CHAPTER 1. PHYLOGENETIC POSITIONS OF THE COLORLESS, COLONIAL IRON-FLAGELLATE ANTHOPHYSA VEGETANS AND POLYKARYON PYRENOIDOSUM GEN. ET COMB. NOV. (HETEROKONTOPHYTA)

Introduction

Anthophysa vegetans (O.F. Müller) Stein is known to be the first chrysophyte ever described (Müller, 1786). This common freshwater colonial flagellate forms (hemi-) spherical colonies that are free-swimming or attached by a distinctive stalk that contains iron and manganese compounds (Pringsheim, 1952; Starmach, 1985). *A. vegetans* is among a lesser number of chrysophytes that have either lost their chloroplasts or retain nonphotosynthetic leucoplasts (Starmach, 1985; Cavalier-Smith *et al.*, 1995/96; Preisig & Andersen, 2000). Placement of *Anthophysa* within the Chrysophyceae is not a subject of debate; Belcher & Swale (1972a), as well as subsequent studies of other chrysophytes, have demonstrated that the ultrastructure of the species is very similar to that of other *Ochromonas*-like flagellates (Hibberd, 1976; Andersen, 1982; Kristiansen, 1990; Moestrup & Andersen, 1991; Wujek, 1996; Preisig & Andersen, 2000). For example, *A. vegetans* bears two slightly subapically inserted flagella of unequal length and the anteriorly directed longer flagellum bears tripartite hairs (mastigonemes); the shorter, posteriorly directed flagellum is smooth. Also, subunits of the basal body are oriented perpendicularly (or at an acute angle) to one another like those found in most, but not all, members of the Chrysophyceae (Belcher & Swale, 1972a; Andersen, 1989, 1990; Preisig & Andersen, 2000). Although *Anthophysa* is obligately phagotrophic, it possesses a leucoplast that lies against the nucleus in the anterior portion of the cell that is surrounded

by the chloroplast endoplasmic reticulum (CER)(Belcher & Swale, 1972a). Also, a conspicuous eyespot (stigma) composed of numerous osmiophilic droplets is found within the leucoplast and is positioned opposite a flagellar swelling found on the smooth, short (mature) flagellum. Together, these ultrastructural characters support placement of *Anthophysa* within the Ochromonadaceae (Fott, 1956; Starmach, 1985; Preisig, 1995; Preisig & Andersen, 2000).

However, relationships among genera and species within the Chrysophyceae are poorly known and there is convincing evidence that the Ochromonadaceae is not a monophyletic taxon (Preisig, 1995; Andersen *et al.*, 1999; Preisig & Andersen, 2000). Thus, two questions are asked: 1) To what other species is Anthophysa most closely related? and, 2) Has *Anthophysa* lost the capacity to photosynthesize independently of other taxa including *Oikomonas*, *Paraphysomonas*, and *Spumella*? Also included in this investigation are new data for an alga originally discovered and described by Trenkwalder (1975) as *Botrydiopsis pyrenoidosa* and placed in the Xanthophyceae.

In this study the morphologies of *A. vegetans* and *B. pyrenoidosa* were reexamined using light and fluorescence microscopy. Also, nuclear small subunit ribosomal RNA (18S rRNA) gene sequences were determined for both species and used to reassess their phylogenetic positions and taxonomy. Based upon this data a new heterokont algal genus, *Polykaryon*, is established.

Materials and Methods

Isolates

Anthophysa vegetans was collected from Indian Creek, US Route 219, Monroe Co., West Virginia, USA (037º 32' 47.8" N, 080º 34' 33.6" W) on 22 July 2001. Serial dilution methods were used to establish unialgal cultures in DYIV medium containing a single rice grain (Andersen *et al.*, 1997) that were maintained on a 12:12 light:dark photoperiod at 15ºC. Cultures of *Botrydiopsis pyrenoidosa* (strain SAG 31.83) were obtained from the Sammlung von Algenkulturen (SAG), Germany (www.epsag.uni-goettingen.de) and maintained in DYIV (without rice) as described above.

Brightfield and fluorescence microscopy

Cells were examined, measured and photographed using an Olympus BX-60 microscope. For fluorescence microscopy, pelleted cells were fixed, stained with the DNA-binding fluorochrome DAPI (4',6-diamidino-2-phenylindole: 0.5 µg/mL, Sigma Chemical Co., St. Louis, MO) as described in Kapraun *et al.* (1992). Brightfield and fluorescence micrographs were taken using an Olympus PM-20 35 mm camera using Kodak T-Max ISO 200 film.

DNA extraction, PCR amplification and cycle sequencing

DNA was extracted using a CTAB method (Doyle & Doyle, 1987), as modified in Bailey *et al.*, (1998). The entire 18S rRNA gene was amplified as previously described (Bailey *et al*., 1998) using primers GO1 and GO7 (Saunders & Kraft, 1994). The

thermocycling profile included an initial denaturation step at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 50 °C for 30 s and primer extension at 72 °C for 1.5 min. This cycle was followed by a final extension step for 7 min at 72 °C and a 4 °C soak. Amplified products were checked for correct length and yield on 0.8% ethidium bromide-stained agarose gels and purified using the GeneClean II Kit (Qbiogene, Carlsbad, CA). PCR products were sequenced on both strands using the BigDye Terminator cycle sequencing kit (v. 2, Applied Biosystems, Foster City, CA) according to the manufacturer's specifications and analyzed on an ABI3100 automated DNA sequencer (Applied Biosystems, Foster City, CA). The 18S rRNA gene sequence for *Botrydiopsis pyrenoidosa* (strain SAG 31.83) was determined twice, on both strands, using DNA extracted from separate subcultures.

Phylogenetic analyses

The 18S rRNA sequences obtained for *A. vegetans* and *B. pyrenoidosa* were aligned by eye with 18S rRNA sequences for 74 other species of heterokont algae (Table 1) using the SeqApp program (Gilbert, 1994). The alignment includes representatives of all presently recognized classes of heterokont algae and is available from the corresponding author upon request. Regions of the sequence matrix that could not be confidently aligned were excluded from subsequent analyses.

The model of nucleotide substitution that best fit the data was determined using the ModelTest (v. 3.06) program (Posada & Crandall, 1998). A TrN+I+G model was selected as the model of best fit for the global heterokont algal data set and was used to build trees under the optimality criteria of parsimony and maximum likelihood (ML).

Phylogenetic analyses were conducted using PAUP*4.0b10 (Swofford, 2002). For parsimony all positions were equally weighted and gaps were treated as missing data. Heuristic searches were performed using TBR branch swapping and repeated using 100 random orders of sequence addition. Bootstrap proportion (BP) values were calculated based upon 5000 resamplings of the data set using the "fast stepwise addition" option in PAUP. The ML likelihood analysis was repeated with 10 random orders of sequence addition and BP values were obtained based upon analyses of 10 pseudoreplicate data sets. The global heterokont algal tree(s) were rooted using sequences for the oomycetes *Achlya bisexualis* and *Lagenidium giganteum* (Table 1).

Results

Polykaryales ord. nov.

Sicut pro familia.

As in the family.

Polykaryaceae fam. nov.

Sicut pro genus.

As in the genus. Genus typicum: *Polykaryon* Bailey et Misner

Polykaryon gen. nov. Bailey *et* Misner

Cellulae virides ad fulvae cum parietibus cellularum laevibus et tenuibus. Chloroplasti duo ad multi, parietales, saepe polyedrici et aliquando cum pyrenoide tumescens. Cellulae maximam partem multinucleatae, cellulis juvenibus et parvioribus

unus ad aliquot nucleos continentibus, cellulis vetioribus majoribusque 30 vel plus.

Green to yellowish-brown cells, round with smooth, thin cell walls and two to many parietal chloroplasts. Chloroplasts often polygonal in shape and may contain a bulging pyrenoid. Most cells are multinucleate: smaller, young cells contain one or a few nuclei whereas larger, older cells may possess 30 or more nuclei.

Polykaryon pyrenoidosum (Trenkwalder) Bailey *et* Misner *comb. nov.*

Basionym: *Botrydiopsis pyrenoidosa* Trenkwalder, *Ber. Naturwiss. –Med. Vereins*, Innsbruck **62**: 8, fig. 1. 1975.

Solitary or clustered cells, round 7.5 – 50 (-85) μm in diameter with smooth cell walls. Reproduction by formation of autospores within the parent cell wall or the formation of zoospores. Zoospores (15 μm long) may be amoeboid or pear-shaped, possess two chloroplasts, and lack an eyespot. Zoospores possess one emergent flagellum visible in the light microscope.

Note: The specific epithet "*pyrenoidosa*" *sensu* Trenkwalder (1975) is declined to "*pyrenoidosum*" to agree with the gender of genus name (Gr., "karyon" = kernel), which is neuter.

Brightfield and fluorescent microscopy

Brightfield microscopic observations of cultured *A. vegetans* cells were generally consistent with those recorded in Starmach (1985). Briefly, colonies were stalked or unstalked, typically 30 μm in diameter (Figs 1-5) and individual cells were 7-10 μm long (Figs 1-8). Unicells liberated from colonies were pear-shaped or oval, but most were not as acutely pyriform as the cell illustrated in Starmach (1985; Figs 1-4, Figs 6-8). Most

cultured *A. vegetans* colonies examined possessed 30 or fewer cells (Figs 1-5) although colonies containing 60 or more cells have been previously reported (Starmach, 1985). It is assumed that these differences reflect intra- and not interspecific diversity.

Cell diameters for *Polykaryon pyrenoidosum* (=*Botrydiopsis pyrenoidosa*) were determined for 334 cells drawn from three separate subcultures from 14 days to one month old (Figs 9-13). Cell diameters ranged from 7.5 – 50 μm and the mode and mean diameters for this sample were 12.5 μm and 17 μm, respectively. Autospores, zoospores and vegetative cells reaching diameters of up to 85 μm were not observed in the cultures (Trenkwalder, 1975; Ettl, 1978). Coccoid cells possessed a smooth cell wall (Figs 11-13) and many parietal chloroplasts that were lenticular or polygonally shaped (Figs 9-11). Fluorescence microscopy of DAPI-stained cells revealed numerous nuclei in older cells of *P. pyrenoidosum* (Figs 14, 15). Smaller, presumably newly formed, cells (e.g., 7.5 – 10 μm) usually contained a single nucleus (not shown) while conservative counts found as many as 40 nuclei in larger cells (e.g., Fig. 15). DAPI-stained nuclei appear round, ovate or elongated and are often paired (Figs 14, 15). Although there were no observations of cells in sizes upwards of 80 μm, autospores or zoospores all other characteristics of cells are consistent with the description given in Trenkwalder (1975).

A priori phylogenetic analyses

The global heterokont algal 18S rRNA alignment contained data for 74 ingroup taxa and 1859 aligned characters of which 530 were parsimony informative. Cladistic analysis of these data yielded 12 equally parsimonious trees (L=3043, CI=0.40, RI=0.67). The majority rule consensus of these 12 trees is depicted in Figure 16. ML analysis of

these data resulted in the tree shown in Figure 17 (ln likelihood = -17637.92034). Ten of the 13 classes of heterokont algae were resolved as monophyletic with robust BP (=91% - 100%) values in both trees, including the Bacillariophyceae, Bolidophyceae, Chrysomerophyceae, Dictyochophyceae, Eustigmatophyceae, Pelagophyceae, Phaeophyceae, Pinguiophyceae, Raphidophyceae, and Xanthophyceae (Figs 16, 17). Both trees imply that the classes Chrysophyceae and Synurophyceae are sister taxa and this observation is robustly supported (BP=99%, 90%) by the data. In the ML tree the Chrysophyceae and Synurophyceae are resolved as monophyletic lineages with weak (<50%) or moderate (70%) bootstrap support, respectively (Fig. 17). The monophyly of the Synurophyceae is moderately supported (BP=72%) in the parsimony analysis; however, the chrysophyte species did not form a single clade in this analysis (Fig. 16). With the exception of the chrysophyte/synurophyte clade, support for relationships among the remaining 11 classes was absent in both trees although the two trees were in many respects topologically consistent (cf. Figs 16, 17).

Nucleotide BLASTn searches as well as global analyses of 18S rRNA gene sequences confirm that *A. vegetans* is a member of the Chrysophyceae (Figs 16, 17). In these 'global' trees A. *vegetans* is resolved as sister to *Ochromonas danica* with solid bootstrap support (=81%, 100%).

The phylogenetic position of *Polykaryon pyrenoidosum* (= *Botrydiopsis pyrenoidosa* Trenkwalder, strain SAG 31.83) among other heterokont algae could not be unambiguously determined. Trenkwalder (1975) classified the alga in the Xanthophyceae but *P. pyrenoidosum* was not resolved within that clade or sister to *Botrydiopsis intercedens* (Figs 16, 17). These analyses imply that the closest sampled

relatives of *P. pyrenoidosum* possibly include species placed in the classes Phaeophyceae, Phaeothamniophyceae or Xanthophyceae. For example, *P. pyrenoidosum* is positioned at the base of Phaeophyceae in both trees but this node is weakly supported (BP=<50%). In short, the 18S rRNA analyses do not provide definitive evidence for placing *P. pyrenoidosum* in any of the classes included in this study.

Anthophysa vegetans: a posteriori phylogenetic analyses

Global heterokont parsimony and ML trees indicate that *A. vegetans* is a member of the class Chrysophyceae. To more accurately determine the position of *A. vegetans* within that class parsimony and ML analyses were performed using a second, less inclusive data set including 48 18S rRNA gene sequences for species belonging to the classes Chrysophyceae and Synurophyceae. Sixty-seven positions within the alignment were excluded from phylogenetic analyses because they could not be confidently aligned. The ModelTest program was used to determine the best fit model of nucleotide substitution for these data and a GTR+I+G model was selected. Parsimony and bootstrap analyses of these data were performed as described above. ML analyses and bootstrapping were also performed as described above except that the trees were constructed using five different orders of random sequence addition. Five species belonging to the class Eustigmatophyceae were chosen to serve as outgroup taxa (*Eustimatos magna*, *Monodopsis subterranea*, *Nannochloropsis granulata*, *Nannochloropsis salina*, and *Pseuocharaciopsis minuta*; Table 1).

The chrysophyte + synurophyte alignment included 1901 characters. Cladistic analysis produced nine equally parsimonious trees (L=2284, CI=0.39, RI=0.57) and the

majority rule of these nine trees is depicted in Figure 18. ML analysis of these data resulted in the tree shown in Figure 19 (ln likelihood = -13446.223). Both trees are poorly resolved, particularly at internal nodes (Figs 18, 19). The synurophytes formed a clade among chrysophyte lineages in both trees but this clade is not well supported (BP=<50%, 70%). These trees imply that the closest sampled relatives of *Anthophysa* include *Poterioochromonas* spp. and *Ochromonas sphaerocystis* (Figs 18, 19).

Discussion

Light microscopic analyses confirm the identities of the algae, *Anthophysa vegetans* and *Polykaryon pyrenoidosum*, examined in this study. Observations made on *A. vegetans* are in agreement with previous reports (Starmach 1985; Belcher & Swale 1972a) and with the more detailed account describing intraspecific variability provided by Pringsheim (1946). Although no autospores or zoospores in cultures of strain SAG 31.83, other data are consistent with Trenkwalder's (1975) description of *Botrydiopsis pyrenoidosa*. Most importantly, it has been confirmed that the species possesses many chloroplasts and that most cells are multinucleate (Trenkwalder, 1975). Among most heterokont algae, particularly excepting some members of the Xanthophyceae (Ettl, 1978), this combination of features is relatively rare. There is than no reason to suspect that the authentic strain deposited by Trenkwalder (SAG 31.83) is no longer representative of the alga originally described as *Botrydiopsis pyrenoidosa* (Trenkwalder, 1975).

The phylogeny of *Anthophysa*

Global parsimony and ML analyses verify that *Anthophysa vegetans* is a member of the Chrysophyceae *sensu stricto* (Figs 16, 17). Within the Chrysophyceae *A. vegetans* is resolved as most closely related to *Poterioochromonas* spp. and *Ochromonas sphaerocystis* (Figs 18, 19). There are few differences among individual swimming cells of *Anthophysa*, *Ochromonas*, *Poterioochromas*; characters used to separate these taxa from one another are subtle and are sometimes exhibited only transiently (Table 2; Pringsheim, 1952; Peterfi, 1969; Preisig & Andersen, 2000).

Like *Anthophysa*, *Poterioochromonas* spp. are sometimes colorless whereas *Ochromonas* species are pigmented. *Anthophysa* possesses an eyespot (stigma) (Pringsheim, 1946; Belcher & Swale, 1972a) that may or may not be present in *Ochromonas* and is absent in *Poterioochromonas* (Andersen, 1982; Peterfi, 1969; Preisig & Andersen, 2000). *Poterioochromonas* and *Ochromonas* species are phototrophic and/or phagotrophic, whereas *Anthophysa* is an obligate phagotroph. When not swimming, the posterior end of *Poteriochromonas* cells are situated in a lorica composed of interwoven chitin fibrils (Peterfi, 1969; Preisig & Andersen, 2000). Cells (~30) may be clustered and attachment stalks emanating from individual loricas may or may not be fused (Preisig & Andersen, 2000). Some *Ochromonas* species, like *Anthophysa* and *Poterioochromonas*, tend to form small clusters (Pringsheim, 1952; Preisig & Andersen, 2000). In summary, when all variation within the genera *Anthophysa*, *Poteriochromonas* and *Ochromonas* are taken into account, there appear to be more similarities among these taxa than differences. It is possible that the presence or absence of pigments, eyespots, loricas or whether or not cells are normally free-swimming or sedentary and clustered are

not always reliable characters for distinguishing among ochromonadalean flagellates that might otherwise be placed in the same genus (Andersen *et al*., 1999).

Parsimony and ML trees including chrysophyte and synurophyte species are poorly resolved (Figs 18, 19). Even so, it seems reasonable to conclude that *Anthophysa* is not closely related to other chrysophytes that have lost chloroplasts or that no longer photosynthesize (i.e., *Oikomonas*, *Paraphysomonas*, and *Spumella*) (Belcher & Swale, 1976; Preisig & Hibberd, 1983; Preisig & Andersen, 2000). Thus, these analyses indicate that photosynthetic capabilities have been independently lost in at least four chrysophyte lineages (Figs 18, 19).

The taxonomic status of *Polykaryon pyrenoidosum*

Phylogenetic analyses indicate that the alga described as *Botrydiopsis pyrenoidosa* and placed in the Xanthophyceae by Trenkwalder (1975) is not closely related to *Botrydiopsis intercedens* (Figs 16, 17). Although the 18S rRNA data doubtless indicate that the alga belongs in the Heterokontophyta, they do not place the alga in any class represented in this examination. Neither is there support for recognizing this taxon as sister to any previously recognized class (Figs 16, 17). Based on this information a new genus (*Polykaryon*), family (Polykaryaceae), and order (Polykaryales) have been established to accommodate *Polykaryon pyrenoidosum*. No class assignments has been given to the species for several reasons. First, the ultrastructure of the species excludes it from some heterokont algal classes, but not necessarily others (Bailey & Misner, submitted). Second, sequences for the large subunit of RuBisCO (*rbc*L) have proven useful for systematics studies of heterokont algae, but a complete *rbc*L sequence for

Polykaryon is presently unavailable (Daugjberg & Andersen, 1997a, 1997b; Bailey *et al*., 1998; Guillou *et al*., 1999; Daugbjerg & Guillou, 2001; Bailey & Misner, unpubl. data). And, third, there has not yet been an investigation of the photosynthetic pigment composition of the alga and pigments are often, but not always, useful taxonomic indicators at the rank of class (Jeffrey, 1989; Bjørnland & Liaaen-Jensen, 1989).