Molecular Phylogeny of the Ocelloid-Bearing Dinoflagellates *Erythropsidinium* and *Warnowia* (Warnowiaceae, Dinophyceae)

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ABSTRACT. Members of the family Warnowiaceae are unarmored phagotrophic dinoflagellates that possess an ocelloid. The genus *Erythropsidinium* (= *Erythropsis*) has also developed a unique dynamic appendage, the piston, which is able to independently retract and extend for at least 2 min after the cell lyses. We provide the first small subunit ribosomal RNA gene sequences of warnowiid dinoflagellates, those of the type *Erythropsidinium agile* and one species of *Warnowia*. Phylogenetic analyses show that warnowiid dinoflagellates branch within the *Gymnodinium* sensu stricto group, forming a cluster separated from the *Polykrikos* clade and with autotrophic *Pheopolyprikikos beachampii* as closest relative. This reinforces their classification as unarmored dinoflagellates based on the shape of the apical groove, despite the strong ecological and ultrastructural diversity of the *Gymnodinium* s.s. group. Other structures, such as the ocelloid and piston, have no systematic value above the genus level.

Key Words. Dinoflagellata, Gymnodiniales, photoreceptor organelle, protist evolution, SSU rRNA phylogeny.

The warnowiids are the only known unicellular organisms bearing a photoreceptor system with cornea, lens, and pigment cup-like structures, a complex organization that, elsewhere, can only be found in metazoan eyes. Hertwig (1884) described the first warnowiid dinoflagellate from the western Mediterranean Sea. His delicate *Erythropsidinium* P.C. Silva (= *Erythropsis* Hertwig) possessed an “eye” and also a piston, which was easily lost. This description provoked the incredulity of earlier protozoologists who did not believe that unicellular organisms could have such complex organelles. *Erythropsidinium* was not admitted in taxonomic schemes and it disappeared from the scientific literature until 1896 (see a review in Gómez 2008; Kofoid and Swezy 1921). Later, Kofoid and Swezy (1921) described many new species of *Erythropsidinium*, likely overestimating their number, through the observation of single or few specimens, using the color and position of the ocelloid as diagnostic characters. By studying the ultrastructure of the complex organelles of warnowiid and polykrikoid dinoflagellates in the 1970s, Greuet (1987 and references therein) demonstrated that these features were variable throughout the life cycle of a single individual. He showed that the ocelloid exhibited a striking similarity to the metazoan eye, as it possessed, at the subcellular level, analogous structural components. Taylor (1980) speculated that it might serve to detect shadow effects provoked by the passage of a potential prey, but also that it could focus and serve as “range finder.” According to this hypothesis, the dinoflagellate would measure the distance to the prey and fire the nematocysts only when a clear image was received on the retinoid.

Some protists are able, by virtue of special organelles, to contract parts of their cell bodies: for example, the stalk of *Vorticella*, the “tail” of *Tontonia*, and the tentacle of noctiluic dinoflagellates. In addition to the ocelloid, the warnowiid dinoflagellates *Erythropsidinium* and *Greeutodinium* A.R. Loeblich III (= *Leucopsis* Greuet) display the piston, an extensible appendage that is unique among the protists (Greuet 1987).

Do dinoflagellates, such as warnowiids and some polykrikoids, containing complex extrusomes and the ocelloid-bearing warnowiids have a common ancestor? Or are they, on the contrary, polyphyletic? The general oceanic distribution of the warnowiids and their delicacy had prevented the determination of gene sequences of these unique protists, especially of the type species, and their phylogenetic position remains untested with molecular phylogenetic data. We have been able to collect these protists and here provide the first small subunit ribosomal RNA gene (SSU rDNA) sequences of warnowiid dinoflagellates, the type *Erythropsidinium agile*, collected from the western Mediterranean Sea, its type locality, and an unidentified species of *Warnowia*. We used phylogenetic analyses to test hypotheses about the origin of complex characters, such as ocelloids and pistons, and discuss the evolutionary origin of such structures and their value as diagnostic characters for systematics.

MATERIALS AND METHODS

Specimen collection and isolation. Individual warnowiid cells were collected by slowly filtering surface seawater taken from the pier of the Station Marine d’Endoume at Marseille (43°16’48”N, 5°20’57”E, bottom depth 3 m) from October 2007 to September 2008. A strainer of 20, 40, or 60-µm netting aperture was used to collect planktonic organisms from water volumes ranging between 10 and 100 L, depending on particle concentration. In addition, we also studied samples collected during several monitoring research cruises to the SOMLIT (Service d’Observation en Milieu Littoral) station in the Bay of Marseille (43°14’30”N, 5°17’50”E, bottom depth 60 m). Seawater samples were collected with a 12-L Niskin bottle at 40 and 55-m depth and filtered as described above. The plankton concentrate was scanned in settling chambers at 100X magnification with a Nikon Eclipse TE200 inverted microscope (Nikon, Tokyo, Japan). Cells were photographed alive at 200X or 400X magnification with a Nikon Coolpix E995 digital camera (Nikon, Tokyo, Japan). The selected specimens were individually micropipetted with a fine needle from the plug of the Niskin bottle at 40 and 55-m depth and transferred to 1 mL filtered and sterilized seawater. Finally, each specimen was picked up and deposited into a 1.5-ml Eppendorf tube filled with several drops of 100% ethanol. The sample was kept at laboratory temperature and in darkness until the molecular analyses could be performed.

Polymerase chain reaction amplification of small subunit rRNA genes (SSU rDNAs) and sequencing. The single cells of *E. agile* and Warnowia sp. fixed in ethanol were centrifuged gently for 5 min at 504 g. Ethanol was then evaporated in a vacuum desiccator and single cells were resuspended directly in 25 µl of Ex TaKaRa (TaKaRa, distributed by Lonza Cia., Levallois-Perret, France) polymerase chain reaction (PCR) reaction mix containing 10 pmol of the eukaryotic-specific SSU rDNA primers EK-42F (5’-CTCAAARGYTAAGCCATGCA-3’) and EK-1520R (5’-CTAAARGYTAAGCCATGCA-3’)
The PCR reactions were performed under the following conditions: 2 min denaturation at 94 °C; 10 cycles of “touch-down” PCR (denaturation at 94 °C for 15 s; a 30 s annealing step at decreasing temperature from 65 down to 55 °C employing a 1 °C decrease with each cycle, extension at 72 °C for 2 min); 20 additional cycles at 55 °C annealing temperature; and a final elongation step of 7 min at 72 °C. A nested PCR reaction was then carried out using 2–5 µl of the first PCR reaction in a GoTaq (Promega, Lyon, France) polymerase reaction mix containing the eukaryotic-specific primers EK-82F (5'-GAAACTGCGAATGGCTC-3') and EK-1498R (5'-CAC CTACGGAAACCTTGTTA-3') and similar PCR conditions as above. A third, semi-nested, PCR was carried out using the dinoflagellate-specific primer DIN464F (5'-TAACAATACAGGCGATTCATCCAT-3'). Amplicons of the expected size (~1,200 bp) were then sequenced bidirectionally using primers DIN464F and EK-1498R (Cogenics, Meylan, France). The sequences obtained were 1,211 bp long and deposited in GenBank with Accession numbers FJ467491–FJ467492.

**Phylogenetic analyses.** The new warnowiid sequences were aligned to a large multiple sequence alignment containing 890 publicly available complete or nearly complete (>1,300 bp) dinoflagellate SSU rDNA sequences using the profile alignment option of MUSCLE 3.7 (Edgar 2004). The resulting alignment was manually inspected using the program ED of the MUST package (Philippe 1993). Ambiguously aligned regions and gaps were ex-
cluded in phylogenetic analyses. Preliminary phylogenetic trees with all sequences were constructed using the neighbor joining method (Saitou and Nei 1987) implemented in the MUST package (Philippe 1993). These trees allowed identifying the closest relatives of our sequences, which were selected, together with a sample of other dinoflagellate species, to carry out more computationally intensive maximum likelihood (ML) and Bayesian Inference (BI) analyses. Maximum likelihood analyses were done with the program TREEFINDER (Jobb, von Haeseler, and Strimmer 2004) applying a GTR+Γ+I model of nucleotide substitution, taking into account a proportion of invariable sites, and a Γ-shaped distribution of substitution rates with four rate categories. This model was chosen using the model selection tool implemented in TREEFINDER (Jobb et al. 2004) with the Akaike information criterion (AIC) as fitting criterion. Bootstrap values were calculated using 1,000 pseudoreplicates with the same substitution model. The BI analyses were carried out with the program PHYLOBAYES applying a GTR+CAT Bayesian mixture model (Lartillot and Philippe 2004), with two independent runs and 1,000,000 generations per run. This model was chosen after several preliminary runs as the model providing the best likelihood estimates. After checking convergence (maximum difference between all bipartitions <0.01) and eliminating the first 1,500 trees (burn-in), a consensus tree was constructed sampling every 100 trees. To test if different tree topologies were significantly different, we carried out the approximately unbiased (AU) test (Shimodaira 2002) implemented in TREEFINDER (Jobb et al. 2004).

RESULTS

Observations of live specimens. Despite one year of intensive sampling, the records of Erythropsidinium were very scarce. We observed only two specimens in late autumn 2007 (Fig. 1) and 20 specimens in June 2008 (Fig. 2–9). The highest abundance was observed at an offshore station in the Bay of Marseille in June 10, 2008 at 55 m depth. Although the general immobility of Erythropsidinium facilitated its capture with a micropipette, the specimens were highly delicate and disintegrated easily during manipulation. Occasionally, the piston vigorously extended and retracted and then the cell quickly displaced in a straight line. Seven specimens resisted the procedure of isolation, washing, and retraction and then the cell quickly displaced in a straight line.

DISCUSSION

Warnowiids within the Gymnodiniales sensu stricto. The members of Gymnodinium s.s. showed a high ultrastructural diversity and also a high degree of ecological specialization when compared with other groups of dinoflagellates. They are present in freshwater, brackish, and marine habitats, exhibiting both planktonic and benthic forms within the same genus (e.g. Polykrikos). The morphology may vary from unicellular to pseu-

Fig. 12. Maximum likelihood phylogenetic tree of dinoflagellate small subunit rDNA sequences, based on 1,078 aligned positions. Names in bold represent sequences obtained in this study. Numbers at the nodes are bootstrap support (values under 50% were omitted). Nodes supported by posterior probabilities >0.9 in Bayesian inference analyses are indicated by black circles. The branch leading to three fast-evolving Amphidinium species has been shortened to one-third (indicated by 1/3). Accession numbers are provided between brackets. The scale bar represents the number of substitutions for a unit branch length. The symbols represent the ocelloid, piston, and nematocyst.
doclonial (multinucleate) and colonial forms. The trophic behavior varies from strictly autotrophic, parasitic, mixotrophic to phagotrophic species with complex organelles (Daugbjerg et al. 2000; Hoppenrath and Leander 2007a; Kim, Iwataki, and Kim 2008).

Based on morphological arguments, three sequences retrieved from GenBank as belonging to the genus Gymnodinium, G. dorsum, G. instriatum, and G. uncatenatum should be also considered as members of the Gymnodinium s.s. despite the low statistical support for their position in our tree. In fact, it has been shown that the two latter species have the typical loop-shaped apical groove characterizing the group (Coats and Park 2002; Hallergaaff 2002). The type of the genus Gymnodinium, G. fusum, also possesses a loop-shaped apical groove running counterclockwise (Daugbjerg et al. 2000). Based on scanning electron microscopy, Takayama (1985) showed the presence of this type of apical groove in Polykrikos, Warnowia, and Erythropсидinium. The apical groove of Gymnodinium and Polykrikos turns around once, while it makes more than one turn in several species of Warnowia, Nematodınum, and Erythropсидinium (Takayama 1985). In contrast to the conservation of the apical groove morphology, the occurrence of complex organelles, such as nematocytes, ocelloïds or pistons, the presence of chloroplasts, the habitat, the colonial behavior, or the number of zooids in the multinucleate species are not taxonomically significant for the definition of taxa above the genus level (i.e. family or higher).

**Origin of the complex organelles.** In addition to trichocysts and mucocysts, some warnowiid and phagotrophic Polykrikos species possess extrusive organelles (i.e. nematocytes). Because these ejective bodies for prey capture are remarkably similar to those in cnidarians (cnidoblasts), the hypothesis of a symbiogenetic origin for these stinging structures in metazoans has been advanced (Shostak and Kolluri 1995). A symbiogenetic origin could be speculated based on the precedent that some ciliates have defensive “extrusomes” derived from bacterial ectosymbionts (Petroni et al. 2000). More difficult is to establish a tentative symbiotic origin of the ocelloid and piston. A complex photoreceptor with cornea, lens, and pigment cup is an organization that can only be found in the metazoan eyes. As some unarmored dinoflagellates (i.e. Symbiodinium) are symbionts in cnidarians, Gehring (2004, 2005) speculated, without corroborative evidence, that ocelloid-bearing dinoflagellates, the warnowiid Erythropсидinium and Warnowia, might have transferred their photoreceptor genes to cnidarians and be at the origin of photoreceptor cells in metazoans.

Although horizontal gene transfer is acknowledged for some “evolutionary jumps” in eukaryotes (Nosenko and Bhattacharya 2007; Raymond and Blankenship 2003), a high number of genes is required for the development of a metazoan eye, which makes its acquisition from unicellular eukaryotes (or the other way round) very improbable. The ontogenesis of the organelle might help unraveling its evolutionary origin. Greuet (1977) found that the retinoid/pigment cup complex (all within the same membranous compartment) dedifferentiated to the point that they appeared to derive from a chloroplast during binary fission in warnowiids. These observations suggested that the warnowiids were able to transform a chloroplast into a complex organelle that is morphologically convergent with the metazoan eye. The origin of the chloroplast in the heterotrophic warnowiids is itself uncertain. However, dinoflagellates have a remarkable facility to acquire and replace chloroplasts from diverse microalgal groups (Saldarriaga et al. 2001), and even non-photosynthetic dinoflagellates have plastid genes (Sánchez-Puerta et al. 2007). Kleptoplastidy in dinoflagellates may be the origin of this high diversity of plastids through secondary or tertiary endosymbiosis (Koike et al. 2005).

The warnowiids branched within the Gymnodinium s.s. group, which has a high diversity of chloroplasts (Daugbjerg et al. 2000; Hansen et al. 2007; Hoppenrath and Leander 2007b). Although the support was relatively moderate in our phylogenetic analysis, the closest relative to the ocelloid-bearing dinoflagellates appears to be P. beauchampii, whose chloroplasts have not been studied in detail. In contrast to Polykrikos, P. beauchampii has one nucleus in each zooid and it may disassociate into single uninucleate cells (Chatton 1933, 1952). The warnowiids are considered strictly heterotrophic. Dodge (1982) reported that Nematodınum armatum contains scattered yellow chloroplasts. Sournia (1986) commented that Dodge’s observations of N. armatum are inconsistent with the generic description and require further clarification. Considering that the closest relative to the warnowiids appears to be the photosynthetic P. beauchampii, the potential for an ocelloid-bearing dinoflagellate with chloroplasts should not be discarded.

The evolutionary origin of the piston is also enigmatic. No other known organism possesses this kind of organelle. Based on transmission electron microscopy, Greuet (1987) illustrated a myofibril system and mitochondrial papillae in the piston. The numerous mitochondria associated with the piston likely provide the energy to maintain its movement even after cell lysis. While the division of the ocelloid is synchronized with the cell division, the piston is retained by one of the daughter cells (Elbrächter 1979; Greuet 1977). Greuet (1969) observed the regeneration of the piston from the bulb. Considering that the warnowiids have been able to develop complex organelles, such as the eye-like structure, the development of a relatively simpler structure, such as the piston, is not unexpected. Consequently, the evolution from simpler structures would explain the origin of complex organelles (ocelloid and piston).

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**LITERATURE CITED**


