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MOLECULAR PHYLOGENY OF SELECTED SPECIES OF THE ORDER DINOPHYSIALES (DINOPHYCEAE)—TESTING THE HYPOTHESIS OF A DINOPHYSIOID RADIATION[1]

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Almost 80 years ago, a radiation scheme based on structural resemblance was first outlined for the marine order Dinophysiales. This hypothetical radiation illustrated the relationship between the dinophysioid genera and included several independent, extant lineages. Subsequent studies have supplied additional information on morphology and ecology to these evolutionary lineages. We have for the first time combined morphological information with molecular phylogenies to test the dinophysioid radiation hypothesis in a modern context. Nuclear-encoded LSU rDNA sequences including domains D1-D6 from 27 species belonging to *Dinophysis* Ehrenb., *Ornithocercus* F. Stein, *Phalacroma* F. Stein, *Amphisolenia* F. Stein, *Citharistes* F. Stein, and *Histioneis* F. Stein were obtained from the Indian Ocean. Previously, LSU rDNA has only been determined from one of these. In Bayesian analyses, *Amphisolenia* formed a long basal clade to the other dinophysioids. These diverged into two separate lineages, the first comprised species with a classical *Phalacroma* outline, also including the type species *P. porodictyum* F. Stein. Thus, we propose to reinstate the genus *Phalacroma*. The relationship between the genera in the second lineage was not well resolved. However, the molecular phylogeny supported monophyly of *Histioneis* and *Citharistes* and showed the genus *Dinophysis* to be polyphyletic and in need of a taxonomic revision. Species of *Ornithocercus* grouped with *Citharistes*, but this relationship remained unresolved. The phylogenetic trees furthermore revealed convergent evolution of several morphological characters in the dinophysioids. According to the molecular data, the dinophysioids appeared to have evolved quite differently from the radiation schemes previously hypothesized. Four dinophysioid species had identical LSU rDNA sequences to other well-established species.

Key index words: ***Amphisolenia*; *Citharistes*; Dinophysiales; *Dinophysis*; Galathea 3 Expedition; *Histioneis*; molecular phylogeny; *Ornithocercus*; *Phalacroma***

Abbreviations: **bp, base pairs; LSL, left sulcal lists; ML, maximum likelihood; pp, posterior probabilities; R1–3, ribs 1–3**

Members of the dinoflagellate order Dinophysiales are distributed worldwide in the marine environment. However, a vast majority of the nearly 300 recognized species are found in tropical waters (Kofoid and Skogsberg 1928, Taylor 1976, Gomez 2005a). Even though the dinophysioids seldom are abundant in numbers, incidents of severe seasonal blooms of *Dinophysis* species have been recorded in some areas (Kofoid and Skogsberg 1928, Maestrine et al. 1996, Guillou et al. 2002, Gomez 2007). Production of toxins associated with diarrhetic shellfish poisoning (DSP) has furthermore implied that this genus has great economic and public health importance (Lee et al. 1989, Hallegraeff 1993, Giacobbe et al. 2000, MacKenzie et al. 2005).

The order comprises both autotrophic and heterotrophic pelagic species (Hackett et al. 2003, Koike et al. 2005), the only exception being the benthic genus *Sinophysis* D. S. Nie et C. C. Wang (Hoppenrath 2000, Selina and Hoppenrath 2004). Several genera appear morphologically adapted to accommodate cyanobacteria as ectosymbionts, and for some *Amphisolenia* species, the presence of an intracellular eukaryote together with various prokaryotic endosymbionts has been reported (Hallegraeff and Jeffrey 1984, Hallegraeff and Lucas 1988, Lucas 1991, Foster et al. 2006). Numerous studies have included morphological and biogeographical information on members of Dinophysiales. However, since all culturing attempts prior to 2006 were unsuccessful, the general knowledge of ecophysiology, life cycle, biochemistry, and genetics is limited (Jørgensen 1923, Kofoid and Skogsberg 1928, Taylor 1976, Hallegraeff and Lucas 1988, Edvardsen et al. 2003, Park et al. 2006, Hart et al. 2007, Reguera et al. 2007, Escalera and Reguera 2008).

Even though the order comprises a broad morphological diversity, the number and arrangement of thecal plates is highly conservative. Only the difficult accessible sulcal plates and small hypothecal plates provide diagnostic information (Tai and Skogsberg 1934, Abé 1967a,b,c). The structural plan and plate tabulation are thus of limited taxonomic

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value in separating taxa of Dinophysiales. Other morphological characters, such as cell size and outline, thecal ornamentation, presence of spines, and the development of the sulcal and cingular lists into fins and sails have been used to separate both species and genera (Kofoid 1926, Kofoid and Skogsberg 1928, Tai and Skogsberg 1934, Hallegraeff and Lucas 1988, Fensome et al. 1993, Steidinger and Tangen 1996). Considerable intraspecific variation in these characters has been reported between individuals and life-cycle stages (Jørgensen 1923, Böhm 1936, Norris and Berner 1970, MacKenzie 1992, Hansen 1993, Zingone et al. 1998, Reguera and González-Gil 2001, Koike et al. 2006). Since some dinophysioid genera were erected based on few morphological characters, the validity of these has been questioned (Tai and Skogsberg 1934, Abé 1967b, Balech 1967, 1988, Taylor 1976, 1980, Dodge 1982). For example, the genus *Phalacroma* was erected to include species previously assigned to *Dinophysis* but possessing a prominent epitheca visible above the horizontal cingular lists (Stein 1883). Another example is *Parahistioneis* Kof. et Skogsb. that was excluded from *Histioneis* based mainly on the lack of a submarginal cross-rib on the posterior cingular list (Kofoid and Skogsberg 1928, Gomez 2007). Both *Phalacroma* and *Parahistioneis* have subsequently been considered congeners with the genus from which they originally were separated (Abé 1967b, Balech 1967, 1988).

The intergeneric relationship within the Dinophysiales has been investigated by several authors (e.g., Kofoid and Skogsberg 1928, Tai and Skogsberg 1934, Abé 1967a, Taylor 1976, 1980, Hallegraeff and Lucas 1988, Edvardsen et al. 2003). Structural resemblance and degree of specialization have been regarded as being highly valuable for obtaining information on the evolution within Dinophysiales. On the basis of this information, Kofoid and Skogsberg (1928) were the first to propose a hypothetical dinophysioid radiation along a number of ascending lineages going from simple to more complex, and including only extant species. One of the major evolutionary lineages began with *Phalacroma* and ended with *Citharistes* (Fig. 1, nos. 11–18). The characters evolved were shape and size of the cingular and sulcal lists. Another lineage involved elongation and posterior bifurcation of a *Dinophysis*-like species into *D. caudata* Saville-Kent and *D. tripos,* and this later led to *D. miles* Cleve or *Amphisolenia* and *Triposolenia* Kof. (Fig. 1, nos. 19–26) (Kofoid and Skogsberg 1928). These evolutionary lineages have later on been supplied with information on ventral plate arrangement, thecal reticulation, plastids, symbionts, and distribution (Tai and Skogsberg 1934, Abé 1967a, Taylor 1980, Hallegraeff and Lucas 1988).

The aim of the present study was to elucidate in a modern context the proposed radiation scheme drawn in Figure 1. This goal was achieved by determining nuclear-encoded LSU rDNA sequences from a diverse assemblage of genera including *Dinophysis, Phalacroma, Ornithocercus, Histioneis, Citharistes,* and *Amphisolenia.* The resulting gene tree (genealogy) will be used to discuss the originally hypothesized dinophysioid evolution.

For quality control of the sequence data used here, we determined LSU rDNA sequences from at least two single cells (up to four in some cases) belonging to the same species, for as many Dinophysiales as possible. For identification purposes, the cells isolated for single-cell PCR were first photodocumented. Hence, micrographs are provided of all species for which LSU rDNA has been determined.

MATERIALS AND METHODS

Sampling site and collection of material. Samples were collected during a cruise across the Indian Ocean going from South Africa to Western Australia in the period October–November 2006. The cruise was part of the Danish Galathea 3 Expedition onboard HDMS *Vædderen.*

Water samples were collected using a 20 μm plankton net (Aquanet, Copenhagen, Denmark) attached to a 130 m long rope. A small amount (~10 mL) of the collected sample was immediately fixed with acid Lugol's iodine (Merck, Darmstadt, Germany) (final concentration ~3%) and kept cold until returning to the Phycology Laboratory, University of Copenhagen.

LM. During the cruise across the Indian Ocean, live cells were observed using an Olympus BX51 light microscope (Olympus, Tokyo, Japan) equipped with differential interference contrast (DIC). Micrographs were recorded digitally with a Colorview II camera (Olympus). At the Phycology Laboratory, University of Copenhagen, the fixed cells were observed using an Olympus Provis AX70 microscope equipped with DIC. Digital micrographs were taken with an Axio Cam (Zeiss, Oberkochen, Germany).

Single-cell isolation. LSU rDNA sequences were determined from single cells isolated either from live or Lugol's-fixed material. Live cells were isolated onboard HDMS *Vædderen* from freshly collected plankton samples using drawn Pasteur pipettes. For identification purposes, isolated single cells were recorded using a Sony High Definition video camera model HC1E (Sony, Tokyo, Japan) mounted on an Olympus stereo microscope SZX 12. Following photodocumentation, the single cells were washed twice in 0.2 μm filtered seawater and placed in 0.2 mL PCR tubes (StarLab, Ahrensburg, Germany), which were immediately frozen at −20°C.

Lugol's-fixed cells were isolated using a drawn Pasteur glass pipette under an Olympus stereomicroscope SZX 12. Micrographs were obtained as described for LM of fixed cells. Following the documentation step, the single cells were washed at least three times in ddH_2O under the stereomicroscope, transferred to a 0.2 mL PCR tube (StarLab, Ahrensburg, Germany), and kept frozen at −20°C until further processing.

PCR amplification and LSU rDNA sequence determination. To ensure cell disruption, either chemical or physical treatments were applied prior to PCR. Chemical disruption was conducted using either Proteinase K (Boehringer, Mannheim, Germany) treatment (Ki and Han 2005, Ki et al. 2005) or Proteinase K and SDS (Sigma-Aldrich, Gillingham, UK) treatment according to a modified version of the protocol provided by Carolyn Troeger (http://www.protocol-online.org/cgi-bin/prot/view_cache.cgi?ID=2743). Physical disruption was conducted using either a sterile needle (Moestrup et al. 2006) or glass beads (Sigma-Aldrich) (Frommlet and Iglesias-Rodríguez 2008). The

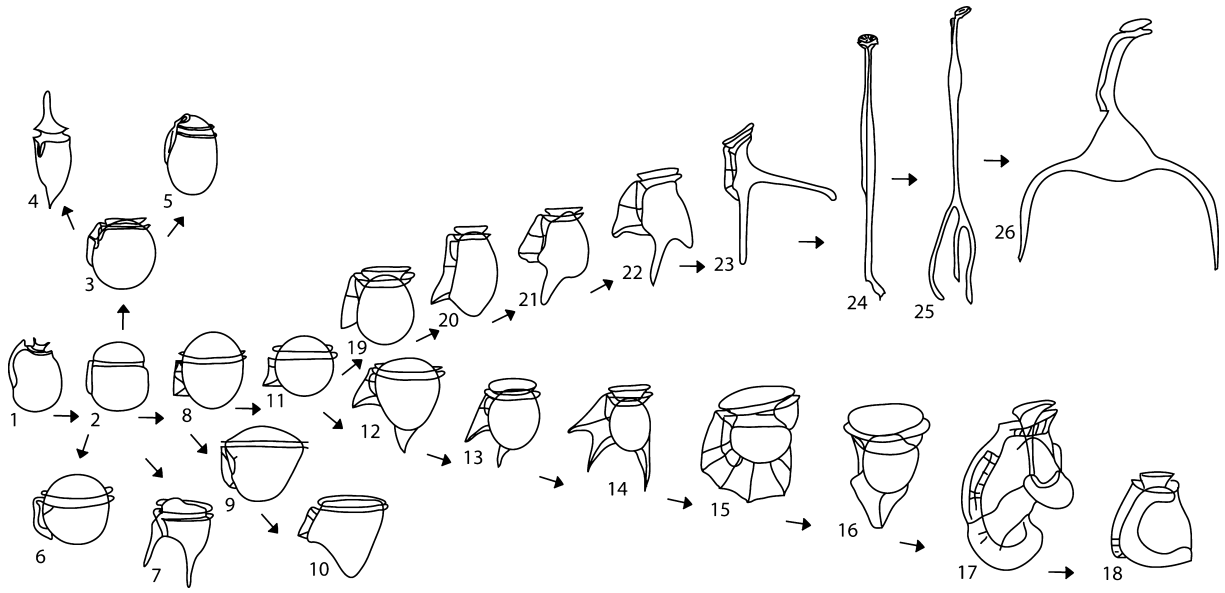

FIG. 1. Hypothecal dinophysioid radiation based on compiled information from Kofoid and Skogsberg (1928), Tai and Skogsberg (1934), Taylor (1980), and Hallegraeff and Lucas (1988). (1) *Sinophysis* sp. (2) *Phalacroma contractum* Kof. et Skogsb. (3) *Metaphalacroma* sp. L. S. Tai. (4) *Oxyphysis oxytoxoides* Kof. (5) *Pseudophalacroma* sp. E. Jørgensen. (6) *Heteroschisma* sp. Kof. et Skogsb. (7) *Dinofurcula* sp. Kof. et Skogsb. (8) *Phalacroma porodictyum.* (9) *Phalacroma cuneus* F. Schütt. (10) *Phalacroma rapa* E. Jørgensen. (11) *Phalacroma parvulum* (F. Schütt) E. Jørgensen. (12) *Phalacroma doryphorum* F. Stein. (13) *Dinophysis odiosa* (Pavill.) L. S. Tai et Skogsb. (14) *Dinophysis schuettii* G. Murray et Whitting. (15) *Ornithocercus steinii* F. Schütt. (16) *Parahistioneis* sp. (17) *Histioneis* sp. (18) *Citharistes apsteinii* F. Schütt. (19) *Dinophysis similis* Kof. et Skogsb. (20) *D. schroederi* Pavill. (21) *Dinophysis caudata* Saville-Kent. (22) *Dinophysis tripos.* (23) *Dinophysis miles* Cleve. (24) *Amphisolenia bidentata* Schöd. (25) *Amphisolenia thrinax* F. Schütt. (26) *Triposolenia intermedia* Kof. et Skogsb. Not drawn to scale.

TABLE 1. LSU rDNA primer combinations for the three different PCR approaches.

	Primary PCR	Seminested PCR	Nested PCR
Primer combination	D1R + Dino-ND D1R + ND28-1483R	D1R + D3B D3A + Dino-ND D3A + ND28-1483R	D2Ra + D3B D3A + ND28-1483R

See Scholin et al. (1994), Hansen and Daugbjerg (2004), and Hansen et al. (2007) for primer sequences.

physical disruption methods were performed in some experiments prior to the PCR followed by heating for 11 min at 94°C with 5 μL TQ buffer.

Single-cell PCR amplification was preformed as previously outlined in Hansen and Daugbjerg (2004) with combinations of eukaryotic LSU rDNA primers as listed in Table 1. PCR conditions, purification of PCR products, and LSU rDNA sequence determination were as described in Moestrup et al. (2008). If several bands were present on the agarose gel, the band of correct length was exercised and used as template for a nested PCR (Moestrup et al. 2008).

Alignment and phylogenetic analyses of nuclear-encoded LSU rDNA. LSU rDNA sequences from the Dinophysiales were assembled with *Dinophysis* sequences available in GenBank (Table S1 in the supplementary material) using Bioedit (v 7.0.5) (Hall 1999). The sequences were aligned with ClustalX. Two alignments, A and B, were constructed for this study. Alignment A comprised 28 LSU rDNA sequences from 27 taxa obtained during this investigation in addition to four dinophysioid sequences available in GenBank. This alignment consisted of 1,441 bp and included the domains D1-D6 sensu Lenaers et al. (1989). Alignment B comprised 700 bp and included the domains D1 and D2 of the LSU rDNA gene. Alignment B comprised 37 sequences from dinophysioid species; of these, 29 were obtained in this study, and eight were retrieved from GenBank (Table S1). Alignment B was generated to include previously obtained partial LSU rDNA sequences of two *Dinophysis* species, which we considered of relevance when testing the dinophysioid radiation scheme. *Dinophysis odiosa* represented an important link in one of the large evolutionary lineages (Fig. 1, no. 13). *Dinophysis rotundata* was previously assigned to *Phalacroma* and has been observed to be genetically very different from other *Dinophysis* species (Guillou et al. 2002, Edvardsen et al. 2003, Hart et al. 2007, Hastrup Jensen and Veland 2008). It was included in alignment B to elucidate the relationship to the *Phalacroma* species from the Indian Ocean. The partial LSU rDNA sequences available in GenBank for these two species, unfortunately, only included the domains D1 and D2 (Edvardsen et al. 2003, Hart et al. 2007). For both *D. odiosa* and *D. rotundata,* the available LSU rDNA sequences were not identical, and therefore two sequences for each species were included (Table S1).

The dinoflagellates *Prorocentrum micans* and *P. minimum* were used in both alignments to polarize the in-group. These species were selected since prorocentroids and dinophysioids share several unique morphological characters, such as the megacystic growth zone and the sagittal suture dividing the typically flattened theca into two almost identical halves (Fensome et al. 1993). The two LSU rDNA data matrices were analyzed using Bayesian analysis and maximum likelihood

(ML) as it is implemented in PhyML 3.0 (Guindon and Gascuel 2003). MrModeltest 2.3 (Nylander 2004) suggested GTR+I+G as the best-fit model for alignment A and GTR+G for alignment B. In Bayesian analysis, two simultaneous Monte Carlo Markov chains (MCMC; Yang and Rannala 1997) were run from random trees for a total of 2,000,000 generations (metropolis-coupled MCMC). Trees were sampled for every 50th generation, and ''burn-in'' evaluated as described in Hansen et al. (2007). AWTY (Wilgenbusch et al. 2004) was used to graphically evaluate the extent of the MCMC analysis. For both alignments, burn-in occurred after 15,050 generations. Hence, the first 301 trees were discarded, leaving 39,700 trees for estimating posterior probabilities (PP). Thus, PP values were obtained from a 50% majority-rule consensus of the kept trees. In PhyML, we used the parameter settings proposed by MrModeltest; PhyML analyses were run using the online version available on the Montepellier bioinformatics platform at http://www.atgc-montpellier.fr/phyml. Evaluation of branch support was performed by bootstrapping with 100 replications.

RESULTS

Species identification. Identification of the 28 species (Table S1 and Figs. 2–4) followed Kofoid and Skogsberg (1928), Taylor (1976), and Balech (1988). All genera and species were named according to Gomez (2005b). Due to lack of details from the LM micrographs, three species could only be identified to genus level. The differentiation between *Dinophysis* and *Phalacroma* was based on *Phalacroma* species having a prominent epitheca and more or less distinct horizontal cingular lists (Stein 1883, Kofoid and Skogsberg 1928). The identification of several specimens was uncertain due to difficulties in obtaining detailed information mainly on lists and ribs. Furthermore, the Lugol's-fixation can cause a considerable volume increase, and comparison of morphometrics could be biased (Menden-Deuer et al. 2001). All provided cell measurements were obtained from the single sequenced cells.

Fortunately, the majority of the included dinophysioid species from the Indian Ocean (in addition to a single species from the west coast of Greenland) had a distinct morphology corresponding to the descriptions by Kofoid and Skogsberg (1928), Taylor (1976), and Balech (1988). Therefore, only specimens for which the identification was uncertain or difficult will be briefly commented on below. Additional arguments for the identification of the type species of *Phalacroma* (viz. *P. porodictyum*) are provided in the discussion.

Numerous variant forms have been reported for several of the species included in this study, for example, *Phalacroma doryphorum, Dinophysis miles,* and *Ornithocercus quadratus* (Kofoid and Skogsberg 1928, Norris and Berner 1970, Taylor 1976). Differentiation between these variants was not of relevance to our examination of the radiation scheme and therefore not attempted here.

Dinophysis braarudii *(Fig. 2, D and E).* This heterotrophic species was observed and sequenced twice while doing a survey of marine dinoflagellates in the vicinity of Disko Island, West Greenland (Hastrup Jensen and Veland 2008). The LSU sequences and micrographs (Fig. 2, D and E) of this rarely observed species were included in the present study to illustrate key arguments in the discussion of the dinophysioid radiation. In ventral view, the shape of the live cell was ellipsoid, ~23 μm long and 15 μm wide. The epitheca was small but visible above the cingular lists. The cingulum was wide and deep and had horizontal lists without ribs. The sulcus was wide and with lists either lacking ribs or with very delicate ribs (Nordli 1951, Hastrup Jensen and Veland 2008). The smooth thecal plates had small scattered pores that were observed in SEM (Hastrup Jensen and Veland 2008). This species was described as belonging to *Phalacroma* (Nordli 1951). However, Gomez (2005b) supported Balech (1967) in transferring this species to *Dinophysis*.

Dinophysis *cfr.* similis *(Fig. 2, K and L).* In fixed material, the cell body was circular in lateral outline, more convex dorsally than ventrally. Widest at the midline, ~49 μm long and 36 μm wide. Both funnel-shaped cingular lists were inclined anteriorly above the epitheca. The left sulcal list (LSL) was rounded posteriorly and lacked the third rib (R3). The length of the second rib (R2) was half the width of the LSL. The included specimen differed from the description of *D. similis* in cell outline and in the posterior portion of the LSL (Kofoid and Skogsberg 1928). Furthermore, ribs were present on the posterior cingular list of the included specimen, a character not observed by Kofoid and Skogsberg (1928). The observed cell outline showed resemblance to *D. shaerica* F. Stein illustrated by Jørgensen (fig. 29 in Jørgensen 1923). Kofoid and Skogsberg (1928) included Jørgensen's (1923) *D. shaerica* as *D. similis* and concluded that body size and the posterior portion of the LSL vary.

Histioneis *sp. (Fig. 2, P and Q).* In fixed material, the cell body was ~44 μm long and 35 μm wide, and the entire length was ~71 μm. The small epitheca was vaulted ventrally, and the cingulum was widest dorsally. No submarginal cross-rib was present on the posterior cingular list. The posterior portion of the hypotheca was tapered in a concave outline. The LSL was concave between R2 and the pointed R3, which extended straight in an approximately vertical axis from the cell body. The LSL had a fine reticulation and extended dorsally from the R3. These characters placed the specimen in the *Histioneis garrettii* Kof. et J. R. Michener group suggested by Gomez (2007). Several characters were shared with especially two members of this group, *H. garrettii* and *H. diomedea* Kof. et J. R. Michener. However, complete similarity to either of these was not observed (Kofoid 1907, Kofoid and Michener

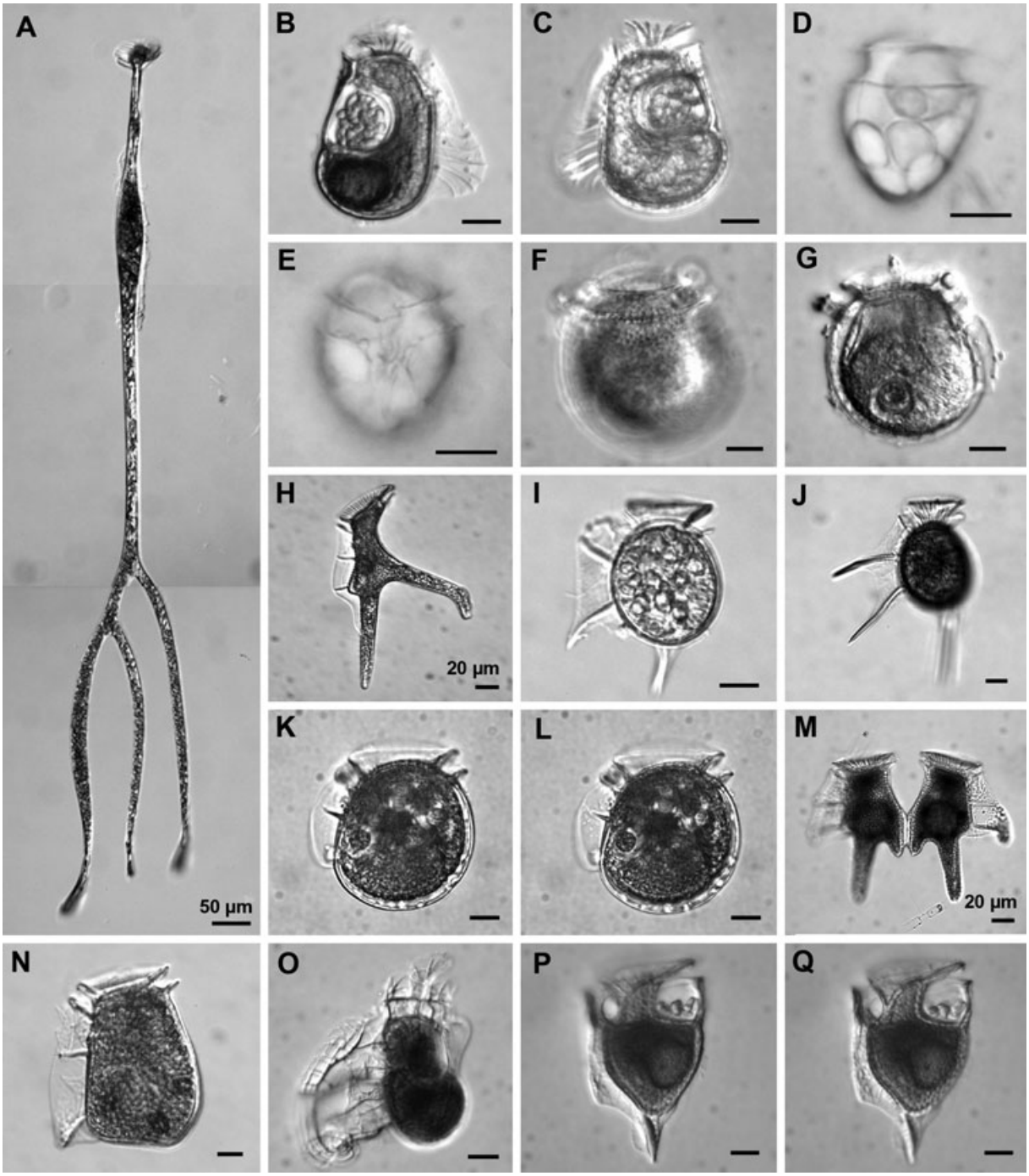

FIG. 2. Light micrographs (differential interference contrast, DIC) of the cells used as template in single-cell PCR determination of nuclear-encoded LSU rDNA. Scale bars 10 µm if not otherwise stated. (A) *Amphisolenia thrinax*. (B) *Citharistes regius* specimen 1, right lateral view. (C) *Citharistes regius* specimen 2, left lateral view. (D) *Dinophysis braarudii*, dorsal view, high focus (from Hastrup Jensen and Veland 2008). (E) *Dinophysis braarudii*, ventral view, high focus (from Hastrup Jensen and Veland 2008). (F) *Dinophysis brevisulcus*, right lateral view, high focus. (G) *Dinophysis brevisulcus*, right lateral view, low focus. (H) *Dinophysis miles*, left lateral view. (I) *Dinophysis pusilla*, left lateral view. (J) *Dinophysis schuettii*, left lateral view. (K) *Dinophysis* cfr. *similis*, left lateral view, low focus. (L) *Dinophysis* cfr. *similis*, left lateral view, low focus. (M) *Dinophysis tripos*, two cells connected at the megacystic bridge. (N) *Dinophysis truncata*, left lateral view. (O) *Histioneis milneri*, left lateral view. (P) *Histioneis* sp., left lateral view, low focus. (Q) *Histioneis* sp., left lateral view, high focus.

1911, Gomez 2007). Both *H. garrettii* and *H. diomedea* have previously been included in the genus *Parahistioneis* (Kofoid and Skogsberg 1928).

Phalacroma *cfr.* argus *(Fig. 3H)*. In fixed material, the large specimen was widest just below the cingulum, ~93 µm long and 73 µm wide. The rounded

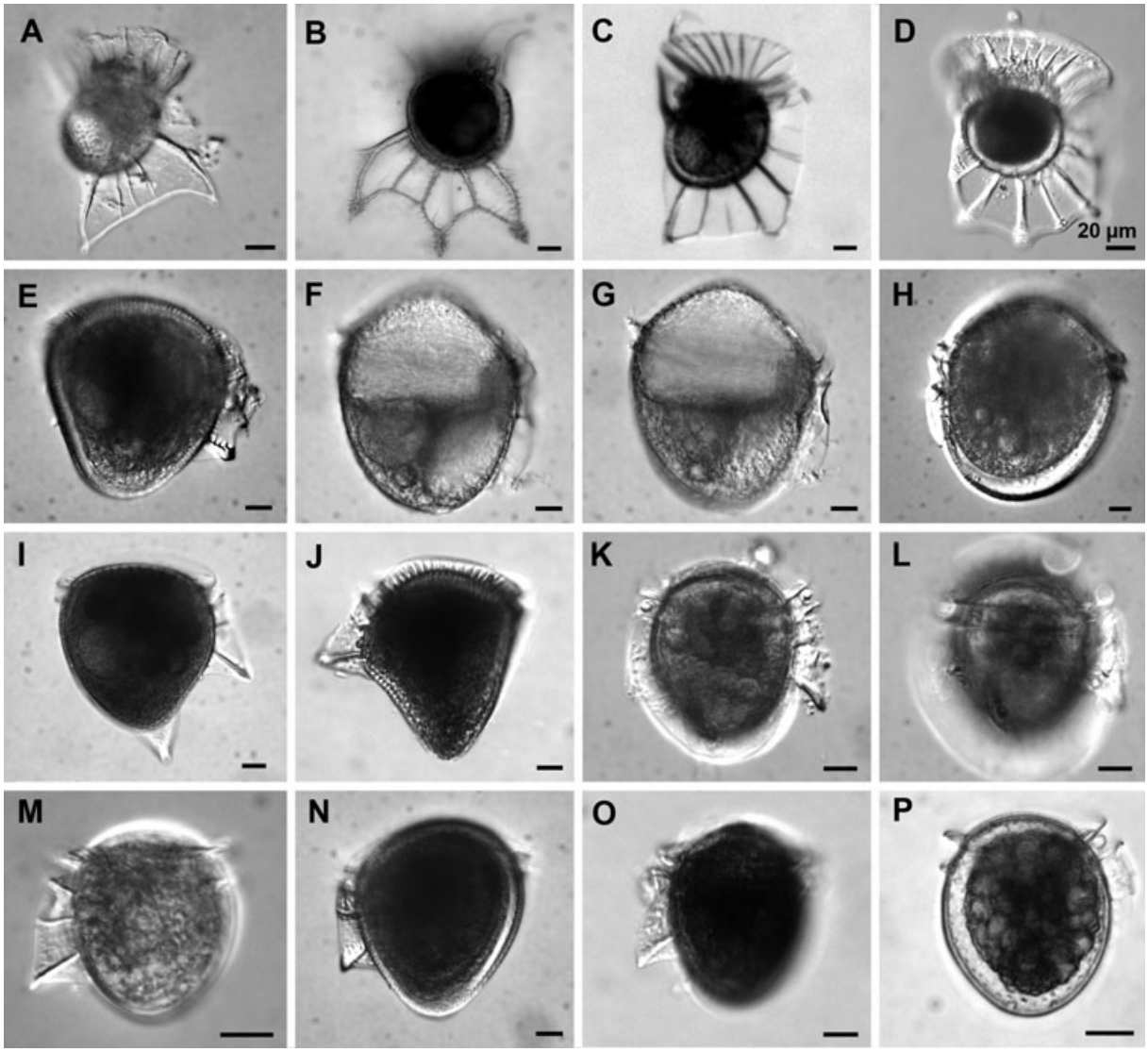

FIG. 3. Light micrographs (differential interference contrast, DIC) of the cells used as template in single-cell PCR determination of nuclear-encoded LSU rDNA. Scale bars 10 µm if not otherwise stated. (A) *Ornithocercus heteroporus*, right lateral view. (B) *Ornithocercus magnificus*, left lateral view. (C) *Ornithocercus quadratus*, right lateral view. (D) *Ornithocercus steinii*, right lateral view. (E) *Phalacroma acutum*, right lateral view. (F) *Phalacroma apicatum*, right lateral view, low focus. (G) *Phalacroma apicatum*, right lateral view, high focus. (H) *Phalacroma* cfr. *argus*, left lateral view. (I) *Phalacroma doryphorum*, right lateral view. (J) *Phalacroma mitra*, left lateral view. (K) *Phalacroma* cfr. *ovum*, right lateral view, low focus. (L) *Phalacroma* cfr. *ovum*, right lateral view, high focus. (M) *Phalacroma* cfr. *parvulum*, left lateral view. (N) *Phalacroma porodictyum*, left lateral view. (O) *Phalacroma* sp. 1, left lateral view. (P) *Phalacroma* sp. 2, right lateral view.

epitheca was >¼ of the total cell length. The LSL was rounded posteriorly and appeared to be without distinct ribs. Contradicting previous descriptions (i.e., Stein 1883, Kofoid and Skogsberg 1928), Taylor (1976) observed specimens that lacked the R3. Our observed specimen was similar to *P. argus* as it was shown on fig. 121 by Steidinger and Williams (1970), which also lacked a distinct R3. It was not identical to *Dinophysis argus*, shown as fig. 5 in Hernández-Becerril et al. (2008).

Phalacroma *cfr.* ovum *(Fig. 3, K and L).* In fixed material, the outline of the cell was slightly elliptical, ~59 µm long and 50 µm wide. The epitheca was prominent and of the same width as the hypotheca. The cingulum was wide and with horizontal delicate ribs. Information on the LSL was difficult to obtain, but the R3 was distinct and inclined posteriorly. The thecal plates appeared to be finely reticulated. Schütt (1895) erected *P. ovum* together with several other dinophysioid species, including *Dinophysis ovum* F. Schütt. When Balech (1967) merged *Phalacroma* and *Dinophysis*, *P. ovum* was transferred to *D. amydula* Balech, which was erected in the same work. According to Sournia (1973), following the International Code of Botanical Nomenclature, *D. amydula* was too similar to *D. amygdulus*

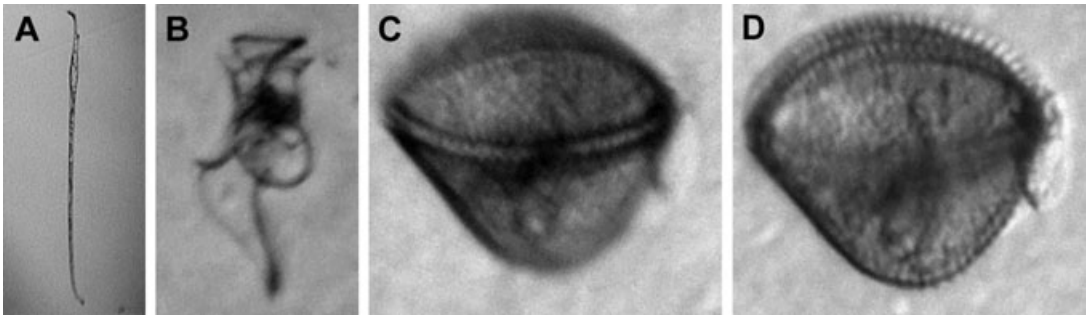

FIG. 4. Frame grabbed images from a high-definition video sequence of live cells used as template in single-cell PCR determination of nuclear-encoded LSU rDNA. (A) *Amphisolenia bidentata* in right lateral view. (B) *Histioneis elongata* in left lateral view. (C) *Phalacroma cuneus* right lateral view, in high focus, showing the cingulum. The thecal reticulation may be discerned. (D) *Phalacroma cuneus,* right lateral view, in midfocus, showing the cell outline. Unfortunately, the scale was not noted during video recording in the stereomicroscope.

Paulsen. The species originally described as *P. ovum* was therefore transferred to *D. amandula* (Balech) Sournia (Sournia 1973). Gomez (2005b) recognized *D. amandula*, but the encountered specimen showed classical *Phalacroma* characters and has therefore been named *P.* cfr. *ovum*.

Phalacroma *cfr.* parvulum *(Fig. 3M).* In fixed material, the cell shape was approximately circular in lateral outline, ~35 μm long and 30 μm wide. The epitheca was small but visible above the anteriorly inclined cingular lists. The cingulum was wide and possessed lists with numerous ribs. The LSL was wide and had distinct ribs. In structural outline, the specimen resembled several of the species in the *P. rotundatum* group described by Kofoid and Skogsberg (1928). On the basis of cell and epitheca size, the encountered specimen was *P. parvulum*. It differed from the description of *P. parvulum* by a more extended R3 and by the presence of ribs on the cingular lists (Jørgensen 1923, Kofoid and Skogsberg 1928, Taylor 1976).

Phalacroma *sp. 1 (Fig. 3O).* In fixed material, the cell shape was ellipsoid, ~57 μm long and ~43 μm wide. The specimen was observed from an oblique lateral view, and the width might have been underestimated. The epitheca was slightly conical and visible above the horizontal cingular lists. The LSL was wide with a distinct and posteriorly curved R3. The LSL appeared to be extremely convex and lacked both R1 and R2. This has not been observed for other members of Dinophysiales. Based on the shape and size of the cell and the R3, the encountered specimen showed most resemblance to the *P. rotundatum* group described by Kofoid and Skogsberg (1928).

Phalacroma *sp. 2 (Fig. 3P).* In fixed material, the cell shape was ellipsoid in lateral outline and widest at the midline, ~39 μm long and 32 μm wide. The epitheca was prominent, and the cingulum was wide with horizontal lists. The encountered specimen lacked the LSL, and information was insufficient for correct species identification. The small size, the shape of the cell, and lack of spines might indicate that the specimen belonged to the *P. rotundatum* group (Kofoid and Skogsberg 1928). The thecal plates had scattered and fairly large pores.

The type species Phalacroma porodictyum. Specimens of the type species of *Phalacroma* (viz. *P. porodictyum*) were observed both in live and Lugol's-fixed material (Fig. 5, A–C). The live cell was elliptical in lateral view and widest just below the cingulum, ~58 μm wide and 71 μm long (Fig. 5A). Cells were to some extent laterally flattened (Fig. 5). The large epitheca was convex, and

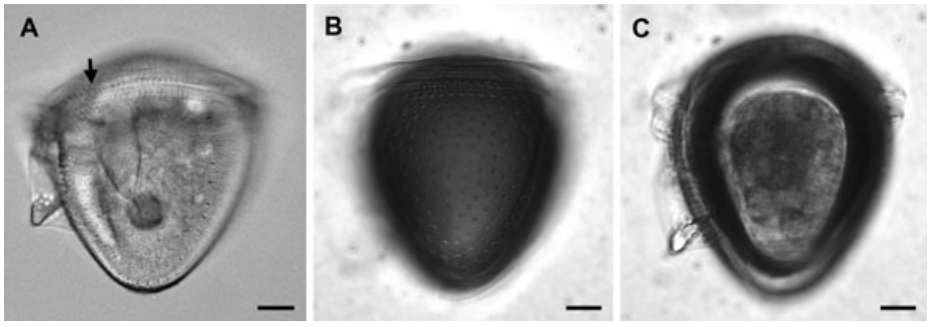

FIG. 5. Light micrographs (differential interference contrast, DIC) of *Phalacroma porodictyum*. All cells are in left lateral view. (A) Live cell; arrow shows indications of ribs on the anterior cingular list. (B) Lugol's-fixed cell in high focus showing the reticulation. (C) Lugol's-fixed cell in low focus showing the cell shape. Scale bars, 10 μm.

the hypotheca was tapered posteriorly (Fig. 5, A and C). The cingulum was wide and located ~1/5 of the cell length from the apex (Fig. 5C). Cingular lists were horizontal and with some indications of numerous ribs (Fig. 5A, arrow). Unfortunately, sufficient information to verify the presence of ribs could not be obtained from the micrographs. The LSL had straight margins and scattered reticulation (Fig. 5, A and C). The R3 was of moderate thickness, either straight or slightly inclined posteriorly. Micrographs provided little information on the right sulcal list, which appeared to end between R2 and R3. The theca was finely areolated with scattered pores (Fig. 5, A and B).

LSU rDNA sequences. A total of 28 partial LSU rDNA sequences of ~1,441 bp, from 27 different dinophysioid species, were included in the phylogenetic analyses (Table S1). The sequences determined for *D. truncata* only comprised 700 bp and were therefore not included in alignment A.

Two sequences differing by 3 bp were obtained for *Citharistes regius* (Fig. 2, B and C), and both were therefore included in the phylogenetic analyses (Figs. 7 and 8). Identical sequences were determined independently at least twice for 11 species: *Dinophysis miles*, *D. schuettii*, *D. tripos*, *Histioneis milneri*, *Ornithocercus heteroporus*, *O. magnificus*, *O. quadratus*, *Phalacroma doryphorum*, *P.* cfr. *parvulum*, *P. porodictyum, P. acutum*. Three of the included sequences were determined from live cells, that is, *Amphisolenia bidentata*, *Histioneis elongata*, and *P. cuneus* (Fig. 4).

Surprisingly, the sequences for *D. tripos* and *D. miles* (Fig. 2, H and M) were identical for all 1,441 bp determined. These specimens from the Indian Ocean were identical to the partial sequences of *D. tripos* from both France and Scotland (GenBank accession nos. AF318238, AY259242, respectively).

Identical sequences were also obtained from *P. porodictyum* (Fig. 3N) and *P. acutum* (Fig. 3E). Similarly, there was no difference in the sequences from *P. cuneus* (Fig. 4, C and D) and *Phalacroma* sp. 1 (Fig. 3O), and *O. quadratus* (Fig. 3C) and *Ornithocercus steinii* (Fig. 3D), respectively.

The two *O. heteroporus* in Figure 6 represented specimens with different morphology but identical LSU rDNA sequences. The specimen in Figure 6, C and D, was similar to the description and illustration of *Ornithocercus biclavatus* E. J. F. Wood (Wood 1954). This species is regarded as being synonymous with *O. heteroporus* (Abé 1967c, Taylor 1976, Gomez 2005b).

Phylogenetic analysis on LSU rDNA including domains D1-D6 (alignment A): Alignment A consisted of 28 sequences determined in the present study and four sequences available in GenBank: *D. acuminata*, *D. acuta*, *D. braarudii,* and *D. norvigica* (Table S1). The molecular phylogeny based on this alignment and inferred from Bayesian analysis yielded the tree topology shown in Figure 7. The two *Prorocentrum* species rooted the tree. The *Amphisolenia* species formed a highly supported basal lineage (Clade A in Fig. 7). The remaining dinophysioids divided into two highly supported lineages labeled Clade B and Clade C, respectively. Clade B was strongly supported by posterior probabilities (1) and bootstrap (99%) and included the type species (viz. *P. porodictyum*) and eight other *Phalacroma* species. The relationship within the *Phalacroma* clade (Clade B) was not well resolved, and most branches were only weakly or moderately supported either by posterior probabilities or bootstrap values.

The phylogenetic analysis based on alignment A did not provide a robust resolution between the genera in Clade C (Fig. 7), which was supported by posterior probabilities (1.0) and bootstrap (92%). Yet, the molecular phylogeny showed the genus *Dinophysis* to be polyphyletic as species from this genus formed four lineages within Clade C.

The five chloroplast/kleptochloroplast-bearing *Dinophysis* species, including the type species (viz. *D. acuta*), formed the strongly supported Subclade I (Fig. 7). The branch lengths within Subclade I were very short, resulting in little support for the

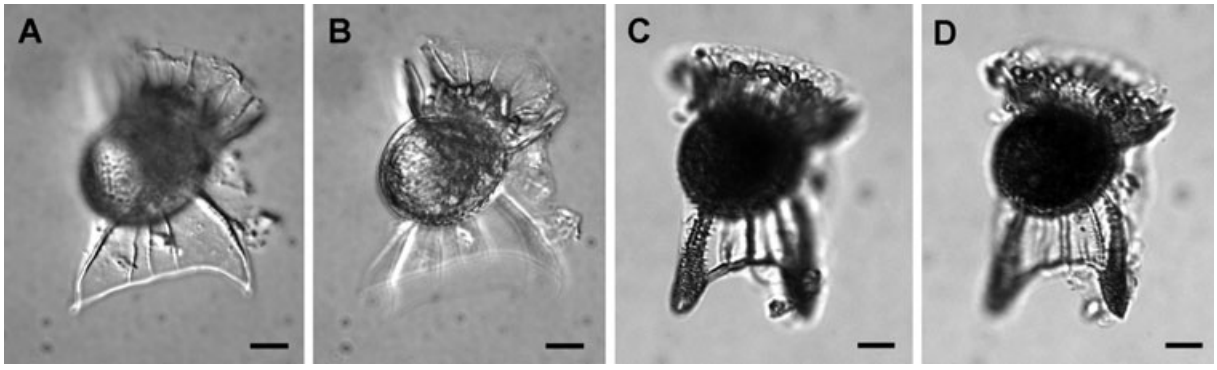

FIG. 6. Light micrographs (differential interference contrast, DIC) of Lugol's-fixed cells with identical LSU rDNA sequences. All cells in right lateral view. (A, B) *Ornithocercus heteroporus.* (C, D) *Ornithocercus heteroporus,* which Wood (1954) described as *Ornithocercus biclavatus* (see additional explanation in text). Scale bars, 10 μm.

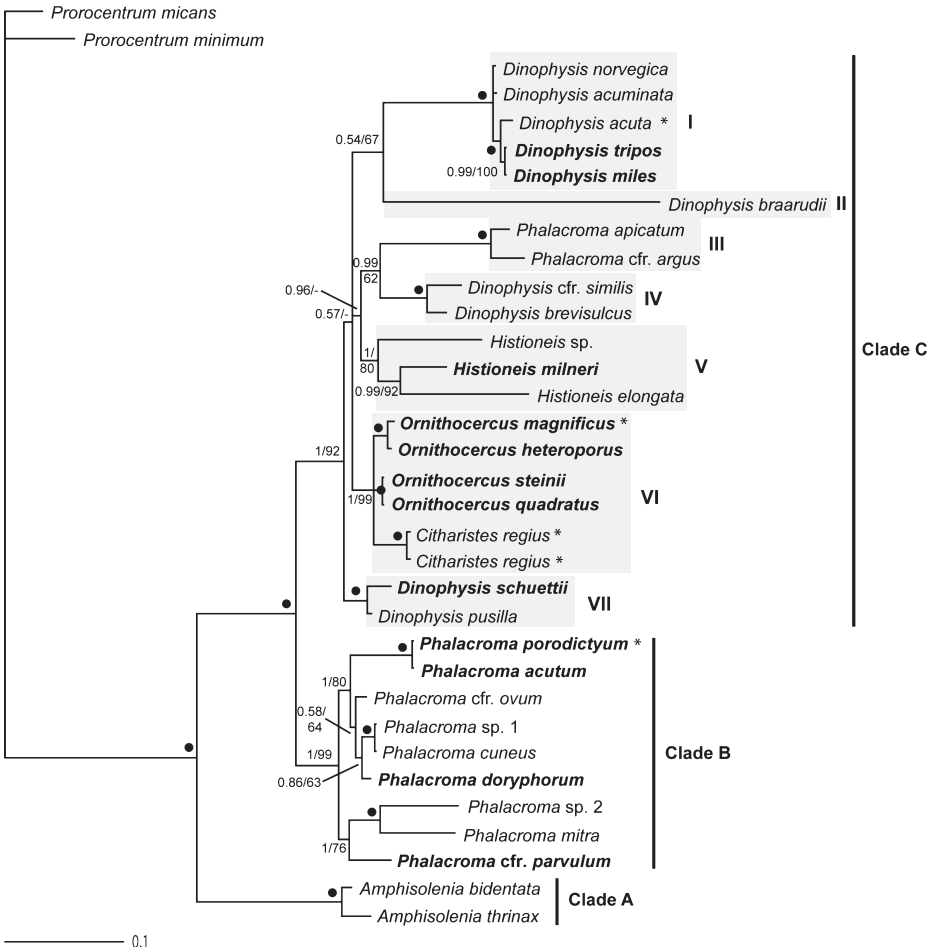

FIG. 7. Phylogeny of 31 members of the Dinophysiales based on nuclear-encoded LSU rDNA sequence including domains D1-D6 (1,441 bp) and inferred from Bayesian analysis. Two prorocentroids (*Prorocentrum micans* and *Prorocentrum minimum*) constituted the outgroup. Branch support was obtained from Bayesian posterior probabilities and bootstrap (100 replicates) in maximum-likelihood (ML) analyses. At internodes, posterior probabilities (≤1) are written first, followed by bootstrap values (in percentage) from ML. ●, indicates the highest possible posterior probability (1.0) and bootstrap value (100%). Asterisks indicate type species. Species in boldface were determined twice. For reasons of discussion, the dinophysioids have been divided into three clades (A–C). Clade C has been further subdivided into seven subclades.

branching patterns. The minute and heterotrophic *Dinophysis braarudii* (Subclade II) diverged as a long-branched sister taxon to the autotrophic *Dinophysis* species (Subclade I). Subclades III and IV, formed by *Phalacroma apicatum* together with *P.* cfr. *argus* (Fig. 3, F–H) and *Dinophysis* cfr. *similis* together with *D. brevisulcus* (Fig. 2, F, G, K, and L), were both supported by posterior probabilities of 1.0 and bootstrap values of 100. These two subclades were situated within a clade supported by posterior probabilities of 0.96 and bootstrap value of 62. Subclade VII comprised the morphologically similar *D. schuettii* and *D. pusilla* (Fig. 2, I and J), which diverged as a sister group to the other dinophysioids in Clade C. However, there was very little branch support for this (pp = 0.57).

According to the phylogenetic analyses, *Ornithocercus* and *Citharistes* were closely related and shared a common ancestor (Subclade VI, Fig. 7). Monophyly was strongly supported for *Citharistes*, while for the genus *Ornithocercus*, it was unresolved due to the low branch support. Still, the four *Ornithocercus* species clustered together in pairs according to cell outline (Fig. 3, A–D). The three *Histioneis* species formed a clade supported by a posterior probability of 1.0 and bootstrap value of 80 (Subclade V, Fig. 7). Within this clade, the narrow-winged *Histioneis* sp. (Fig. 2, P and Q) diverged first. According to the Bayesian analysis, the *Histioneis* species clustered with *D.* cfr. *similis*, *D. brevisulcus*, *P. apicatum,* and *P.* cfr. *argus* (Subclades III and IV).

Phylogenetic analysis on LSU rDNA including domains D1-D2 (Alignment B): The phylogenetic analyses of the 37 dinophysioids comprised in alignment B and inferred from Bayesian analysis yielded the tree topology illustrated in Figure 8. The tree topologies in Figures 7 and 8 were similar, but support for internodes was generally lower in the second tree (compare Figs. 7 and 8). The divergence of *D. pusilla* and *D. schuettii* as a basal branch within

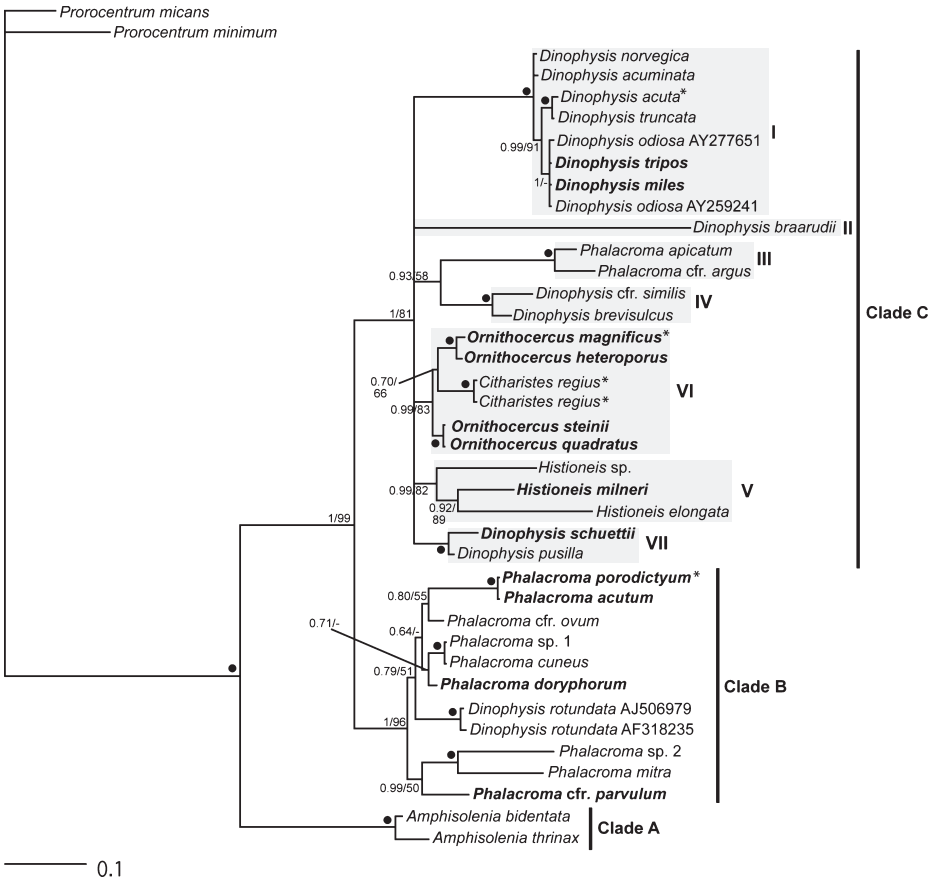

FIG. 8. Phylogeny of 36 members of the Dinophysiales based on nuclear-encoded LSU rDNA sequence including domains D1-D2 (700 bp) and inferred from Bayesian analysis. Two prorocentroids (*Prorocentrum micans* and *Prorocentrum minimum*) constituted the outgroup. Branch support was obtained from Bayesian posterior probabilities and bootstrap (100 replicates) in maximum-likelihood (ML) analyses. At internodes, posterior probabilities (≤1) are written first, followed by bootstrap values (in percentage) from ML. ●, indicates the highest possible posterior probability (1.0) and bootstrap value (100%). Asterisks indicate type species. Species in boldface were determined twice. For reasons of discussion, the dinophysiods have been divided into three clades (A–C). Clade C has been further subdivided into seven subclades.

Clade C was not supported by the phylogeny based on alignment B (Subclade VII in Fig. 8). Also, the positions of *D. braarudii* (Subclade II) and the *Histioneis* clade (Subclade V) were inconsistent between the two phylogenies (Figs. 7 and 8). The relationships of these subclades were unresolved as in Figure 8. The two included *Dinophysis rotundata* sequences branched within the *Phalacroma* group (Clade B in Fig. 8). *D. truncata* (Fig. 2N) and the two *D. odiosa* (fig. 3, n and o, in Hart et al. 2007) clustered with *D. acuta*, *D. tripos,* and *D. miles,* supported by a posterior probability of 0.99 and bootstrap value of 91. Among these species, a close relationship between *D. truncata* and *D. acuta* was strongly supported, while the position of the heterotrophic *D. odiosa* was less well resolved.

DISCUSSION

Phylogenetic relationships and the radiation of Dinophysiales. The present study is the first attempt to elucidate the molecular phylogeny and evolutionary history of the tropical members of Dinophysiales, and the LSU rDNA gene from 28 tropical dinophysioids has been determined by single-cell PCR. According to the LSU rDNA gene, the dinophysioids evolved into three major lineages, here designated Clades A, B, and C, respectively (Figs. 7 and 8). A dinophysioid radiation scheme as suggested by the LSU rDNA gene is illustrated in Figure 9 and will be discussed below. The phylogenetic analyses, including a diverse assemblage of the Dinophysiales, do not support the evolutionary radiation, going from a less to a more complex cellular outline (Fig. 1). Rather, branching patterns based on the LSU rDNA gene propose an evolutionary history comprising several short lineages (Fig. 9).

Diagnostic characters typically applied in the taxonomy of thecate dinoflagellates are highly conservative within the Dinophysiales. Species belonging to this order have therefore been arranged in genera according to their structural resemblance (Kofoid and Skogsberg 1928, Fensome et al. 1993). Even though the relationship between some of the

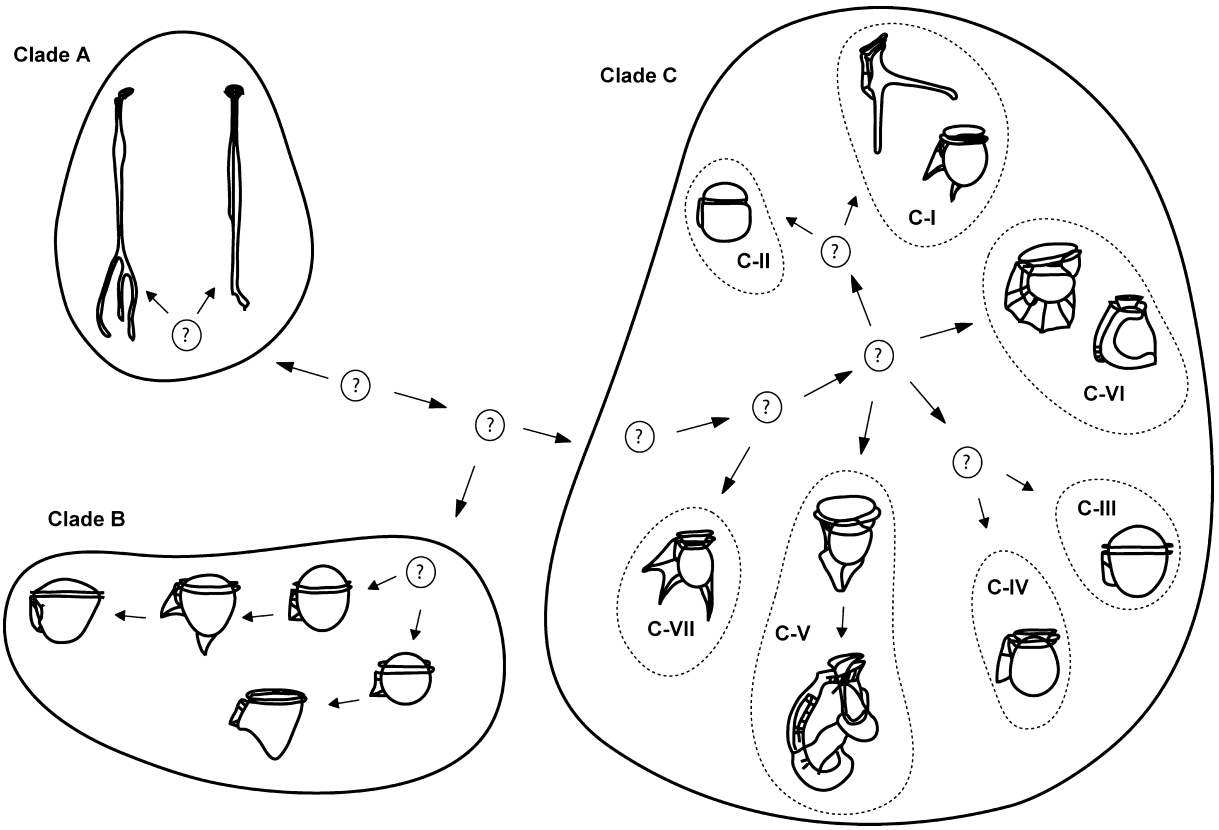

FIG. 9. Dinophysioid radiation as proposed by a phylogeny based on nuclear-encoded LSU rDNA sequences. Clades A, B, and C correspond to the three clades in Figures 7 and 8, respectively. The same species are included in Figure 1. The only addition is *Phalacroma argus* in Subclade C–III. ? = unknown ancestral organism. See text for further explanation. Not drawn to scale.

dinophysioid genera is unresolved in the molecular phylogeny, the present analyses demonstrate that structural resemblance is an insufficient marker of relationship, and supplementary diagnostic characters are necessary to separate the dinophysioid genera. Several characters previously regarded of taxonomic value within the Dinophysiales appear to be subject to convergent evolution, for example, the prominent epitheca and the funnel-shaped cingular lists (Steidinger and Tangen 1996). If the LSU rDNA–based phylogenies mirror the true evolutionary history of the Dinophysiales, the taxonomy of this order is urgently in need of revision. Due to limited molecular and morphological data, a complete revision is premature. Therefore, the proposed relationships from the phylogenetic trees are merely outlined below.

The dinophysioid families. Numerous suggestions for assembling the dinophysioid genera into families have been proposed through the last 125 years, for example, Stein (1883), Kofoid and Skogsberg (1928), Abé (1967a,b,c), Fensome et al. (1993), and Gomez (2005b). Though only including two species of the genus *Amphisolenia*, the molecular phylogenies seem to verify the taxonomic status of *Amphisoleniaceae* Er. Lindem. (Clade A). However, future determination of nuclear-encoded LSU rDNA from the supposedly closely related *Triposolenia* will have to demonstrate the monophyly of Amphisoleniaceae. On the contrary, the *Dinophysiaceae* F. Stein (Clade B and C) is well supported by the molecular analyses. These results are therefore in agreement with those of Fensome et al. (1993), which, on the basis of ventral plate tabulation, suggested three dinophysioid families, *Amphisoleniaceae*, *Dinophysiaceae,* and *Oxyphysiaceae* Sournia.

Clade A: Amphisolenia. The elongated *Amphisolenia* species (Figs. 2A and 4A) form a basal group within the Dinophysiales, revealing an early divergence (Clade A in Figs. 7 and 8). The evolutionary distant relationship of *Amphisolenia* to the other dinophysioids is indicated both morphologically, by the arrangement of the two small hypothecal plates, and physiologically, by the presence of intracellular symbionts of both bacterial and eukaryotic origin (Abé 1967a, Lucas 1991, Foster et al. 2006). Since the elongated genera *Triposolenia* and *Amphisolenia* show similar sulcal and ventral hypothecal plate arrangements, they are suggested to be closely related (Abé 1967c, Fensome et al. 1993).

In this basal lineage, the hypotheca increased extremely in length and was furthermore bifurcated in some species (Fig. 2A). These features were also observed for *D. miles* (Fig. 2H), and we conclude,

on the basis of molecular phylogeny, that elongation and posterior bifurcation have evolved at least twice independently within Dinophysiales (Fig. 9, Clade A and Subclade C–I).

Clade B: Phalacroma. Ten *Phalacroma* species, including the type species (viz. *P. porodictyum*), formed a highly supported separate lineage within the phylogenetic tree (Clade B in Fig. 8). On the basis of these results and the diagnostic morphological characteristics (see below), we conclude that the disputed *Phalacroma* is indeed a valid genus, and we therefore propose a reinstatement.

The genus was erected by Stein (1883) who regarded the horizontal cingular lists and the large epitheca observed in some *Dinophysis* species as being so different from the concept of *Dinophysis* that he erected *Phalacroma*. Stein (1883) did notice that some species assigned to this new genus were difficult to separate from *Dinophysis*. Studies on thecal plate arrangement showed *Dinophysis* and *Phalacroma* to be almost identical (Tai and Skogsberg 1934), and in 1967, Abé (1967b) and Balech (1967), simultaneously, merged the two genera. Previous studies including physiological, ecological, and molecular data have suggested that *Phalacroma* and *Dinophysis* are separated at the genus level (Hallegraeff and Lucas 1988, Guillou et al. 2002, Edvardsen et al. 2003, Hart et al. 2007, Hastrup Jensen and Veland 2008). However, to the best of our knowledge, this is the first study to present a molecular phylogeny comprising the type species of *Phalacroma* and several other *Phalacroma* species.

Identity of Phalacroma porodictyum. Except for a slight variation in cell outline, the specimens of *P. porodictyum* from the Indian Ocean (Figs. 3N and 5) were similar to the original drawings (in Stein 1883, figs. 11–14, pl. XVIII). Compared to more recent studies, the encountered specimens showed an overall similarity in cell outline to the drawing made by Taylor (1976, fig. 45), also based on Indian Ocean material. The thecal plate reticulation and the shape of the LSL were similar to the specimen observed in the scanning electron microscope by Hallegraeff and Lucas (1988, fig. 3). The observed specimens (Figs. 3N and 5) are narrower than the specimen in fig. 22 in Hernández-Becerril et al. (2008).

Phalacroma F. Stein 1883

Dinophysis Ehrenb. partim sensu Abé (1967) and Balech (1967) Stein 1883, p. 23, plate XVIII, figs. 11–14.

Type: Phalacroma porodictyum F. Stein 1883.

Lecto type (designated here): Die Naturgeschichte der arthrodelen Flagellaten, plate XVIII, fig. 11.

Epitype (designated here): Figure 3N from the Indian Ocean, NW of Australia (16°01′668 S, 119°20′233 E), 16 November 2006.

Emended description of Phalacroma. Small to medium-sized cells. Cell outline is typically circular to ellipsoid and can be tapered posteriorly. Cells are more or less laterally depressed. Epitheca is large but <¼ of the cell length. It is visible above the cingular lists, and flattened to convex in outline. Cingular lists are narrow and horizontal and can possess ribs. Left sulcal list typically widens posteriorly and always possesses three distinct ribs. Thecal ornamentation is of different types, from smooth with scattered pores to deep and strongly areolated. Cytoplasm is colorless, pink or yellow-brown. Chloroplasts of haptophyte origin are observed in a few species.

The typical *Phalacroma* cell outline with a distinct epitheca and horizontal cingular lists has arisen several times within Dinophysiales [e.g., Clade B, Subclade III, *P. apicatum* and *P.* cfr. *argus* (Fig. 3, F–H), and Subclade II, *Dinophysis braarudii* (Fig. 2, D and E)]. A narrow and delicate LSL, which lacks distinct ribs, distinguishes these three species from the reinstated *Phalacroma* as proposed here. Consequently, we conclude that the structure and width of the LSL are important diagnostic characters in delimitating the genus *Phalacroma*.

Gomez (2005b) recognized 41 species of *Phalacroma*. The taxonomic situation concerning *Phalacroma* cfr. *ovum* (see above) indicates that even more species should be assigned to this genus. Due to lack of molecular and occasionally insufficient morphological data, we are reluctant to transfer these to the reinstated *Phalacroma* genus. Confident identification was obtained for five species situated with the *Phalacroma* clade (Clade B in Fig. 8) and thus closely related to the type species *P. porodictyum*. Therefore, these are transferred to the reinstated genus.

Phalacroma acutum (F. Schütt) Pavill.
Synonym: Dinophysis acutoides Balech
Phalacroma cuneus F. Schütt
Synonym: Dinophysis cuneus (F. Schütt) T. H. Abé
Phalacroma doryphorum F. Stein
Synonym: Dinophysis doryphora (F. Stein) T. H. Abé
Phalacroma mitra F. Schütt
Synonym: Dinophysis mitra (F. Schütt) T. H. Abé
Phalacroma rotundatum (Clap. et J. Lachm.) Kof. et J. R. Michener
Synonym: D. rotundata Clap. et J. Lachm.

The molecular phylogenies showed *P. mitra* (Fig. 3J), the only included autotrophic *Phalacroma* species to be recently diverged, thereby suggesting that the chloroplasts of haptophyte origin have been obtained as an independent evolutionary event in this species only. The maximum sequence divergences within the *Phalacroma* clade (Clade B in Fig. 7), based on alignment A, were 7.9% in uncorrected distances and 8.3% based on the Kimura-2-parameter model. These values are higher than what previously have been reported for the autotrophic *Dinophysis*, where the sequence divergence in the D1 and D2 domains usually lies below 3% (Edvardsen et al. 2003, Hart et al. 2007, Hastrup Jensen and Veland 2008). This finding indicates

either an early divergence of the genus *Phalacroma* or a more rapid substitution rate within this genus, compared to the autotrophic *Dinophysis* species.

Clade C: Dinophysis, Phalacroma, Histioneis, Ornithocercus, *and* Citharistes. In Clade C, a great morphological and ecological diversity has arisen from a common ancestor. The phylogenetic relationship of this lineage is, as mentioned previously, unresolved between seven subclades (Figs. 7 and 8). We are therefore unable to elucidate the order of evolutionary events within this lineage but can merely discuss them independently for the different subclades (C–I to C–VII in Fig. 9).

Dinophysis *(Subclades I, II, IV, and VII).* Based on the results shown in Figures 7 and 8, the genus *Dinophysis* was polyphyletic, as it formed four separate clades (i.e., Subclades I, II, IV, and VII in Figs. 7–9). These results indicate that besides *Dinophysis* sensu stricto (Subclade I), *Dinophysis* should be divided into at least three new genera (Subclades II, IV, and VII).

The sequence divergence estimates (Table 2) support that there are four *Dinophysis* subclades within the Dinophysiales. *D. braarudii* in Subclade II shows the highest divergence from the three other subclades; the results lie in the range of 12.9% to 15.6% uncorrected distances and 14.3% to 17.6% distance estimations based on the Kimura-2-parameter model. This distant relationship of *D. braarudii* was also indicated by the length of the branch in the phylogenetic tree (Fig. 7). The sequence divergences among the three remaining subclades were lower, and between Subclade IV and Subclade VII, the maximum distances were 6.9% and 7.3%, respectively (Table 2). These results indicate that the genetic evolution has been more rapid in *D. braarudii* than in the three other *Dinophysis* subclades.

Dinophysis *sensu stricto (Subclade I).* Subclade I comprised the type species *D. acuta* and six other *Dinophysis* species (Fig. 8). Thus, this group represents *Dinophysis* sensu stricto. The relationship between these mainly autotrophic species is, as previously observed, not well supported (Guillou et al. 2002, Edvardsen et al. 2003, Saldarriaga et al. 2004, Hart et al. 2007), and the molecular phylogenies cannot unravel the evolution within *Dinophysis* s.s. The domains D1-D6 LSU rDNA variation within Subclade I was relatively low as the interval was within 0% to 1.3% in both uncorrected distances and distance estimations based on the Kimura-2-parameter model. This low sequence divergence between the *Dinophysis* s.s. species suggests a rapid morphological evolution within this group (Edvardsen et al. 2003, Hart et al. 2007). Shared characters for these *Dinophysis* species are the thecal plate reticulation, an extreme laterally flattened theca, and funnel-shaped cingular lists projected above the small epitheca (Hallegraeff and Lucas 1988, Steidinger and Tangen 1996). The molecular phylogeny showed, as previously observed, *D. odiosa* (Fig. 1, no. 13, this study; fig. 3, n and o, in Hart et al. 2007) to be belonging to *Dinophysis* s.s., despite the fact that this species is heterotrophic and possesses a different thecal ornamentation, a different cell shape, and an antapical spine (Steidinger and Tangen 1996, Hart et al. 2007). Whether *D. odiosa* is early or recently diverged within *Dinophysis* s.s. cannot be established based on the present data. The latter would imply that the chloroplasts, extremely flat cell body, and thecal ornamentation are secondarily lost within this species.

Dinophysis *(Subclade II).* *D. braarudii* (Fig. 2, D and E), in the analysis based on alignment A, formed a long-branched sister taxon to *Dinophysis* s.s. (Fig. 7). This minute species is morphologically very distinct from *Dinophysis* s.s. in cell outline, thecal reticulation, and by lacking chloroplasts (Nordli 1951, Hastrup Jensen and Veland 2008). Based on the original hypothesis by Kofoid and Skogsberg (1928), the small size and the simple cell outline of this species would imply an ancestral position within Dinophysiales. *Phalacroma contractum* (Figs. 1 and 2), which was regarded as one of the most primitive members of Dinophysiales, shares several characters with *D. braarudii* (Kofoid and Skogsberg 1928, McMinn and Scott 2004). This similarity could be explained by either convergent evolution or by a recent diversification where the minute size, the horizontal cingular lists, and the narrow LSL without ribs are results of secondary reductions.

Dinophysis *(Subclade IV).* *D.* cfr. *similis* and *D. brevisulcus* formed a highly supported and long-branched clade (Figs. 7 and 8). The two species in

TABLE 2. Sequence divergence in percent interval of the four *Dinophysis* subclades as they were defined based on Figures 7 and 8.

	Subclade I	Subclade II	Subclade IV	Subclade VII
Subclade I	–	14.5–17.6	9.2–12.1	7.9–10.6
Subclade II	13.1–15.6	–	15.1–15.8	14.3–14.5
Subclade IV	8.6–11.1	13.6–14.1	–	6.7–7.3
Subclade VII	7.5–9.9	12.9–13.1	6.4–6.9	–

Divergence estimated based on 1,441 bp of the LSU rDNA, corresponding to domains D1 to D6, sensu Lenaers et al. (1989). Uncorrected distances are given below the diagonal and distance estimations based on the Kimura-2-parameter model above the diagonal.

Subclade IV shared anterior inclined cingular lists, a rounded outline, and lack of a distinct R3 on the LSL (Fig. 2, F, G, K, and L). The two latter characters distinguish them from *Dinophysis* s.s. *D.* cfr. *similis* and *D. brevisulcus* have been suggested to be closely related to *P. protuberans* Kof. et Skogsb. and *P. expulsum* (Kof. et J. R. Michener) Kof. et Skogsb., which both lack an R3 (Kofoid and Skogsberg 1928, Tai and Skogsberg 1934, Taylor 1976).

Dinophysis *(Subclade VII).* *D. schuettii* and *D. pusilla* (Fig. 2, I and J) have a round cell outline, a long antapical spine sometimes with sails and large distinct ribs on the sail-like LSL. The anterior cingular list in this subclade is furthermore inclined to an extreme extent (Kofoid and Skogsberg 1928, Steidinger and Tangen 1996). These characters distinguish them from the typical *Dinophysis* s.s. outline (Subclade I) (Kofoid and Skogsberg 1928, Steidinger and Tangen 1996). Previous studies based on morphology and ecology have regarded *D. schuettii* and *D. pusilla* to be closely related to *D. hastata* F. Stein (Kofoid and Skogsberg 1928, Norris and Berner 1970, Hallegraeff and Lucas 1988). However, *D. hastata* is morphologically very similar and possibly conspecific with *D. odiosa* (Taylor 1976), which clustered within *Dinophysis* s.s. (Subclade I in Fig. 8). Since *D. pusilla* and *D. schuettii* are not closely related to *D. odiosa*, the more or less round cell outline with an antapical spine has been subject to convergent evolution in these heterotrophic *Dinophysis* species.

Phalacroma *(Subclade III).* *P. apicatum* and *P.* cfr. *argus* (Subclade III) are not included in the reinstated *Phalacroma* genus as here proposed (Clade B in Figs. 7 and 8). These two species have previously been suggested to be closely related and can be differentiated from the *Phalacroma* species by an epitheca being >¼ of the cell length and by the lack of distinct ribs on the LSL (Kofoid and Skogsberg 1928, Steidinger and Williams 1970, Taylor 1976). Subclade III is highly supported, and due to the distant relationship of these two species to the other *Phalacroma* species, they most likely should be transferred to a new genus. *P. apicatum* and *P.* cfr. *argus* are different from most other species in Clade C in having a large epitheca and horizontal cingular lists (Fig. 3, F–H). *P. apicatum* and *P.* cfr. *argus* clustered within a moderately supported clade with *D.* cfr. *similis* and *D. brevisulcus* (Subclade IV, discussed above), suggesting a relationship of a yet unknown taxonomic level. The four species all possess a round cellular outline and a narrow LSL without distinct ribs (Figs. 2, F, G, K, L; and 3, F–H). We therefore assume that these characters are ancestral for Subclades III and IV. The sequence divergence estimates between Subclades III and IV lie in the range of 8.7% to 10.1% uncorrected distances and 9.3% to 10.9% distance estimations based on the Kimura-2-parameter model. This finding indicates the same degree of relationship between these two subclades as among the three *Dinophysis* subclades (I, IV, and VII).

Ornithocercus, *Histioneis, and* Citharistes *(Subclades V and VI).* Two lineages became morphologically adapted to accommodate ectosymbionts (i.e., Subclade V comprising *Histioneis* and Subclade VI comprising *Ornithocercus* and *Citharistes*). *Ornithocercus* and *Histioneis* have been hypothesized to be very closely related, and *Parahistioneis* has been speculated to represent an intermediate form between these two (Fig. 1) (Murray and Whitting 1899, Kofoid and Skogsberg 1928, Tai and Skogsberg 1934, Gomez 2005a). *Citharistes* is clearly distinct from *Histioneis* and *Ornithocercus* both morphologically and by the presence of relatively small lists, two additional small hypothecal plates, and the fact that the dorsal cell wall forms the chamber accommodating the prokaryotic ectosymbionts. In both *Ornithocercus* and *Histioneis,* the ectosymbionts are kept between the cingular lists (Kofoid and Skogsberg 1928, Balech 1988, Steidinger and Tangen 1996).

Surprisingly, *Ornithocercus* and *Citharistes* formed a highly supported clade (Subclade VI) where monophyly was only supported for *Citharistes*. Based on the low branch support in Subclade VI and the morphological similarities, it is still reasonable to assume that *Ornithocercus* is a monophyletic genus. This assumption is confirmed by the sequence divergence estimates where the difference between the two *Ornithocercus* groups—that is, *O. magnificus* together with *O. heteroporus* (Fig. 3, A and B) and *O. quadratus* together with *O. steinii* (Fig. 3, C and D)—lie in the range of 1.5% to 1.7% both in uncorrected distances and distance estimations based on the Kimura-2-parameter model. The values between the two *Citharistes* specimens and the *Ornithocercus* species are somewhat higher, in the range of 2.5% to 3.2% uncorrected distances and 2.5% to 3.3% distance estimations based on the Kimura-2-parameter model.

The monophyly of *Histioneis* (Subclade V) and the position separate from *Ornithocercus* and *Citharistes* (Subclade VI) is supported by the composition of the ectosymbiont present in *Histioneis,* which is different from the ones observed in *Ornithocercus* and *Citharistes* (Lucas 1991, Foster et al. 2006). We therefore conclude that *Histioneis* not is an evolutionary intermediate between *Citharistes* and *Ornithocercus* (compare Figs. 1 and 9).

Histioneis sp. (Fig. 2, P and Q) possesses morphological features characteristic of the previously accepted genus *Parahistioneis*. This species diverged as a sister taxon to the two other *Histioneis* species (Figs. 2O and 4B), which possess the typical *Histioneis* morphology (Kofoid and Skogsberg 1928). The results, therefore, to some extent support *Parahistioneis* as a valid genus. But additional molecular data are needed to justify this.

Phenotypic variation being genetically undetectable. The differences observed in cell outline between the specimens in Figures 3, E and N, and 6 were undetectable in the LSU rDNA gene. These molecu-

lar results and the high morphological similarity indicate that *Phalacroma acutum* is synonymous with *P. porodictyum* (Fig. 3, E and N), and furthermore verify that Abé (1967c) and Taylor (1976) were correct in considering *Ornithocercus biclavatus* synonymous with *O. heteroporus* (Fig. 6).

Unexpectedly, the morphologically distinguishable species *D. tripos* and *D. miles* (Fig. 2, H and M), and *O. quadratus* and *O. steinii* (Fig. 3, C and D), respectively, also had identical LSU rDNA sequences. The differences in thecal plate reticulation, cell outline, and the LSL shape between *Phalacroma cuneus* (Fig. 4, C and D) and *Phalacroma* sp. 1 (Fig. 3O) were likewise untraceable in the LSU rDNA sequences. Additional genetic markers (e.g., mitochondrial genes) should enlighten the genetic relationship among these species.

Identical LSU rDNA sequences for *D. acuminata* and *D. sacculus* F. Stein, and for *D. tripos* and *D. odiosa*, respectively, have previously been observed (Guillou et al. 2002, Hart et al. 2007). Our data further imply no genetic differentiation in the LSU rDNA gene among *D. odiosa, D. tripos,* and *D. miles.* The slightly longer branch of *D. odiosa* (AY277651) is due to a two base pair difference compared to *D. tripos* and *D. miles* at the very end of our alignment B (Fig. 8). Intermediate morphotypes and an overlapping range of variability exist between the *D. acuminata* and *D. sacculus* (Zingone et al. 1998), whereas the autotrophic *D. tripos* and *D. miles* are clearly distinguishable from each other and the heterotrophic *D. odiosa.* These obvious phenotypical variations being undetectable in the LSU rDNA gene indicate rapid morphological evolution and recent evolutionary diversification among at least three dinophysioid genera.

The ancestral organisms: Based on the minute size and simple morphology, *D. braarudii* would be regarded as very close to the dinophysioid ancestral organism (Kofoid and Skogsberg 1928, Tai and Skogsberg 1934, Hallegraeff and Lucas 1988). Yet according to the molecular phylogenies, *D. braarudii* has recently diverged within the Dinophysiales (Figs. 7 and 8). Another candidate to be closely related to the ancestral organism is the microcephalic genus *Sinophysis* (Fig. 1, no. 1) (Taylor 1980, Hoppenrath et al. 2007). This heterotrophic genus is hypothesized to be closely related to the genus *Sabulodinium* R. D. Saunders et J. D. Dodge and the extinct *Nannoceratopsis* Deflandre, which both possess dinophysioid and peridinioid characters (Piel and Evitt 1980, Selina and Hoppenrath 2004, Hoppenrath et al. 2007). Based on the ventral plate tabulation *Amphisolenia* is the dinophysioid most closely related to *Nannoceratopsis* (Piel and Evitt 1980). This is in agreement with the basal position of *Amphisolenia* in the dinophysioid molecular phylogeny. Future molecular studies including LSU rDNA sequences from *Sinophysis* and *Sabulodinium* could possibly reveal the enigma of the ancestral organism, from which all the dinophysioids arose.

Since chloroplasts are present only within the most recently diverged dinophysioids, the ancestral organism most likely lost its chloroplast secondarily and therefore was heterotrophic. This is further supported by the chloroplasts being of different origin and positioned in two different clades (Moestrup and Daugbjerg 2007). The only included autotrophic species not comprised in *Dinophysis* s.s. (Subclade I in Figs. 7 and 8) is *Phalacroma mitra* (Fig. 3J and Clade B in Figs. 7 and 8). This species, as the closely related and possible conspecific *P. rapa*, possesses a plastid of haptophyte origin, while the typically dinophysioid chloroplasts are of cryptophyte origin (Taylor 1976, Hallegraeff and Lucas 1988, Hackett et al. 2003, Koike et al. 2005). On the basis of tree topologies, we conclude that the chloroplasts in the autotrophic *Dinophysis* and in *P. mitra* and *P. rapa* are the result of two independent evolutionary events.

The ancestral organism probably possessed rhabdosomes, as these organelles are distinct for Dinophysiales and present in all genera included in the phylogenetic analysis (Vesk and Lucas 1986, Hallegraeff and Lucas 1988).

CONCLUSION

Combinations of LSU rDNA sequences with micrographs of a single cell enabled us to produce the first molecular phylogeny including a wide assemblage of the tropical dinophysioid genera. The phylogenetic analyses revealed that the evolutionary history within Dinophysiales is to a great extent much more complicated than previously hypothesized. Furthermore, structural resemblance is an insufficient marker of genetic relationship between the dinophysioid genera. Future investigations should include more dinophysioid species and additional genetic markers to completely unravel the evolutionary history within the Dinophysiales. Additional morphological diagnostic characters should be applied in the separation of the dinophysioid genera and to the characterization of at least four new genera alluded to in this study.

NOTE ADDED IN PROOF

Concomitant with our study, Handy et al. (2009) have also addressed the phylogenetic relationship within the Dinophysiales using a molecular approach. Based on sequences of the rDNA operon, their data, with representatives from four genera, similarly support that *Phalacroma* and *Dinophysis* represent separate and distinct genera.

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Supplementary Material

The following supplementary material is available for this article:

Table S1. List of dinoflagellates included in the phylogenetic analyses. Positions and sampling dates are given for those dinophysioids collected in the Indian Ocean. GenBank accession numbers and references to micrographs of specimens used for single-cell PCR are also provided.

This material is available as part of the online article.