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AMPHIDINIUM REVISITED. II. RESOLVING SPECIES BOUNDARIES IN THE *AMPHIDINIUM OPERCULATUM* SPECIES COMPLEX (DINOPHYCEAE), INCLUDING THE DESCRIPTIONS OF *AMPHIDINIUM TRULLA* SP. NOV. AND *AMPHIDINIUM GIBBOSUM*. COMB. NOV.[1]

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Amphidinium operculatum **Claparède et Lachmann, the type species of the dinoflagellate genus *Amphidinium*, has long had an uncertain identity. It has been considered to be either difficult to distinguish from other similar species or a morphologically variable species itself. This has led to the hypothesis that *A. operculatum* represents a "species complex." Recently, the problem of distinguishing *A. operculatum* from similar species has become particularly acute, because several morphologically similar species have been found to produce bioactive compounds of potential interest to the pharmaceutical industry. In this study, we cultured and examined existing cultures of several species of *Amphidinium*, most of which have been previously identified as *A. operculatum* or as species considered by some to be synonyms or varieties of *A. operculatum*. Thirty strains were examined using comparative LM, SEM, and partial large subunit (LSU) rDNA sequence data. Through morphological and molecular phylogenetic analyses, six distinct species were identified, including *Amphidinium trulla* sp. nov. and *Amphidinium gibbosum* comb. nov. *Amphidinium operculatum* was redescribed based on four cultures. Genetic variability within the examined *Amphidinium* species varied greatly. There was little difference among strains in partial LSU rDNA for most species, but strains of *A. carterae* and *A. massartii* Biencheler differed by as much as 4%. In both *A. carterae* and *A. massartii*, three distinct genotypes based on partial LSU rDNA were found, but no morphological differences among strains could be observed using LM or SEM. In the case of *A. carterae*, no biogeographically related molecular differences were found.**

Key index words: ***Amphidinium belauense*; *Amphidinium carterae*; *Amphidinium gibbosum*; *Amphidinium klebsii*; *Amphidinium operculatum*; *Amphidinium* sensu stricto; *Amphidinium trulla*; Dinophyceae; LSU rDNA analysis; molecular phylogeny**

Abbreviations: **BA, Bayesian analysis; LSU rDNA, nuclear-encoded large subunit rRNA coding gene; ML, maximum likelihood; PP, posterior probability**

Within the benthic and endosymbiotic dinoflagellate genus *Amphidinium*, identification of the type species *Amphidinium operculatum* Claparède et Lachmann and closely related species has long been considered problematic. The main reasons for this are the morphological similarity between *A. operculatum* and at least 10 other nominal species, and the fact that the original descriptions of *A. operculatum* and species similar to *A. operculatum* were often incomplete or did not clearly state the distinguishing characters. Many later taxonomists did not appreciate that distinguishing characters existed and used the names of these species interchangeably. Failure to differentiate between species has led to hypotheses that descriptions of most other named species actually represent *A. operculatum*, which is therefore a very morphologically variable species throughout its range or life cycle (Herdman 1924, Dodge 1982, Barlow and Triemer 1988), or that an *A. operculatum* species complex exists (Larsen 1985, Hoppenrath 2000, Al-Qassab et al. 2002, Murray and Patterson 2002). A previous attempt to resolve the identity of these species (Taylor 1971b) provided some clarification but was hampered by a lack of access to a large diversity of cultures or to modern techniques such as nucleotide sequencing.

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The identification of species of *Amphidinium* is particularly relevant because strains of two species of *Amphidinium* sensu stricto, one of which is described as a variety of the type species, *A. operculatum* var. *gibbosum* and *A. carterae*, have been reported to produce ichthyotoxins called amphidinols, amphidinolides, and caribenolides (Kobayashi et al. 1991, Satake et al. 1991, Bauer et al. 1994, 1995a, Paul et al. 1996, Houdai et al. 2001). Amphidinolides have shown potential antitumor activity and are being investigated for use in the development of drugs against leukemia (Bauer et al. 1994, 1995b, Williams and Meyer 2001), whereas amphidinols have shown antifungal activity (Satake et al. 1991). The identification of species closely related to these would make new organisms available for future screening of bioactive substances.

In addition, some *Amphidinium* strains, especially of species identified as *A. carterae* and *A. operculatum*, have high growth rates and are easily grown in culture, making them ideal study objects. For this reason, studies of the unique structure of the dinoflagellate nucleus (Oakley and Dodge 1974, Barlow and Triemer 1986, 1988), of the peridinin-chloroplast A-protein light-harvesting antenna complex (Damjanovic et al. 2000, Kleima-Foske et al. 2000a,b), of the UV-protective substances known as mycosporine amino acids (Banaszak et al. 2000), and of the unique form II of RUBISCO (Jenks and Gibbs 2000) have been carried out on strains of *Amphidinium*. Considerable effort is going into research using these strains, resulting in the unfortunate situation that the identity of the species on which important research has been carried out is not clear. Finally, the ecological significance of *Amphidinium* species in their role as primary producers and primary consumers in the microbenthos is largely unknown. Identifiable species are a prerequisite for future ecological investigations.

In an effort to clarify species identification, we cultured or obtained 30 strains of *Amphidinium* sensu stricto, many of which had been initially identified as "*Amphidinium operculatum*" or as "*Amphidinium carterae*," and investigated all strains using LM and some strains using SEM. We sequenced the partial large subunit (LSU) rDNA, covering the domains D1 to 20 bp downstream of D6 (Lenaers et al. 1989). We also examined the species *A. operculatum*, *Amphidinium steinii*, and *A. carterae* from field-collected sediment samples from sites in southeastern Australia, western Australia, and several sites in Denmark by LM and SEM. We critically examined all previous taxonomic information about these species in light of our observations. To facilitate species identification in future studies, species boundaries were established by applying a typological (morphological) species concept, that is, morphological synapomorphies were considered critical for species identification, and where genetic differences were found between morphologically indistinguishable organisms, these were considered to represent genotypes.

MATERIALS AND METHODS

Cultures. Sediment and water column samples for isolation of species into culture were collected from marine sites in several areas of New Zealand, southeastern Australia, the state of Rio de Janeiro, Brazil, and Denmark (Table 1). Cultures of species of *Amphidinium* were obtained by isolation using a fine pipette and brought into nonaxenic unialgal cultures using TL media (Larsen et al. 1994) or F/2 media (Guillard 1983). Where possible, new cultures were lodged in recognized culture collections: the Cawthron Collection of Microalgae (CAWD strain numbers) in Nelson, New Zealand; the Scandinavian Culture Collection for Algae and Protozoa (K numbers) in Copenhagen, Denmark; or the CSIRO Collection of Living Microalgae (CS numbers) in Hobart, Australia. The strain codes SM and MFJ are held by the authors. Cultures were also obtained from the Provasoli-Guillard National Center for Marine Phytoplankton (CCMP numbers) and from Imojen Pearce, from the University of Tasmania, Australia (AKLVO1 and AKLSPO1). A DNA sample of *A. gibbosum* strain SI-36-5 was obtained from Y. Shimizu (University of Rhode Island, Kingston, RI, USA). The descriptions of the species *A. operculatum*, *A. steinii*, and *A. carterae* also incorporated information from these species examined and photographed live from approximately 60 sediment samples from nine sites in Australia and Denmark (for collection methods, sites, and dates see Al-Qassab et al. 2002, Flø Jørgensen 2002, Murray and Patterson 2002, Murray 2003).

LM. Micrographs were obtained using a BX 60 microscope with a DP10 digital camera (Olympus, Tokyo, Japan), Olympus Provis AX70 microscope with an AXIOCAM digital camera (Zeiss, München-Hallbergmoss, Germany), or a DMR microscope (Leica, Wetzlar, Germany) with a 6006 camera (Nikon, Tokyo, Japan) using Kodak Tech Pan emulsion film (Nikon). Images were generally taken using DIC optics (Nikon). Negatives were digitalized using a Nikon Cool Scan (Nikon).

SEM. Cells were pipetted individually from samples, rinsed three times in filtered seawater, and placed on polylysine-coated coverslips or taken from cultures and filtered onto 8-μm filters (Millipore, Bedford, MA, USA) using a Swinnex filter holder. They were fixed in 2% osmium tetroxide (Proscitech, Kirwan, Australia) in seawater for 20 min, rinsed in distilled water, and dehydrated in a series of increasing ethanol concentrations (15%, 30%, 50%, 70%, 90%, and 100%). The cells were critical point dried. They were observed using a model 505 (Philips, Eindhoven, The Netherlands) or a model 6330 F (JEOL, Tokyo, Japan) field emission scanning electron microscope at 5–20 kV.

DNA extraction, amplification, and sequencing of LSU rDNA domains D1–D6. DNA extraction, amplification, and sequencing of partial LSU rDNA were done as described in Flø Jørgensen et al. (2004).

Sequence alignment and phylogenetic analyses. Sequences were aligned as described in Flø Jørgensen et al. (2004). As outgroup species, the two dinoflagellates *Woloszynskia pseudopalustris* (GenBank accession no. AF260402) and *Alexandrium fundyense* (GenBank accession no. AF200666) were used; several additional ingroup sequences were used: *Amphidinium carterae* JL3 (GenBank accession no. AF260380), *A. herdmanii* (GenBank accession no. AY455675), *A. herdmanii* (GenBank accession no. AY460595), and *A. mootonorum* (GenBank accession no. AY455676). The data matrix comprised 1196 aligned positions, including introduced gaps but excluding the highly variable D2 region (alignment available at www.bi.ku.dk/staff/nielsd/jphycol2004b.htm). In total, 544 aligned positions were considered unambiguous and examined using maximum likelihood (ML) and Bayesian analysis (BA).

ML analysis was performed using PAUP* version 4.0b10 (Swofford 2000) using heuristic searches with 10 random

TABLE 1. Strains of the six species compared in this study, their collection site, and date.

Species name	Culture number	GenBank accession number no.	Collection site and date (if known)	Isolated by
Amphidinium carterae	CS-740	AY460578	Sediment from Port Botany, Sydney, Australia, December 1999	S. Murray
	SM10	AY460579	Sediment from Canal de Joatinga, Rio de Janeiro, Brazil, December 2000	S. Murray
	SM11	AY460580	Niteroi, Rio de Janeiro, Brazil	S. Lourenço
	CAWD22	AY460581	Water column from Wellington, New Zealand	L. Rhodes
	CAWD23	AY460582	Water column from Marlborough, New Zealand	L. Rhodes
	CAWD57	AY460583	Water column from Kauauora, New Zealand	L. Rhodes
	CCMP124	AY460584	Puerto Penasco, Sonora, Mexico 1966	R. Norris
	CCMP121	AY460585	Endosymbiont in the jellyfish *Cassiopeia xamachana* from the Caribbean Sea	R. Kinzie
	CCMP1748	AY460586	Main Lagoon, Carrie Bow Cay, Belize, Caribbean Sea	S. L. Morton
	K-0654	AY455669	Hirsholmene Harbour, Denmark, from sediment, August 2000	M. F. Jørgensen
Amphidinium gibbosum	SI-36-50	AY460587	Interstitial water from U.S. Virgin Island, St. Thomas, April 1990	L. Maranda
	CCMP120	AY455672	Endosymbiont in the pelagic flatworm *Amphiscolops langerhansii* in an artificial seawater tank, Miami, FL, USA	D. L. Taylor
Amphidinium massartii	AKLSP01	AY460588	Tasmania, Australia	I. Pearce
	AKLV01	AY460589	Tasmania, Australia	I. Pearce
	CCMP1821	AY455670	Kingston, Rhode Island, USA, October 1997	P. Hargraves
	CCMP1342	AF260381	Knight Key, Florida, USA, 1985	J. Bomber
Amphidinium operculatum	K-0663	AY455674	Sediment from Port Botany, Sydney, Australia, March 2000	S. Murray
	CAWD42	AY460590	Water column from Cable Bay, New Zealand	L. Rhodes
	CAWD55	AY460591	Water column from Parengarenga, New Zealand	L. Rhodes
	CAWD56	AY460592	Water column from Parengarenga, New Zealand	L. Rhodes
Amphidinium steinii	SM12	AY460593	Sediment from Angra dos Reis, Rio de Janeiro, Brazil December 2000	S. Murray
	CS-741	AY455673	Sediment from Port Botany, Sydney, Australia, March 2001	S. Murray
Amphidinium trulla	K-0657	AY455671	Sediment from Jægerspris, Isefjorden, Denmark, June 2002	M. F. Jørgensen
	CAWD68	AY460594	Water column from Rangaunu, New Zealand	L. Rhodes

addition replicates and a TBR branch swapping algorithm. Modeltest version 3.04b (Posada and Crandall 1998) was used to estimate the optimal model, which appeared to be general time reversible (GTR + G + I) using a 0.01 level of significance. The exact parameters were estimated from consecutive heuristic searches until the values of the parameters converged. The optimal parameters were as follows: substitution matrix of (1, 2.602, 1, 1, 6.3935, 1), proportion of invariable sites = 0.1951, gamma distribution shape $\alpha = 0.6395$, and nucleotide frequencies of a = 0.2736, c = 0.1864, g = 0.2797, and t = 0.2603.

BA was conducted using the program MrBayes version 2.01 (Huelsenbeck and Ronquist 2001, Huelsenbeck et al. 2001) set to operate with a general time reversible model with a gamma distribution and three heated chains supplementing the cold chain as described by Hall (2001). A total of 2,020,000 generation were calculated with a tree sampled every 50 generations and a prior burn-in of 20,000 generations equaling 400 trees. The ln likelihood value converged at a value of approximately -7.4×10^9. Forty thousand sampled trees were imported into PAUP* version 4.0b10 (Swofford 2000) and used to calculate a consensus phylogram, as described in Flø Jørgensen et al. (2004).

RESULTS

Amphidinium operculatum Claparède et Lachmann
Figs. 1, A–F, 2A, 3A

Synonym: Amphidinium elegans Grell et Wohlfarth-Bottermann

Description. Cells are ovoid to ellipsoidal in shape, with the greatest width between the cell center and the posterior end and dorsoventrally flattened. Cells are 29–50 μm long and 21–28 μm wide (for individual strains see Table 2). The right side of the hypocone is convex, whereas the left is almost straight (Fig. 1A). A small epicone overlays the anterior central part of the hypocone. In ventral view, the epicone is irregular triangular with the anterior left tip clearly deflected to the left. The epicone is 7–10 μm wide and anteriorly fairly flat (Fig. 1A). The angle of the anterior right tip is almost 90 degrees, with the anterior left tip forming an angle of approximately 30 degrees. The deeply

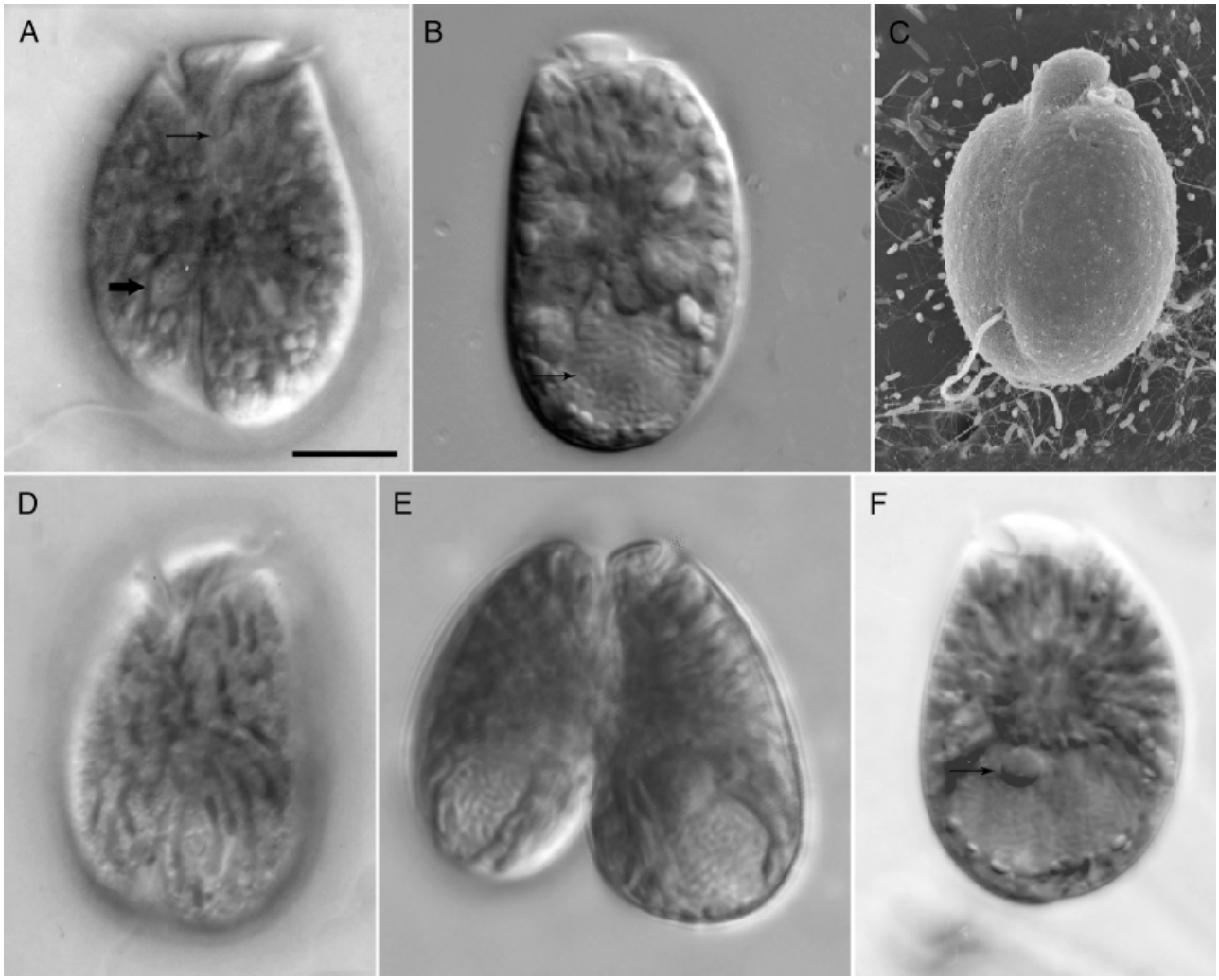

FIG. 1. *Amphidinium operculatum*, LM and SEM. (A) Cell from natural sample from western Australia, thick arrow points to pusule and sulcal origin, thin arrow points to cingular shape. (B) Dorsal view of cell from culture CAWD42, thin arrow points to posterior nucleus. (C) Cell from K-0663 showing posterior flagellar insertion. (D) Cell from natural sample from Sydney showing elongated plastids. (E) Cells from culture K-0663 showing cell division by binary fission. (F) Cell from natural sample from Sydney showing round inclusion ("stigma"). Scale bar, 10 μm.

incised cingulum originates 0.3 cell lengths from the anterior of the cell. The cingulum is displaced and slightly descending, with an angle of approximately 45 degrees between the proximal and distal ends. The sulcus originates in the lower one third of the cell and just to the right of the central axis (Fig. 1, A and C). It is initially narrow but widens as it approaches the antapex. A narrow ventral ridge runs between the two points of flagellar insertion. The multiple plastids are yellow-brown and elongated, appearing to radiate to the cell periphery, but scattered near the center (Figs. 1D and 3A). No pyrenoid is visible. The nucleus is crescent shaped or oval, in the posterior of the cell, and with delicate thread-like chromosomes (Fig. 1B). Just above the nucleus, one orange-yellow, round, globular inclusions is often visible (Fig. 1F), with a diameter of 6–8 μm. Rarely, several inclusions were observed. Two pusules are present, lying in close proximity to the origin of the transverse and longitudinal flagella, with a diameter of approximately 2 μm (Fig. 1A). Colorless globules ;are sometimes present, as are assimilation granules. Asexual division is by binary fission in the motile cell (Fig. 1E).

Distribution. Species we consider to represent *A. operculatum* have been reported from the vicinity of Bergen, Norway (Claparède and Lachmann 1859); Woods Hole, Massachusetts, USA (as *A. elegans*, Grell and Wohlfarth-Bottermann 1957); Ryukyu Islands, Japan and New Caledonia (as *A. klebsii*, Fukuyo 1981); several sites in New Zealand (Table 1, CAWD55, CAWD56, CAWD42); and northern, southeastern, and western Australia (as *A. elegans*, Al-Qassab et al. 2002, Murray and Patterson 2002, and as *A. herdmanii*, Larsen and Patterson 1990).

Descriptions that we do not consider to represent *A. operculatum* are the following: *A. operculatum* sensu

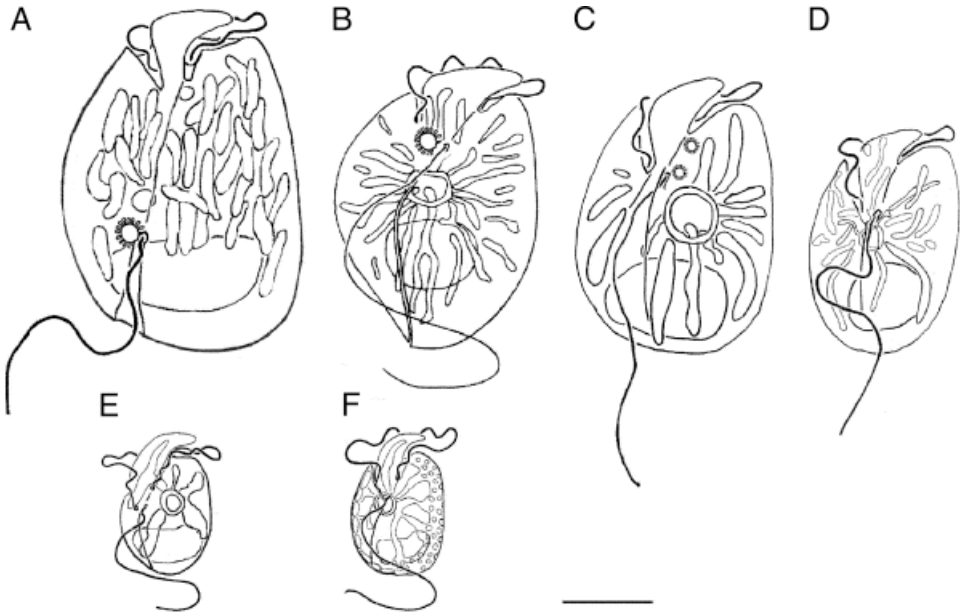

FIG. 2. Line drawings of species included in this study. (A) *Amphidinium operculatum*, (B) *Amphidinium gibbosum*, (C) *Amphidinium steinii*, (D), *Amphidinium trulla*, (E) *Amphidinium massartii*, (F) *Amphidinium carterae*. Scale bar, 10 μm. Drawings show comparative sizes of the cells; epicone shape; points of flagellar insertion; pusules, if present, as circular structures with radiating vesicles; shape of the plastid, if present; size and position of the ring-shaped starch-sheathed pyrenoid, if present; ventral ridges between the points of flagellar insertion; and nuclei. In F, the small circles represent the perforated single chloroplast appearing as a row of small bricks along the edge of a cell.

Diesing (1866), *A. operculatum* sensu Stein (1883), *A. operculatum* sensu Klebs (1884), *A. operculatum* sensu Calkins (1902), *A. operculatum* sensu W. Herdman (1911), *A. operculatum*, sensu Kofoid and Swezy (1921), *A. operculatum* sensu Lebour (1925), *A. operculatum* sensu Conrad (1926), *A. operculatum* sensu Zimmermann (1930), *A. operculatum* sensu Conrad and Kufferath (1954), *A. operculatum* sensu Dodge (1982), *A. operculatum* sensu Larsen (1985), *A. operculatum* sensu Steidinger and Tangen (1997), *A. operculatum* sensu Daugbjerg et al.

TABLE 2. Intraspecific variation in morphometric features among clones of species of *Amphidinium*. The measurements of SI-36-5 were taken from Maranda and Shimizu (1996).

		Length				Width			
Species	Culture	Maximum	Minimum	Mean	SD	Maximum	Minimum	Mean	SD
Amphidinium carterae	K-0654	20	14	17	1.1	14	10	12	1.1
	CS-740	16	11	14	1.4	11	8	9	.96
	SM10	17	13	15	1.1	13	7	10	1.3
	SM11	18	12	15	1.8	13	7	10	1.7
	CAWD22	20	12	17	2.4	15	8	11	1.9
	CAWD23	16	11	14	1.3	11	8	9	1.2
	CAWD57	17	10	14	2.0	11	6	9	1.3
	CCMP1748	—	—	—	—	—	—	—	—
	CCMP121	17	12	14	1.2	13	9	10	0.8
	CCMP124	18	12	15	1.3	12	9	10	0.8
Amphidinium gibbosum	SI-36-5	43	31	—		23	19	—	
	CCMP120	34	24	29	2.3	23	15	19	1.9
Amphidinium massartii	AKLVO1	20	13	16	2.0	17	7	11	2.4
	AKLSPO1	20	13	16	2.0	15	9	10	1.7
	CCMP1342	—	—	—	—	—	—	—	—
	CCMP1821	18	13	15	1.3	14	9	15	1.3
Amphidinium operculatum	K-0663	50	29	35	3.1	21	28	24	2.4
	CAWD42	48	32	39	4.7	30	18	25	3.4
	CAWD55	46	32	37	3.8	36	15	25	4.1
	CAWD56	43	30	36	4.0	30	15	24	3.7
Amphidinium steinii	CS-741	37	20	31	4.2	24	10	20	3.0
	SM12	38	23	29	3.6	32	15	23	6.9
Amphidinium trulla	CAWD68	30	18	24	3.7	22	12	17	2.8
	K-0657	31	18	25	2.7	25	13	19	2.1

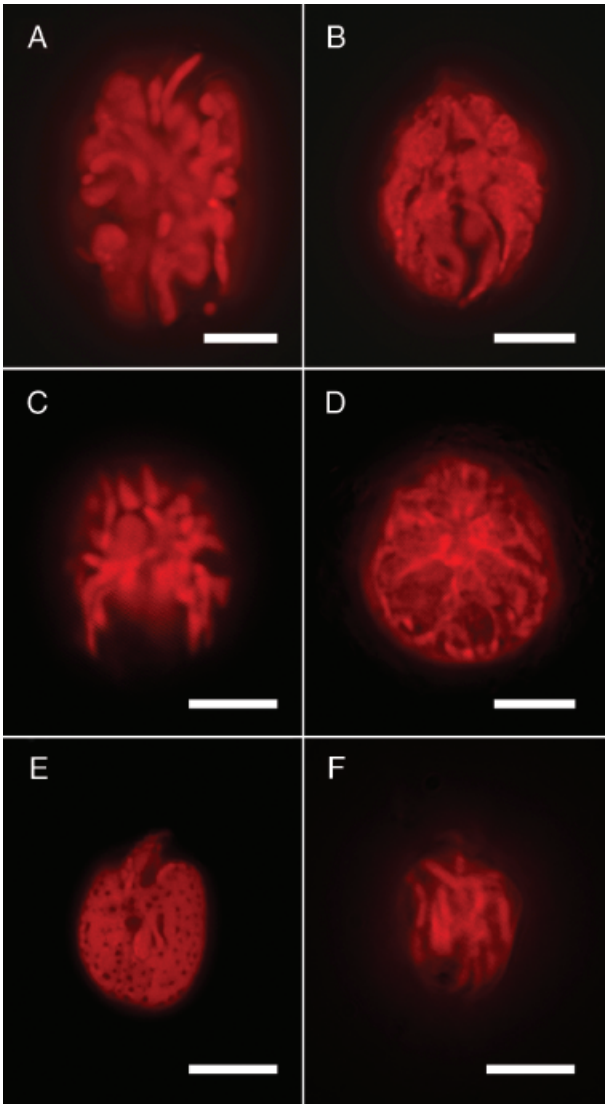

FIG. 3. Epifluorescence LM of species of the *Amphidinium operculatum* species complex. (A) Ventral view of *A. operculatum* K-0663 showing multiple plastids. (B) Dorsal view of *A. gibbosum*. Conspicuous chloroplast lobes radiate from a centrally located pyrenoid. (C) Dorsal view of *A. trulla* showing plastid lobes radiating. (D) Ventral view of *A. steinii*. Multiple slender plastids radiate. (E) Ventral view of *A. carterae*. Plastid lobes are continuous with a superficial perforated plastid. (F) Dorsal view of *A. massartii*, showing slender plastid lobes radiating.

(2000), *A. operculatum* sensu Hoppenrath (2000), *A.* cf. *operculatum* sensu Murray and Patterson (2002), and *A. operculatum* sensu Al-Qassab et al. (2002).

Remarks. Based on the posterior position of the origin of the sulcus, hypocone size and shape, the flatter anterior shape of the epicone, the absence of a ring-like starch-sheathed pyrenoid in the center of a single plastid, and the possession of a round colored inclusion (''stigma'') situated just above the nucleus, *A. operculatum* was identified with reference to the original description (Claparède and Lachmann 1859, Flø Jørgensen et al. 2004). *Amphidinium elegans* was found to be a synonym (Flø Jørgensen et al. 2004). An ultrastructural study of this species, under the name *A. elegans*, has been conducted by Grell and Wohlfarth-Bottermann (1957).

Klebs (1884) described a similar species as *A. operculatum*, which differed from the original description in its anterior position of the sulcus, ''tongue-like'' epicone, more posterior radiation of the plastids, and the possession of ''furrows'' or corrugations on the cell surface. Based on this description, *Amphidinium klebsii* was named as a new species (Kofoid and Swezy 1921). Many authors have since reported *A. klebsii*, whether the species observed by them possessed exactly the same characters as in the original description or not. Many recent authors (Dodge 1982, Larsen 1985, Hoppenrath 2000) have considered *A. klebsii* to be a synonym of *A. operculatum*, and the names have been used interchangeably by other authors. However, based on the original descriptions, we do not consider these two species to be synonymous. In particular, furrows are not present in *A. operculatum* or in *A. steinii*, *A. gibbosum*, or *A. trulla* sp. nov. Another *Amphidinium* species, *A. corrugatum* Larsen and Patterson, has been well documented to possess furrows. Although there is also a possibility that the observation of furrows in *A. klebsii* was an error, we prefer to take a conservative approach and not synonymize these two species.

Amphidinium steinii Lemmermann

Figs. 2C, 3D, 4, A–J

Synonyms: Amphidinium wislouchi Hulburt, *Amphidinium rostratum* Proškina-Lavrenko

Description. Cells are oval from the ventral side and dorsoventrally flattened. Cells are 20–38 μm long, 10–32 μm wide (for individual strains see Table 2). The epicone is minute, triangular, curved anteriorly, and clearly deflected to the left (Fig. 4A). The cingulum originates 0.2 cell lengths from the apex and is displaced and descending, with the distal end 2–4 μm below and to the right of the proximal end. The sulcus begins just to the right of the mid-ventral line and is initially deep and wide (2–3 μm), becoming less distinct as it nears the posterior of the cell. Two small pusules are present, each approximately 1 μm diameter, one below the origin of the cingulum and the other to the right of the origin of the sulcus (Fig. 4B). A narrow ventral ridge runs between the two points of flagellar insertion. The longitudinal flagellum arises in a pocket just to the left of and below the origin of the sulcus. The nucleus is round to oval, approximately 10 μm diameter and in the posterior part of the hypocone (Fig. 4, C, D, and G). The plastid appears to be single and is yellow-brown, with strands radiating from the ring-like starch-sheathed pyrenoid, which is 4–5 μm diameter (Figs. 3D and 4D). Oil or starch droplets are often found in cultures (Fig. 4B). Asexual division occurs in hyaline-covered cysts (''zoosporangia''), in which either two or more daughter cells may be formed (Fig. 4E). Cells with a very similar appearance but which were metabolic (i.e. amoeboid) in their movements were also seen

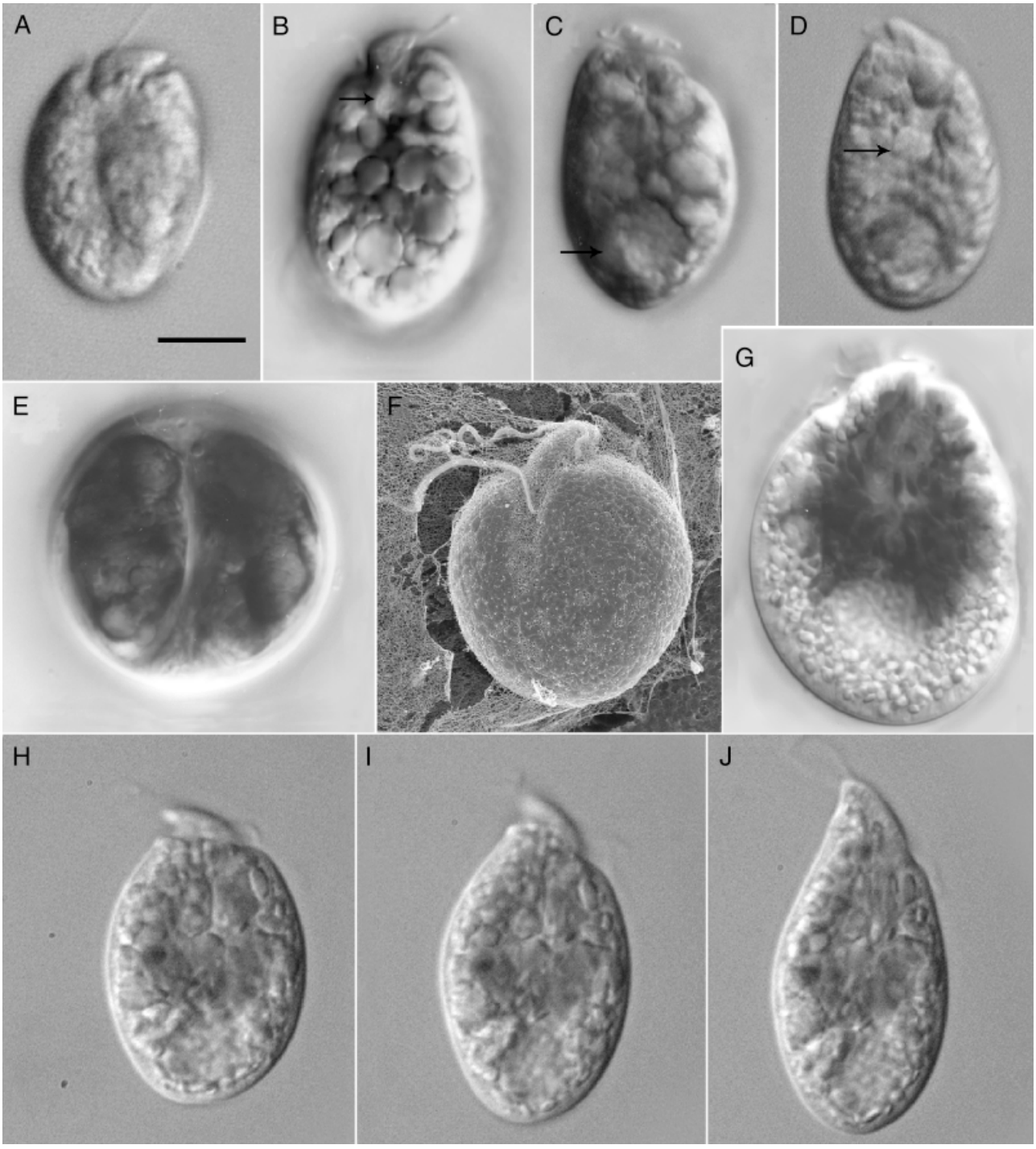

FIG. 4. *Amphidinium steinii*, LM and SEM. (A) Cell from culture CS-741 showing shape of the epicone. (B) Cell from culture CS-741, arrow points to pusule next to longitudinal flagellar insertion. (C) Dorsal view of cell from CS-741, arrow points to posterior nucleus. (D) Dorsal view of cell from CS-741, arrow points to ring-like pyrenoid. (E) Division cyst from CS-741. (F) SEM of cell from CS-741 showing anterior longitudinal flagellar insertion and the gymnodinoid pattern of amphiesmal vesicles. (G) Cell from natural sample from Sydney, Australia to illustrate metabolic movement. (H–J) Sequence of images showing metabolic movement in a single cell from CS-741. Scale bar, 10 μm.

rarely in clonal cultures (Fig. 4, H–J) and in cells from natural samples (Fig. 4G). These cells were a little larger than the normal vegetative swimming cells, about 45 μm in diameter when rounded and up to 50 μm by 15 μm when elongated ($n = 5$). The epicone of these cells changed in shape with the cell movements but was usually "tongue shaped" and deflected to the left. The cingulum of these cells was difficult to

distinguish, and the sulcus was not visible. The two flagella were positioned the same as in the normal vegetative cells but did not run within the cingulum or the sulcus. The two pusules, located near the positions of flagellar insertion, were clearly visible (Fig. 4G). The plastids, pyrenoid, and nucleus position were identical to that of the normal vegetative cells.

Distribution. Descriptions of species that we consider to be *A. steinii* have been recorded from the following sites: marine sediment in southeastern and western Australia (Al-Qassab et al. 2002, Murray and Patterson 2002), marine sediment in Rio de Janeiro, Brazil (unpublished data), brackish water ponds in Wismar, Germany (Stein 1883), salt marsh in New Jersey, USA (Barlow and Triemer 1988), and a freshwater swamp in Maryland, USA (Thompson 1950).

Remarks. This species has sometimes been identified as *Amphidinium operculatum* or *Amphidinium klebsii* and was recently described as *Amphidinium* cf. *operculatum* (Murray and Patterson 2002). Stein (1883) was the first author to describe a species as *A. operculatum* that had a sulcus which differed from that of the original description, originating just posteriorly to the insertion of the transverse flagellum rather than in the lower part of the hypocone, a difference he commented on. Stein's description showed the species dividing by means of a division cyst (Stein 1883) and showed the lobes of the plastid radiating down over the posterior nucleus. Lemmerman (1910) introduced the name *Amphidinium steinii* for the species in Stein's (1883) description; however, the differences between *A. steinii* and *A. operculatum* were not stated. Based on the description of Stein (1883), *A. steinii* has a clear identity, with the following characters distinguishing it from similar species: asexual division by means of a division cyst, sulcus originating just anterior to the point of transverse flagellar insertion, plastid radiating from a central pyrenoid, with lobes curving down over the posterior nucleus, and a curving rather than anteriorly flat left deflected epicone. Some reports of *A. operculatum* or *A. klebsii* have shown all these characters, including the division cysts (Zimmermann 1930, Thompson 1950, Barlow and Triemer 1988) and they therefore represent *A. steinii*. *Amphidinium wislouchi* Hulburt was described thoroughly and, as with *Amphidinium rostratum* Proškina-Lavrenko, was a similar size, had an anterior position of sulcal origin, possessed a pyrenoid, and had a similar shape and a similar plastid to *A. steinii*. In the original descriptions, the differences between these species and *A. steinii* or *A. operculatum* were not mentioned (Hulburt 1957, Proškina-Lavrenko 1945 cited in Popovský and Pfiester 1990). These species had all the characters listed above, with the exception that the division cyst was not described. Because these two species were identical to *A. steinii* in all reported characters, we consider it most likely that they are synonymous with *A. steinii*.

The observation of metabolic (amoeboid) movement in this species was very unexpected. To our knowledge, this is the only species of dinoflagellate in which vegetative cells with a dinokont morphology have been shown to move metabolically. We are unsure as to how to interpret these observations. They may be cells that were about to undergo encystment or had just undergone excystment. However, the metabolic cells appeared to be larger than the standard vegetative swimming cells, suggesting that they may represent a separate perhaps sexual stage in the life cycle of this species. These cells have been observed in both cultures and live samples (Fig. 4, G–J). More research needs to be conducted to determine the role of these cells in the life cycle of *A. steinii*.

Amphidinium gibbosum comb. nov. (Maranda et Shimizu) Fløe Jørgensen et Murray

Figs. 2B, 3B, 5, A–D

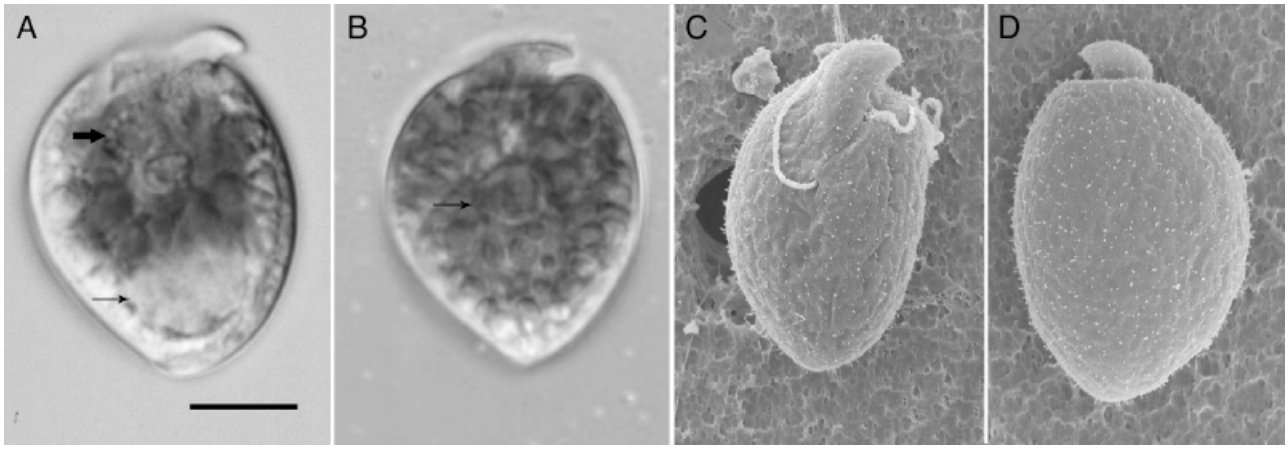

FIG. 5. *Amphidinium gibbosum*, LM and SEM. (A) Cell from culture CCMP120, thick arrow points to pusule, thin arrow points to posterior nucleus. (B) Cell from culture CCMP120, thin arrow points to pyrenoid. (C) SEM of cell from culture CCMP120, showing anterior longitudinal flagellar insertion. (D) Dorsal view of cell from culture CCMP120 showing gymnodinoid pattern of amphiesmal vesicles. Scale bar, 10 μm.

Basionym: Amphidinium operculatum var. *gibbosum* Maranda et Shimizu

Description. Cells are asymmetrical ellipsoid, with a slightly pointed antapex, and dorsoventrally flattened. Cells are 24–43 μm long, 17–23 μm wide (for individual strains see Table 2). The hypocone shape varies from oval to heart shaped. The right side is strongly convex. The posterior part of the right side is often concave, giving the right side of the cell a sigmoid shape and the cell itself a characteristic "hump-backed" appearance (Fig. 5A). The minute epicone overlays the anterior part of the hypocone, its tip deflected to the left (Fig. 5A). In ventral view, the epicone is triangular, almost flattened at the apex with the left upper tip deflected so much that it reaches to the left lateral side of the cell. The right side of the epicone tends to have a slight inclination to the left, whereas the left has a bend just above the anterior edge of the hypocone, the lower part of it being approximately straight (Fig. 5D). The right upper tip has an angle of slightly more than 90 degrees, whereas the posterior tip of the epicone is situated to the right of the mid-ventral line, about 0.4 of the cell length from the apex. The cingulum is deeply incised, with the ends displaced. The sulcus originates just posteriorly to the cingulum (Fig. 5C) and runs all the way to the antapex. There is a large 4 μm diameter pusule located beside the origin of the sulcus. The sulcus has a pronounced right bend after about one third of its length, curving slightly to the left for the remainder of its course. The olive-brown plastid contains several radiating slender lobes (Fig. 3B). A large ring-like pyrenoid, approximately 5 μm in diameter, is located just anteriorly to the center of the hypocone (Fig. 5B). The round nucleus is positioned posteriorly (Fig. 5A). Assimilation granules and occasionally red oil droplets are found in the cells. Asexual division is by binary fission in the motile cell, splitting in the vertical plane, with the epicones last to separate.

Distribution. This species has been found as an endosymbiont in the flatworms *Amphiscolops langerhansii*, *Amphiscolops* sp., and *Haplodiscus* sp. isolated from Florida, USA (Taylor 1971a,b, Blanco and Chapman 1987, Trench and Winsor 1987) as well as free-living in the plankton from the U.S. Virgin Islands (Maranda and Shimizu 1996).

Remarks. This species has been previously identified under the names *A. operculatum* var. *gibbosum* (Maranda and Shimizu 1996), *A. klebsii* (Taylor 1971a,b, Blanco and Chapman 1987), and *A.* "belauense" (McNally et al. 1994). Several ultrastructural studies have been conducted on this species (Taylor 1971a, Blanco and Chapman 1987, Maranda and Shimizu 1996). Maranda and Shimizu (1996) listed the differences between *A. operculatum* var. *gibbosum* and other species or strains; however, they refrained from naming a new species while the identity of *A. operculatum* was still uncertain. The name *A.* "belauense" was never officially published for the purposes of the ICBN or ICZN, and so even though the use of this name in the literature predates the name *A. operculatum* var. *gibbosum*, the correct name is the latter. We propose a new combination because morphological and molecular genetic data show that this species is not closely related to *A. operculatum*.

Amphidinium trulla sp. nov. Murray, Rhodes et Flø Jørgensen

Figs. 2D, 3C, 6, A–D

Cellulae ovatae, inter dorsum et ventrem complanatae, 18–30 μm longae, 12–22 μm latae. Epiconus minutus, lunatus et ad sinistram deflectus. Epiconus in regione antica curvatus et ad dextram inclinatus. Cingulum incipiens in loco 0.1–0.15 ab apici longitudinis cellulae, in media facie ventrale. Ulterior exitus cinguli situs in loco 0.3–0.4 longitudinis cellulae ab apici, et paulum ad dextram mediae lineae ventralis. Pusulae invisae. Sulcus incipiens in sacculo 1–2 μm in regione antica et ad sinistrum proximi exitus cinguli. Sulcus procedens ad regionem posticam. Fulvus chloroplastus, singularis, cum auriculis numerosis qui ab media cellula diffugient. Pyrenoides in forma anuli, recondita in amyli vagina, 4–5 μm diametro, crassa, 1–2 μm, media. Nucleus rotundus, in parte postica hypoconi. Divisio per fissionem in partibus duo, in cellula mobile.

Etymology: From the Latin *trulla*, a scoop, and *trua*, a stirring spoon, referring to the shape of the epicone.

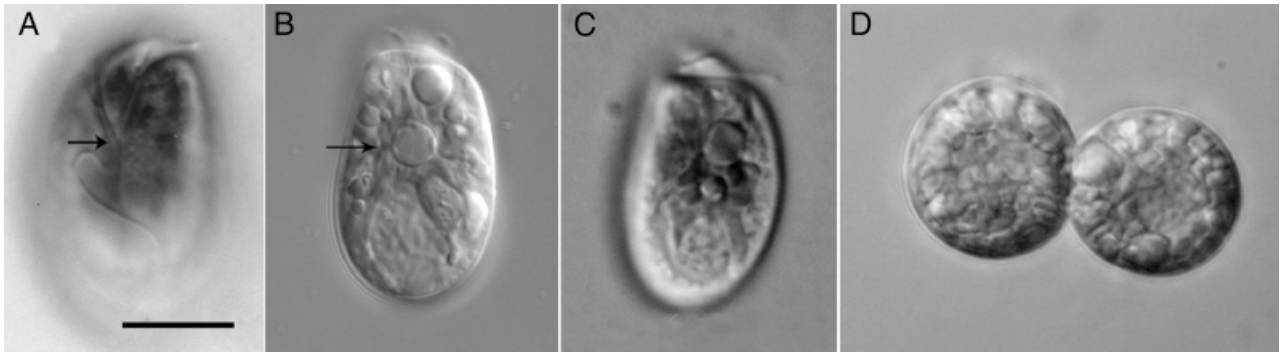

FIG. 6. *Amphidinium trulla*, LM. (A) Cell from culture CAWD68, arrow points to longitudinal flagellar insertion. (B) Dorsal view of cell from culture CAWD68, arrow points to pyrenoid, posterior nucleus also visible. (C) Dorsal view of cell from CAWD68 showing shape of the plastid. (D) Dividing cells from CAWD68. Scale bar, 10 μm.

TABLE 3. Percent difference in the D1–D6 regions of the LSU rDNA.

Species	Intraspecific or within genotype difference in aligned sites	Interspecific difference in aligned sites between this species and all other species included in this study
Amphidinium carterae genotype 1	0%	
Amphidinium carterae genotype 2	0–0.2%	
Amphidinium carterae all genotypes	0–1.7%	4.5%–35.2%
Amphidinium gibbosum	0.2%	3.3%–34.8%
Amphidinium cf. *massartii* genotype 1	0.2%	
Amphidinium massartii all genotypes	0.2%–4.4%[a]	5.9%–40%[a]
Amphidinium operculatum	0–1.0%	34.1%–36.8%
Amphidinium steinii	0.5%	18.3%–35.4%
Amphidinium trulla	0.2%	6.9%–34.4%

The D2 region was not included because it could not be aligned unambiguously.

[a]These sequences were compared only over the first 712 bp of the alignment because of a shorter sequence being included.

Holotype: Figure 2D. The culture on which the holotype is based is lodged in the Cawthron Collection, no. CAWD68.

Type locality: Rangaunu, New Zealand

Description. Cells are oval from the ventral side and dorsoventrally flattened. Cells are 18–31 μm long, 12–22 μm wide (for individual strains see Table 2). The minute epicone is crescent-shaped from the ventral side and overlays the anterior part of the hypocone (Fig. 6A). The epicone is anteriorly curved rather than flat and slopes to the right. The epicone is clearly deflected toward the left. The cingulum begins 0.1–0.15 of the cell length from the apex, midway across the ventral face, rises initially, and then descends on the ventral side (Fig. 6A). The cingulum is displaced and descending, with the distal end 0.3–0.4 of the cell length from the apex and just to the right of the mid-ventral line (Fig. 6A). Pusules were not seen. The sulcus originates in a pocket 1–2 μm anteriorly and to the left of the proximal end of the cingulum and continues to the posterior (Fig. 6A). A narrow ventral ridge runs between the two points of flagellar insertion. The yellow-brown plastid is single with multiple lobes, radiating out from the center of the cell (Figs. 3C and 6C). There is a ring-like starch-sheathed pyrenoid that is approximately 5 μm in diameter and approximately 1 μm thick, located about centrally or just anterior to the center of the cell (Fig. 6, B and C). The round nucleus is in the posterior part of the hypocone and is 7–9 μm in diameter (Fig. 6B). Asexual division is by binary fission in the motile cell (Fig. 6D).

Distribution. *Amphidinium trulla* has been cultured from a water column sample from Rangaunu, New Zealand and a sediment sample from Jægerspris Beach, Isefjorden, Denmark.

Remarks. The vegetative swimming cell of *A. trulla* is most similar to that of *A. steinii* in epicone shape and size, cell size, possession of a central pyrenoid from which plastid lobes radiate, and the anterior position of the sulcus. The main morphological character separating these two species is the mode of asexual division, which takes place in the motile cell in *A. trulla* and in division cysts in *A. steinii*. In addition, cells with metabolic movement have only been observed in *A. steinii*. Nonmotile cells are seldom or never found in cultures of *A. trulla*, whereas they frequently occur in cultures of *A. steinii*. Substantial differences in LSU rDNA sequences were also found between these two species (see *Phylogenetic analysis based on partial LSU rDNA*, below; Table 3). Without culturing or molecular genetic sequencing of cells from samples *in situ*, these two species may be difficult to distinguish.

Amphidinium massartii Biecheler

Figs. 2E, 3F, 7, A–E

Synonym: Amphidinium höfleri Schiller et Diskus

Description. Cells are oval from the ventral side and dorsoventrally flattened. Cells are 12–20 μm long and 7–17 μm wide (for individual strains see Table 2). The hypocone is slightly pointed at the antapex. In ventral

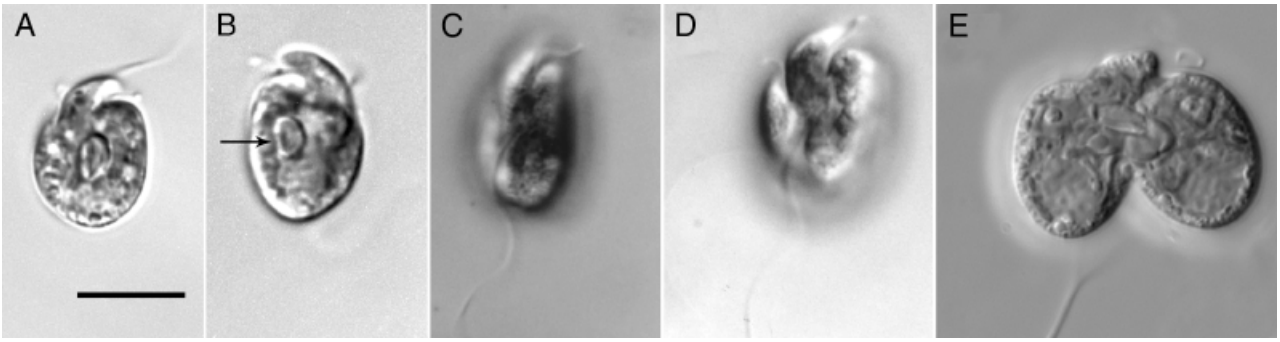

FIG. 7. *Amphidinium massartii* and *Amphidinium* cf. *massartii*, LM. (A) Cell from culture CCMP1821, showing epicone shape. (B) Dorsal view of cell from culture CCMP1821, arrow points to pyrenoid. (C) *Amphidinium* cf. *massartii*, culture AKLVO1, showing longitudinal flagellar insertion. (D) *Amphidinium* cf. *massartii*, culture AKLVO1, showing epicone shape. (E) *Amphidinium* cf. *massartii*, culture AKLVO1, dividing cells. Scale bar, 10 μm.

view, the minute epicone is crescent shaped and the apex flattened slightly and pointing toward the left (Fig. 7, A, B, and D). The cingulum begins 0.3 of the cell length from the apex, midway across the ventral face, rising initially, and then descending on the ventral side. The cingulum is displaced and descending, with the distal end 0.5–0.6 of the cell length from the apex and about 2 μm from the right margin of the ventral face. There is a narrow ventral ridge that runs between the two points of flagellar insertion. The sulcus originates 4 μm below the proximal end of the cingulum and becomes less distinct as it continues to the posterior (Fig. 7, C and D). The rounded or crescent-shaped nucleus is in the posterior part of the hypocone (Fig. 7, B and E). The yellow-green plastid is single with several narrow lobes, radiating out from the center of the cell (Fig. 3F). There is a central ring-like starch-sheathed pyrenoid, which is 2–4 μm in diameter, from which the plastid lobes radiate (Fig. 7, A and B). Asexual division is by binary fission in the motile cell (Fig. 7E).

Distribution. *Amphidinium massartii* has been described from the water column from Sete, France (Biecheler 1952), from the sediment from British Columbia, Canada (Baillie 1971), from a salt marsh in Rhode Island, USA (CCMP1821), from the water column from Florida, USA (CCMP1342), and from the water column from Tasmania, Australia (AKLVO1, AKLSPO1). The species identified as *A. operculatum* in Daugbjerg et al. (2000) was actually a strain of *A. massartii* (CCMP1342). The species referred to as *A. massartii* by Saldarriaga et al. (2001) is here considered to be a genotype of *A. carterae* (strain CCMP124).

Remarks. *Amphidinium massartii* has previously been considered a synonym of *A. klebsii* (Taylor 1971b) or *A. operculatum* (Dodge 1982, Larsen 1985, Hoppenrath 2000). *Amphidinium massartii* is much closer to *A. carterae* than to *A. operculatum* in size range, which was originally given as 15–20 μm long (Biecheler 1952). It is also distinguished from *A. operculatum* by its differently shaped epicone and anterior position of sulcal origin. *Amphidinium massartii* overlaps completely in size range with *A. carterae* and can therefore be very difficult to distinguish with certainty based on LM. The morphological characters that distinguish the two species are the shape of the plastid, which is more centrally located and less dense in *A. massartii*, compared with the peripherally located reticulated plastid in *A. carterae*, a structure that was consistent in all genotypes and growth phases of *A. carterae*. It is also distinguished by the generally slightly longer and wider epicone to body length ratio in *A. massartii* compared with that of *A. carterae* (in *A. massartii* the epicone is 0.5–0.6 of the cell length, whereas in *A. carterae* it is about 0.5 of the cell length). Carter (1937) described a species that was later referred to the new species *A. carterae*. Carter (1937) also described "impoverished specimens," meaning cells with a smaller plastid, which were embedded in mucilage. We believe these cells may represent *A. massartii*, because cultured cells of *A. massartii* appear to have a less dense plastid than those of *A. carterae*, and the culture CCMP1821 was found to produce mucilage.

We emend the original description of Biecheler (1952) in that we additionally describe the plastids as connected to the centrally located pyrenoid. We consider that the later described *A. höfleri* Schiller et Diskus is identical to *A. massartii* (Schiller and Diskus 1955), in particular in its long epicone shape, and they therefore represent the same species. The original description of *A. rynchocephalum* Anissimowa was similar to the present species in that it possessed a similarly shaped epicone, a similar plastid and pyrenoid, and was 17–23 μm (Anissimowa 1926). However, the long V shape of the cingulum and the variable deflection of the epicone appear to be different to that of *A. massartii*. We did not find in the literature a record of a species with this exact shape since its description.

Amphidinium carterae Hulburt

Figs. 2F, 3E, 8, A–F

Synonym: Amphidinium microcephalum Norris

Description. Cells are oval from the ventral side and dorsoventrally flattened. Cells are 10–20 μm long, 9–13 μm wide, and approximately 6 μm in breadth (for individual strains see Table 2). The epicone is crescent shaped from the ventral side and clearly deflected toward the left (Fig. 8, A and D). The

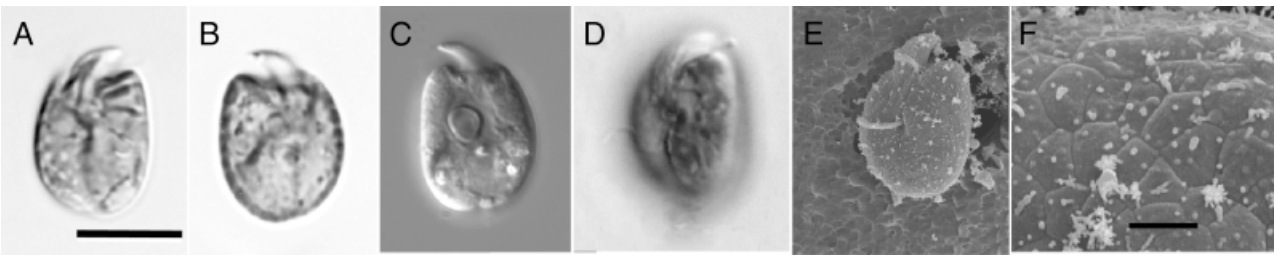

FIG. 8. *Amphidinium carterae*, LM and SEM. (A) Cell from culture K-0654, genotype 1, showing general cell shape. (B) Mid-plane focus of dorsal side of cell from culture K-0654, showing plastid shape. (C) Dorsal view of cell from culture CAWD22, genotype 1, showing pyrenoid. (D) Cell from culture CAWD57, genotype 2, showing longitudinal flagellar insertion. (E) SEM of cell from culture CCMP124, genotype 2. (F) SEM of cell surface of cell from culture CCMP124, showing gymnodinoid pattern of amphiesmal vesicles. Scale bar, 10 μm in A–D and 1 μm in E.

TABLE 4. Summary of the main morphological differences between the species discussed.

	General cell shape	Position of origin of the sulcus	Position of distal end of the cingulum	Asexual division	Starch-sheathed ring-shaped pyrenoid	Form of the plastid
Amphidinium carterae	Oval	Close to the cingulum	0.5 of the cell length from the apex	In motile cells	Present	Single, lobes radiating, and superficial with perforations
Amphidinium gibbosum	"Hump-backed," with a pointed antapex	Close to the cingulum	0.3 of the cell length from the apex	In motile cells	Present	Single, strands radiating from the center
Amphidinium operculatum	Oval to pear shaped	Far from the cingulum, in the lower 1/3 of the cell	0.1–0.2 of the cell length from the apex	In motile cells	Absent	Appear to be multiple, elongated, scattered
Amphidinium massartii	Oval	Close to the cingulum	0.5–0.6 of the cell length from the apex	In motile cells	Present	Single, lobes radiating from the center
Amphidinium steinii	Oval, some cells can move metabolically and change their shape	Close to the cingulum	0.3–0.4 of the cell length from the apex	In cysts	Present	Single, strands radiating from the center
Amphidinium trulla	Oval	Close to the cingulum	0.3–0.4 of the cell length from the apex	In motile cells	Present	Single, lobes radiating from the center

cingulum begins 0.3–0.4 of the cell length from the apex, midway across the ventral face, rising initially, and then descending on the ventral side. The cingulum is displaced and descending, with the distal end 0.5 of the cell length from the apex, 1–2 μm from the right margin of the ventral face (Fig. 8E). The sulcus originates 1–2 μm below the proximal end of the cingulum, continuing to the posterior (Fig. 8, D and E). A narrow ventral ridge runs between the two points of flagellar insertion. The rounded nucleus is in the posterior part of the hypocone (Fig. 8C). The yellow-brown plastid is single, with multiple lobes radiating out from the center of the cell, and also connects to a superficially positioned plastid containing numerous perforations (Figs. 2F, 3E and 8B). There is a central ring-like starch-sheathed pyrenoid, approximately 3 μm diameter (Fig. 8C). Asexual division is by binary fission in the motile cell.

Distribution. *Amphidinium carterae* has been recorded from arctic (Okolodkov 1998) to tropical waters (Fukuyo 1981) and is a truly cosmopolitan species, present in marine sediments and the water column. It has also been found as a symbiont in the jellyfish *Cassiopeia xamachana* from the Caribbean Sea (clone CCMP121).

Remarks. *Amphidinium carterae* is a common and well-known species that was first described (Carter 1937) as *A. klebsii* but was later considered to be a new species (Hulburt 1957), based on its smaller size and plastid shape. A thorough ultrastructural study of *A. carterae* has been conducted by Dodge and Crawford (1968), including a description of the peripheral reticulated plastid. *Amphidinium carterae* overlaps slightly in size range with *A. trulla* and *A. steinii* and overlaps completely in size range with *A. massartii* (Table 2). *Amphidinium carterae* is distinguished from *A. steinii* in its shape and because in culture it does not divide by means of division cysts and does not normally produce nonmotile cells in mid-logarithmic growth phase cultures. *Amphidinium carterae* may be distinguished from *A. trulla* by the comparative length of the epicone relative to the total cell length, because *A. carterae* has a comparatively longer epicone to total cell length ratio (Table 4, Fig. 2). For a comparison of *A. carterae* with *A. massartii*, see the above description. *Amphidinium microcephalum* Norris was described as being very similar to the present species in size (9–12 μm), shape, and the possession of a single plastid (Norris 1961), and it is considered here to be a synonym.

The species *Amphidinium herdmanii* Kofoid et Swezy and *A. mootonorum* Murray et Patterson are distinct morphologically from species of the *Amphidinium operculatum* species complex and have been thoroughly described (Larsen 1985, Hoppenrath 2000, Flø Jørgensen 2002, Murray and Patterson 2002); full descriptions are therefore not included here.

Phylogenetic analysis based on partial LSU rDNA. The phylogenetic analyses based on partial LSU rDNA using BA (Fig. 9) and ML (Fig. 10) resulted in trees with similar topologies. In both analyses, most branches were very well supported by either bootstrap analysis (Fig. 10) or high posterior probability (PP) values (Fig. 9). The main difference between the two analyses was in the sister-group relationship of the clade containing *A. carterae*, *A. massartii*, *A. gibbosum*, and *A. trulla*. In the BA analysis, this clade was found to be the sister group to the clade containing *A. steinii*, *A. mootonorum*, and *A. herdmanii* (Fig. 9), whereas in the analysis using ML, this clade was found to be the sister group to *A. operculatum* (Fig. 10).

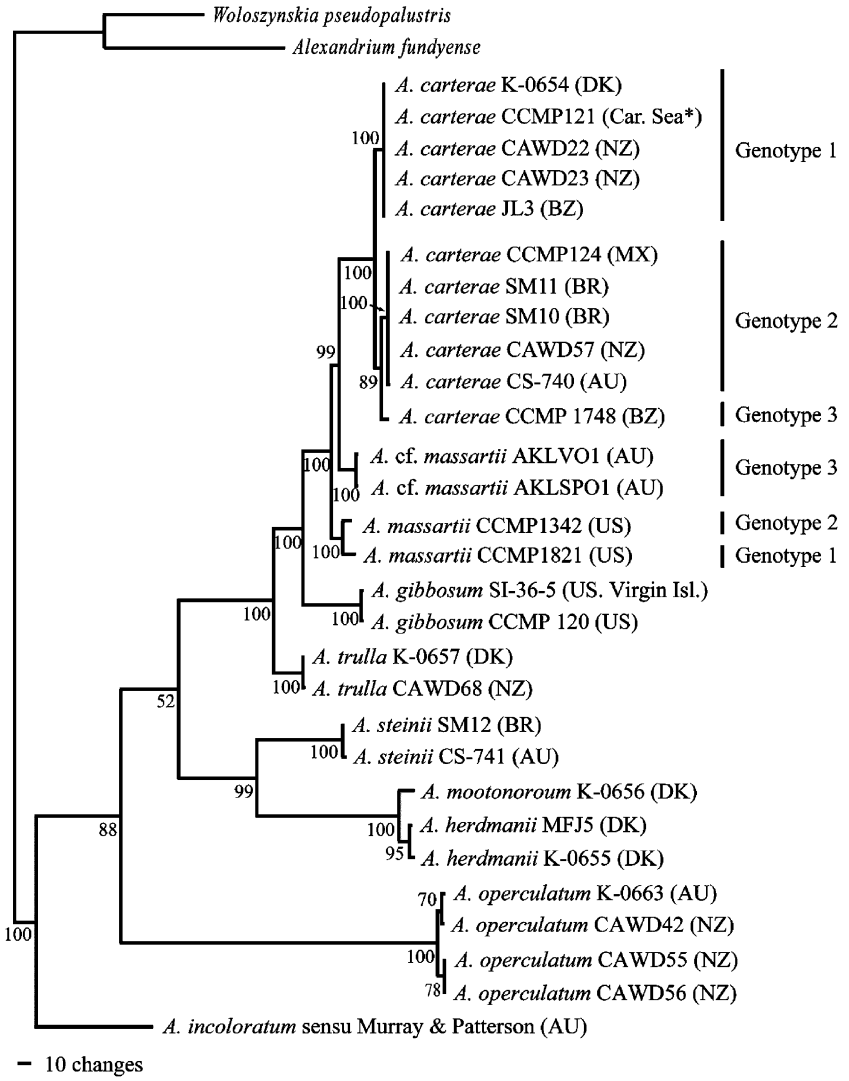

FIG. 9. Consensus phylogram of 40,000 sampled trees obtained by Bayesian analysis of partial LSU rDNA sequences covering the domains D1–D6. Posterior probability (PP) values above 50% are shown. *The sampling site of strain CCMP121 is not specified further.

In both phylogenetic analyses, the species *A. steinii*, *A. operculatum*, *A. gibbosum*, *A. carterae*, and *A. trulla* formed clades that were clearly separated from one another (Figs. 9 and 10). Within these clades, some variation was found, with *A. steinii* strains differing from each other by 0.5%, excluding the hypervariable D2 region, and *A. operculatum* strains differing by 0–1.0% (Table 3). The species *A. carterae* formed a clade, but within that three distinct clades were found that differed from each other by about 40 fixed base differences (up to 1.7% excluding the D2 region) (Figs. 9 and 10, Table 3). The strains of *A. massartii* did not form a single clade. In both analyses, two lineages of *A. massartii* were found to diverge at the base of the *A. carterae* clade (Figs. 9 and 10). The two *A.* cf. *massartii* strains from Tasmania were found to form a sister group to the *A. carterae* clades, whereas the other two strains were a sister group to *A. carterae* and the other two *A. massartii* strains.

DISCUSSION

The confusion regarding species identity within the *A. operculatum* species complex has resulted from both the uncertain identity of the type species *A. operculatum* and the failure to distinguish between species that are relatively similar, morphologically, to *A. operculatum*. After examining multiple clonal cultures and comparing the sequences of LSU rDNA, we were able to distinguish several species from one another.

Identity of Amphidinium operculatum *and other species*. We redescribed the type species *A. operculatum* in detail based on four cultures from Australia and New Zealand, in comparison with the original description of Claparède and Lachmann (1859) (Flø Jørgensen et al. 2004). This species is clearly distinguished from all other species examined in this study in its possession of a sulcus that originates posteriorly in the cell and the lack of a ring-like starch-sheathed

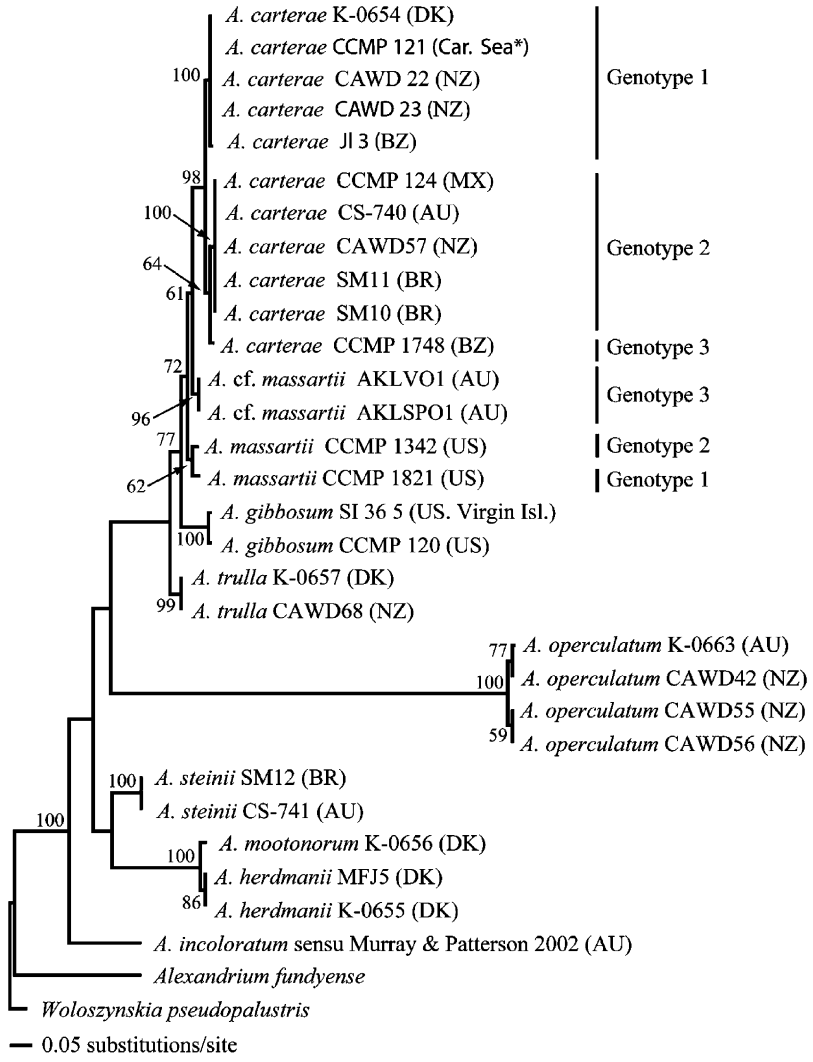

FIG. 10. One of four trees with equal likelihood score obtained by maximum likelihood analysis of LSU rDNA covering domains D1–D6. The difference in topology of the four most likely trees was restricted into the clades of *Amphidinium carterae* genotype 1 and 2. Bootstrap support (BS) values above 50% are shown. *The sampling site of strain CCMP121 is not specified further.

pyrenoid as part of the plastid. The species *A. trulla*, *A. steinii*, *A. gibbosum*, and *A. massartii* all possess a ring-like starch-sheathed pyrenoid from which the plastid lobes radiate but differ from one another in both morphological features and partial LSU rDNA. The new species *A. trulla* is most morphologically similar to *A. steinii*, differing because it does not divide in division cysts and does not commonly produce nonmotile cells in culture. *Amphidinium gibbosum* is quite distinct morphologically, because the right side is convex, giving it a hump-backed shape. *Amphidinium steinii* and *A. trulla* differ both in size and epicone shape compared with *A. massartii* and *A. carterae*. However, in natural samples it may be difficult to distinguish between *A. trulla* and *A. steinii* as well as between *A. carterae* and *A. massartii* based on microscopy of vegetative motile cells alone.

We do not consider the species *A. klebsii* to be a synonym of *A. operculatum*, because the original descriptions of these species differ. However, we note that none of the species described in the literature as *A. klebsii* was said to possess the distinguishing characters for this species as included in the original description (Klebs 1884, Kofoid and Swezy 1921), in particular the possession of furrows or corrugations on the cell surface.

Phylogenetic analyses of LSU rDNA and sequence divergence. From the phylogenetic analyses of partial LSU rDNA sequences, species of the *A. operculatum* species complex were clearly differentiated into separate clades with high support. The morphological similarity of the species was only partly reflected in the phylogenetic analyses. In its LSU rDNA sequence, *A. operculatum* was very divergent compared with other relatively morphologically similar species, differing by at least 34%. In addition, *A. steinii* was not found to be a member of the clade including *A. trulla*, *A. gibbosum*, *A. massartii*, and *A. carterae* and

instead was found to be a sister species of a clade consisting of *A. mootonorum* and *A. herdmanii*, both with 100% BS and PP support. This is surprising given that the morphological differences between *A. steinii* and *A. trulla* are minimal, with the most important feature being the mode of asexual reproduction. The sequence difference between *A. steinii* and *A. trulla* in the D1–D6 regions of their aligned LSU rDNA was found to be at least 19%, which was large considering that the difference between *A. mootonorum* and *A. herdmanii*, which have more apparently divergent morphologies, was 3.9%.

Genotypes of Amphidinium carterae *and* Amphidinium massartii. Both *A. carterae* and *A. massartii* were found to form three separate clades, here referred to as genotypes. The sequence divergence in the aligned LSU rDNA between *A. carterae* genotypes was at least 1.7%, excluding the hypervariable D2 region, whereas the *A. massartii* genotypes were up to 4.4% different, excluding the D2 region. The relatively large difference among strains is contrary to what we observed in the other *Amphidinium* species, in which different strains of the same species were 0.2%–1.0% different. Two strains identified as *A.* cf. *massartii* AKLSPO1 and AKLVO1 were a sister group to the *A. carterae* clade rather than forming a clade with the other two *A. massartii* strains in both the ML and BA analyses. These two strains were identified as *A. massartii* primarily by their plastid shape, with lobes radiating from a central pyrenoid, their epicone shape, and their size.

A possible explanation for this could be that the genotypes in fact represent cryptic species that appear identical when observed in LM. On the basis of our present data, we do not believe the evidence is sufficient to name further new species. Estimating species boundaries using sequence divergence alone is problematic, because it is unlikely that all existing genotypes would have been sampled. However, it is possible that future ultrastructural studies will reveal additional characters that clearly separate these genotypes.

The *A. carterae* genotypes 1 and 2 showed no biogeographically related distribution pattern. Strains of genotype 1 have been isolated from water samples in Denmark, Belize, and New Zealand and as an endosymbiont in *Cassiopeia xamachana* sampled in the Caribbean Sea, whereas genotype 2 has been encountered in Mexico, Brazil, New Zealand, and Australia. Hence, it is likely that both genotypes have a cosmopolitan distribution. In a further species included in this study, *A. gibbosum*, a strain was examined that lived endosymbiotically in a flatworm. These endosymbiotic strains of *A. carterae* and *A. gibbosum* were found to be almost identical in LSU rDNA to free-living strains from elsewhere.

Identity of biotoxin producing species. The strains analyzed in this study previously found to produce amphidinolides or amphidinols, biotoxins produced by some *Amphidinium* species, were the strain of *A. carterae*, CAWD57 (Houdai et al. 2001), and the strain of *A. gibbosum* SI-36-5 (Maranda and Shimizu 1996). A further strain, which appears to be of *A. gibbosum*, has also been reported in the literature to produce amphidinolides (Kobayashi et al. 1991). In the past, *A. operculatum* and/or "*A. klebsii*" were suggested to be potentially ichthyotoxic species (Taylor et al. 1995). This appears to be a product of the taxonomic confusion over the identification of *A. operculatum*, because we could find no published evidence that *A. operculatum*, *A. steinii*, or *A. trulla*, as described here, produce toxins. This study has demonstrated that the species of *Amphidinium* that have been reported so far to produce amphidinolides, *A. carterae* and *A. gibbosum*, can be distinguished both morphologically and by nucleotide sequences from other species of *Amphidinium* that have not been reported to produce toxins. The species *A. massartii* and *A. trulla*, which were found to belong to the same clade as *A. carterae* and *A. gibbosum* in phylogenies generated using LSU rDNA data, are therefore possible future candidates for screening for bioactive compounds.

Morphological plasticity. The concept that *Amphidinium* species are very morphologically plastic originates from observations (Massart 1920) of cells of a species identified as *A. operculatum* that were capable of changing shape. Herdman (1924) also illustrated a series of intermediate forms between "*A. klebsii*" and *A. herdmanii* from wild samples. Our confirmation of metabolic movement in some cells of *A. steinii* may explain the observation of Massart (1920) and possibly also the description of a "plastic morphology" in a species in culture identified as *A. klebsii* (Barlow and Triemer 1988). However, it is questionable whether the observations of Herdman (1924) are the result of shape variability in one species or of observations of several different species from the *A. operculatum* species complex.

We did not observe metabolic movement in other species of *Amphidinium* sensu stricto. Extensive morphometric analyses of *Amphidinium* species in culture found the sizes of individual vegetative motile cells of to be in the range of 30%–35% of the mean (Fløe Jørgensen 2002), with cell length and width strongly correlated. This indicates that morphological plasticity in *Amphidinium* species is probably not common and may be restricted to the life cycle of one species, *A. steinii*. However, alterations in conditions might invoke species to produce cells with metabolic movement, and as cultures were generally kept under stable conditions, these cells may not have appeared. Further studies are needed to clarify this issue.

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