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RESEARCH NOTE

Cryptic diversity of small-sized species of *Phalacroma* (Dinophysales, Dinophyceae) from Denmark Strait (Eastern Arctic Greenland)

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SUMMARY

Phalacroma currently comprises 69 species of mostly marine heterotrophic dinophysoids. With a round cell body and short sulcal and cingular lists, *Phalacroma* spp. have a simple morphology compared to other dinophysoid genera. Therefore, species identification is not always a trivial matter. A few Arctic species have been described and, with the exception of *Ph. rotundatum*, they are not only infrequently recorded but also occur in low cell abundances. Here, we studied a Lugol fixed sample from Denmark Strait. From this sample 13 cells with a *Phalacroma*-like morphology were isolated under a stereo microscope, photo documented, and used for single-cell PCR determination of nuclear-encoded LSU rDNA sequences. The sequences fell in three groups defined by their ribotype. A single ribotype sequence representing each group was added to an alignment containing a diverse assemblage of dinophysoids and analyzed using Bayesian analysis and maximum likelihood. Based on comparative light microscopy one of the ribotypes (ribotype 1) was similar but not identical to *Ph. ruudi* and ribotype 2 could not be matched to a known species of *Phalacroma.* In the phylogeny both clustered with other species of *Phalacroma* including the type species. The morphology of ribotype 3 was similar but not identical to *Ph. braarudii.* In the phylogeny ribotype 3 clustered outside the core group of *Phalacroma* species. Hence, ribotype 3 was designated '*Phalacroma'* sp. 3 and has to be described as a new genus pending additional data. This study has revealed the existence of cryptic species diversity within the *Phalacroma* morphotype boundary. Two additional *Phalacroma* species (*Ph. apicatum* and *Ph.* cf. *argus*) also clustered outside *Phalacroma* sensu stricto further emphasizing the presence of cryptic species within the genus. Future studies with a polyphasic approach are needed to better address the taxonomy of *Phalacroma* and *Phalacroma*-like dinophysoids.

Key words: Cryptic species diversity, morpho-species, *Phalacroma*, phylogeny, ribotype, taxonomy.

Dinophysoids, a group of marine thecate dinoflagellates, comprise many remarkable genera with well-developed cingular and sulcal lists (e.g. *Ornithocercus* F. Stein, *Histioneis* F. Stein, *Parahistoneis* Kofoid & Skogsberg), elongated cell bodies (e.g. *Amphisolenia* Stein, *Triposolenia* Kofoid), or a dorsal concavity comprising cyanobacterial ectosymbionts (e.g. *Citharistes* F. Stein). In contrast, dinophysoids of the mostly heterotrophic genus *Phalacroma* F. Stein exhibit a simplified morphology with short cingular and sulcal lists (Kofoid & Michener 1911; Kofoid & Skogsberg 1928). Of the currently 69 species assigned to *Phalacroma* (Gómez 2012), 50 species have been described prior to 1929 and therefore illustrated by line drawings only. Thirty species (~43% of the total species diversity) have been described in just four publications: Stein (1883, four species), Schütt (1895, seven species), Kofoid and Michener (1911, eight species) and Kofoid and Skogsberg (1928, 11 species). The somewhat limited documentation and low frequency of observations for many of the species, some even considered rare at the time of description, continues to make correct identification of the small-sized species of *Phalacroma* a challenging undertaking. *Phalacroma* is characterized by a clearly visible epitheca (>1/4 of the cell length) and short and horizontal cingular lists (Steidinger & Tangen 1997; Jensen & Daugbjerg 2009). The majority of *Phalacroma* species are restricted in their distribution to tropical, subtropical or warm-temperate areas (Kofoid & Skogsberg 1928). However, a few species (*Ph. braarudii* Nordli, *Ph. rotundatum* (Claparéde & Lachmann) Kofoid & Michener and *Ph. ruudi* Braarud) are known to occur in cold temperate to Arctic waters. Except for *Ph. rotundatum*, which can be abundant (N. Daugbjerg, unpubl. data, 2013), most other cold-water species also appear in low cell numbers (Kofoid & Skogsberg 1928; Braarud 1935; Braarud *et al.* 1958; Harvey *et al.* 1997).

Here we used an integrated water sample (0–40 m) from Denmark Strait collected 4 September 2012 near the east coast of Greenland (67°32.00′N, 27°17.28′W, Appendix S1 in the Supporting Information) to elucidate the species diversity and phylogeny of small-sized, cold water cells with a *Phalacroma*-like morphology. A sequence comparison based on partial LSU rDNA sequences (952 bp) determined by single-cell PCR of 13 individuals revealed a division into three ribotype groups. Within each of these groups, the sequences were 100% identical. Henceforth these groups are referred to as ribotypes 1–3 and their morphology is discussed below and illustrated in Figs. 1–3. The material was examined 2 months after the cruise ended in September 2012. A detailed

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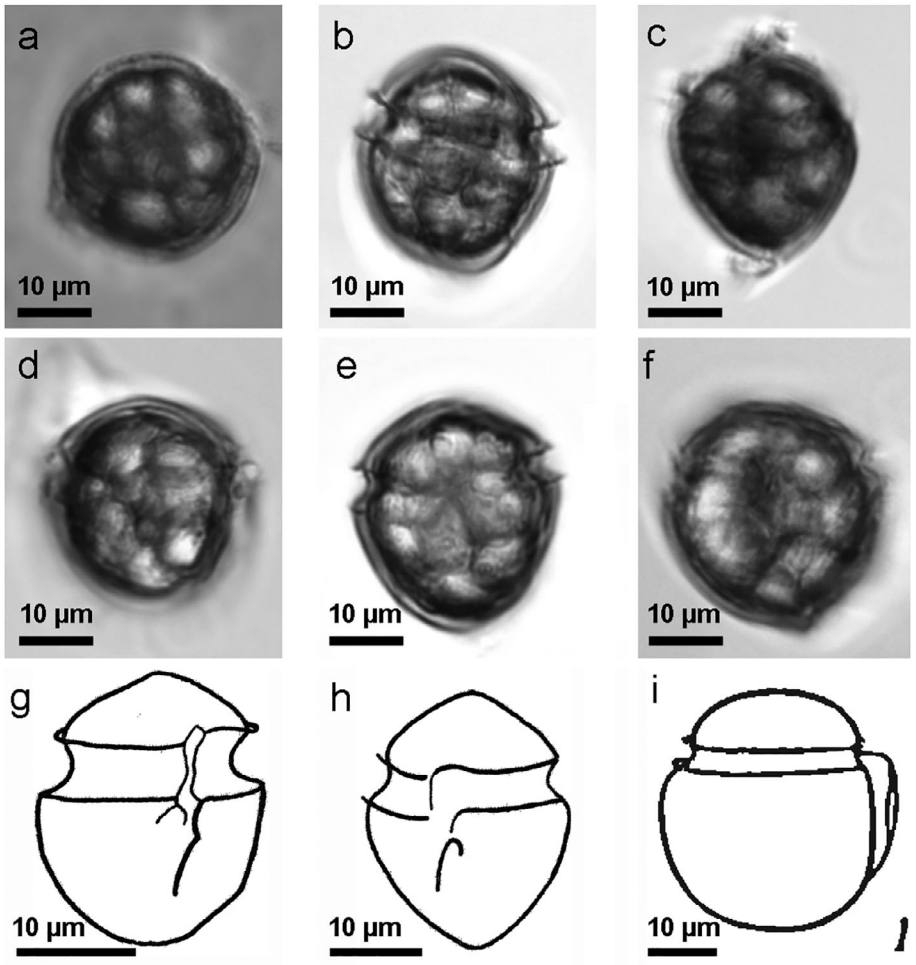

Fig. 1. Light micrographs of *Phalacroma* sp. 1 (ribotype 1). (a–f): Nomarski interference contrast of Lugol fixed cells. (a): Oblique view from above. (b, d): Lateral view. (c): Dorsal view. (e, f): Ventral view. (g, h): Drawings of *Ph. ruudi* from the original description by Braarud (1935), his figs e and g). (i): Drawing of *Ph. contractum* from the original description by Kofoid and Skogsberg (1928), their fig 3.1. Drawings are included for reasons of comparison.

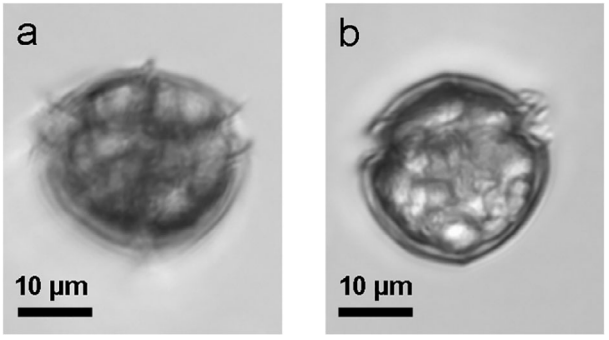

Fig. 2. Light micrographs of *Phalacroma* sp. 2 (ribotype 2). (a, b): Nomarski interference contrast of Lugol fixed cells in lateral view.

description of the material and methods is provided in Appendix S2 in the Supporting Information.

PHALACROMA SP. 1 (RIBOTYPE 1)

The morpho-species description of ribotype 1 was based on six Lugol fixed cells and morphometric measurements included length, depth (lateral view) and width (dorso-ventral view). From an oblique angle, the cell in Fig. 1a appeared round when viewed from above and broadly egg-shaped in both lateral (Fig. 1b,d) and dorso-ventral (Fig. 1c,e,f) views. The average length was 29.4 µm ± 1.4 (range 28.8–30.6 µm, $n = 5$), the depth was 25.0 µm ± 0 ($n = 2$) and the width was 27.1 µm ± 1.9 (range 25.0–28.8 µm, $n = 3$). This gave a length to depth ratio of 1.1:1. Cells of ribotype 1 possessed a prominent epitheca that measured 9.8 µm ± 0.7. This corresponded to approximately 33% of the total cell length. In lateral view, the epitheca was more or less round (Fig. 1b,d) and, in dorso-ventral view, the apex was slightly pointed (Fig. 1c,e,f). The width of the lower part of the epitheca was more narrow compared to the upper part of the hypotheca (Fig. 1b,e). Cells were widest in the cell middle (Fig. 1b–f). The girdle was left-handed and displaced approximately one girdle width (Fig. 1e). The girdle lists were narrow and parallel and the girdle, itself, markedly constricted (Fig. 1b,e,f). The left side of the hypotheca was less curved than the right, giving the antapex a pointed end (Fig. 1c,e,f). Cells had no visible chloroplasts and contained several large vacuoles, 6–7 µm in diameter. In material collected June–August 1929 from the same sampling area as studied here (Denmark Strait, but North of Iceland), Trygve Braarud described a new species of *Phalacroma* (viz. *Ph. ruudi*). The short description (10 lines on page 112; Braarud 1935) was accompanied by seven drawings of which two are included

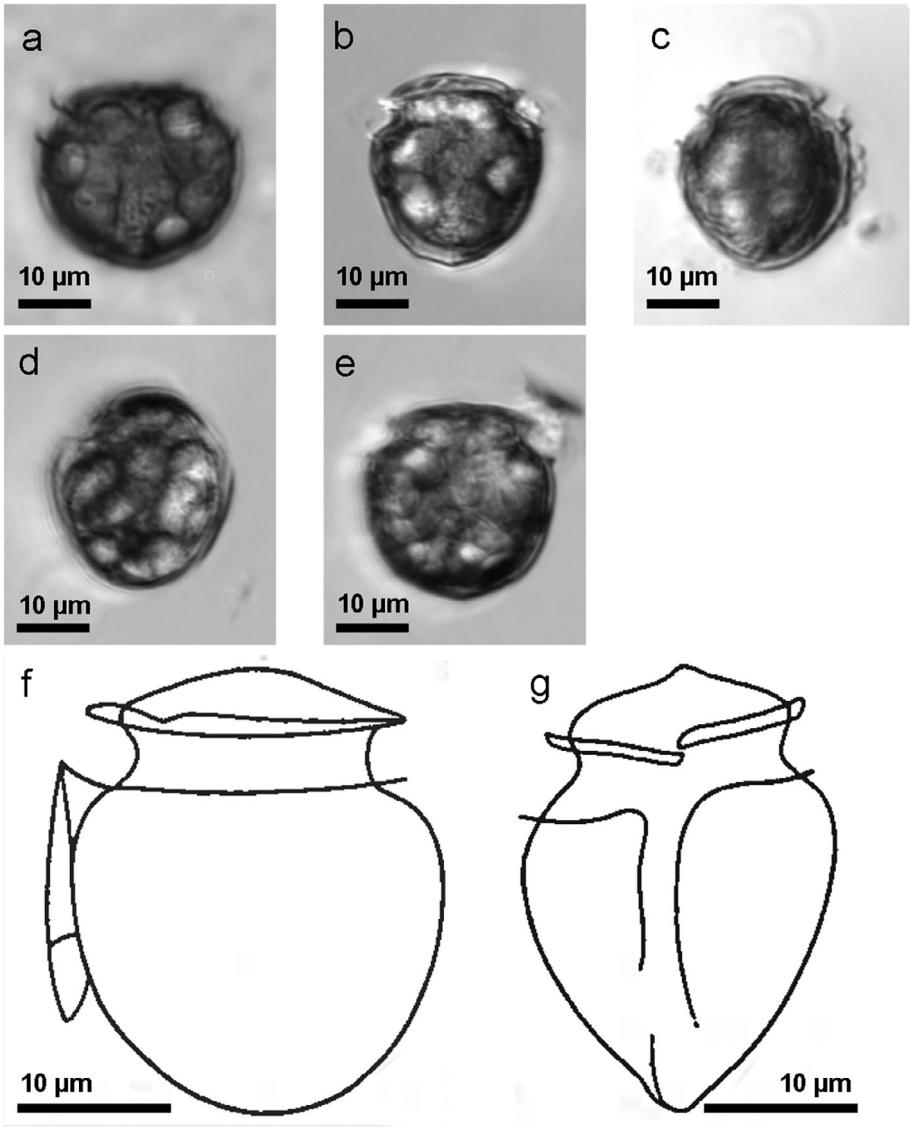

Fig. 3. Light micrographs of '*Phalacroma*' sp. 3 (ribotype 3). (a–e): Nomarski interference contrast of Lugol fixed cells. (a, b, e): Dorso-ventral view. (c, d): Lateral view. (f, g): Drawings of *Ph. braarudii* from original description by Nordli (1951), his figs b (left lateral view) and a (ventral view), respectively. Drawings are included for reasons of comparison.

here for reasons of comparison (Fig. 1g,h). *Phalacroma* sp. 1 and *Ph. ruudi* do share a number of morphological features (e.g. similar size and outline of the cell, identical length : depth ratio and a wide cingulum) but they also possess a distinct difference in the displacement of the girdle. In *Ph. ruudi* it is not displaced (Fig. 1g,h), whereas in *Phalacroma* sp. 1 it is displaced approximately one girdle width. This difference is taxonomically important, and we do not consider them conspecific, albeit they are collected from approximately the same location. *Phalacroma* sp. 1 was also of similar size to *Ph. contractum* described by Kofoid and Skogsberg (1928, fig. 3.1 and reproduced here as Fig. 1i). *Ph. contractum* was collected in the eastern tropical Pacific, where the water temperature was 24°C. However, in addition to the ecological difference (water temperature), the shape of the cell body and the narrow cingulum in *Ph. contractum* (~10% of cell length) compared to approximately 20% in *Phalacroma* sp. 1 also distinguishes these two taxa. *Phalacroma* sp. 1 clustered with 11 other species of *Phalacroma* (including the type species) and this monophyletic clade was highly supported by posterior probabilities and bootstrap values (PP = 1.0 and bootstrap support = 99%) (Fig. 4). Specifically, *Phalacroma* sp. 1 formed a sister taxon to *Phalacroma* sp. 2 also from this study and their relationship was highly supported (PP = 1.0 and BS = 100%).

PHALACROMA SP. 2 (RIBOTYPE 2)

The morpho-species description of ribotype 2 was based on two cells. Cells were 24.7 ± 0.4 µm long ($n = 2$) and 25.7 ± 0.9 µm in depth ($n = 2$). Hence, the length : depth ratio was 1:1. The cell outline was round and with the widest part in the middle. The epitheca measured approximately 33% of the total cell length. The apical and antapical end of the cell was slightly pointed (Fig. 2a,b). Cells were most likely heterotrophic and contained several vacuoles (~ 5 µm in diameter). Looking through the literature (e.g. Stein 1883; Schütt 1895; Kofoid & Michener 1911; Kofoid & Skogsberg

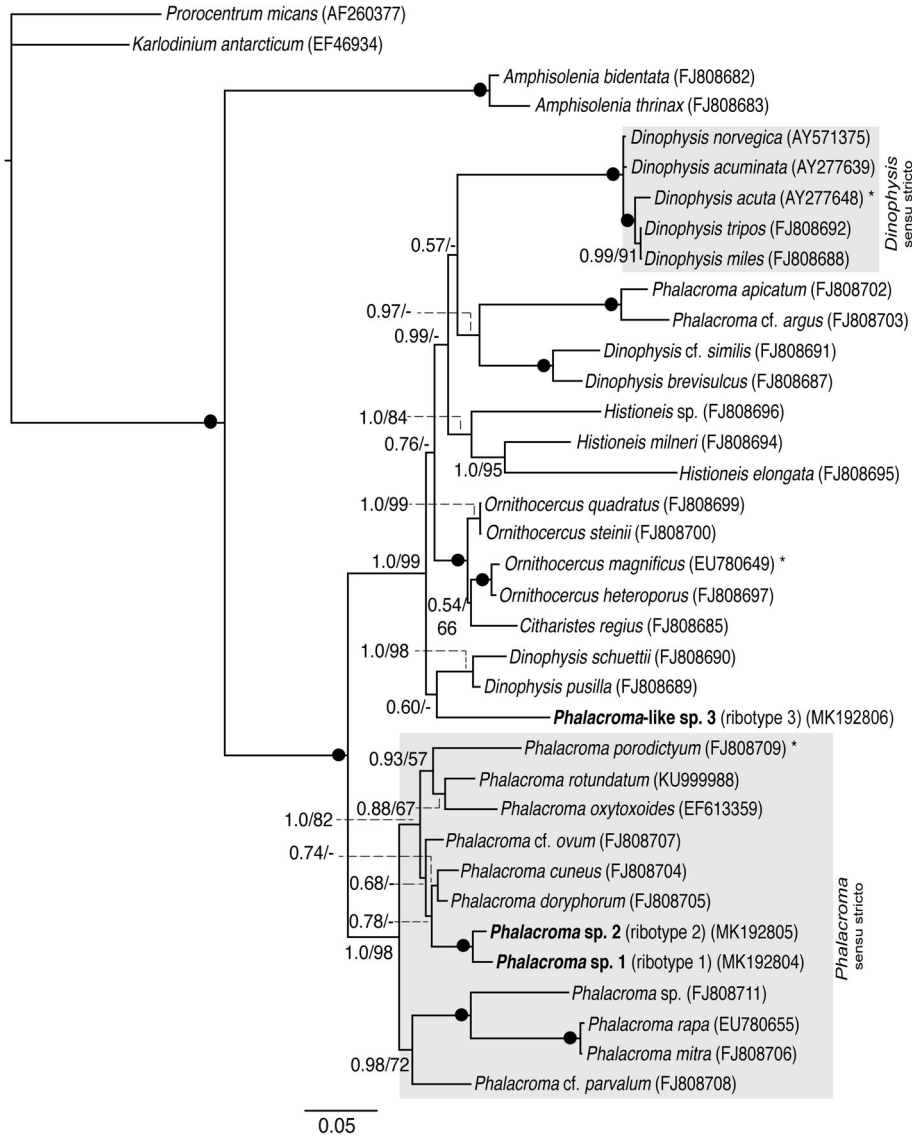

Fig. 4. Molecular phylogeny of three Arctic marine *Phalacroma* and *Phalacroma*-like morpho-species based on single-cell PCR determination of nuclear-encoded LSU rDNA sequences (985 base pairs including introduced gaps). The phylogenetic reconstruction was inferred from Bayesian Analysis (BA) and the outgroup comprised *Prorocentrum micans* and *Karlodinium antarcticum*. Numbers before slashes are posterior probabilities (≥ 0.5) from BA and numbers after the slashes are bootstrap support values from 1000 replications using maximum likelihood (> 50%). The highest possible branch support values in BA and ML (1.0 and 100%, respectively) are marked by filled circles. GenBank accession numbers are provided in brackets. The monophyletic genera *Phalacroma* s.s. and *Dinophysis* s.s. are marked in grey. Type species are indicated by an asterisk (*). The branch lengths are proportional to the number of changes per site.

1928; Braarud 1935; Nordli 1951), we have not been able to find a *Phalacroma* species with a morphology that matches ribotype 2. Therefore, this ribotype was identified as *Phalacroma* sp. 2 until additional data can be obtained. In the phylogeny (Fig. 4) *Phalacroma* sp. 2 clustered within the monophyletic genus *Phalacroma* s.s. (including the type species *Ph. porodictyum*).

'*PHALACROMA*-LIKE' SP. 3 (RIBOTYPE 3)

The morpho-species description of ribotype 3 was based on 5 Lugol fixed cells. In dorso-ventral view cells were round to broad oval and the epitheca was flat (Fig. 3a,b,e). In lateral view cells were oval and the epitheca was rounded (Fig. 3c,d). Cells measured 26.3 μm ± 0.9 in length (range 25.0–27.5 μm, $n = 5$), 23.8 μm ± 1.8 in depth (range 22.5–25.0 μm, $n = 2$) and 25.9 μm ± 1.9 in width (range 23.8–27.5 μm, $n = 3$). The length:depth ratio was 1.1:1 and the epitheca was approximately 17% of the total cell length. Cells were widest in the cell middle and the nucleus was positioned in the lower part of the hypotheca (Fig. 3a,b). This species was presumably heterotrophic and contained several large vacuoles, 5–8 μm in diameter (Fig. 3a–e). Most likely these represented food vacuoles. The morphology of the sulcal lists on the ventral side of the cell could barely be discerned (to the right in Fig. 3c and the left in Fig. 3d). Nordli (1951) described *Ph. braarudii* (25–32 μm long, 21–29 μm deep and 18–20 μm wide) from the West Fjord in the Lofoten area (Norway). This species to some extent overlapped in cell size with '*Phalacroma*-like' sp. 3. Two of the Nordli's original drawings are reproduced here as Fig. 3f,g. However, the general outline of the cells differed, particularly with respect to the shape of the epitheca. In '*Phalacroma*-like' sp. 3 it is flat in dorso-ventral view, whereas it is pointed in *Ph. braarudii.* The sulcal lists also differ in morphology as they were hardly visible in '*Ph.*-like' sp. 3, and clearly observed in *Ph. braarudii* by

Nordli (1951). In the phylogenetic inference (Fig. 4) '*Phalacroma*-like' sp. 3 did not cluster with either *Phalacroma* s.s. or the lineage encompassing *Ph. apicatum*/*Ph.* cf. *argus.* Rather, it took an unresolved position with lineages comprising *Citharistes regius*, *Dinophysis* spp., *Histioneis* spp., *Ornithocercus* spp., *Phalacroma apicatum* and *Ph.* cf. *argus.* Despite this unresolved relationship ribotype 3 cannot be regarded as a species of *Phalacroma.* Thus, ribotype 3 has to be described as a new genus following additional morphological observations.

To further explore the LSU rDNA sequences of *Phalacroma* and *Phalacroma*-like species included in Figure 4, the sequence divergence for all pairwise comparisons using the Kimura-2-parameter model was estimated. The results are shown in Appendix S3 in the Supporting Information. The maximum sequence divergence between all core *Phalacroma* species (i.e. those that clustered with the type species in a monophyletic clade) was 12% (*Ph. porodictyum* compared to *Ph. mitra* and *Ph. porodictyum* compared to *Ph. rapa*). The minimum sequence divergence was 1.9% when *Phalacroma* sp. 1 and *Phalacroma* sp. 2 were compared. The divergence between *Ph. apicatum* and *Ph.* cf. *argus*, which were not related to the core group of *Phalacroma* species, was 4.1% and, when comparing the min. and max. sequence divergences of these to the core species of *Phalacroma*, values of 15.2–15.6% (*Ph. doryphorum* compared to *Ph. apicatum*/*Ph.* cf. *argus*) and 19.5–20.1% (*Ph. rapa*/*Ph. mitra* compared to *Ph. apicatum*/*Ph.* cf. *argus*), respectively, were estimated. The sequence divergence between all core *Phalacroma* species and '*Phalacroma*-like' sp. 3 ranged from 12.3 to 18.0%. Thus, the estimated sequence divergences supported the LSU rDNA-based phylogeny indicating a unique position of *'Phalacroma*-like' sp. 3. The sequence divergence between *Phalacroma* sp. 1 and *Phalacroma* sp. 2 was somewhat higher compared to all pairwise comparisons of core (monophyletic) species of *Dinophysis* (i.e. the clade comprising the type species). Here, the values ranged from 0.3–1.6% when based on the same LSU rDNA fragment. Comparing the sister taxa *Dinophysis brevisulcus* and *D.* cf. *similis*, the sequence divergence was 4.2% and with *D. schuettii* to *D. pusilla* (also sister taxa) the divergence was 2.3%. Comparing the three separate clades of *Dinophysis* spp., the divergence estimates ranged from 9.5–14.0% (see Appendix S4 in the Supporting Information). Based on all of these estimates and the phylogenetic inference, we argue that *Phalacroma* sp. 1 and *Phalacroma* sp. 2 are two distinct species.

Isolation of individual cells from Denmark Strait with a *Phalacroma*-like morphology proved, by LSU rDNA sequencing (ribotyping), to present two presently unidentified species of *Phalacroma* and new a dinophysoid lineage which we named '*Phalacroma*-like' sp. 3. We are reluctant to describe these taxa until additional morphological observations become available. As already stated by Kofoid and Skogsberg (1928) and confirmed here, *Phalacroma* and *Phalacroma*-like cold water species are small in size, round and with a simplified morphology. From the phylogenetic inference, it seemed that selection had favored the independent evolution of cells with a small size and a simple dinophysoid outline of cells. It is still unclear what determines this morphological selection, but cold water is less fluid and thus thicker. Sinking rates are, therefore, reduced in cold water and the great sulcal extensions and wings that we see in many tropical dinophysoids may not be selected for.

The results from this and other studies (e.g. Jensen & Daugbjerg 2009) have exposed the taxonomic problems with delineation between species of *Phalacroma* and those with a *Phalacroma*-like morphology. Here, at least three lineages comprising species with a *Phalacroma*-like morphology have been observed. Future studies will have to examine how many of the approximately 50 species of *Phalacroma* known from the literature are true *Phalacroma* species, or if they should be transferred to other or new genera of dinophysoids. This is not an easy undertaking considering their rare occurrence and difficulty in keeping them in the laboratory for detailed morphological characterization.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Map showing collection site of station 1.12 (67°32.00′N, 27° 17.28′W) where a net-plankton sample (20 μm plankton net) was collected in Denmark Strait, 4 September 2012. The station depth was 262 m and the integrated sample depth was 0–40 m

Appendix S2. Detailed description of material and methods

Appendix S3. Sequence divergence in percent between *Phalacroma* and *Phalacroma*-like species based on 952 base pairs of nuclear-encoded LSU rDNA. Distances calculated using the Kimura-two-parameter model. All pairwise distances were calculated using PAUP* ver. 4.01 (build 161). Species are color-coded according to their phylogeny as shown in Fig. 4

Appendix S4. Sequence divergence in percent between *Dinophysis* and *Dinophysis*-like species based on 952 base pairs of nuclear-encoded LSU rDNA. Distances calculated using the Kimura-two-parameter model. All pairwise distances were calculated using PAUP* ver. 4.01 (build 161). Species are color-coded according to their phylogeny as shown in Fig. 4