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# *AMPHIDINIUM* REVISITED. I. REDEFINITION OF *AMPHIDINIUM* (DINOPHYCEAE) BASED ON CLADISTIC AND MOLECULAR PHYLOGENETIC ANALYSES[1]

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**Members of *Amphidinium* Claparède and Lachmann constitute a major part of sand-dwelling benthic dinoflagellates worldwide. The genus is traditionally defined by its small epicone size, not exceeding one third of the total cell length. It has long been suspected that this functional definition does not reflect phylogeny, yet the problem of identifying the type species *A. operculatum* and closely related species has until now hindered attempts to redefine the genus. In this study 12 *Amphidinium* species were examined using phylogenetic analyses based on nuclear-encoded, partial, large subunit (LSU) rDNA, with a further six *Amphidinium* species being included in a morphological cladistic analysis. The species selected represented taxa with a range of morphological dissimilar epicone forms. Both cladistic analysis and analyses based on partial LSU rDNA revealed that *Amphidinium* species with minute left-deflected epicones formed a monophyletic clade that included the type species. *Amphidinium* species with other epicone types were found to be unrelated to this clade. The type species *A. operculatum* was identified based on general cell shape and size, position of a dark organelle previously defined as a stigma, and origin of the sulcus. The description of *A. elegans* by Grell and Wohlfarth-Bottermann was found to be identical to it. A species fitting the original description of *A. operculatum* was cultured and included in the analyses. Based on cladistic and molecular analyses, it grouped together with all other species with minute left-deflected epicones, and this group constitutes the true genus *Amphidinium* sensu stricto. An emendation of the genus definition is presented.**

***Key index words:* *Amphidinium*; *Amphidinium operculatum*; Bayesian analysis; cladistic analysis; Dinophyceae; Gymnodiniales; molecular phylogeny; LSU rDNA gene; single-cell PCR**

***Abbreviations:* BA, Bayesian analysis; BS, bootstrap support; LSU, large subunit; MP, maximum parsimony; PP, posterior probability**

Members of *Amphidinium* are among the most abundant and diverse sand-dwelling benthic dinoflagellates worldwide (Dodge 1982, Larsen 1985, Larsen and Patterson 1990, Hoppenrath 2000). Some species can become so abundant as to cause sand discoloration (Herdman 1911, Herdman 1922, Dragesco 1965), and together with benthic diatoms and cyanobacteria, autotrophic *Amphidinium* species are likely to be some of the most important contributors to primary production in the interstitial zone. Their ecological importance still needs to be investigated, as ecological studies of benthic protists have focused on the role and abundance of heterotrophic species (Fenchel 1967, 1986, Patterson et al. 1989, Larsen and Patterson 1990, Lee and Patterson 2002, Al-Qassab et al. 2002).

The genus *Amphidinium* consists of athecate dinoflagellates with a small to minute epicone as compared with the size of the hypocone (Kofoid and Swezy 1921), recently specified as one third or less of the total cell length (Steidinger and Tangen 1997). As presently defined, *Amphidinium* encompasses a variety of morphologically very dissimilar organisms. It includes marine and freshwater species; autotrophic, mixotrophic, and heterotrophic modes of nutrition; and pelagic and benthic forms. It has long been suspected that the definition does not reflect phylogeny (Schiller 1933) but is part of the functional division between the athecate genera *Amphidinium*, *Gymnodinium* F. Stein, *Gyrodinium* Kofoid and Swezy, and *Katodinium* Fott that traditionally comprise the order Gymnodiniales (Kofoid and Swezy 1921, Fensome et al. 1993).

Defining the athecate genera based on both morphological and molecular phylogenetic evidence has

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only recently commenced. Daugbjerg et al. (2000) found the traditional definition of *Gymnodinium* to encompass four genera, *Gymnodinium* sensu stricto, *Akashiwo* G. Hansen and Moestrup, *Karenia* G. Hansen and Moestrup, and *Karlodinium* J. Larsen, defined primarily on the basis of apical groove shape and partial nuclear-encoded large subunit (LSU) rDNA sequences. *Gyrodinium* was redefined to consist of only heterotrophic species with an elliptical apical groove, but the phylogenetic position of this genus still needs to be established. Finally, Hoppenrath (2000) demonstrated that some common *Katodinium* species have fine thecal plates, making their position within Gymnodiniales clearly artificial.

A major obstacle in the investigation of *Amphidinium* has been that the identification of the type species *A. operculatum* and supposedly closely related species has been notoriously difficult. First described by Claparède and Lachmann (1859) from the vicinity of Bergen, Norway, the name *A. operculatum* has been used for *Amphidinium* species with radiating chloroplasts and a minute left-deflected epicone ever since, even if illustrations revealed differences in shape and size compared with the original description. The erection of the species *A. klebsii* Kofoid and Swezy (1921) and *A. steinii* Lemmermann (1910) based on descriptions of *A. operculatum* by Klebs (1884) and Stein (1883), added to the confusion, as it resulted in the names *A. operculatum*, *A. klebsii*, and *A. steinii* being used almost interchangeably because of a lack of detailed species specific characters in the original descriptions of these species.

In the present study, the phylogenetic relationships of 20 distinct species of *Amphidinium* were investigated using cladistic analysis of morphological and ultrastructural characters, with a further 12 distinct *Amphidinium* species being analyzed using partial LSU rDNA sequences covering the domains D1 and 20 bp downstream of D6. These species possess a range of different epicone forms and morphological shapes (Fig. 1). Species boundaries were established by a combination of morphological characters obtained by LM and by partial LSU rDNA sequence differences (Murray et al. 2004). Hence, we use a combination of morphological features and molecular data to redefine the genus *Amphidinium*.

## MATERIALS AND METHODS

*Cultures.* Autotrophic species of *Amphidinium* were isolated by micropipetting and brought into nonaxenic unialgal cultures (K strain numbers, deposited at the Scandinavian Culture Collection for Algae and Protozoa in Copenhagen, Denmark, and CS strain numbers, deposited at the CSIRO Collection of Living Microalgae in Hobart, Australia) using either TL media (Larsen et al. 1994) or F/2 media (Guillard 1983) or obtained from the following sources: The Provasoli-Guillard National Center for Marine Phytoplankton (CCMP strain numbers) and the Culture Collection of Algae, University of Texas (LB strain numbers).

Heterotrophic *Amphidinium* species were collected at Isefjorden at Jægerspris Beach, Denmark and at Port Botany, Sydney, Australia by sampling the upper sediment layer from a sand/mud flat exposed at low tide. Cells were collected by the frozen seawater method (Uhlig 1964, Fenchel 1967) using a 100-μm mesh or by sampling directly from the interstitial seawater.

*LM.* Micrographs were obtained using either a BX 60 microscope (Olympus) with a DP10 digital camera (Olympus, Tokyo, Japan) or a Provis AX70 microscope (Olympus) either mounted with an Olympus PM 20 using Kodak Tech Pan (Kodak, Rochester, NY, USA) emulsion film or with an Axiocam digital camera (Zeiss, Munchen-Hallbergmoos, Germany). Negatives were digitalized using a Nikon Cool Scan (Nikon, Tokyo, Japan).

*Cladistic analysis of morphological characters.* A total of 37 species representing 14 dinoflagellate genera was scored for 39 morphological and ultrastructural characters. Of the 37 species, 20 belonged to *Amphidinium* as traditionally defined. Characters and their score matrix are listed in Tables 1 and 2, respectively. Characters were coded as multistate. Unknown characters were coded as "?", and characters were given equal weight. For taxa where characters were inapplicable, states were coded as "-" and treated as missing. This way of treating inapplicable characters has some inherent problems, as they might lead to spurious optimizations and over-resolved cladograms (Forey and Kitching 2000). However, of the 10 characters in the cladistic analysis where some taxa were coded as inapplicable, only the characters 16–18 (chloroplast-derived features) are likely to be influenced by the problems described by Maddison (1993). The possible effects of this are mentioned in the Results.

Cladistic analysis were performed in PAUP* version 4.0b10 (Swofford 2000). Tree searches were performed using the heuristic search addition command using 10 replicates. To assess the robustness of clades, a jackknife search was performed using 10,000 replicates and 33% character deletion, and Bremer support indices were calculated. *Oxyrrhis marina* Dujardin was chosen as outgroup for this analysis. The lack of the typical dinokaryon nucleus and the possession of an atypical flagellum with hairs have led researchers to believe this organism is in between dinoflagellates and other eukaryotes (Loeblich 1976, Taylor 1980, Fensome et al. 1993), a notion supported by phylogenetic studies of small subunit rDNA (Lenaers et al. 1991, Saldarriaga et al. 2001). The use of *O. marina* made it possible to compare more morphological features with other dinoflagellates than would have been possible if the outgroup had consisted of the morphologically more divergent alveolate sister groups, the apicomplexans and ciliates.

Ingroup taxa were chosen to cover as wide a range of genera as possible, but availability of data did influence the taxa chosen. Ultrastructural features have only been recorded for 40 species of dinoflagellates (Dodge and Lee 2000), and because these are potentially useful characters, species described ultrastructurally were preferably chosen. Taxa of genera that lack the typical dinokont flagella configuration (such as *Prorocentrum*, *Mesoporos*, *Kofoidinium*, and members of Phytodiniales) were not included as too many characters were impossible to score. Because these genera are not believed to be closely related to *Amphidinium*, their omission was expected to have little impact on the final analysis. A discussion of the characters used is available at *www.bi.ku.dk/staff/nielsd/jphycol2004a.htm*. Mapping of morphological characters on to the phylogeny was conducted using MacClade v.4.03 (Maddison and Maddison 2000).

*DNA extraction, amplification, and sequencing of LSU rDNA domains D1–D6.* For species kept in culture, a volume of 10–80 mL of culture was centrifuged for 10 min at 1500–3000 rpm at room temperature and the pellet and 1 mL supernatant transferred to a 1.5-mL Eppendorf tube

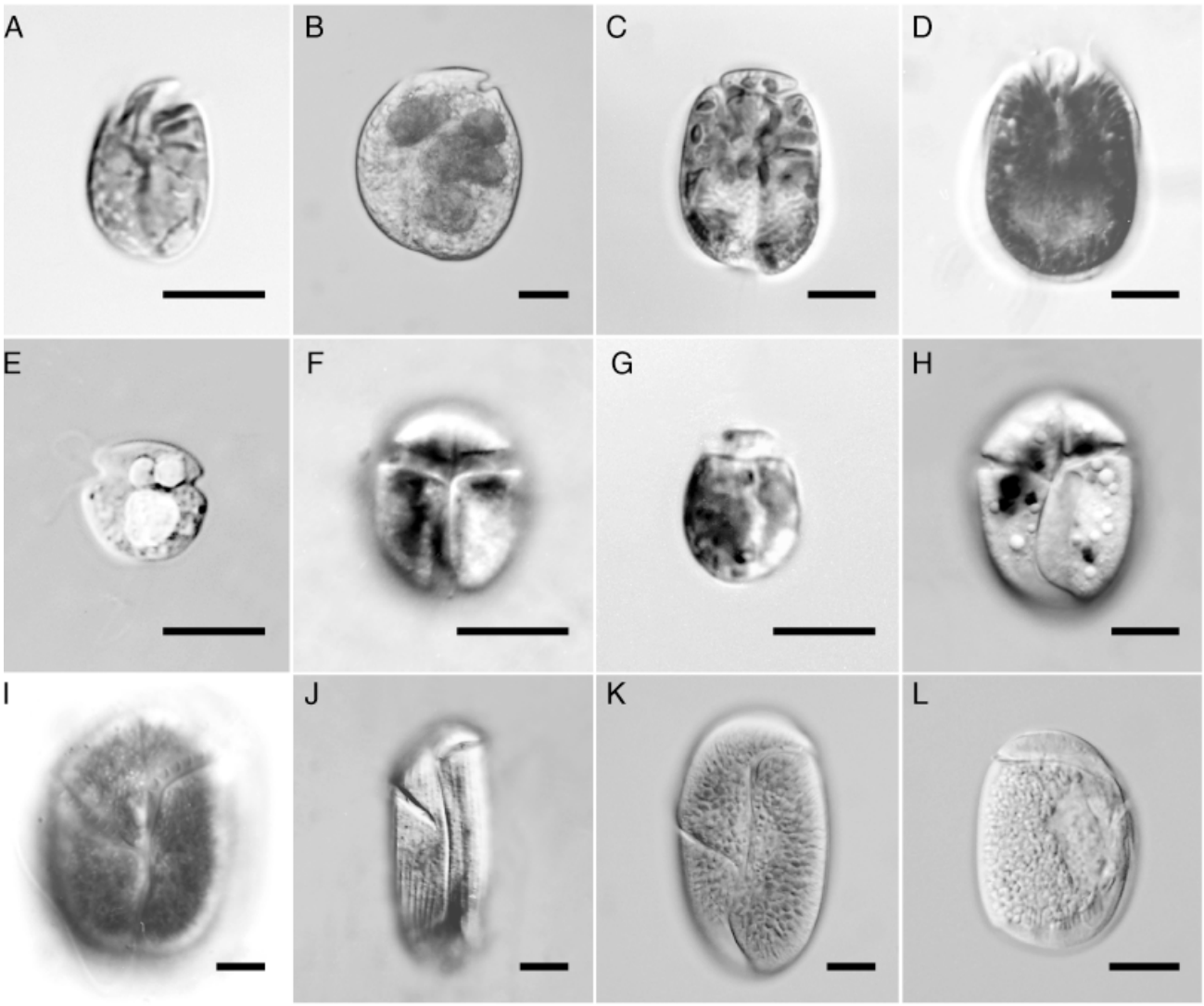


FIG. 1. Micrographs illustrating the morphological difference in epicone shape encompassed within *Amphidinium* as presently defined. (A) *Amphidinium carterae* Hulburt, an autotrophic species with a small crescent shaped left-deflected epicone. (B) *Amphidinium incoloratum* Campbell sensu Murray and Patterson (2002), a heterotrophic species with a small crescent shaped left-deflected epicone. (C) *Amphidinium herdmanii* Kofoid and Swezy, having a small triangular shaped epicone. (D) *Amphidinium corrugatum* Larsen and Patterson. This species has a small, triangular, left-deflected to symmetrical epicone. (E) *Amphidinium lacustre* Stein, a small heterotrophic species with a large straight epicone. (F) *Amphidinium latum* Lebour, having a large straight epicone with short sulcal extension and apical groove counter-clockwise encircling the apex. (G) *Amphidinium poecilochroum* Larsen, a small heterotrophic species with a straight epicone and sulcal extension reaching the apex. (H) *Amphidinium pellucidum* Herdmann, a heterotrophic species with a large epicone with short sulcal extension and an apical groove encircling the apex in a counterclockwise direction. (I) *Amphidinium boggayum* Murray and Patterson, an autotrophic species with displaced cingulum and an apical groove encircling the apex counterclockwise. (J) *Amphidinium scissum* Kofoid and Swezy, a heterotrophic species with displaced cingulum, surface striations, and an apical groove encircling the apex in a spiral. (K) *Amphidinium britannicum* Lebour, a species with a pronounced descending cingulum resulting in a highly asymmetrical epicone. (L) *Amphidinium semilunatum* Herdman. This heterotrophic species has a large curving epicone when seen in lateral view. Scale bar, 10 μm. (Micrograph of *A. lacustre* was kindly provided by Dr. A. Calado. Micrographs D, F, H, and I are reproduced from Murray, S. & Patterson, D. J. 2002. The benthic dinoflagellate genus *Amphidinium* in south-eastern Australian waters, including three new species. *Eur. J. Phycol.* 37:279–98, with permission.)

(Greiner bio-one, Kremsmuenster, Austria). To facilitate cell lysis, tubes were frozen at –20° C for a minimum of 1 day. Total genomic DNA was extracted using the CTAB method (Doyle and Doyle 1987). Extracted DNA was used as a template to amplify approximately 1450 bp of the LSU rDNA gene covering the variable domains D1–D6 downstream (Lenaers et al. 1989), using the primers D1R (Scholin et al. 1994) and 28-1483R (Daugbjerg et al. 2000). Double-stranded DNA was amplified either as described by Lundholm et al. (2002) using 5 μL tetramethylammonium chloride to unfold the secondary structure of the LSU rDNA gene or using a PCR reaction mix consisting of 2.5 mM $MgCl_2$, 1 unit DNA polymerase (Bioline, Astral Scientific, Caringbah, Australia), 10 × $NH_4$ buffer, 0.2 mM of mixed dNTPs, and 10 pmol of either of the primers. The thermal cycles were as follows: one initial denaturing step at 94° C for 3 min followed

TABLE 1. Characters used in the morphological cladistic analysis.

| Character | States | Coded as |
|---|---|---|
| General shape | | |
| 1. Flattening | Not flattened | 0 |
| | Dorso-ventral | 1 |
| | Lateral | 2 |
| 2. Epicone/total length ratio | Epicone 1/3 or less of total cell length | 0 |
| | Epicone greater than 1/3 of the total cell length | 1 |
| 3. Epicone deflection | Not deflected | 0 |
| | Deflected to the left | 1 |
| 4. Epicone not protruding above hypocone on dorsal side | Absent | 0 |
| | Present | 1 |
| Cell covering | | |
| 5. Thecal vesicle deposits | No inclusions | 0 |
| | Thin deposits | 1 |
| | Heavy deposits | 2 |
| 6. Tabulation | Gymnodinoid | 0 |
| | Gonyaulacoid | 1 |
| | Peridinoid | 2 |
| 7. Scales | Absent | 0 |
| | Present | 1 |
| Cingular characters | | |
| 8. Cingulum | Single | 0 |
| | Multiple | 1 |
| 9. Cingular displacement | Absent | 0 |
| | Present | 1 |
| 10. Cingular overhang | Absent | 0 |
| | Present | 1 |
| Sulcal characters | | |
| 11. Position of sulcal origin | Close to cingulum | 0 |
| | In lower 1/3 of the cell, not connected to the cingulum | 1 |
| 12. Sulcal extension on the epicone | Absent | 0 |
| | Present | 1 |
| Apical groove | | |
| 13. Apical groove | Absent | 0 |
| | Present | 1 |
| 14. Apical groove direction | Counterclockwise encircling apex | 0 |
| | Clockwise encircling apex | 1 |
| | Straight and wide | 2 |
| Plastids | | |
| 15. Plastids (not kleptochloroplasts) | Absent | 0 |
| | Present | 1 |
| 16. Plastid number | Single and radiating from centre | 0 |
| | Multiple small plastids | 1 |
| 17. Major pigment | Peridinin | 0 |
| | Fucoxanthin | 1 |
| 18. Large, central, starch sheathed pyrenoid | Absent | 0 |
| | Present | 1 |
| Nucleus | | |
| 19. Nucleus position | Anterior | 0 |
| | About central | 1 |
| | Posterior | 2 |
| 20. Nuclear envelope with vesicular chambers | Absent | 0 |
| | Present | 1 |
| 21. Nuclear histones | Absent | 0 |
| | Present | 1 |
| 22. Mitotic apparatus | Intranuclear | 0 |
| | Extranuclear | 1 |
| Peduncle | | |
| 23. Peduncle or microtubular basket indicating a peduncle | Absent | 0 |
| | Present | 1 |
| Stigma | | |
| 24. Stigma | Absent | 0 |
| | Present | 1 |
| Pusule | | |
| 25. Pusule | Absent | 0 |
| | Present | 1 |
| 26. Pusule type | 1 | 0 |
| | 2 | 1 |
| | 3 | 2 |
| | 4 | 3 |
| | 5 | 4 |

TABLE 1. Continued.

| Character | States | Coded as |
|---|---|---|
| Flagellar apparatus | | |
| 27. Flagellar apparatus with striated collars | Absent | 0 |
| | Present | 1 |
| 28. Nuclear fibrous connector in microtubular root 1 | Absent | 0 |
| | Present | 1 |
| 29. Two striated collar connectives | Absent | 0 |
| | Present | 1 |
| 30. Basal body angle | Less than 45 degrees | 0 |
| | 90– 100 degrees | 1 |
| | greater than 120 degrees | 2 |
| Life history | | |
| 31. Asexual division in division cyst | Absent | 0 |
| | Present | 1 |
| Surface features | | |
| 32. Striations | Absent | 0 |
| | Present | 1 |
| 33. Dorsal groove | Absent | 0 |
| | Present | 1 |
| 34. Dorsal corrugations | Absent | 0 |
| | Present | 1 |
| 35. Indentation inside rim of hypocone on ventral side | Absent | 0 |
| | Present | 1 |
| Apical pore | | |
| 36. Apical pore | Absent | 0 |
| | Present | 1 |
| 37. Canal plate | Absent | 0 |
| | Present | 1 |
| Horns and spines | | |
| 38. Apical horn | Absent | 0 |
| | Present | 1 |
| 39. Antapical spine/s | Absent | 0 |
| | Present | 1 |

by 35 cycles each consisting of 94° C for 30–60 s, 50–55° C for 30–60 s, and 72° C for 1–3 min and a final cycle of 72° C for 5–6 min.

The single-cell PCR method described by Edvardsen et al. (2003) was used in a slightly modified version to amplify DNA from heterotrophic species. Cells of heterotrophic *Amphidinium* were isolated from natural samples by capillary isolation and identified using an inverted microscope (Labovert FS, Leitz, Wetzlan, Germany) with a 40 × lens. Before collection, digital pictures were obtained of the species targeted in the sample using an Olympus BX60 microscope with an Olympus DP10 digital camera to ensure that no misidentification with any other organism in the particular sample would occur. The morphology of the heterotrophic species was in accordance with the descriptions of these species given in Murray and Patterson (2002). After identification, the cell was washed a minimum of five times by transferring it to drops of 0.2 µm pore-filtered seawater to avoid contamination and finally transferred to approximately 10 µL of sterile $H_2O$ in a 0.5-mL thin-walled Eppendorf tube. In some tubes up to three cells were pooled together so as to increase the total amount of DNA. Eppendorf tubes were kept frozen a minimum of 1 day at −20° C to facilitate cell lysis. The PCR reagents described by Lundholm et al. (2002) were added directly to the ice-cooled Eppendorf tubes with the modification that the dinoflagellate specific primer Dinospec. (unpublished data) was used instead of 28-1483R to avoid amplification of DNA from any nondinoflagellate food organism. The thermal cycles run were the same as described above.

PCR products were visualized either on EtBr-stained 2% Nusieve gels or 0.7% agarose gels. Purification were achieved using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) on the total PCR product or by cutting out the PCR product band of appropriate size and cleaning it using the Ultraclean 15 kit (Geneworks, Adelaide, Australia), both as recommended by the manufacturers. Nucleotide sequences were determined using Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and sequence reactions run on ABI PRISM 377 DNA sequencer (Perkin Elmer) at the Botanical Institute, University of Copenhagen or at the Sydney University Prince Alfred Macromolecular Analysis Centre. Primers used for cycle sequencing were D1R, D2C-R Scholin et al. (1994), 28-1483R (Daugbjerg et al. 2000), and D3A, D3B-R (Nunn et al. 1996).

*Sequence alignment and phylogenetic analyses*. Sequence fragments were assembled and proofread with the program Sequencher V® version 3.0 (Gene Codes, Ann Arbor, MI, USA) and aligned in Bioedit version 5.09 (Hall 1999), using information on the secondary structure of the LSU rDNA molecule for alveolate taxa obtained from the rRNA World-Wide Web server (De Rijk et al. 2000). As outgroup species three apicomplexans and two ciliates were chosen (*Eimeria tenella*, *Toxoplasma gondii*, *Plasmodium falciparum, Tetrahymena pyriformis*, and *Euplotes aediculatus*, GenBank accession numbers AF026388, X75429, U21939, X01533, and AF223571, respectively). The data matrix comprised 1300 aligned positions, including introduced gaps after excluding domain D2. The D2 region was defined as the sequence parts situated between positions 411–713, both included. This part of the LSU rDNA gene is highly variable, indicating a high mutational rate not suitable for molecular analyses at the species and genera defining level. It was therefore excluded from the analyses (alignment available at *www.bot.ku.dk/staff/nielsd/jphycol2003a.htm*). In total, 654 aligned positions were

TABLE 2. Matrix of morphological characters used in the cladistic analysis.

| Taxa | Character number | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 |
| *Oxyrrhis marina* | ?[a] | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | —[b] | 0 | — | — | — | 1 | 0 | 1 | 0 | 0 | 0 | 0 | — | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | — | — | — | — |
| *Akashiwo sanguinea* | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | — | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 2 | ? | 0 | 0 | 0 | 0 | — | — | — | — |
| *Alexandrium catenella* | 0 | 1 | 0 | 0 | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | — | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 4 | 1 | 0 | 1 | 1 | ? | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| *Amphidinium boggayum* | ? | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | ? | ? | 0 | ? | 0 | 1 | ? | 0 | 1 | ? | ? | ? | ? | ? | 0 | 0 | 0 | 0 | 0 | — | — | — | — |
| *Amphidinium britannicum* | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | — | 1 | 1 | ? | ? | 1 | 0 | 0 | 1 | 0 | 0 | 1 | ? | ? | ? | 0 | ? | 1 | 0 | 0 | 0 | 0 | — | — | — | — |
| *Amphidinium carterae* | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | — | 1 | 0 | 0 | 1 | 2 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | — | — | — | — |
| *Amphidinium corrugatum* | 1 | 0 | ? | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | — | 1 | 1 | ? | ? | 2 | ? | 0 | 1 | ? | 0 | 1 | ? | ? | ? | ? | ? | 0 | 0 | 0 | 1 | 1 | — | — | — | — |
| *Amphidinium gibbosum* | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | — | 1 | 0 | 0 | 1 | 2 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | ? | 0 | 0 | 0 | 0 | 0 | — | — | — | — |
| *Amphidinium herdmani* | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ? | ? | 1 | 0 | ? | 1 | 2 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | ? | 0 | ? | ? | ? | 0 | 0 | 0 | 0 | — | — | — | — |
| *Amphidinium incoloratum* | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | — | 0 | — | — | — | 2 | ? | 0 | 1 | ? | 0 | 1 | ? | ? | ? | ? | ? | 0 | 0 | 0 | 0 | 0 | — | — | — | — |
| *Amphidinium lacustre* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | — | 0 | — | — | — | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 3 | 1 | 0 | 0 | 2 | 1 | 0 | 0 | 0 | 0 | — | — | — | — |
| *Amphidinium latum* | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | — | — | — | 1 | ? | 0 | 1 | ? | 1 | ? | ? | ? | ? | ? | ? | ? | 0 | 0 | 0 | 0 | — | — | — | — |
| *Amphidinium massartii* | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | — | 1 | 0 | 0 | 1 | 2 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | ? | ? | ? | ? | 0 | 0 | 0 | 0 | 0 | — | — | — | — |
| *Amphidinium mootonorum* | 1 | 0 | 1 | ? | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ? | 1 | 1 | ? | ? | 1 | ? | 0 | 1 | ? | 0 | 1 | ? | ? | ? | ? | ? | 1 | 0 | 0 | 0 | 0 | — | — | — | — |
| *Amphidinium operculatum* | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | — | 1 | 0 | ? | ? | 2 | ? | 0 | 1 | ? | 0 | 1 | ? | ? | ? | ? | ? | 0 | 0 | 0 | 0 | 0 | — | — | — | — |
| *Amphidinium pellucidum* | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | — | — | — | 1 | ? | 0 | 1 | ? | 0 | ? | ? | ? | ? | ? | ? | ? | 0 | 0 | 0 | 0 | — | — | — | — |
| *Amphidinium poecilochroum* | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ? | 1 | ? | 0 | — | — | — | 1 | ? | 0 | 1 | 1 | 0 | 1 | 0 | 1 | ? | ? | ? | 0 | 0 | 0 | 0 | 0 | — | — | — | — |
| *Amphidinium scissum* | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | — | — | — | 1 | ? | 0 | 1 | ? | 0 | ? | ? | ? | ? | ? | ? | 0 | 1 | 0 | 0 | 0 | — | — | — | — |
| *Amphidinium semilunatum* | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ? | 0 | 1 | ? | 0 | — | — | — | 1 | ? | 0 | 1 | ? | 0 | ? | ? | ? | ? | ? | ? | 0 | 0 | 0 | 0 | 0 | — | — | — | — |
| *Amphidinium* sp. (*britannicum*-like) | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | — | 1 | 1 | ? | 0 | 1 | ? | 0 | 1 | 0 | 0 | 1 | 0 | ? | 0 | ? | ? | 1 | 0 | 0 | 0 | 0 | — | — | — | — |
| *Amphidinium steinii* | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | — | 1 | 0 | ? | ? | 2 | ? | 0 | 1 | ? | 0 | 1 | ? | ? | ? | ? | ? | ? | 0 | 0 | 0 | 0 | — | — | — | — |
| *Amphidinium testudo* | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | — | 1 | 1 | ? | 1 | 2 | ? | 0 | 1 | ? | 0 | ? | ? | ? | ? | ? | ? | 1 | 0 | 1 | 0 | 1 | — | — | — | — |
| *Bysmatrum arenicola* | 1 | 1 | 0 | 0 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | — | 1 | 1 | ? | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | ? | ? | ? | ? | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 |
| *Ceratium furcoides* | 1 | 1 | 0 | 0 | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | — | 1 | 0 | ? | 0 | ? | 0 | 0 | 1 | 1 | 0 | 1 | 4 | 1 | 0 | 0 | 2 | ? | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 |
| *Gonyaulax spinifera* | 0 | 1 | 0 | 0 | 2 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | — | 1 | 0 | ? | 0 | ? | 0 | 0 | 1 | 1 | 0 | 1 | 4 | 1 | 0 | 1 | 2 | ? | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 |
| *Gymnodinium aureolum* | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | ? | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 2 | 1 | 1 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | — | — | — | — |
| *Gymnodinium catenatum* | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | ? | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 2 | 1 | 1 | 0 | ? | 0 | 0 | 0 | 0 | 0 | — | — | — | — |
| *Gymnodinium cryopilum* | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | ? | — | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 2 | 1 | 1 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | — | — | — | — |
| *Gymnodinium fuscum* | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | ? | — | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 2 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | — | — | — | — |
| *Heterocapsa pygmaea* | 0 | 1 | 0 | 0 | 1 | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | — | 1 | 0 | ? | ? | 1 | 0 | 0 | 1 | 0 | 0 | 1 | ? | 1 | 0 | 0 | 0 | ? | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 |
| *Heterocapsa rotundata* | 0 | 1 | 0 | 0 | 1 | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | — | 1 | 0 | ? | ? | 1 | 0 | 0 | 1 | 0 | 0 | 1 | ? | 1 | 0 | 0 | 0 | ? | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 |
| *Karenia mikimotoi* | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 2 | 1 | 1 | 1 | 0 | ? | 0 | 0 | 1 | 0 | 0 | 1 | ? | 1 | 0 | 0 | ? | ? | 0 | 0 | 0 | 0 | — | — | — | — |
| *Karlodinium micrum* | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 2 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | ? | ? | 0 | 0 | 0 | 0 | — | — | — | — |
| *Peridiniella catenata* | 0 | 1 | 0 | 0 | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | — | 1 | 1 | ? | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 4 | 1 | 0 | 1 | 1 | ? | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 |
| *Peridiniopsis beriolense* | 0 | 1 | 0 | 0 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | — | 0 | — | — | — | 2 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | ? | 1 | ? | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 |
| *Polykrikos kofoidii* | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | — | — | — | — | ? | 0 | 1 | 1 | 0 | 1 | ? | 0 | 0 | 0 | 2 | ? | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Protoceratium reticulatum* | 0 | 1 | 0 | 0 | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | — | 1 | 0 | 0 | 0 | 2 | 0 | 0 | 1 | 1 | 0 | 1 | 4 | 1 | 0 | 1 | 1 | ? | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |

[a]Unknown characters.
[b]Character is not applicable for specific taxon.

TABLE 3. List of dinoflagellate taxa included in the partial LSU rDNA analyses with corresponding GenBank accession numbers.

| Taxa | Strain no. | Accession no. |
|---|---|---|
| *Akashiwo sanguinea* (Hirasaka) G. Hansen and Moestrup | K-950003 | AF260396 |
| *Alexandrium catenella* (Whedon and Kofoid) Balech | A3 | AF200667 |
| *Alexandrium fundyense* Balech | K-0270 | AF200666 |
| *Amphidinium carterae* Hulburt | K-0654 | AY455669 |
| *Amphidinium britannicum* Lebour | K-0658 | AY455679 |
| *Amphidinium herdmanii* Kofoid and Swezy | K-0655 | AY455675 |
| *Amphidinium incoloratum* Campbell (sensu Murray and Patterson 2002) | | AY455677 |
| *Amphidinium gibbosum* (Maranda and Shimizu) Fløe Jørgensen and Murray | CCMP 120 | AY455672 |
| *Amphidinium massartii* Biecheler | CCMP 1821 | AY455670 |
| *Amphidinium mootonorum* Murray and Patterson | K-0656 | AY455676 |
| *Amphidinium operculatum* Claparède and Lachmann | K-0663 | AY455674 |
| *Amphidinium trulla* Murray, Rhodes and Fløe Jørgensen | K-0657 | AY455671 |
| *Amphidinium semilunatum* C. Herdman | | AY455678 |
| *Amphidinium* sp. (*britannicum*-like) | LB 1562 | AY455680 |
| *Amphidinium steinii* Lemmermann | CS-741 | AY455673 |
| *Ceratium fusus* (Ehrenberg) Dujardin | | AF260390 |
| *Ceratium lineatum* (Ehrenberg) Cleve | | AF260391 |
| *Ceratium tripos* (O. F. Müller) Nitzsch | | AF260389 |
| *Gonyaulax baltica* Ellegaard, Lewis and Harding | K-0487 | AF260388 |
| *Gymnodinium catenatum* L. W. Graham | | AF200672 |
| *Gymnodinium fuscum* F. Stein | CCMP 1677 | AF200676 |
| *Gymnodinium impudicum* (Fraga and Bravo) G. Hansen and Moestrup | JL30 | AF200674 |
| *Gymnodinium pellucidum* (Herdman) Fløe Jørgensen and Murray | | AY455681 |
| *Heterocapsa rotundata* (Lohmann) G. Hansen | K-0479 | AF260400 |
| *Heterocapsa triquetra* (Ehrenberg) F. Stein | K-0447 | AF260401 |
| *Karenia brevis* (Davis) G. Hansen and Moestrup | K–880001 | AF200677 |
| *Karenia mikimotoi* Miyake and Kominami *ex.* Oda) G. Hansen and Moestrup | K-0579 | AF200682 |
| *Karlodinium micrum* (Leadbeater and Dodge) J. Larsen | K-0522 | AF200675 |
| *Peridinella catenata* (Levander) Balech | K-0543 | AF260398 |
| *Peridinium bipes* F. Stein | AJC8-847 | AF260385 |
| *Peridinium cinctum* Ehrenberg | AJC4cl-a | AF260394 |
| *Peridinium willei* Huitfeld-Kaas | AJC2–675 | AF260384 |
| *Prorocentrum micans* Ehrenberg | K-0335 | AF260377 |
| *Prorocentrum minimum* (Pavillard) Schiller | K-0010 | AF260379 |
| *Protoceratium reticulatum* Bütschli | K-0485 | AF260386 |
| *Scrippsiella* sp. | K-0399 | AF260392 |
| *Scrippsiella trochoidea* (F. Stein) Loeblich var. *aciculifera* | K-0500 | AF260393 |
| *Woloszynskia pseudopalustris* (Woloszynska) Kisselew | AJC12cl-915 | AF260402 |

If available in culture, strain numbers are indicated. (K-xxxxxx, cultures of Dr. K. Steidinger, A3, Culture of Dr. C. Scholin, K-xxxx, Scandinavian Culture Collection of Algae and Protozoa (SCCAP), CCMP, Provasoli-Guillard National Center for Culture of Marine Phytoplankton, LB, The Culture Collection of University of Texas (UTEX), JL, Culture of Dr. J. Larsen, AJC, culture of Dr. A. J. Calado. *Amphidinium trulla* sp. nov. and *A. gibbosum* comb. nov. are described in Murray et al. (2004).

considered unambiguous and examined using maximum parsimony (MP) and Bayesian analysis (BA) methods. All species included in the molecular analyses with their corresponding GenBank accession numbers are listed in Table 3.

MP was performed using PAUP* version 4.0 b8a (Swofford 1998), using the heuristic search option with a random addition of sequences (1000 replicates) and a branch-swapping algorithm (tree-bisection-reconnection). Characters were weighted equally, and gaps were treated as missing data. To determine the robustness of the tree, bootstrap replicates (bootstrap support [BS]) were conducted (Felsenstein 1985) using a reweighted consistency index over an interval of 1–1000.

BA was performed using the program MrBayes 2.01 (Huelsenbeck and Ronquist 2001, Huelsenbeck et al. 2001) set to operate with a general time reversible model with a gamma distribution and with three heated chains supplementing the cold chain, following the recommendations by Hall (2001). A total of 1,040,000 generations were calculated with trees sampled every 50th generation and with a prior burn-in of 40,000 generations equalling 800 sampled trees. The ln likelihood value converged at a value of approximately $-1806 \times 10^7$. The 20,000 sampled trees were imported into PAUP* version 4.0b10 (Swofford 2000) and used to calculate a consensus phylogram. This was constructed by using the branch lengths of those sampled trees that display the most commonly encountered branching pattern for the particular node in question. Hence, the number of trees used to calculate the average length of a given branch is directly correlated to the posterior probability (PP) support of the node. PP values were obtained by calculating a majority rule consensus cladogram, as described by Hall (2001). Because of computational constraints, no maximum likelihood analysis was performed.

## RESULTS

*Identification of the type species* Amphidinium operculatum *Claparède and Lachmann*. Identification of *A. operculatum* was achieved by reexamination of the first description and illustrations of Claparède and Lachmann (1859). An English translation of the French text is available at *www.bi.ku.dk/staff/nielsd/jphycol2004a.htm*. Because several *Amphidinium* species were found to be about the same size, with a similar shape and a centrally located pyrenoid with radiating

chloroplast lobes (Murray et al. 2004), making these characters unsuitable at the species level, emphasis was given to characters previously neglected: specific shape of the epicone, "stigma" shape, and the position of the origin of the sulcus.

In the original dorsoventral illustration of *A. operculatum* by Claparède and Lachmann (Fig. 2A), the epicone does not overlay the anterior part of the hypocone, implying that it is a dorsal depiction of the cell. After enlarging the illustration, it became clear that the anterior part of the epicone was deflected to one side. If seen in dorsal view, this would imply that the epicone was deflected to the right, contrary to what is observed in all other known *Amphidinium* species with minute deflected epicones. However, if Claparède and Lachmann made the error of focusing through the cell, this explains why the overlaying posterior part of the epicone was not illustrated. The illustration drawn by Claparède and Lachmann therefore represents a low focus image of the cell from the ventral side ("reverse image"). This is also supported by the fact that the origin of the longitudinal flagellum is depicted. Claparède and Lachmann showed it to originate from a pore-like opening in the lower left part of the hypocone (Fig. 2A).

When seen in a ventral position, some conspicuous features are notable. The general shape of the cell illustrated is pear shaped, the broadest width lying lower than the center of the cell. The right side of the cell is pronounced convex, whereas the left side is only slightly so and in the description stated as being almost straight (Claparède and Lachmann 1859). The epicone width is less than half the cell width, and the anterior right bend of the epicone has an angle of slightly less than 90 degrees.

The cells contain a compact circular dark spot (Fig. 2A). Some later authors have interpreted this spot to be a pyrenoid. However, in most *Amphidinium* species with minute left-deflected epicones, the pyrenoid forms a ring-like structure, not a compact one. Finally, the longitudinal flagellum was shown to arise from a pore-like opening in the lower left side of the hypocone. In their description Claparède and Lachmann (1859) stated that a sulcus did exist but "is difficult to spot as the upper and lower edges of it do not give any change to the surface." They observed it to be positioned near one of the sides created by the compression of the cell and furthermore stated that "it looks like it does not stretch all the way up to the transverse furrow."

Claparède and Lachmann (1859) reported *A. operculatum* to be abundant at times; thus, it seems unlikely that it should not have been encountered since first being described. A thorough examination of previous records of *Amphidinium* species with minute epicones revealed that the species described as *A. elegans* by Grell and Wohlfarth-Bottermann (1957) (Fig. 2B) was in the same size range (approximately 50 μm); had the characteristic pear-shaped cell outline with the right side convex while the left was almost straight, an identical epicone shape, and a sulcus that originated in the lower one third of the hypocone; and

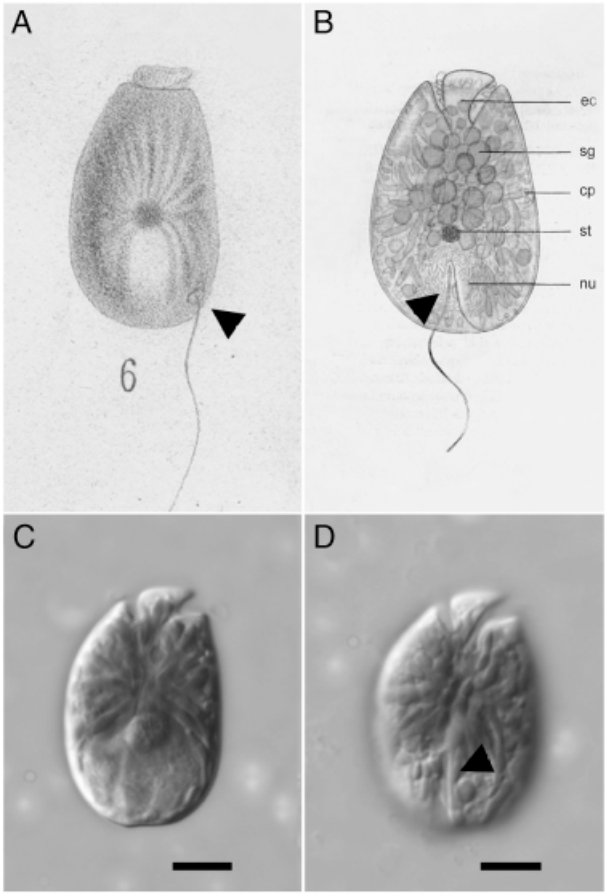


FIG. 2. Illustrations and micrograph of *Amphidinium operculatum*. (A) Original illustration by Claparède and Lachmann (1859). Arrowhead points to sulcal "pore." (B) Original illustration of *Amphidinium elegans* by Grell and Wohlfarth-Bottermann (1957). Original German abbreviations are as follows: ec, epicone; sk, starch grains; pl, chloroplasts; st, stigma; nu, nucleus. Arrowhead points to origin of sulcus. (C and D) Micrographs of *Amphidinium operculatum*, strain SM06. Arrow indicates sulcus position. Scale bar, 10 μm.

was described as being partly covered by the protruding right edge. Grell and Wohlfarth-Bottermann clearly stated that the places of origin for both flagella were widely separated, equalling the description by Claparède and Lachmann (1859) for *A. operculatum*.

Situated just above the posterior nucleus, Grell and Wohlfarth-Bottermann described an organelle (the so-called stigma) that they illustrated as a compact circular structure, closely resembling the "dark spot" observed by Claparède and Lachmann (1859). The only difference between the two descriptions is that Grell and Wohlfarth-Bottermann observed the stigma to be yellow to orange in color, whereas Claparède and Lachmann described a dark spot. This discrepancy could be the result of different degrees of light sensibility in the microscopes used, and we consider the two species to be indistinguishable and therefore synonyms.

In Figure 2, C and D are micrographs taken of *A. operculatum* (strain SM06 [identical to K-0663] Murray 2003). They are in accordance with the description of

*A. elegans* by Grell and Wohlfarth-Bottermann, having an orange stigma, the characteristic small epicone, and a sulcus that originates in the lower one third of the hypocone. However, we found that the general cell shape tends to vary from pear shaped to a more ovoid shape, as in the cells depicted. A detailed description of *A. operculatum* including the full synonym list is presented in Murray et al. (2004).

*Cladistic analysis of morphological and ultrastructural characters.* The strict consensus of the 5234 equally parsimonious trees is given in Figure 3, with characters and states listed in Table 1 and character scores for the species included listed in Table 2. In general, few clades on the tree were strongly supported with Bremer support values greater than 0 or jackknife values greater than 50% (Fig. 3).

The genus *Amphidinium* as currently defined was found to be polyphyletic, but a large group of *Amphidinium* species—*A. carterae*, *A. operculatum*, *A. incoloratum*, *A. massartii*, *A. gibbosum* (comb. nov.; see Murray et al. 2004), *A. steinii*, *A. testudo*, *A. corrugatum*, *A. mootonoroum*, and *A. herdmanii*—did form a monophyletic group with a Bremer support value of 1 and a jackknife value of 43.6% (only values above 50% is shown on the tree). These species all possess minute left-deflected epicones with the possible exception of *A. corrugatum*, where the epicone varies from slightly left-deflected to symmetrical.

*Amphidinium britannicum*, which has a highly asymmetrical epicone (Fig. 1 K), grouped together with a species previously misidentified as *A. corpulentum* (UTEX strain LB 1562) with a relatively high jackknife support of 62%. This clade was found to be sister group to the *Amphidinium* group with minute left-deflected epicones but lacked jackknife support.

All other *Amphidinium* species with their varying large type epicones (Fig. 1, E–L) grouped within the *Gymnodinium* clade, except the minute freshwater species *Amphidinium lacustre*, which did not group together with any other of the genera included in the analysis. The analysis found *A. scissum*, *A. semilunatum*, *A. latum*, *A. poecilochroum*, and *A. pellucidum* to form an unresolved clade, with the species *A. boggayum* as sister group together with the other gymnodinoids and *Polykrikos kofoidii*, but neither of these groupings was supported. The grouping of most of the heterotrophic *Amphidinium* species into one clade might, however, be the result of these species having a relatively high percentage of inapplicable character states, artificially bringing the autotrophic species closer together as described by Maddison (1993).

*Phylogenetic analysis of partial LSU rDNA.* The results of the MP and BA phylogenetic analyses of partial LSU rDNA sequences are presented in Figures 4 and 5 (species included in the analyses with corresponding GenBank accession numbers are listed in Table 3). In both analyses *Amphidinium* was polyphyletic as presently defined. The clade consisting of the species with minute left-deflected epicones was found to form a monophyletic clade by either

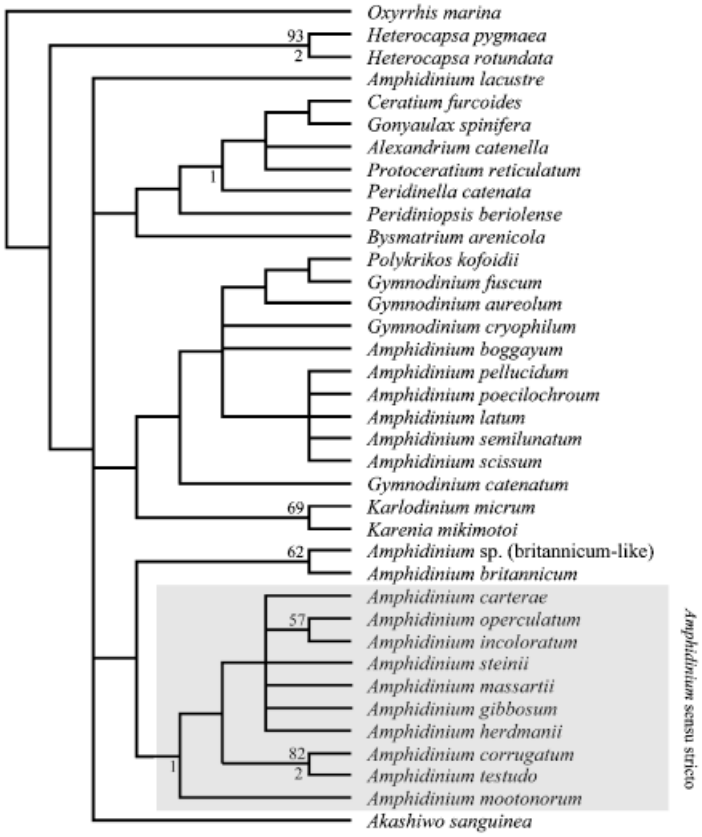


FIG. 3. Strict consensus tree of the 5234 most parsimonious trees based on morphological and ultrastructural characters. Tree length = 86, consistency index = 0.558, retention index = 0.787. Data matrix comprised 39 characters, of which 31 were parsimony informative. Jackknife values from 10,000 replicates with 33% character deletion are given above branches, Bremer support indices below.

analysis, supported with BS and PP of 100%, respectively. The type species *A. operculatum* was found to belong to this clade, grouping together with a clade consisting of *A. carterae*, *A. massartii*, *A. gibbosum*, and *A. trulla* (sp. nov.; see Murray et al. 2004). Using MP analysis, this group was supported by a high BS of 99%, whereas in the analysis using BA it was found to have a PP support of 76% (Figs. 4 and 5). In analyses using both MP and BA *A. operculatum* was found to have a high degree of specific mutational changes compared with closely related *Amphidinium* species (Figs. 4 and 5). Another clade comprising *A. herdmanii* and *A. mootonorum* was highly supported (100% BS and PP) in both MP and BA, with *A. steinii* as sister species to this group supported with 100% BS and PP, respectively (Figs. 4 and 5).

The only heterotrophic species with minute left-deflected epicone included in the analyses, *A. incoloratum* (sensu Murray and Patterson 2002), formed a sister species to all of the autotrophic *Amphidinium* species with left-deflected minute epicones with high support (BS and PP of 100%).

In analyses using both MP and BA, *A. pellucidum* was found to be a sister species to the type species of *Gymnodinium*, *G. fuscum*, with a high BS of 98% and PP of 100%. This clade grouped with the other two gymnodinoids in the analyses, *G. catenatum* and *G. impudicum*, and together these four species formed the *Gymnodinium* clade supported by a high BS and PP of 100%, respectively.

The position of *A. britannicum* differed depending on the analysis. In analyses using both MP and BA, it formed a sister species to the species previously misidentified as *A. corpulentum*, *Amphidinium* sp., with a BS of 69% and PP of 86% (Figs. 4 and 5). However, in 10 repetitive BA analyses run to assess the robustness of the obtained tree, PP support for the clade varied from below 50% to a 100% support in one analysis. In analyses using MP, *Akashiwo sanguinea* was found to be a sister species to the *britannicum* clade but lacked support. In contrast, in the BA analysis the *britannicum* clade was found to be sister group to the gymnodinoids with a relatively high PP of 75%.

The heterotrophic species *A. semilunatum* did not seem to be closely related to any of the genera included in the present analysis. In MP analysis, *A. semilunatum* formed a sister species to both the *Gymnodinium* clade and the clade consisting of *Akashiwo sanguinea* and *A. britannicum* but lacked support. In the BA analysis, *A. semilunatum* was related to a clade consisting of the gonyaulacoids and the two recently erected athecate genera, *Karenia* and *Karlodinium*, but with a low PP support of 62%.

*Identifying the morphological characters defining* Amphidinium. In both cladistic and molecular phylogenetic analyses, a monophyletic group of *Amphidinium* species including the type species *A. operculatum* was identified (Figs. 3–5). In Figure 6 changes in morphological states have been plotted onto a majority consensus cladogram obtained by BA as a way to visualize the unique characters for the *Amphidinium*

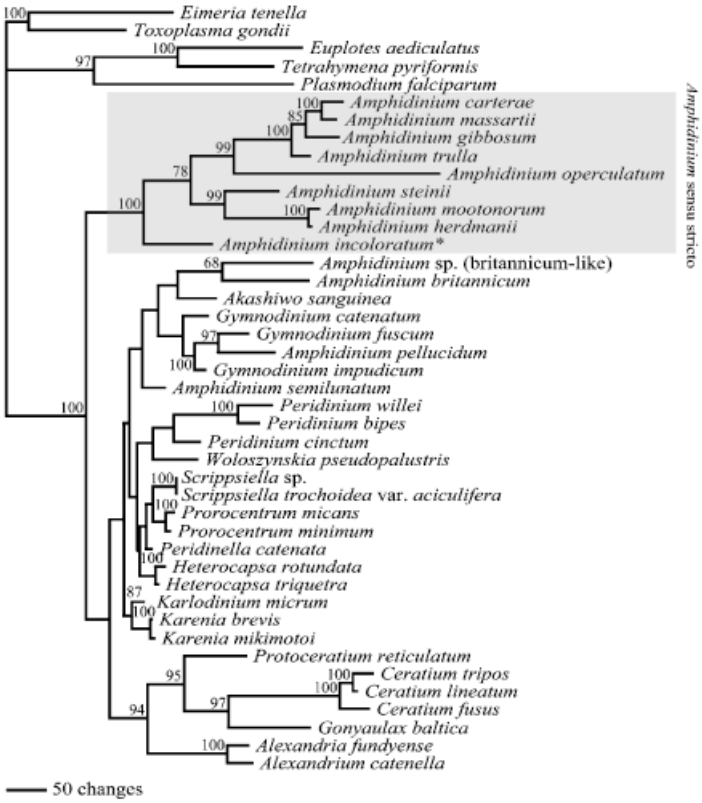


FIG. 4. The most parsimonious tree obtained by PAUP* using the heuristic search option, based on partial LSU rDNA covering domains D1–D6. Tree length = 3998, consistency index = 0.414, retention index = 0.526. Bootstrap values were inferred from MP analysis using a weighted rescaled consistency index over an interval of 1–1000. Values less than 50% are not shown. *sensu Murray and Patterson (2002).

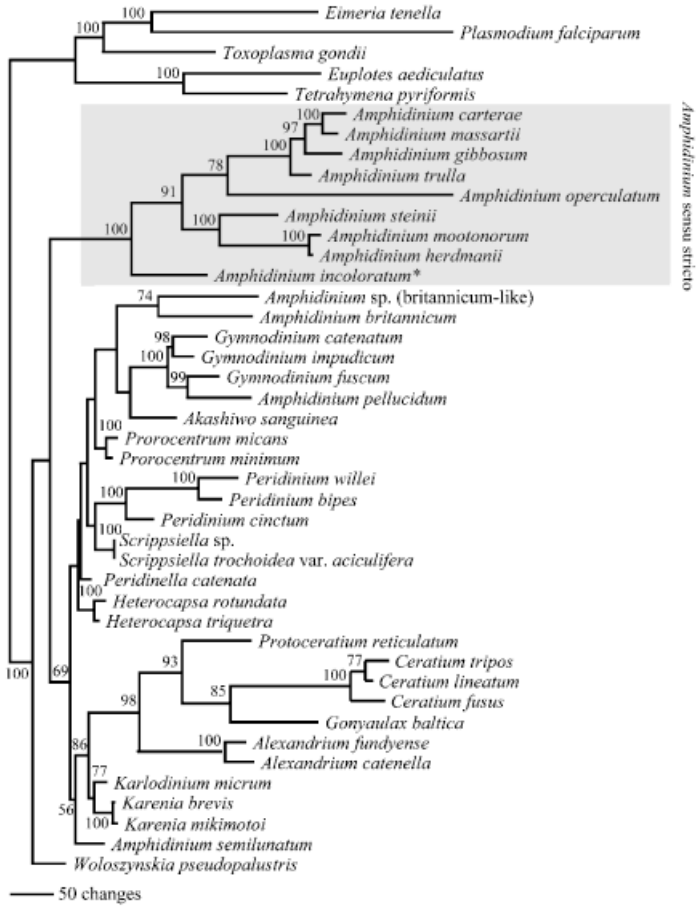


FIG. 5. Consensus phylogram constructed from 40,000 sampled trees obtained by MrBayes, based on partial LSU rDNA sequences covering domains D1–D6 and using a general time reversible model with a gamma distribution. A total of 2,040,000 generations were calculated with a tree sampled each 50 generations and with a prior burn-in of 40,000 generations. Numbers above or below nodes are PP values; values below 50% are not shown. *sensu Murray and Patterson (2002).

clade and the characters that represent homoplasies. Four morphological characters from the cladistic analysis were found to relate to the *Amphidinium* clade (1 = dorsoventral flattening, 2 = epicone equal or less than one third of total cell length, 3 = epicone minute and left deflected, and 19 = posterior located nucleus). Of these four characters, only character 3 was found to be unique to the clade, with *A. corrugatum* representing a border case in regard to the degree of left deflection, which in some specimens seems to be absent. Dorsoventral flattening was present in the genera *Karenia* and *Ceratium* as well as in the species *A. britannicum* and *A. pellucidum*. Because dorsoventral flattening is not observed in the other gymnodinoids included in the analyses, it must have evolved at least twice and therefore represents a homoplasy. The same line of argument applies for the character of the possessions of epicones of one third or less the total cell length, found in *A. pellucidum*, *A. semilunatum*, and arguably *A. britannicum*. Finally, the possession of a posterior located nucleus is not synapomorphic for all species in the *Amphidinium* clade, because *A. mootonorum* has a centrally located nucleus.

For states 5 (thecal vesicle deposits) and 6 (tabulation, Table 1) the ancestral state in Figure 6 was assumed to be no inclusions of thecal vesicle deposits (0) and gymnodinoid tabulation (0), equalling the states present in *O. marina*. Seen as ancestral character states, they represent plesiomorphies and therefore are not unique for the *Amphidinium* clade.

The recent study of plastid evolution by Yoon et al. (2002) indicated that the latest common ancestor of dinoflagellates most likely possessed a fucoxanthin containing chloroplast and that heterotrophic species such as *O. marina* has secondarily lost their plastids. However, even if the latest common ancestor of the dinoflagellates would have had thecal vesicle deposits and a different type of tabulation than gymnodinoid, the two character states would represent homoplasies, because they are found both in the *Amphidinium* clade, *Gymnodinium*, *Karenia*, and *Karlodinium*.

Based on cladistic and molecular phylogenetic evidence, we emend the genus definition of *Amphidinium* as follows:

***Amphidinium*** Claparède and Lachmann emend. Flø Jørgensen, Murray and Daugbjerg

Athecate benthic or endosymbiotic dinoflagellates with minute irregular triangular- or crescent-shaped epicones. Epicone overlaying anterior ventral part of hypocone. Epicone deflection to the left. Cells dorsoventrally flattened, with or without chloroplasts.

*Type species: Amphidinium operculatum* Claparède and Lachmann

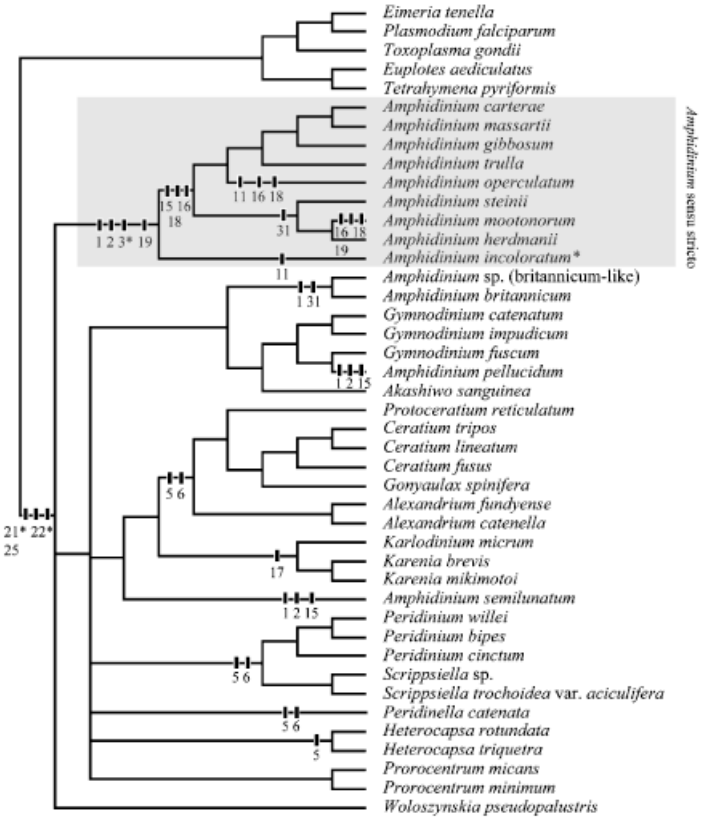


FIG. 6. Morphological and ultrastructural character state changes plotted onto a majority consensus cladogram constructed from the 40,000 trees sampled by MrBayes. Only characters relevant to the definition of *Amphidinium* are included. For list of character with different states, see Table 1. *sensu Murray and Patterson (2002).

*Synonym: Amphidinium elegans* Grell and Wohlfarth-Bottermann

Species transferred to other genera (see discussion).

***Gymnodinium pellucidum*** (C. Herdman) Flø Jørgensen & Murray *comb. nov.*

*Basionym: Amphidinium pellucidum* C. Herdman

## DISCUSSION

The present study has shown the existence of a monophyletic *Amphidinium* clade, defined primarily by the presence of a minute and left-deflected epicone. This corresponds with the proposed genus defining character for *Amphidinium* by Daugbjerg et al. (2000), who suggested that the presence of a "finger-like" epicone could define the true *Amphidinium*. However, Daugbjerg et al. abstained from redefining the genus because of the low number of *Amphidinium* species included in their study and the uncertainty regarding the type species identity. In this study, 9 of an estimated total of 20 *Amphidinium* species with minute left-deflected epicones were included in the molecular analyses and were found to form a strongly supported monophyletic clade, including the reidentified type species *A. operculatum*. Thus, we consider the evidence strong enough to emend the genus definition.

The type species *A. operculatum* has remained enigmatic for almost 150 years. The primary reason for this has been the failure to establish which of the described characters were significant at the species level. The use of molecular genetic data provide a powerful tool for solving such taxonomical "elusive" species, as genetically distinct cultures can be identified, facilitating the identification of plesiomorphic characters at the species level. In the case of identifying *A. operculatum*, before this study the presence of a centrally located pyrenoid from which chloroplasts radiates toward the cell perimeter has generally been regarded as the main identifying character together with a minute left-deflected epicone. However, these characters are observed in *A. massartii*, *A. gibbosum*, *A. trulla*, and *A. steini*, explaining why *A. operculatum* has long been thought to show a high degree of morphological plasticity, as the above mentioned species differ in size and shape (Murray et al. 2004). With the exception of *A. gibbosum*, all have been misidentified as *A. operculatum* (Dodge 1982, Larsen 1985, Daugbjerg et al. 2000, Hoppenrath 2000, Murray and Patterson 2002, Al-Qassab et al. 2002).

As is the case with the type species of *Gymnodinium* (*G. fuscum*), *A. operculatum* is quite divergent compared with the other species included in *Amphidinium*. The nucleus has delicate thread-like chromosomes, the "dark spot" seems to be a unique organelle, and the sulcus origin in the lower one third of the hypocone is only found in *A. incoloratum* Campbell sensu Murray and Patterson (2002). The divergent nature is also supported by the partial LSU rDNA MP and BA

phylograms, where *A. operculatum* shows the greatest number of specific nucleotide changes observed, both within *Amphidinium* and when compared with all other dinoflagellate species included in the analysis. This could indicate that *A. operculatum* has a higher LSU rDNA mutational rate than other *Amphidinium* species, raising the concern that the grouping could be influenced by long branch attraction (Philippe 2000). However, removing the sequence of *A. operculatum* or any other *Amphidinium* sensu stricto species did not alter the tree topology in regard to the monophyly of *Amphidinium* sensu stricto in a series of additional MP and BA analyses run to test the robustness of the *Amphidinium* sensu stricto clade. This indicates that long branch attraction does not influence the results.

The divergent nature of *A. operculatum* raises the question of whether morphologically distinguishable clades exist within *Amphidinium* sensu stricto that could justify the erection of more genera on behalf of other characters. Both MP and BA analyses found two strongly supported clades within *Amphidinium*. One clade consists of *A. carterae*, *A. massartii*, *A. gibbosum*, *A. trulla*, and *A. operculatum* (*A. carterae* clade) and one consists of *A. steinii*, *A.mootonorum*, and *A. herdmanii* (*A. herdmanii* clade). The only exception between analyses was that BA did not find high support for the inclusion of *A. operculatum* in the *A. carterae* clade. For neither of these clades were we able to find a unique character that could define them as a group. All species in the *A. carterae* clade except *A. operculatum* share two characters: central pyrenoid with chloroplast lobes radiating toward the perimeter and posterior situated nucleus. However, these characters are also shared with the species *A. steinii* and *A. herdmanii*.

The *A. herdmanii* clade consists of very dissimilar organisms. *Amphidinium steinii* differs in that the vegetative division cysts seem to be the primary life stage and that it has a life stage exhibiting extreme cellular morphological plasticity. *Amphidinium mootonorum* has a unique morphology with multiple chloroplasts, no visible pyrenoid (in LM), and a centrally located nucleus, all characters that differentiate it from both *A. steinii* and *A. herdmanii*. That *A. steinii* shows a high degree of nucleotide divergence compared with *A. herdmanii* and *A. mootonoroum* was expected, taking the morphological differences into consideration, but the close relationship between *A. herdmanii* and *A. mootonorum* is puzzling considering the difference in morphology between the two species. However, as only *A. carterae* (Dodge and Crawford 1968), *A. gibbosum* (as *A. klebsii*, Blanco and Chapmann 1987, Maranda and Shimizu 1996), and *A. massartii* (Flø Jørgensen 2002) have been investigated in detail using TEM, it cannot be ruled out that ultrastructural characters exist that could be unique for either clade.

*Amphidinium incoloratum* Campbell sensu Murray and Patterson (2002) formed a sister species to all the autotrophic *Amphidinium* species in both MP and BA analyses. Arguably, one might consider if the lack or presence of chloroplasts could be sufficient to separate *Amphidinium* as we define it into two genera. We abstain from this for two reasons. Other heterotrophic species of "true" *Amphidinium* exist (e.g. *A. incoloratum* Campbell sensu Flø Jørgensen 2002, *A. yuroogurrum* Murray and Patterson 2002) that have not been examined in this study but have similar morphologies to autotrophic *Amphidinium* species. Without knowledge of their phylogenetic position, we consider a split premature. Furthermore, evidence points to the fact that chloroplasts have been lost and gained multiple times within the dinoflagellates (Saldarriaga et al. 2001, Yoon et al. 2002), making it unsuitable as a genus-defining character. The fact that *A. pellucidum*, which is heterotrophic, was found to be included within the *Gymnodinium* sensu stricto clade in both morphological and molecular analyses further supports this point of view.

The traditional functional definition of *Amphidinium* includes approximately 120 species (Murray and Patterson 2002). Of these, only an estimated 20 species are of the minute left-deflected epicone type, leaving more than 100 species to have their generic affiliation reexamined. Because this is likely to be a lengthy undertaking, we suggest that the term *Amphidinium* sensu lato be used for species traditionally belonging to *Amphidinium* but not falling within the new definition.

Of the *Amphidinium* species with large type epicones (e.g. Fig. 1, E–L), some clearly belong to other genera. As mentioned above, *A. pellucidum* formed a sister species to *G. fuscum*, the type species of *Gymnodinium*. As *A. pellucidum* has a horseshoe-shaped apical groove encircling the apex counterclockwise (Murray and Patterson 2002), it falls within the emended definition of *Gymnodinium* (Daugbjerg et al. 2000), making it the first heterotrophic *Gymnodinium* species. The newly described species *Amphidinium boggayum* Murray and Patterson (2002) possess a similar type of apical groove and most likely belong to *Gymnodinium*, but without sequence data we abstain from transferring it at the present time. Based on morphology alone, *Amphidinium scissum* Kofoid and Swezy belong to *Gyrodinium* as presently defined, as it has fine striations on its amphiesma and is a naked heterotrophic dinoflagellate, but again we abstain from transferring it due to lack of sequence data.

The position of all other *Amphidinium* species with large type epicones that were included in the analyses could not be established satisfactorily. *Amphidinium latum* and *A. poecilochroum* are both minute heterotrophic species feeding on cryptomonads and capable of retaining prey chloroplasts for some time (Larsen 1988, Horiguchi and Pienaar 1992). However, their overall morphology differs somewhat, and it is doubtful whether the similarity in nutrition mode implies a common ancestry. *Amphidinium lacustre* Stein is a minute heterotrophic freshwater species also feeding on cryptomonads and other small flagellates but lacking the sulcal extension to the apex observed in both *A. poecilochroroum* and *A. latum* (Calado et al. 1998). These three species have a cell outline that can vary substantially in shape, suggesting that cell outline

may not be a useful character for defining these species.

*Amphidinium semilunatum* showed no clear relationship toward other genera included in both the MP and BA molecular analyses. This corresponds with the findings of Saldarriaga et al. (2001) in which *A. semilunatum* was found to be a sister group to the rest of the dinoflagellate taxa included, based on analysis of complete small subunit rDNA data, using maximum likelihood. As it has a distinct morphology, it could represent a new genus. The same applies for *A. britannicum*, which grouped together with a smaller similar species in the molecular analyses. The smaller species was also included in the analyses of Saldarriaga et al. (2001) as *Amphidinium corpulentum* Kofoid and Swezy and was found to be a distant sister species to the suessoid clade, but this topology lacked support.

The placement of *Amphidinium* in the Gymnodiniales was not supported by our analyses. In the analysis of LSU rDNA data using MP, *Amphidinium* was found to be the earliest diverging group of the dinoflagellate genera included, similar to the results of Daugbjerg et al. (2000). However, because most of the internal nodes lacked support, the early divergence is uncertain. In the analysis using BA, *Amphidinium* was also found to be the earliest diverging group together with *Woloszynskia pseudopalustris*, with a PP of 74%. Neither in the analyses of LSU rDNA nor in the morphological cladistic analysis was *Amphidinium* a sister group to *Gymnodinium*. The definition of the Gymnodiniales therefore clearly needs to be emended. However, as the phylogenetic position of other athecate dinoflagellate genera are still uncertain, further work is needed before the phylogenetic relationship of genera currently placed in the Gymnodiniales can be established.

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