9-2006

Morphological and Phylogenetic Description of an Unusual Amphidinium (Dinophyceae) Species

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Abstract

Amphidinium carterae, an important harmful algal species that produces powerful antifungal and hemolytic compounds (amphidinols) and cytotoxic macrolides (amphidinolides) is ubiquitous in coastal waters. Samples from coral rubble contained an unusual and previously unreported *Amphidinium* (D2) with a circular outline. Genetic analysis of clone D2 of this species, involving the sequencing of large subunit (LSU) rDNA, revealed a relationship between *Amphidinium* sp. D2 and both *A. carterae* and *A. massartii*. However, morphological and genetic differences suggest that *Amphidinium* sp. D2 is not conspecific with *A. carterae* or *A. massartii*. Further studies to describe this species are presently underway.

Introduction

Amphidinium Claparède & J. Lachmann is a genus of epibenthic dinoflagellates with approximately 120 described species (Murray and Patterson 2001). The concept of the genus has recently been narrowed by Flø Jørgensen *et al.* (2004), to the exclusion of many species, including all known freshwater species (Calado and Moestrup 2005). Hypotheses used to explain past systematic confusion about the species within *Amphidinium sensu stricto* included the concept of an *Amphidinium operculatum* Claparède & J. Lachmann species complex as well as the notion that there were many forms of *A. operculatum* (Barlow and Triemer 1988; Al-Qassab *et al.* 2002; Murray and Patterson 2002; Murray *et al.* 2004). Murray *et al.* (2004) used phylogenetic analysis combined with morphological descriptions to show at least 9 distinct species within *Amphidinium sensu stricto*.

Within the genus *Amphidinium* the ubiquitous *Amphidinium carterae* Hulburt is probably the most studied species. Along with *A. klebsii* Kofoid & Swezy (=*A. gibbosum sensu* Murray *et al.* 2004), *A. carterae* is known to produce amphidinols, compounds having both antifungal and hemolytic properties and cytotoxic macrolides called amphidinolides (Satake *et al.* 1991; Ishibashi and Kobayashi 1997; Echigoya *et al.* 2005). Ecologically, the role of *Amphidinium* species as primary producers is likely to be significant in coastal, benthic communities (Flø Jørgensen *et al.* 2004).

The defining morphological characteristic of the genus *Amphidinium* is an epicone smaller than the hypocone; within *Amphidinium sensu stricto* the epicone is minute, triangular or crescent-shaped, and deflected to the left. Morphological characters used to differentiate species include epicone size, presence or absence of plastids, plastid appearance, positioning of the sulcus and cingulum, and general cellular proportions (Flø Jørgensen *et al.* 2004). *Amphidinium* species within the smallest size range (10-20 x 7-17μm) include *A. carterae* and *A. massartii* Biecheler. The two species are similar in size and appearance and are often difficult to differentiate using light microscopy (Murray *et al.* 2004).

The objective of this study is the identification to the species level of the two clonal isolates of *Amphidinium* (D1 and D2) which have a circular morphology. Morphological and phylogenetic results were compared to known cultures of *A. carterae* and *A. klebsii*, as well as data from Murray *et al.* (2004).

Materials and Methods

All cultures of *Amphidinium* species were established using single cell pipette isolation and maintained non-axenically. *Amphidinium* clones D1 and D2 were isolated from a sample of live rock obtained at a local aquarium store and *A. carterae* UNCW and *A. klebsii* UNCW were both isolated from water samples taken in the Bahamas. All cultures were maintained in K media (Keller and Guillard 1987) at 39‰ and 28 °C on a 14:10 h L:D cycle.

Micrographs were obtained using a Zeiss Axio Imager Z1. Images for measurements were taken on a Nikon Diaphot inverted microscope and measured in Axio Vision v4.5.0.0. Chloroplast epifluorescence was observed using a Rhodamine filter.

Moestrup, Ø. *et al.* (eds) Proceedings of the 12th International Conference on Harmful Algae. International Society for the Study of Harmful Algae and Intergovernmental Oceanographic Commission of UNESCO, 2008 Copenhagen

Table 1. Morphometric variation among species of *Amphidinium*

Species	n	Length (µm)								W/L	
		Maximum	Minimum	Mean	SD	Maximum	Minimum	Mean	SD	Mean	SD
*A. massartii**	-	19	13	16	1.8	15	8	12	1.8	0.77	-
*A. carterae**	-	16	11	14	1.2	11	7	9	1.0	0.67	-
A. carterae UNCW	50	18	12	15	1.5	13	8	10	1.2	0.67	0.05
Amphidinium sp. D2	102	15	10	12	1.1	14	10	12	1.0	0.97	0.05

*From Murray *et al.* 2004.

A volume of 8-10 ml of culture was centrifuged at 2,000 rpm for 15 min. The resulting pellet was frozen in liquid nitrogen and kept frozen in a -80 °C freezer until DNA extraction. Extraction of genomic DNA was done using a Power Soil DNA kit (MoBio Labs, Inc.). PCR of the LSU rDNA gene was performed with previously published primers (Flø Jørgensen *et al.* 2004). LSU rDNA, sites D1-D6, was sequenced using internal primers (D1R, D3B, D3AC, D2C, D2Ra, and 1483R) and read on an Applied Biosystem's Hitachi 3100 Genetic Analyzer (Tokyo, Japan).

Sequences were assembled using the program Sequencher v4.6 and aligned using Clustal W (European Bioinformatics Institute). Aligned sequences were proofread using MacClade v4.06. The hypervariable D2 region was removed according to the alignment of Murray *et al.* (2004) resulting in sequences of 1189 base pairs.

Maximum likelihood analysis was done with PAUP v4.0b10 (Swofford 2000) using heuristic searches with 10 random addition replicates and a TBR branch swapping algorithm. The optimized model chosen by Modeltest version 3.06 (Posada and Crandall 1998) was a general time reversal (GTR+I+G). The specific parameters were a rate matrix of (1, 2.8547, 1, 1, 6.2407, 1), proportion of invariable sites = 0.1811, gamma distribution shape a= 0.6863, and nucleotide frequencies of a=0.2585, c=0.196, g=0.288, t=0.2567. Bootstrap values were obtained using heuristic searches and 100 replicates.

Results

Width and length measurements of *Amphidinium* sp. D2 overlap in size range with both *A. carterae* and *A. massartii*. The length to width ratio for *A. carterae* and *A. massartii* ranges from 0.67 to 0.77, whereas in the almost circular *Amphidinium* sp. D2 it is 0.97 (Table 1). *Amphidinium carterae* has an ovoid cell shape with a starch-sheathed pyrenoid located in the left anterior region of the hypocone, while *Amphidinium* sp. D2 has a more centrally located pyrenoid (Fig. 1 a, b).

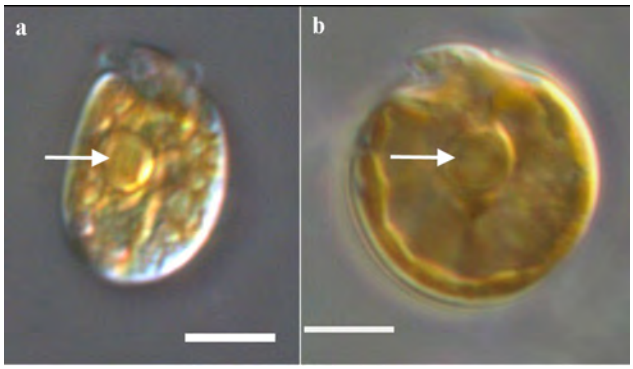

Figure 1. Light micrographs of (a) *A. carterae* and (b) *Amphidinium* sp. D2 in dorsal view. Arrows point to pyrenoid. Scale bars = 5 µm.

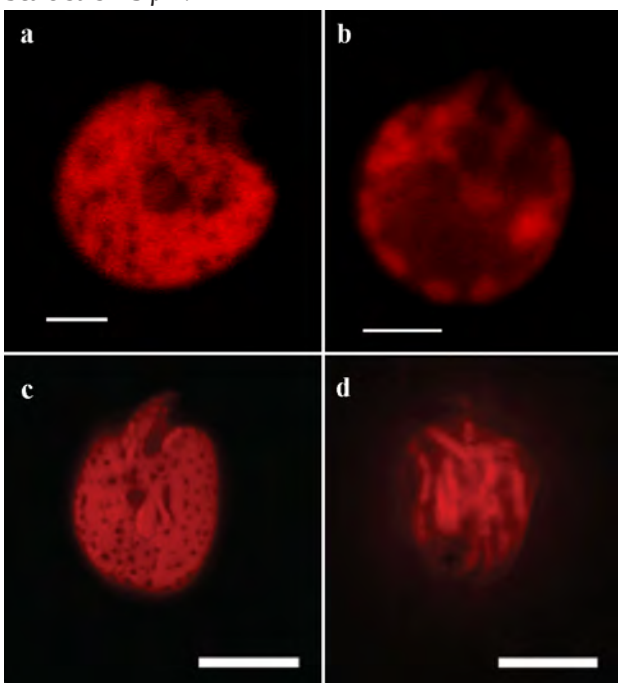

Figure 2. Epifluorescence micrographs of *Amphidinium* species. (a, b) Ventral views of *Amphidinium* sp. D2, (c) ventral view of *A. carterae*, (d) dorsal view of *A. massartii*, *(c,d) from Murray *et al.* 2004. (a, b) Scale bars = 5 µm. (c, d) Scale bars = 10 µm.

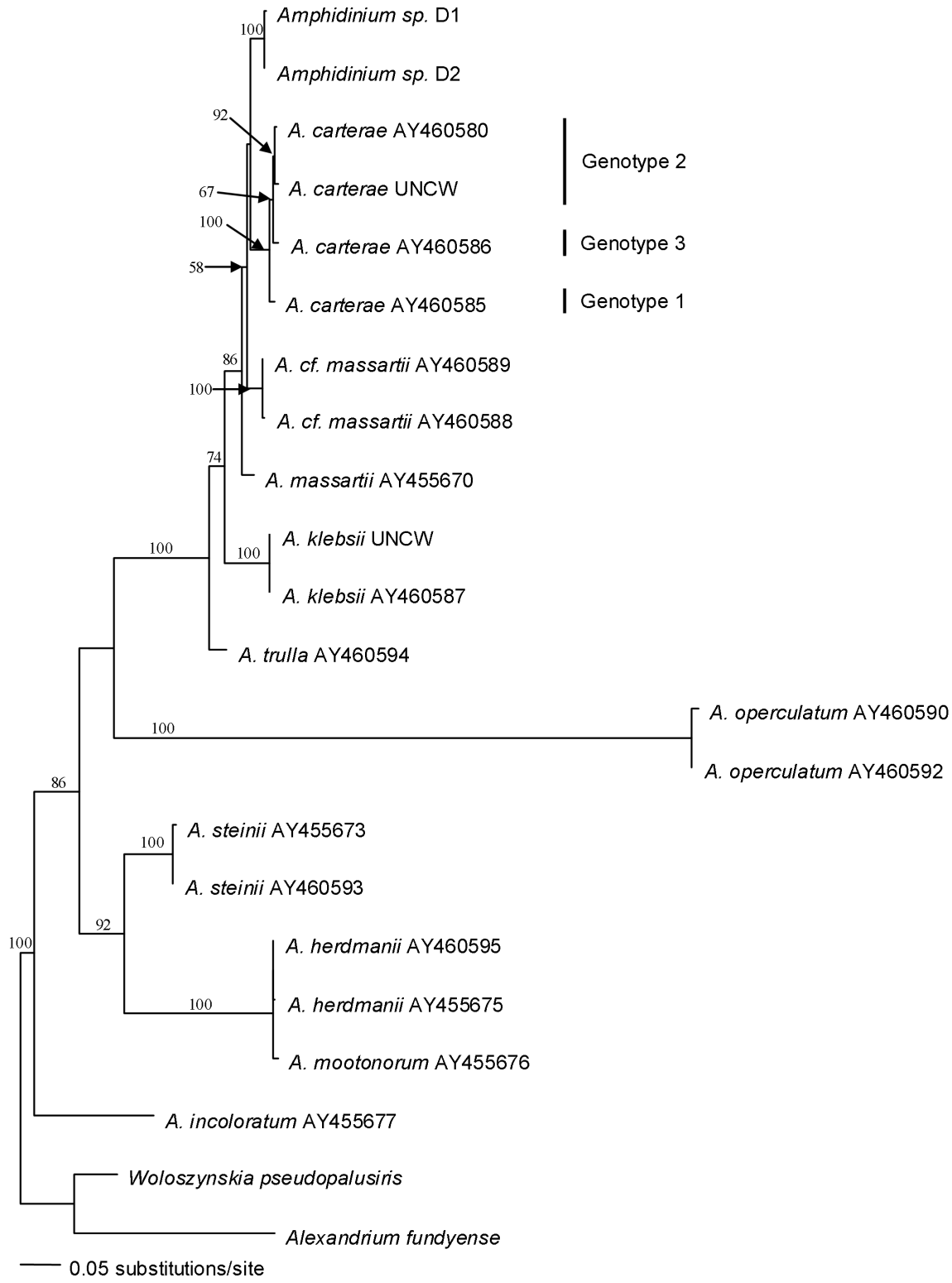

Figure 3. Phylogram obtained by maximum likelihood analysis of LSU rDNA sites D1-D6 excluding the hypervariable site D2. Bootstrap values above 50% are shown.

Amphidinium sp. D2 has a dorsally located, perforated, peripheral plastid which is connected to a central radiating chloroplast (Fig. 2 a, b). This morphology is distinct to *A. carterae* and found within all genotypes of the species (Murray *et al.* 2004). *Amphidinium massartii* (Fig. 2 d) was described as having a more internal plastid with many lobes radiating from the pyrenoid (Murray *et al.* 2004).

Maximum likelihood analysis of LSU rDNA places *Amphidinium* clones D1 and D2 as a sister group to *A. carterae* (Fig. 3). The intraspecific variation of LSU rDNA, domains D1-D6 excluding domain D2, for all *A. carterae* genotypes is 0.4-1.7%. The variation between *Amphidinium* clones D1 and D2 and *A. carterae* (4.1-4.4 %) is higher than *A. carterae*'s intraspecific variation (unpublished). *Amphidinium* D1 and D2 formed their own clade distinct from both *A. carterae* and *A. massartii*.

Discussion

Morphologically *Amphidinium* sp. D2 is distinct from both *A. carterae* and *A. massartii*. Phylogenetic analysis shows that *Amphidinium* sp. D2 is related to *A. carterae* and *A. massartii* but not close enough to call it either species. By combining the morphological and phylogenetic data it is shown that *Amphidinium* sp. D2 is not conspecific to either *A. carterae* or *A. massartii*. More environmental and physiological studies are needed to determine the plasticity of this unusual morphology. The morphological and phylogenetic proximity of *Amphidinium* sp. D2 to *A. carterae* is cause for interest in the production of bioactive compounds. More toxin assays need to be run to determine *Amphidinium* sp. D2's potential to produce toxins.

Acknowledgements

This work was supported by CDC grant # 01-584-4 awarded to C. Tomas. We would like to thank Dr. BK Song for his help with this project.

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