

## ***Sinophysis* and *Pseudophalacroma* are Distantly Related to Typical Dinophysoid Dinoflagellates (Dinophysales, Dinophyceae)**

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**ABSTRACT.** Dinophysoid dinoflagellates are usually considered a large monophyletic group. Large subunit and small subunit (SSU) rDNA phylogenies suggest a basal position for Amphisoleniaceae (*Amphisolenia*, *Triposolenia*) with respect to two sister groups, one containing most *Phalacroma* species plus *Oxyphysis* and the other *Dinophysis*, *Ornithocercus*, *Histioneis*, *Citharistes* and some *Phalacroma* species. We provide here new SSU rDNA sequences of *Pseudophalacroma* (pelagic) and *Sinophysis* (the only benthic dinophysoid genus). Molecular phylogenies support that they are very divergent with respect to the main clade of Dinophysales. Additional molecular markers of these two key genera are needed to elucidate the evolutionary relations among the dinophysoid dinoflagellates.

**Key Words.** Dinoflagellata, *Dinophysis*, Gymnodiniales–Peridinales–Prorocentrales lineage, sand dwelling protist, SSU rDNA phylogeny.

**E**XTANT dinoflagellates show five basic types of tabulation: peridinioid, gonyaulacoid, dinophysoid, prorocentroid and gymnodinioid, which are the basis for a dinoflagellate classification into five major orders (Taylor 1980). The dinophysoid dinoflagellates are considered a monophyletic group in both classical taxonomical schemes and molecular phylogenetic analyses from available data (Handy et al. 2009; Hastrup, Jensen, and Daugbjerg 2009). The dinophysoids have a remarkable variety of forms, ranging from less ornamented species with a *Phalacroma*-like outline, usually regarded as the ancestral forms, to highly ornamented species from warm oceans that harbour diazotrophic symbionts (*Ornithocercus* F. Stein, *Histioneis* F. Stein). Despite major differences in general appearance, the arrangement and number of their thecal plates are highly conserved among the investigated dinophysoid genera (Abé 1967; Balech 1980). Authors who attempted to elucidate the dinophysoid radiation proposed that the ancestral dinophysoid would be a small cell resembling the *Phalacroma* F. Stein outline, with horizontal circular lists and simplified morphology with a narrow sulcal list without ribs (Hallegraeff and Lucas 1988; Kofoid and Skogsberg 1928). In addition, Abé (1967) and Taylor (1980) proposed as an alternative hypothesis an ancestor resembling the microcephalic *Sinophysis* D.S. Nie et Chia C. Wang. Taylor (1980) also suggested a similarity of *Sinophysis* with the fossil *Nannoceratopsis* Deflandre, considered a missing link between dinophysoids and peridinioids.

The oceanic genus *Pseudophalacroma* Jørg. shows little morphological specialization. It possesses an atypical notch in the epitheca with two highly elongated ventral epithecal plates. The microcephalic *Sinophysis* is the only known benthic dinophysoid and superficially resembles a *Prorocentrum* Ehrenb. cell with a small epitheca (Abé 1967). *Pseudophalacroma* and *Sinophysis* are characterized by a reduced sulcal list with highly reduced or absent supporting ribs. They could be considered representative forms of the primitive *Phalacroma* and the microcephalic hypothetical dinophysoid ancestors, respectively (Abé 1967; Taylor 1980).

We determined sequences of small subunit (SSU) rRNA sequences for two *Sinophysis* species, *Sinophysis ebriola* (Herdman) Balech and *Sinophysis grandis* Hoppenrath, collected near their type localities, the British coasts and the North Sea, respectively, and five sequences of the type of

*Pseudophalacroma*, *Pseudophalacroma nasutum* (F. Stein) Jørg., from its type locality, the open Mediterranean Sea, and a second undescribed *Pseudophalacroma* species. Phylogenetic analyses suggest that these species are very divergent with respect to the other dinophysoids.

### MATERIALS AND METHODS

The specimens of *P. nasutum* were collected in open Mediterranean waters (see Fig. S1 and Table S1 as Supporting Information). Ten litres of seawater were collected from the surface with a bucket and filtered using a strainer of 20- $\mu$ m netting aperture. The retained material was fixed with 50% ethanol (final concentration) and examined in Utermöhl chambers with an Olympus (Tokyo, Japan) IX51 inverted microscope with an Olympus DP71 camera. A second *Pseudophalacroma* species was isolated from a sample collected in the Marmara Sea (Table S1). A volume of 36 liters was filtered with a 20- $\mu$ m nylon collector and cells resuspended in 0.22- $\mu$ m-filtered seawater and fixed in ethanol (~80% final concentration). The concentrated sample was examined in Utermöhl chambers with a Nikon (Tokyo, Japan) Eclipse TE2000-S inverted microscope with a Nikon DS-2M camera. *Sinophysis* specimens were isolated from sand samples in a tidal pool on Wimereux beach, English Channel (Fig. S1 and Table S1). The upper centimetre of sand was collected with a spoon, deposited in a bottle, rinsed with seawater and stirred vigorously. The resulting suspension was settled in a composite Utermöhl chamber and examined with a Nikon (Tokyo) Eclipse TE2000-S inverted microscope with a Nikon DS-2M camera.

Each specimen was micropipetted individually with a fine capillary into a clean chamber, washed several times in serial drops of 0.2- $\mu$ m sterilized water and finally deposited into a 0.2-ml Eppendorf tube with several drops of absolute ethanol. SSU rDNAs were amplified from each single cell following three rounds of nested PCR with the primers EK-42F (5'-CTCAARGAYTAAGCCATGCA-3') and EK-1520R (5'-CYGCAGGTTACCTAC-3'); EK-82F (5'-GAAACTGCGAATGGCTC-3') and EK-1498R (5'-CACCTACGGAAACC TTGTTA-3'); and DIN464F (5'-TAACAATACAGGGCATC CAT-3') and EK-1498R. PCR amplification of SSU rRNA genes and sequencing were done as described in Gómez, López-García, and Moreira (2011). Sequences were aligned using MUSCLE 3.7 (Edgar 2004). Maximum likelihood phylogenetic analyses were carried out with TREEFINDER (Jobb, von Haeseler, and Strimmer 2004) applying a GTR +  $\Gamma$  + I model of nucleotide substitution and 1,000 bootstrap replicates.

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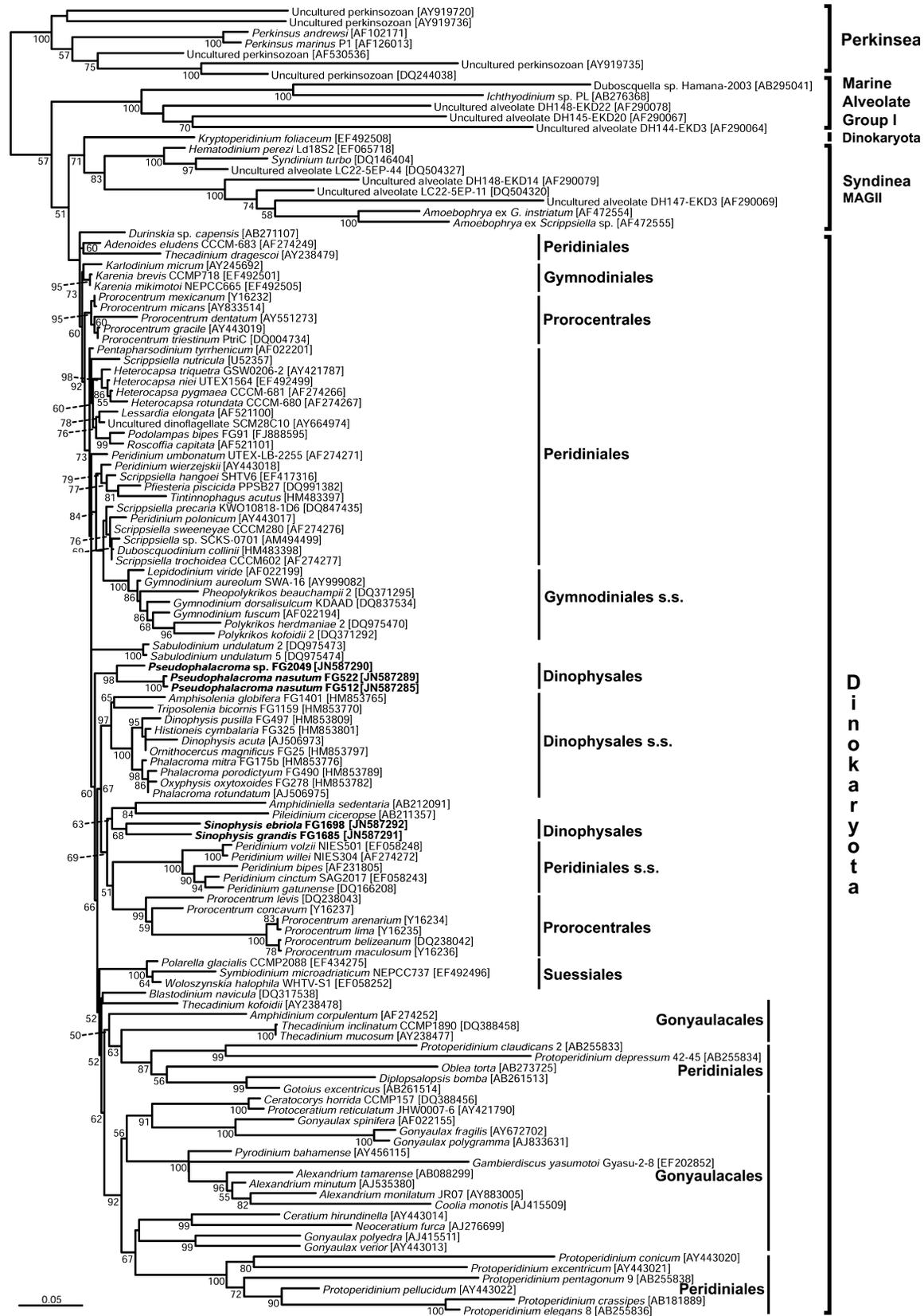


Fig. 1. Maximum likelihood phylogenetic tree of dinoflagellate SSU rDNA sequences, based on 1,214 aligned positions. Names in bold represent sequences obtained in this study. Numbers at the nodes are bootstrap proportions (values under 50% are omitted). Accession numbers are provided between brackets. The scale bar represents the number of substitutions for a unit branch length.

Alignments can be obtained by request to the corresponding author.

## RESULTS AND DISCUSSION

Phylogenetic analysis of SSU rDNAs showed a main dinophysoid clade composed by the genera *Amphisolenia* F. Stein, *Dinophysis* Ehrenb., *Histioneis*, *Ornithocercus*, *Triplosolenia* F. Stein, *Phalacroma* (including *Oxyphysis oxytoxoides* Kof.) with high bootstrap proportion (BP of 97%). As in previous analyses (Gómez et al. 2011), this clade of Dinophysales sensu stricto did not show any strongly supported close affiliation to any other dinoflagellate group, making it difficult to infer its relative position with respect to the other dinoflagellate orders (Fig. 1). However, the new *Sinophysis* spp. and *Pseudophalacroma* spp. sequences appeared in separate clades and also separated from the Dinophysales s.s. Although based on a single molecular marker, this result suggests that all dinoflagellates with dinophysoid morphology might not form a monophyletic group. Four of the five *P. nasutum* sequences were totally identical (Fig. S2). This species formed a highly supported clade (BP 98%) with *Pseudophalacroma* sp. The two *Sinophysis* species sequences (which were quite distantly related with only 94% identity) branched within a weakly supported group (BP 69%) containing the benthic genera of atypical tabulation *Amphidiniella* T. Horig. and *Pileidinium* M. Tamura et T. Horig., the type of *Peridinium* Ehrenb., as well as various benthic species of *Prorocentrum* with a symmetrical valve (i.e. *P. lima* [Ehrenb.] J.D. Dodge). Depending on the taxonomic sampling used, our two species of *Pseudophalacroma* branched sometimes with a very low support within this clade (data not shown) or as an independent lineage among the Gymnodiniales–Peridinales–Prorocentrales lineage (Fig. 1). The large evolutionary distance between the three separate dinophysoid lineages was also observed in an unrooted SSU rDNA tree with only dinophysoid species (Fig. S2). A similar analysis considering the full alignment length (1,646 positions) recovered a similar tree, where *Sinophysis* and *Pseudophalacroma* are also separated from the main dinophysoid clade (Fig. S3).

We further tested the possible monophyly of all dinophysoids using Approximately Unbiased (AU) tests to compare the topology shown in Fig. 1 with alternative trees in which we constrained (i) the monophyly of the main dinophysoid clade plus *Sinophysis*, (ii) the main dinophysoid clade plus *Pseudophalacroma* or (iii) the main dinophysoid clade plus *Sinophysis* and *Pseudophalacroma* (in each case, only the indicated node was constrained and the rest of the tree topology was optimized). The AU tests rejected the two last possibilities (with  $P = 0.045$  and  $P = 0.036$ , respectively) but not the first one ( $P = 0.23$ ). Therefore, we could not discard that *Sinophysis* spp. might be sister group of the main dinophysoid clade, but the separation of *Pseudophalacroma* spp. from this clade appeared to be significantly rejected, though it has to be confirmed by the analysis of additional molecular markers.

Our SSU rDNA phylogeny is compatible with the hypothesis that dinophysoids, at least *Sinophysis*, derives from a peridinioid-like ancestor that would have reduced the number of plates after the fusion of some of its thecal plates. The evolution of the dinophysoids appears to be more complex than previously considered. The *Sinophysis* lineage appears to be potentially very interesting to address the possible evolutionary origin of the dinophysoids and might serve to tentatively explain the relationships with prorocentroids and the hypo-

thetical peridinioid ancestor. More molecular markers of the key genera *Sinophysis* and *Pseudophalacroma* are needed to elucidate the evolutionary relations among the dinophysoid dinoflagellates.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Table S1.** List of new SSU rDNA sequences of *Pseudophalacroma* spp. and *Sinophysis* spp. used for the phylogenetic analysis.

**Fig. S1.** Micrographs of the specimens of *Pseudophalacroma* spp. and *Sinophysis* spp. used for single-cell PCR analysis (see Table S1 for details).

**Fig. S2.** Maximum likelihood phylogenetic tree of dinophysoid SSU rDNA sequences, based on 1,238 aligned positions.

**Fig. S3.** Maximum likelihood phylogenetic tree of dinophysoid SSU rDNA sequences, based on 1,646 aligned positions.

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