary of 30 years of research (Sarma 1982), it is apparent that the karyological research of Trentepohliales has made little progress.

Microspectrophotometry can be used to quantify fluorescence values (I0), provided that a standard with a known DNA content is used and equivalent staining is obtained in both standard and experimental nuclei (Kapraun and Shipley 1990).
Intensity and consistency of DAPI (4′-6′diamidine-2-phenylindole) staining of green algal nuclei and chicken erythrocytes (RBC) have been demonstrated previously (Kapraun 1993, 1994). Comparison of I_r values for algal samples and RBC have resulted in extrapolation of DNA contents for many groups of algae (Kapraun 1994). For species in which both gametophyte and sporophyte specimens were available, variations in nuclear DNA levels associated with ploidy level differences were observed. In such cases, estimated DNA contents for G2-phase haploid gametophyte nuclei and for G1-phase diploid sporophyte nuclei were essentially identical. In addition, DNA levels for 2 C nuclei closely approximated 50% of the 4 C values (López-Bautista and Kapraun 1995, Kapraun and López-Bautista 1997).

A significant new level of interest in the molecular systematics of green algae has developed during the past decade. Much of this interest is based on the phylogenetic importance of these algae as related to vascular plants. Recently, a monograph of the genera *Cephaluros, Phycopeltis,* and *Stomatochroon* was published (Thompson and Wujek 1997), and the family Trentepohliaceae was placed in a new class, the Trentepohliophyceae (van den Hoek et al. 1995). Our efforts to understand this enigmatic group have emphasized their molecular phylogeny, using phylogenetic analysis of the 18S rRNA, the internal transcribed spacer (ITS) regions of the rRNA gene sequences, the chloroplast *rbcL* gene, and mitochondrial rRNA (Chapman et al., 1995; López et al., 1996, 1997, 1998) and immunofluorescence microscopy (Waters et al., 1998). The present cytogenetic investigation was initiated to provide information on nuclear genome sizes for eight species of the order Trentepohliales with cytophotometry using the DNA-localizing fluorochrome 4′,6-diamidine-2-phenylindole (DAPI) and
It was also hoped that the ploidy levels of the gametophytic and sporophytic strains of *Cephaleuros parasiticus* could be determined robustly to clarify the life cycle of this species.

**MATERIAL AND METHODS**

Source of isolates included in this study are recorded in Table 4.1. Specific identifications are based on previously published descriptions and diagnostic criteria (Karsten 1891; Printz 1921, 1939; Thompson and Wujek 1992, 1997). Specimens of Trentepohliales were maintained in culture in MBV-medium (Friedl 1989), and illuminated by 75W cool-white fluorescent tubes on a continuous light regimen at room temperature. Algal material for microspectrophotometry was fixed in Carnoy’s solution (Kapraun *et al.*, 1992) and stored in 70% ethanol at 4°C. Detailed procedures for use of the DNA-localizing fluorochrome DAPI and requirements for reproducible staining have been described previously (Kapraun *et al.*, 1992) and involve rehydration in water and softening with 5% EDTA. Algal specimens were transferred to cover slips treated with subbing solution, air dried and stained with DAPI (0.5 μg/mL 4′-6′ diamidino-2-phenylindol (Sigma Chemical Co., St. Louis, MO 63178); prepared slides were stored in a refrigerator overnight prior to examination. Cytophotometric measurements were made with an Olympus BH2-RFK fluorescence microscope equipped with a high pressure mercury vapor lamp (HBO, 100W), a 340-nm excitation filter, and a 420-nm suppression filter specific for DAPI-bound DNA emissions (Shihira-Ishikawa 1984).

Microspectrophotometric data for chicken erythrocytes (RBC) with a DNA content of 2.4 pg (Clowes *et al.* 1983) were used to quantify mean fluorescence intensity ($I_f$) values for algal specimens (Kapraun *et al.* 1991). Nuclear DNA contents
Table 4.1. Source of specimens

<table>
<thead>
<tr>
<th>Species</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cephaleuros virescens</em></td>
<td>LSU Campus, Baton Rouge, LA, USA. BR-50</td>
</tr>
<tr>
<td>Kunze in Fries</td>
<td></td>
</tr>
<tr>
<td><em>Cephaleuros parasiticus</em></td>
<td>LSU Campus, Baton Rouge, LA, USA. BR-52</td>
</tr>
<tr>
<td>Karsten (gametophyte)</td>
<td></td>
</tr>
<tr>
<td><em>Cephaleuros parasiticus</em></td>
<td>LSU Campus, Baton Rouge, LA, USA. BR-51</td>
</tr>
<tr>
<td>Karsten (sporophyte)</td>
<td></td>
</tr>
<tr>
<td><em>Trentepohlia arborum</em></td>
<td>Univ. Sao Paulo Campus, Brazil</td>
</tr>
<tr>
<td>(Agardh) Hariot</td>
<td></td>
</tr>
<tr>
<td><em>Trentepohlia dialepta</em></td>
<td>Kampala, Uganda. CCAP 483/2</td>
</tr>
<tr>
<td>(Nylander) Hariot</td>
<td></td>
</tr>
<tr>
<td><em>Trentepohlia aurea</em></td>
<td>Aberystwyth, Dyfed, Wales. UTEX LB 429</td>
</tr>
<tr>
<td>(L.) Martius</td>
<td></td>
</tr>
<tr>
<td><em>Trentepohlia odorata</em></td>
<td>Unknown. CCAP 483/4</td>
</tr>
<tr>
<td>(Wiggers) Wittrock</td>
<td></td>
</tr>
<tr>
<td><em>Trentepohlia sp.</em></td>
<td>Cape Cod, MA, USA. UTEX 1227</td>
</tr>
<tr>
<td><em>Physolinum monile</em></td>
<td>Unknown</td>
</tr>
<tr>
<td>(De Wildeman) Printz</td>
<td></td>
</tr>
</tbody>
</table>

for Trentepohliales specimens were estimated by comparing the $I_f$ values of the RBC standard (Kapraun et al. 1991, 1992, Johnson et al. 1987): $RBC I_f/Trentepohlialean algal I_f = 2.4 pg/ x pg$, where $x$=unknown value. The number of algal nuclei examined in each sample and nuclear genome size (pg) ±SD for each isolate were recorded.

RESULTS AND DISCUSSION

Available data for chromosome numbers reported in 16 species of

Trentepohliales are shown in Table 4.2. Chromosome numbers from Trentepohlialean
taxa range from 12 to 56 (Table 4.2). It has been noted (Jose and Chowdary 1978) that in the case of *Trentepohlia (sensu lato)*, several species have identical chromosome numbers. Conversely, a single species can exhibit “chromosomal” or “cytological” races with several chromosome numbers as in the case of *Trentepohlia aurea*, *Cephaleuros solutus*, and *C. virescens* (Chowdary 1978; Jose and Chowdary 1977).

Taxa in the order Trentepohliales show a discontinuous large-scale variation in chromosome numbers (Fig. 4.1). Chromosome numbers for species of Trentepohliales suggest a basic chromosome complement of N = 6. Chromosome numbers in Trentepohliales (Fig. 1) appear to be arranged in an apparent progression, e.g. 1X=6, 2X=12, 3X=18, 4X=24, 8X=48, and 9X=54, with lower numbers probably representing basic (ancestral) chromosome complements, and higher numbers resulting from polyploidy, which is considered (Cole 1990) common throughout the plant kingdom and is a significant factor in plant evolution. Similar polyploid events have been observed in other green algae, such as in the Cladophorales/Siphonocladales complex and Caulerpales (Kapraun 1993). Chromosome numbers other than whole numbers multiples in Trentepohliales could be the result of a process of loss or gain of chromosomes (aneuploidy) during karyokinesis (Sarma 1982). Aneuploidy events are common and have been reported in other green algae, e.g. order Ulvales and Caulerpales (Kapraun 1993). Thus, karyotype evolution in the order Trentepohliales can be visualized as a complex process involving alternating events of genome doubling (polyploidy) and centric fission and/or fussion events resulting in aneuploidy.

Microspectrophotometry with DNA-localizing fluorochromes has been used previously for karyological studies in green algae (Kapraun 1993, 1994). In the present
Table 4.2. Chromosome numbers reported in some members of the Trentepohliales. * Assignment of 1N or 2N is presumptive as chromosome numbers were reported without reference to confirmed ploidy levels. b As cited by Sarma (1982).

<table>
<thead>
<tr>
<th>Species</th>
<th>1N*</th>
<th>2N*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cephalleuros solutus</em> Karsten</td>
<td>12, 14</td>
<td></td>
<td>Jose and Chowdary 1977</td>
</tr>
<tr>
<td><em>C. virescens</em> Kunze in Fries</td>
<td>18</td>
<td>36 (24,27,32)</td>
<td>Jose and Chowdary 1977</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chowdary 1959</td>
</tr>
<tr>
<td><em>Physolinum monile</em> (de Wildeman) Printz</td>
<td></td>
<td>22</td>
<td>Chowdary 1963</td>
</tr>
<tr>
<td><em>Printzina bossei</em> (de Wildeman) Thompson and Wujek</td>
<td></td>
<td>48</td>
<td>Chowdary 1962 b</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Jose and Chowdary 1978</td>
</tr>
<tr>
<td><em>P. dusenii</em> (Hariot) Thompson and Wujek</td>
<td>18</td>
<td></td>
<td>Chowdary 1962 b</td>
</tr>
<tr>
<td><em>P. effusa</em> (Krempelhuber) Thompson and Wujek</td>
<td>18</td>
<td></td>
<td>Jose and Chowdary 1978</td>
</tr>
<tr>
<td><em>P. lagenifera</em> (Hildebrand) Thompson and Wujek</td>
<td>4</td>
<td></td>
<td>Chowdary 1962 b</td>
</tr>
<tr>
<td><em>P. santurcensis</em> (Tiffany) Thompson and Wujek</td>
<td>16</td>
<td></td>
<td>Jose and Chowdary 1978</td>
</tr>
<tr>
<td><em>Trentepohlia abietina</em> (Flotow) Hansgirg</td>
<td>16</td>
<td></td>
<td>Jose and Chowdary 1978</td>
</tr>
<tr>
<td><em>T. aurea</em> (Linnaeus) Martius</td>
<td>16</td>
<td></td>
<td>Suematu 1960</td>
</tr>
<tr>
<td><em>T. aurea</em> (Linnaeus) Martius</td>
<td>18</td>
<td></td>
<td>Abbas and Godward 1964</td>
</tr>
<tr>
<td><em>T. aurea</em> (Linnaeus) Martius</td>
<td></td>
<td>56</td>
<td>Jose and Chowdary 1978</td>
</tr>
<tr>
<td><em>T. cucullata</em> de Wildeman</td>
<td></td>
<td>48</td>
<td>Jose and Chowdary 1978</td>
</tr>
</tbody>
</table>

(table continued)
T. elongata (Feltes) de Toni 24 Chowdary 1962b
T. jucunda (Cesati) Hariot 42 Jose and Chowdary 1978
T. treubiana de Wildeman 56 Jose and Chowdary 1978
T. umbrina (Kutzing) Bornet 12 Chowdary 1962b
T. uncinata (Gobi) Hansgirg 56 Jose and Chowdary 1978

investigation, DAPI staining resulted in a reproducible, intense, and stable nuclear fluorescence with only slight cytoplasmic interference in the whole mount preparations of Trentepohlialean taxa. A summary of the genome size (pg DNA) estimates from I_r values from the eight species of Trentepohliales included in this study is given in Table 4.3. Comparisons of mean fluorescence intensity (I_r) values of Trentepohlialean nuclear genomes to those of RBC resulted in an estimate of 1.1-7.2 pg of DNA content for the Trentepohliales. The lowest values for nuclear genome size were recorded for species of Trentepohlia, ranging from 1.1-3.0 pg of DNA. Calculated values for isolates of Physolinum monile with a value of 4.1 pg of DNA content closely resemble those for Trentepohlia samples. In contrast, estimated nuclear genome content from isolates of Cephaleuros showed the greatest range and highest values, from 2.0 to 7.2 pg of DNA. By comparison, data indicate 2 C nuclear DNA contents of 0.17-4.9 pg in the Caulerpales (Kapraun 1994), 2.6-4.9 pg in the Siphonocladales (Kapraun and Nguyen 1993), 0.2 to 1.4 pg in the Cladophorales (Hinson and Kapraun 1991; Kapraun & Dutcher 1991), 0.6-1.0 pg in the Ulvales (Kapraun and Bailey 1992), and 0.7 to 2.4 pg in the Dasycladales (Kapraun and Buratti 1998).
Variations in nuclear DNA level associated with ploidy level differences in gametophytic and sporophytic phases in green algae have been demonstrated with microspectrophotometry (Kapraun 1994; Kapraun and Shipley 1990). In the present

![Graph showing chromosome numbers reported in 16 species of Trentepohliales and arranged in a theoretical polyploid sequence based on N=6.]

**Fig. 4.1.** Chromosome numbers reported in 16 species of Trentepohliales and arranged in a theoretical polyploid sequence based on N=6.
### Table 4.3. Nuclear genome size (pg) in species of Trentephliales

<table>
<thead>
<tr>
<th>Species</th>
<th>Number of slides</th>
<th>Number of nuclei</th>
<th>Nuclear genome size (pg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physolinum monile</td>
<td>3</td>
<td>95</td>
<td>4.1 ± 0.9</td>
</tr>
<tr>
<td>Cephaleuros virescens</td>
<td>4</td>
<td>146</td>
<td>2.0 ± 0.8</td>
</tr>
<tr>
<td>Cephaleuros parasiticus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gametophyte</td>
<td>3</td>
<td>105</td>
<td>3.9 ± 1.4</td>
</tr>
<tr>
<td>Cephaleuros parasiticus</td>
<td>4</td>
<td>90</td>
<td>7.2 ± 1.3</td>
</tr>
<tr>
<td>Sporophyte</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trentepohlia arborum</td>
<td>4</td>
<td>110</td>
<td>3.0 ± 0.6</td>
</tr>
<tr>
<td>Trentepohlia dialepta</td>
<td>6</td>
<td>202</td>
<td>2.4 ± 0.5</td>
</tr>
<tr>
<td>Trentepohlia aurea</td>
<td>4</td>
<td>141</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>Trentepohlia odorata</td>
<td>4</td>
<td>147</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>Trentepohlia sp. UTEX 1227</td>
<td>3</td>
<td>93</td>
<td>1.2 ± 0.3</td>
</tr>
</tbody>
</table>

Study, *Cephaleuros parasiticus* exhibited DNA levels for 2 C nuclei in gametophytic phase that closely approximate 50% of the 4 C values in the sporophytic phase (Table 4.3). The diplobiontic life cycle of the Trentephliales is not well documented.

Thompson's (1961) observations on life history of Trentepohlialean algae suggest an alternation of isomorphic generations in the genera *Trentepohlia* and *Phycopeltis*, and an alternation of heteromorphic generations in *Cephaleuros* and *Stomatochroon*, with a dwarf sporophyte. In *Cephaleuros virescens*, a tentative ultrastructural confirmation of Thompson's observations was made by the discovery of synaptonemal complexes in sporangia of this alga (Chapman and Henk 1981), indicating evidence of meiosis and sexual reproduction (Lewin 1997). The change in ploidy levels between gametophyte
and sporophyte of *Cephalaleuros parasiticus* in this study confirmed previous
observations based on culture and ultrastructural studies on the presumed sexual life
cycle in this genus.

Recent molecular phylogenetic studies (López-Bautista et al., 1997, 1998)
consistently align the Trentepohliales with the class Ulvophyceae, in which the typical
life cycle exhibits an alternation of generations without the formation of a resistant
zygote. However, the life cycles of Trentepohalean genera (including *Cephalaleuros
parasiticus*) have never been proved satisfactorily. The original description of *C.
parasiticus* (Karsten 1891) lacks observations on its life cycle. Also, the major
systematic monograph on Trentepohliales (Printz 1939) does not provide information
about an alternation of generations in this species. Thompson’s (1961) undocumented
observations on the life cycle of *C. virescens* describe an alternation of heteromorphic
generations with a diploid dwarf sporophyte. Chapman and Henk (1985) reported the
existence of the distinctly different zoosporangium-producing and gametangium-
producing thalli in *C. parasiticus*. Although both thalli occur on the same leaf, one
bears clusters of zoosporangiate branches that seasonally emerge through the ventral
surface of the leaf, whereas the other thallus type bears gametangia that break through
the dorsal leaf surface. Although gametangia and zoosporangia have not been found on
the same thallus, the sporangia-producing thalli of *C. parasiticus* have not been found to
contain synaptonemal complexes. By contrast, synaptonemal complexes have been
detected in the sporangia of a putative dwarf sporophyte in *C. virescens* (Chapman and
Henk 1981), suggesting that the site of meiosis occurs in the sporangium of the putative
“diploid dwarf sporophyte.”
Although confirmation will require direct culture observations of the life cycle, our present results using nuclear genome quantification with DAPI on the change of ploidy levels in gametophyte and sporophyte isolates for *C. parasiticus* strongly support the observations by Chapman and Henk (1981, 1985). The haploid gametophyte thallus in *C. parasiticus* bears gametangia dorsally, and the diploid sporophyte thallus produces zoosporangia ventrally. In the later case, zoospores are presumably produced by meiosis. Since the original taxonomic description by Karsten (1891) and the monographic treatment of the order by Printz (1939) did not report on the two separate thalli for *C. parasiticus*, and since in all other respects the isolates fit well the description in Karsten (1891) and Printz (1939), we are confident that this isolate belongs to the taxon *C. parasiticus*, and therefore a taxonomic emendation is warranted. Localization and examination of the type specimen would also be appropriate in the completion of this story.

Nuclear genome size in species of Trentepohliales shows a pattern of large-scale DNA variation (Fig. 4.2); this pattern is characterized by discontinuity and a regular progression of values. Previous studies (Kapraun et al. 1988, Kapraun and Gargiulo 1987) have reported similar cases in other green algae, e.g., *Cladophora* and *Codium*. There is considerable evidence that both increase and decrease in DNA content have accompanied speciation and phylogenetic advancement in green algae (Kapraun et al. 1988, Kapraun and Gargiulo 1987), and vascular plants (Rees et al. 1966, Price 1976). Since nuclear genome size data for Trentepohliales are restricted to the eight species reported in this study, any speculation concerning the relationship between genome size and evolution of the order Trentepohliales must be considered tentative. However, the
apparent doubling sequence in nuclear DNA content of Trentepohliales (Fig. 4.2) can be explained involving the karyotype data; there is a large-scale variation in chromosome numbers (from [4]12 to 56) in the 16 taxa reported (Table 4.2). Our hypothesis suggests that the doubling sequence (Fig. 4.2) observed in nuclear DNA content of the Trentepohliales could be the result of variation in chromosome numbers (Fig. 4.1), thus indicating that evolution in the order of the subaerial Trentepohliales has involved polyploidy accompanied by doubling of genome size.

![Graph](image)

**Fig. 4.2.** Relationship of nuclear genome size (pg) estimates to a theoretical doubling sequence standardized to $1X = 1.0$ pg
CHAPTER 5
PHYLOGENY OF THE TRENETEPOHLIALES (CHLOROPHYTA) BASED ON THE CHLOROPLAST \textit{rbcL} GENE SEQUENCES
INTRODUCTION

The order Trentepohliales comprises an enigmatic group of subaerial green algae mainly from tropical and subtropical habitats (Chapman 1984). The trentepohlialean algae are of a recent divergence. Eocene fossils identifiable as Trentepohliaceae have been reported by Tappan (1980). Thompson and Wujek (1997) mentioned the report of Dilcher (1965) and Reynolds and Dilcher (1984) of excellent preserved thalli, sporangiophores, and hairs of Cephaleuros, from leaf compressions from the Eocene 40,000 years ago [sic, the period should be 40 million years ago]. O’Kelly and Floyd (1990), based on cytological and ultrastructural features, suggested that the Trentepohliales are a derived group that has arisen relatively recently.

Ultrastructural studies of Trentepohlia (Graham and McBride 1975, Roberts 1984), Cephaleuros (Chapman 1976, 1980, 1981, Chapman and Henk 1982, 1983), Phycopeltis and Stomatochroon (Good 1978) reported a counterclockwise flagellar (CCW) apparatus configuration in these trentepohlialean algae, which has been cited as evidence for an affinity to the Ulvophyceae (Roberts 1984). Phylogenetic analysis of the nuclear-encoded small subunit ribosomal RNA gene (18S rDNA) (López-Bautista and Chapman, in preparation) also support placing the order Trentepohliales within the ulvophycean algae in the chlorophycean lineage. However, ultrastructural (Chapman and Henk 1986) and immunofluorescence (Waters et al. 1998) studies of cell division confirmed the presence of a phragmoplast-type vegetative cell division in the genera Cephaleuros and Trentepohlia. This type of cell division is typically found in the streptophytes lineage, that is in several charophycean algae and the land plants.
The large subunit ribulose-1,5-bisphosphate carboxylase/oxygenase (rbcL) gene is located in the chloroplast genome in photosynthetic organisms and has been well studied (Kellogg and Juliano 1997) in its structure and interactions with its substrates, CO₂, O₂, and ribulose 1,5 bisphosphate (RuBP). The rbcL gene is relatively stable (Manhart and VonderHaar 1991) at the nucleotide and amino acid sequence levels and it has been extensively used by plant systematics to unravel the phylogenetic history of plants, specially land plants (Olmstead and Palmer 1994). The use of rbcL sequences to the systematic of algae have been applied successfully to broad phylogenetic analysis within Rhodophyta (Freshwater et al. 1994), Phaeophyta (Siemer et al. 1998), and Haptophyta (Daugbjerg and Andersen 1997). In Chlorophyta, however, the rbcL sequences for molecular systematics have been applied only within restricted taxonomic groups such as the Prasinophyceae (Daugbjerg et al. 1995), Zyg nematales (McCourt et al. 1995), Characeae (McCourt et al. 1996), and Volvocales (Nozaki et al. 1995, 1998). However, the use of this gene in ascertaining the phylogenetic relationships among the green algal classes has not been assessed. In the present study, amplification and sequencing of the chloroplast-encoded gene rbcL is used to assess the usefulness of rbcL as a phylogenetic marker of the order Trentepohliales within the green algal classes. For this assessment the results from 18S rDNA studies and morphological (including ultrastructural) studies provided a basis for comparison.

MATERIALS AND METHODS

Source and Growing Conditions. Unialgal cultures of Trentepohliales were grown in liquid and or agarized MWH media (Nichols 1973) under continuous cool white fluorescent light. Species of the green algae and vascular plants included in this

**Total DNA Extraction.** Total cellular DNA was extracted from trentepohlialean samples as follows. Aliquots of unialgal cultures were rinsed with Tris-HCl 0.5M pH 7.2. Cells were disrupted by grinding in liquid nitrogen with a chilled mortar and pestle. Samples were treated as described in the Dneasy plant mini handbook for DNA isolation from plant tissue, Dneasy Plant Mini Kit (QIAGEN, Inc. Valencia, CA). The nucleic acid pellet was dissolved in sterile distilled water. DNA samples were stored at -20°C. Integrity of DNA preparation was checked by electrophoresis in agarose gels stained with ethidium bromide. Algal filaments were disrupted in liquid N₂ with a mortar and pestle. The sample was suspended in a lysis buffer and incubated at 65°C with RNase. Total DNA extraction and purification were performed by filtration and membrane-binding DNA protocol with the DNeasy Plant Mini Kit (QIAGEN 69103). Total DNA was concentrated in final volume of 100 uL and visualized on an agarose gel with ethidium bromide.
**DNA Amplification and Sequencing.** DNA amplification via polymerase chain reaction of the chloroplast-encoded *rbcL* gene was performed using the primers designed by Nozaki et al. (1995):

<table>
<thead>
<tr>
<th>Primer</th>
<th>Position</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>rbcL</em> 1</td>
<td>1-20</td>
<td>ATGGTCCACAAACAGAAAC</td>
</tr>
<tr>
<td><em>rbcL</em> 320</td>
<td>320-341</td>
<td>TATTGAAGAGGTCCAGTAAC</td>
</tr>
<tr>
<td><em>rbcL</em> 395</td>
<td>395-376 reverse</td>
<td>GCACGTAAGCTTTGAAACC</td>
</tr>
<tr>
<td><em>rbcL</em> 650</td>
<td>650-671</td>
<td>GTTTCTTTTCGTAAGC</td>
</tr>
<tr>
<td><em>rbcL</em> 803</td>
<td>803-782 reverse</td>
<td>TCGTGCAATAATAATAGGTACAC</td>
</tr>
<tr>
<td><em>rbcL</em> 830</td>
<td>830-809 reverse</td>
<td>TTAGCTGTGAAACCACCTGTA</td>
</tr>
<tr>
<td><em>rbcL</em> 1181</td>
<td>1181-1160 reverse</td>
<td>AAGATTTCAACTAAAGCTGGCA</td>
</tr>
<tr>
<td><em>rbcL</em> 1421</td>
<td>1421-1402 reverse</td>
<td>TTGTCAATAGTATCAAATTTC</td>
</tr>
</tbody>
</table>

Template DNA was amplified in 0.2 mL thin-wall PCR tubes (DOT Scientific Inc., Burton, MI) in a total reaction volume of 50 µL. Each amplification reaction consisted of 2 µL of template DNA, 2 µL of two flanking primers, 5 µL of MgCl₂ (25 mM), 5 µL of Thermophilic DNA Polymerase 10X buffer (Promega Corp., Madison, WI), 4 µL of 1 mM deoxynucleotide mix (containing dATP, dTTP, dCTP, and dGTP), 0.4 µL of *Taq* DNA polymerase in storage buffer B (Promega Corp., Madison, WI), and sterile water. Negative controls without DNA template were included. Amplification cycles were controlled in a GeneAmp PCR System 2400 (Perkin-Elmer) thermocycler, with an initial denaturation step at 94°C for 1 min, followed by forty-five cycles of PCR amplification under the following conditions: denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 68°C for 60 s. Amplified products and a standard 1Kb
DNA ladder (Life Technologies, Inc. Gaithersburg, MD) were visualized by electrophoresis on 0.8% agarose 1X TBE gels stained with ethidium bromide for correct length, yield, and purity. DNA products were purified with Millipore UFC3 TTK 00 (30,000 NMWL) filter units (Millipore Corporation, Bedford, MA) with an Eppendorf 5415 C table-top microcentrifuge. Purified DNA amplification products were sequenced with internal \textit{rbcL} primers and the protocol of the DNA Sequencing Kit, Big-Dye Terminator Cycle Sequencing Ready Reaction ABI PRISM (Perkin Elmer Applied Biosystems, Foster City, CA) in an ABI 377 Prism Automated Sequencer. DNA sequences were captured in text as well as in color-coded electropherograms.

**Sequence Alignment and Data Analysis.** Sequence data were converted with ReadSeq with the Pearson/Fasta output format available at the following web site: http://dot.imgen bcm.tmc.edu:9331/seq-util/readseq.html (Baylor College of Medicine, Houston, TX). Converted sequences were submitted for automatic multiple alignment to MultiAlin, multiple sequence alignment program (Corpet 1988) available at the internet site: http://W3.toulouse.inra.fr/lgc/multalin/multalin.html (Laboratoire du Génétique Cellulaire, Institut National de la Recherche Agronomique, Toulouse, France). The \textit{rbcL} sequences were manually adjusted by using SeqPup v. 0.6, available at ftp://iubio.bio.indiana.edu/molbio/seqpup/. All analyses were performed with a G3 Macintosh computer (Apple Computer Inc., Cupertino, CA). Regions in the data matrix that could not be unambiguously aligned were excluded from the analyses.

A total of twenty-five taxa was used to construct a general data matrix. Distance analyses were performed with the PAUP* 4.0 package (Swofford, 1999). The two-parameter model of Kimura (1980) was used to generate distance matrices, which were
converted to phylogenetic trees with the neighbor joining method (Saitou and Nei 1987). Maximum parsimony analyses were performed in PAUP* 4.0 with the heuristic search option with a branch-swapping algorithm (tree bisection-reconnection) and random sequence addition (100 replicates). For Maximum Likelihood analyses, PAUP* 4.0 program was used. Bootstrap analyses were performed in PAUP* 4.0 to assess the stability/support of nodes with 100-5000 replications for maximum parsimony and neighbor-joining analyses.

RESULTS AND DISCUSSION

Phylogenetic analyses were performed on a set of taxa that included six streptophytes as the outgroup: *Chara connivens*, *Coleochaete orbicularis*, *Nitella translucens*, *Spirogyra maxima*, *Zea mays*, and *Zamia inermis*; seven prasinophytes: *Cymbomonas tetramitiformis*, *Mantoniella squamata*, *Micromonas pusilla*, *Nephroselmis olivacea*, *Pycnococcus provasolii*, *Pseudoscourfieldia marina*, and *Pyramimonas parkeae*; two ulvophycean algae: *Bryopsis maxima* and *Codium fragile*; two trebouxiophycean algae: *Chlorella ellipsoidea* and *Chlorella vulgaris*; six chlorophycean algae: *Astrephomene perforata*, *Chlamydomonas debaryana*, *Dunaliella salina*, *Eudorina elegans*, *Tetrabena socialis*, and *Volvox globator*; and two trentepohlialean taxa: *Trentepohlia* sp. and *Trentepohlia dialepta*.

Three different tree-building algorithms (distance, parsimony, and maximum likelihood) produced basically similar topologies (fig. 5.1 to 5.4), each positioning the trentepohlialean taxa unequivocally within the chlorophycean lineage, the Chlorophyta. This lineage forms a monophyletic group (bootstrap support >93%). All the major groups of the chlorophycean lineage form monophyletic groups; the Ulvophyceae,
represented by *Bryopsis maxima* and *Codium fragile*, forms a clade with a bootstrap support >84%, Trebouxiophyceae with 100%; Prasinophyceae with a bootstrap support >83%; and Chlorophyceae with a bootstrap support of 67% in the parsimony analysis but with 88% support in the distance method (fig. 5.1 and 5.2).

The order Trentepohliales, represented by *Trentepohlia dialepta* and *Trentepohlia* sp., forms a monophyletic group in the maximum likelihood analysis (fig. 5.4) and in the distance bootstrap analysis with a bootstrap support of 78% (fig. 5.1). This clade is not supported in the parsimony bootstrap analysis (fig. 5.2); however, it is present in the maximum parsimony tree (fig. 5.3) as well as in the maximum likelihood tree. It is important to note that trentepohlialean taxa are never associated with the charophycean clade. The branching order of the trentepohlialean taxa in the tree occurs after the divergence of the ulvophycean clade and before the Chorophyceae/Prasinophyceae group. The Trentepohliales arguably derived from ancestral ulvophycean algae. Results of this investigation based on *rbcL* sequences support studies on the 18S rDNA (López-Bautista and Chapman in preparation; Zechman et al. 1990), ultrastructure of the flagellar apparatus (Roberts 1984), and *mt* LSU rDNA (López-Bautista et al., in preparation) which reported the Trentepohliales as clearly in the Chlorophyta and not in to the charophycean lineage. These results underscore the questions raised by the presence of a phragmoplast-type cell division in the Trentepohliales, detected by electron microscopy and immunofluorescence studies (Chapman and Henk 1986, Waters et al. 1998, respectively). There are at least two hypotheses that address this problem: 1) two separate origins of non-homologous phragmoplast-type cell division (one in the charophycean lineage and one in the
Trentepohliales); or 2) multiple losses of phragmoplast-type cell division. It is difficult to explain the independent evolution of such a complex and cytologically fundamental process, probably involving several genes, in two different lineages, the Chlorophyta and Streptophyta. But if the process evolved once in some, but not all, charophycean algae, it is possible that an almost identical process evolved once in some, but not all green algae in the Chlorophyta. A comprehensive ultrastructural and immunological study of the basal charophyceae as well as trentepohlialean algae may provide the answer for this conundrum.

Next in the branching order, the Chlorophyceae and Prasinophyceae form a sister group, in the NJ bootstrap (fig. 5.1) and maximum likelihood trees (fig. 5.4). The position of the Trebouxiophyceae, represented by *Chlorella vulgaris* and *C. ellipsoidea*, is intriguing, if not bizarre. The trebouxiophycean algae appear to be in different branches in the different analyses. In the NJ bootstrap tree (fig. 5.1), they appear basal to the Chlorophycean/Prasinophycean algae groups; in the MP tree (fig. 5.3) they become the sister group of the Prasinophyceae; and in the ML tree (fig. 5.4) the trebouxiophycean algae appear in a more basal situation between the ulvophycean and trentepohlialean algae.

Another intriguing situation in the *rbcL* tree in all tree-building algorithm analyses is the position of the prasinophycean taxa as an "advanced" group and in a sister position with the Chlorophyceae. In 18S rDNA phylogenetic trees (López-Bautista and Chapman in preparation; Nakayama et al. 1998), the prasinophyceae are basal in the chlorophycean lineage; they are not a monophyletic group but rather a polyphylectic group forming a basal grade in the chlorophycean lineage with at least one
representative (*Mesostigma viride*) in the streptophyte lineage (An et al. 1999). Thus, their position in the *rbcL* phylogenetic tree is totally anomalous.

Manhart and VonderHaar (1991) analyzed the *rbcL* sequence of *Codium* (an ulvophycean alga) with *Chlorella* and *Chlamydomonas* (both originally reported as chlorophycean algae). They found an anomalous position of *Codium* between *Chlorella* and *Chlamydomonas*, thus concluding that the Chlorophyceae (*sensu* Mattox and Stewart 1984) was polyphyletic. These results on the polyphyletic nature of the Chlorophyceae sensu Mattox and Stewart have been addressed by Friedl (1995) using 18S rDNA. He concluded that at least one group (including *Chlorella*), emerged as a new class, the Trebouxiophyceae (Friedl 1995) as a sister group of the Chlorophyceae. Thus, the polyphyletic nature of the Chlorophyceae, as detected by Manhart and VonderHaar (1991) and Zechman et al. (1990), has been resolved, but the position of *Codium*, an ulvophycean genus, as an intermediary taxon between *Chlorella* (trebouxiophycean algae) and *Chlamydomonas* (chlorophycean genus) in the *rbcL* tree was anomalous.

Similar anomalies in the *rbcL* gene tree of a broad scope of green plants have been reported by Manhart (1994). In this study, nucleotide and amino acid sequences of the *rbcL* of several species of bryophytes, ferns, flowering plant and a few green algae including *Codium fragile*, *Chlorella ellipsoidea*, *Chlorella* strain N1a, *Chlamydomonas reinhardtii*, *Chlamydomonas moewusii*, *Coleochaete orbicularis*, *Chara connivens*, and *Pedinomonas minor* were analyzed. The green algae were basal in the overall tree; however anomalies were detected. The ulvophycean genus *Codium* was found as a sister group of the charophycean genera *Chara* and *Nitella* among the Streptophyta.
clade and Chlorella was a basal branch followed by the sister group of the
Chlamydomonas spp. and Pedinomonas minor. These results prompted Manhart (1994)
to conclude that the gene rbcL is limited to analyses within the main groups (i.e.,
classes) of taxa.

The present results question the reliability of the rbcL sequences for the analysis
of the phylogenetic relationships among the major groups of green algae, but not to a
more restricted use within the green algal classes or to a more circumscribed group of
green algae. In contrast, the use of rbcL sequences in the phylogenetic analyses of
major groups of red algae (Freshwater et al. 1994) and brown algae (Siemer et al. 1998)
in a broad scope has been found to be reliable.

Similar conclusions on the limited application of the rbcL gene were made by
McCourt et al. (1995), who pointed out that rbcL sequences may be inappropriate in
phylogenetic studies of ancient branching events (unless and until more thorough taxon
sampling is possible). A possible explanation may be that rbcL sequences are too
divergent to test phylogenetic relationships at higher taxonomic ranks such as classes in
ancient groups such as the green algae. Manhart (1994) pointed out that high levels of
homoplasy in the rbcL trees of green plants preclude meaningful phylogenetic analysis
at higher taxonomic levels, as well as other potential problems such as RNA editing,
pseudogenes, unequal rates of evolution, inadequate taxon sampling, and functional
constraints on sequence evolution. In a study by Daugbjerg et al. (1995), rbcL genes
evolved at different rates in two classes of green algae (Prasinophyceae and
Pedinophyceae) with a higher rate recorded for Prasinophyceae. The conclusions of
Daugbjerg et al. (1995) have important implications since it is well known that
heterogenous substitution rates have important implications in phylogenetic analysis, wherein ML performs better than MP under conditions where the rates of nucleotide substitutions among groups or branches is unequal. Albert et al (1994) suggested that \textit{rbcL} evolution is "strongly constrained by function." Kellogg and Juliano (1997) confirmed that observation in studies of the structure and function of RuBisCO. Recognizing the high constraints on RuBisCO, they proposed several implications for the use of \textit{rbcL} in systematics studies: a) the number of potentially useful characters may be many fewer than 1428 bp, b) because of functional constraints, rates of change can vary greatly across the molecule, c) some changes may be correlated, and thus might be down-weighted accordingly, and d) some of the variation in RuBisCO may be adaptive and present insights into the nature of evolutionary change in response to the environment.

The analyses of chloroplast-encoded \textit{rbcL} sequences in this study confirm previous results using 18S rDNA and \textit{mL} LSU rDNA genes in positioning the order Trentepohliales within the Chlorophyta lineage. Our results support the application of the \textit{rbcL} gene within circumscribed groups such as classes, orders and families of the green algae; however we question the reliability of this gene for the analysis of the phylogenetic relationships among the major groups of green algae (i.e., classes).
Fig. 5.1. Phylogeny of the Viridiplantae based on the chloroplast-encoded rbcL gene sequences comparison. Tree inferred from neighbor-joining analysis using a total of 1440 aligned positions. The phylogeny is rooted with the streptophytes Coleochaete orbicularis, Chara connivens, Nitella translucens, Spirogyra maxima, Zea mays, and Zamia inermis. Bootstrap values of distance (neighbor-joining) above the nodes (only values >50% were recorded). A = Chlorophyceae, B = Prasinophyceae, C = Trebouxiophyceae, D = Trentepohliales, E = Ulvophyceae, and F = Outgroup.
Fig. 5.2. Phylogeny of the Viridiplantae based on the chloroplast-encoded rbcL gene sequences comparison. Tree inferred from parsimony analysis using a total of 1440 aligned positions. The phylogeny is rooted with the streptophytes Coleochaete orbicularis, Chara connivens, Nitella translucens, Spirogyra maxima, Zea mays, and Zamia inermis. Bootstrap values of parsimony (neighbor-joining) above the nodes (only values >50% were recorded). A = Chlorophyceae, B = Prasinophyceae, C = Trebouxiophyceae, D = Trentepohliales, E = Ulvophyceae, and F = Outgroup.

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Fig. 5.3. Phylogeny of the Viridiplantae based on the chloroplast-encoded \( rbcL \) gene sequences comparison. Tree inferred from maximum parsimony analysis using a total of 1440 aligned positions. The phylogeny is rooted with the streptophytes *Coleochaete orbicularis*, *Chara connivens*, *Nitella translucens*, *Spirogyra maxima*, *Zea mays*, and *Zamia inermis*. Length of the tree 2626, CI = 0.425, RI = 0.529. A = Chlorophyceae, B = Prasinophyceae, C = Trebouxiophyceae, D = Trentepohliales, E = Ulvophyceae, and F = Outgroup.
Fig. 5.4. Phylogeny of the Viridiplantae based on the chloroplast-encoded rbcL gene sequences comparison. Tree inferred from maximum likelihood analysis using a total of 1440 aligned positions. The phylogeny is rooted with the streptophytes Coleochaete orbicularis, Chara connivens, Nitella translucens, Spirogyra maxima, Zea mays, and Zamia inermis. Ln likelihood = -13129.08375. A = Chlorophyceae, B = Prasinophyceae, C = Trebouxiophyceae, D = Trentepohliales, E = Ulvophyceae, and F = Outgroup.
CHAPTER 6
GENERAL SUMMARY AND CONCLUSIONS
This dissertation examined nuclear, chloroplast, and mitochondrial genes as well as karyology and nuclear genome quantification of the green algal order Trentepohliales (Chlorophyta). Based on ultrastructural features of the flagellar apparatus, cytokinesis, biochemical, biophysical, and physiological characters, conflicting views have been hypothesized about the systematic position of this order among the several classes of green algae. The major discussions have been focused on ulvophycean versus charophycean affinities of the order Trentepohliales. In this study, representatives of the order Trentepohliales were compared with other green plants to further assess the order's phylogenetic position.

The second chapter of this dissertation reports the results of the distance, parsimony and likelihood analyses of the nuclear-encoded small subunit ribosomal DNA (18S rDNA) sequences of 53 taxa and 1733 aligned characters. Two glaucocystophytes were used as the outgroup and fifty-one representatives of the Viridiplantae. The results support the existence of two lineages, the chlorophycean and charophycean groups. The order Trentepohliales was shown to be a monophyletic group and positioned within the chlorophycean lineage, associated with the ulvophycean algae. In order to elucidate the position of the trentepohlialean algae within the class Ulvophyceae, a second analysis was performed with 4 outgroup taxa from the Chlorophyceae and Trebouxiophyceae and twenty-three ulvophycean algae. The order Trentepohliales emerged as a sister taxa to the clade containing the Siphonocladales/Cladophorales complex and Dasycladales.

In the third chapter, the mitochondrial large subunit ribosomal RNA (mtLSU rRNA) of Cephaleuros parasiticus (Trentepohliaceae) was investigated by northern blot
hybridization and compared with previous reports of representatives of three classes of green algae (Chlorophyceae, Trebouxiophyceae, and Prasinophyceae). Both continuous and discontinuous *mt*LSU have been reported in green algae and the distribution of these two types have been shown to be consistent with phylogenetic relationships. An oligonucleotide probe complementary to a highly conserved region of the *mt*LSU rRNA detected and identified clearly a 3Kb fragment with the expected size of a continuous *mt*LSU rRNA in *C. parasiticus*; a result consistent with an ulvophycean affinity. This continuous pattern has been reported in green algae with a CCW flagellar configuration. The continuous *mt*LSU rRNA in *C. parasiticus* supports the previous hypothesis that relates all green algae with a CCW flagellar apparatus to having a continuous *mt*LSU. Our result suggest that the basal body configuration of the flagellar apparatus, as well as the *mt*LSU in green algae are reliable markers assessing phylogenetic affiliations among the green algae.

In chapter four, karyological data and microspectrophotometry techniques based on DAPI were used to provide information on nuclear genome sizes for eight species of the order Trentepohliales. Data available in the literature for chromosome numbers is reported for 16 species. These chromosome numbers show a discontinuous large-scale variation with a basic chromosome complement of $N = 6$ and probably representing a polyploid series. Comparisons for the mean fluorescence intensity values resulted in an estimate of 1.1-4.1 pg for the trentepohlialean algae. In these results, gametophyte and sporophyte samples of *C. parasiticus* were analyzed, the gametophyte levels for 2 C nuclei closely approximate 50% of the 4 C values in the sporophyte phase. This results confirm the presence of a sexual life cycle in this genus. The genome quantification of
the eight trentepohlialean taxa suggest a doubling sequence for nuclear DNA content. Chromosome numbers and genome quantification suggest that evolution in this order might reflect a polyploid series.

The fifth chapter of this dissertation reports the results of the distance, parsimony, and likelihood analyses of the chloroplast-encoded large subunit ribulose-1,5-bisphosphate carboxylase/oxygenase (rbcL) sequences of twenty-five taxa of Viridiplantae and 1733 aligned characters. The results positioned the order Trentepohliales unequivocally within the chlorophycean lineage. Representatives of this lineage, Trentepohliales, Prasinophyceae, Ulvophyceae, Chlorophyceae, and Trebouxiophyceae formed a monophyletic groups. However, topological anomalies were detected in the rbcL tree when compared with the 18S rDNA tree. Specifically, the branching order of the Prasinophyceae and Trebouxiophyceae in every method of analysis (distance, parsimony, and likelihood) resulted in a bizarre topology. This study questions the reliability of the rbcL sequences for the analysis of the phylogenetic relationships among higher groups of green algae (classes), but not to a more restricted use within the algal classes, orders or to a more lower taxonomic rank.

In conclusion, the studies developed in this dissertation clearly indicate that the order Trentepohliales is a monophyletic group. They provide robust support for including the trentepohlialean algae in the chlorophycean lineage within the class Ulvophyceae, and suggest that most probably evolved from a macroscopic and filamentous marine ancestor. There is some evidence to suggest that evolution within the subaerial Trentepohliales has involved polyploidy accompanied by doubling of genome size. More research is needed to explain the enigmatic presence of the
phragmoplast-type cytokinesis in this order. Such complex cytological process has evolved in two separate lineages of green plants, in the streptophytes and the Trentepohliales, both of which interestingly are associated with terrestrial (subaerial) habits. An ultrastructural and immunological research of the evolution of the phragmoplast in the basal lineages of the streptophytes as well as in the Trentepohliales may provide the answer.
REFERENCES


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December 22, 1999

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TO: Juan M. Lopez-Bautista
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Juan Manuel López-Bautista was born on February 8th, 1958, in Madero city, Tamaulipas, México, the son of Manuel López-Tamez and Esther Bautista de López. Juan M. López-Bautista completed a Bachelor of Science degree in Biology, with Honors, at the Universidad Autónoma de Nuevo León, Monterrey, México in 1981. López-Bautista was a student worker at the Phycology laboratory during his Bachelor studies. He worked at the Universidad del Noreste at Tampico, México, teaching phycology, bryology and pteridology and conducting research on the systematics and biogeography of the marine algae of the Gulf of Mexico. In 1986 he moved to México city for one year to continue with his research on seaweeds at the National Polytechnic Institute. In 1987 he taught botany-related courses at the Instituto Tecnológico de Ciudad Victoria, Tamaulipas and conducting research in marine and freshwater algae from northeastern Mexico. He received a scholarship from the Agency of International Development, USAID in Fall of 1991 to conduct research at the Center for Marine Science Research, the University of North Carolina at Wilmington. Juan M. López-Bautista earned a Master of Science degree in Biology at the University of North Carolina at Wilmington in 1994 working with Dr. Donald F. Kapraun, studying the agar analysis, and nuclear genome quantification and characterization of agarophytes (Gracilaria) from the Mexican Gulf Coast. He received an Award for Outstanding Leadership in 1994 from the University of North Carolina at Wilmington. Juan began his doctoral work in Dr. Russell L. Chapman's laboratory in 1994, studying the evolutionary relationships of the subaerial green algal order Trentepohliales.
(Chlorophyta). His scientific interest is in phycology, especially green and red algae with emphasis on their biodiversity, biogeography, systematics and phylogeny.

While studying at LSU, he has earned several awards and honors including the Sigma Xi LSU Chapter and the National Sigma Xi, and Phycological Society of America Grant-in-Aid of Research Awards, the LSU and PSA Hoshaw Travel Awards (1995-1999), the William J. Luke Botany Teaching Assistant Award from the LSU Department of Plant Biology, and the Award for Excellence in Teaching from the LSU College of Basic Sciences. His studies on phycology have been published in recognized journals. The list includes:

"Molecular and microspectrophotometric analyses and phylogeny of the subaerial Trentepohliales (Chlorophyta)." J. Phycol. 35 (Suppl): 21; 1999.


He will receive the degree of Doctor of Philosophy in May, 2000.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Juan Lopez-Bautista

Major Field: Plant Biology

Title of Dissertation: Molecular Systematics of the Green Algal Order Trentepohliales (Chlorophyta)

Approved:

[Signatures]

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures]

Date of Examination:

January 18, 2000