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Polykrikos tanit sp. nov., a New Mixotrophic Unarmoured Pseudocolonial Dinoflagellate from the NW Mediterranean Sea

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Pigmented pseudocolonies initially identified as *Polykrikos hartmannii* Zimmermann were detected at several locations of the Catalan coast (NW Mediterranean Sea) in April–June of 2012 and April–May of 2013. To further explore the several remarkable morphological discrepancies between these organisms and *P. hartmannii*, we carried out a detailed morphological study and used single-cell PCR to obtain partial LSU and SSU rDNA sequences. The resulting phylogenies showed that our isolates occupy a basal position within the *Polykrikos* clade, close to *P. hartmannii*, but do not correspond to any described polykrikoid species. *P. barnegatensis* Martin is controversially considered to be synonymous with *P. hartmannii*. The organisms studied in this work were similar to *P. barnegatensis* but showed significant morphological differences with its original description such as the torsion of the pseudocolony, more pronounced overhanging of the cingula, stepped fusion border of the zooids, and number and shape of nuclei. Consequently, we propose that the isolates constitute a new species, which we named *Polykrikos tanit* sp. nov. The observed characters, pigmented, same number of zooids and nuclei, sulci not fused, and its phylogeny suggest that the species is an early evolutionary *Polykrikos* species.

Key words: Acrobase; mixotrophy; *Pheopolykrikos*; phylogeny; SEM; single-cell PCR.

Introduction

The polykrikoid organisms are included within the Gymnodiniales sensu stricto clade (Hoppenrath and Leander 2007a,b; Kim et al. 2008) and are grouped within two genera: *Polykrikos*, erected by Bütschli (1873), and *Pheopolykrikos*, erected by Chatton (1933) and later emended by Matsuoka and Fukuyo (1986). Both genera comprise unarmoured multinucleated pseudocolonial organisms and autotrophic as well as heterotrophic species are known.

Polykrikos species were primarily characterized on the basis of their even number of zooids, with half the number of nuclei. Every zooid has its own cingulum and a pair of flagella. The sulci of both zooids are fused. In addition to the type species of the genus, *P. schwartzii* Bütschli, members include *P. kofoidii* Chatton, *P. lebourae* Herdman *emend.* Hoppenrath et Leander, *P. herdmanae* Hoppenrath et Leander, and *P. hartmannii* Zimmermann. The validity of *P. grassei* Lecal is highly dubious and *P. auricularia* Bergh is considered to be synonymous

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with *P. schwartzii*. *P. barnegatensis* was erected by Martin (1929) following observations of a single specimen from Barnegat Bay (USA). This two-zooid pseudocolonial photosynthetic organism contains only a single, large nucleus and lacks nematocysts. It was synonymized with *P. hartmannii* by Chatton (1952), although he described two nuclei. However, this nomenclature was not adopted by Hulburt (1957) because of the difference in the number of nuclei. Nevertheless, the synonymy of *P. barnegatensis* with *P. hartmannii* is accepted (Gómez 2012; Guiry and Guiry 2013), albeit with uncertainty (Hoppenrath and Leander 2007a).

The genus *Pheopolykrikos* comprises *Ph. beauchampii* Chatton as the type species, characterized by having four zooids and four nuclei. Although the validity of the genus has been discussed by several authors (Dodge 1982; Loeblich III 1980; Sournia 1986), *Pheopolykrikos* species are defined as having the same number of nuclei as zooids and forming phototrophic pseudocolonies that are able to dissociate (Matsuoka and Fukuyo 1986). *P. hartmannii* was transferred into *Pheopolykrikos* because it agreed with all previously cited characteristics (Matsuoka and Fukuyo 1986). However, phylogenetic analyses of LSU rDNA (Hoppenrath and Leander 2007a; Kim et al. 2008) and SSU rDNA (Hoppenrath and Leander 2007a,b) sequences subsequently showed that *P. hartmannii* clusters with *Polykrikos* species independently of *Ph. beauchampii*, thereby confirming the validity of the genus *Pheopolykrikos*. Moreover, those results together with ultrastructural studies led to the re-classification of *Pheopolykrikos hartmannii* as *Polykrikos hartmannii* (Hoppenrath et al. 2010), although this nomenclature has not been adopted by all authors (Tang et al. 2013)

Recently, in samplings carried out at several locations along the Catalan coast (NW Mediterranean Sea), we detected pigmented pseudocolonies comprising two zooids. Detailed morphological observations and partial LSU and SSU rDNA sequencing were used to determine whether these organisms were *P. hartmannii* or *P. barnegatensis* or constituted a new species. In this study, we show that our isolates indeed belong to a new species, which we have named *Polykrikos tanit* sp. nov.

Results

Polykrikos tanit sp. nov.

Morphology

Unarmoured pseudocolonies consisted of two zooids. They were 46–76 μm long and 26–50 μm wide with a length:width ratio of 1.4–2 (n=20). Pseudocolonies were ovate and nearly circular in cross-section. The sides of the pseudocolonies were convex, with a constriction at the junction of the two zooids (Figs 1A, B; 2A). The border of this junction was stepped, with the left side being higher than the right one (Figs 1A, B; 2A, B). The epicone of the anterior zooid was round (Fig. 1A) to conical (Figs 1F; 2A, B). The apex was blunted and protuberant (Fig. 2A, B). The hypocone of the posterior zooid was round, slightly bilobated, and the antapex was flattened. Pseudocolonies showed torsion of the cell body to the left (Fig. 2A). The cingula were displaced about two to three times their width, with overhanging ends (Figs 1A, B; 2A, B). A large peduncle was always present in each zooid, emerging from the upper intercingular area (the area where both ends of the cingulum meet) (Fig. 2A, B, C). Peduncles were present in all specimens observed by SEM (n=30), but we never observed them under LM. The sulci ran obliquely from right to left (Fig. 2B) and had a sigmoid outline resulting from the overhanging cingula (Figs 1A, B; 2A). The sulcus of the posterior zooid reached the antapex, where it widened. The sulcal anterior end of the anterior zooid penetrated the epicone, in contact with the acrobase, which formed a closed anti-clockwise loop around the apex that re-joined the sulcus, although at a lower position than its proximal end (Fig. 2A, B, D). The sulci of both zooids were not fused and ran independently. Pseudocolonies had two nuclei, one for each zooid, located in the hypocone of the anterior zooid and the epicone of the posterior zooid (Fig. 1C, E). The nuclei were ovate to horizontally lenticular and nearly touched. Some pseudocolonies contained only a single nucleus, located centrally (Fig. 1D). Pseudocolonies had a yellow-greenish colouration, apparently with numerous small ovate chloroplasts (Fig. 1F, G). Neither nematocysts nor taeniocysts were observed. Large ingestion bodies were commonly seen in the posterior zooids (Fig. 1H), in some cases displacing the nuclei. Cyst formation was never observed.

The pseudocolonies differed in width among specimens. Some were wider, with a lower length:width ratio ($\leq$ 1.5), while others were narrower, with a higher length:width ratio (> 1.6). In the wider pseudocolonies (Figs 1H; 2A) the cingula were more deeply overhanging and torsion was greater than in the narrower pseudocolonies (Figs 1A; 2B). Both morphologies were observed in natural samples, but healthy specimens and those possessing visible ingestion bodies tended to be wider, whereas isolated specimens maintained in

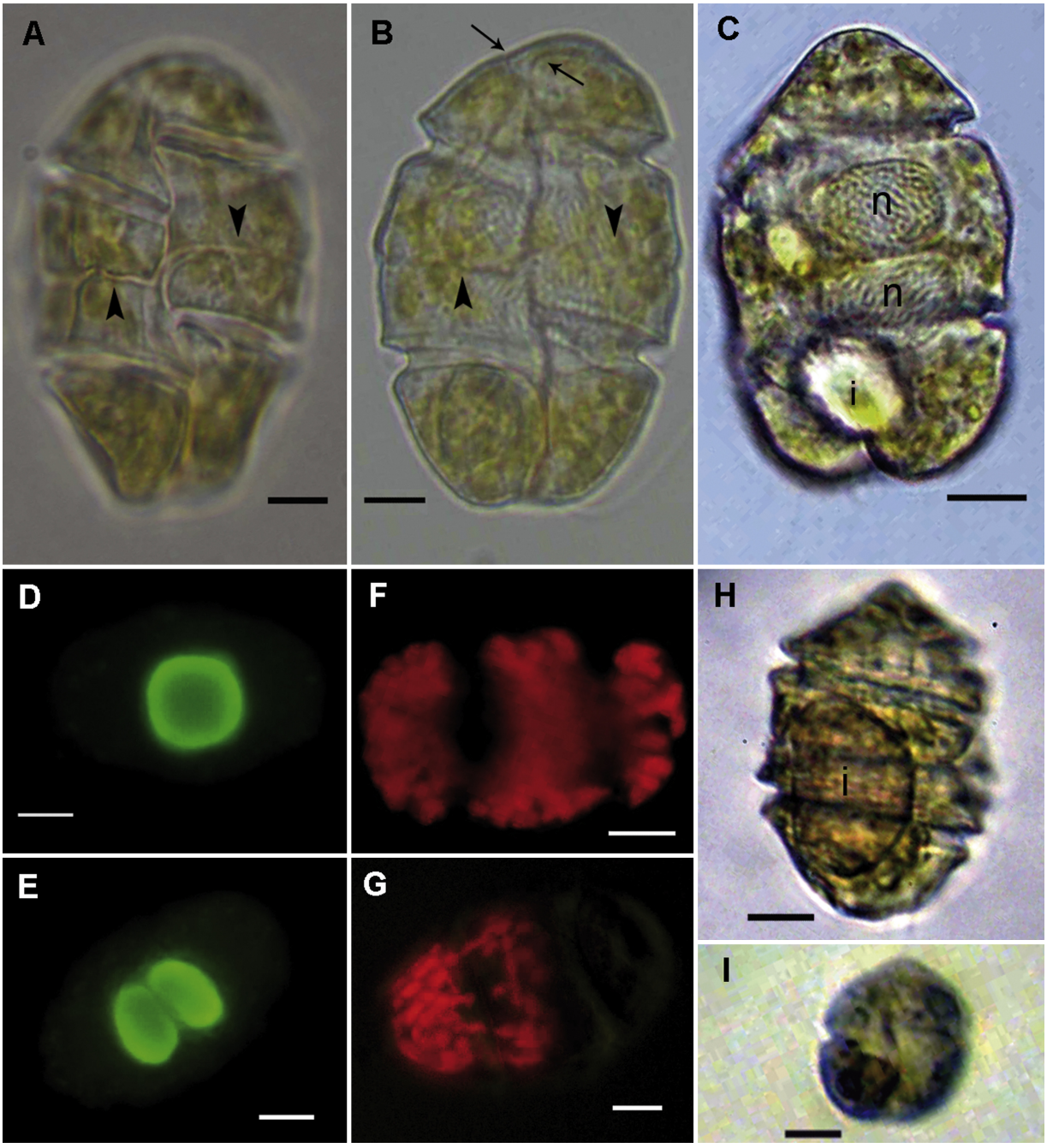

Figure 1. Light microscopy images of *P. tanit* sp. nov. **A**) and **B**) Ventral view of pseudocolonies. Note the sigmoid outline of the sulci and the stepped junction of the two zooids (arrowheads). Arrows indicate the acrobase. **C**) Left lateral view of a pseudocolony showing two nuclei (n) and an ingestion body (i). Epifluorescence images of *P. tanit* sp. nov. **D**) Pseudocolony with one nucleus stained with Sybr Green. **E**) Pseudocolony with two nuclei stained with Sybr Green. **F**) and **G**) Images showing the shape and distribution of the autofluorescent plastids. Light microscopy images of *P. tanit* sp. nov. **H**) Right lateral view of a pseudocolony showing a large ingestion body (i). **I**) Ventral view of a single zooid. Scale bars = 10 μm.

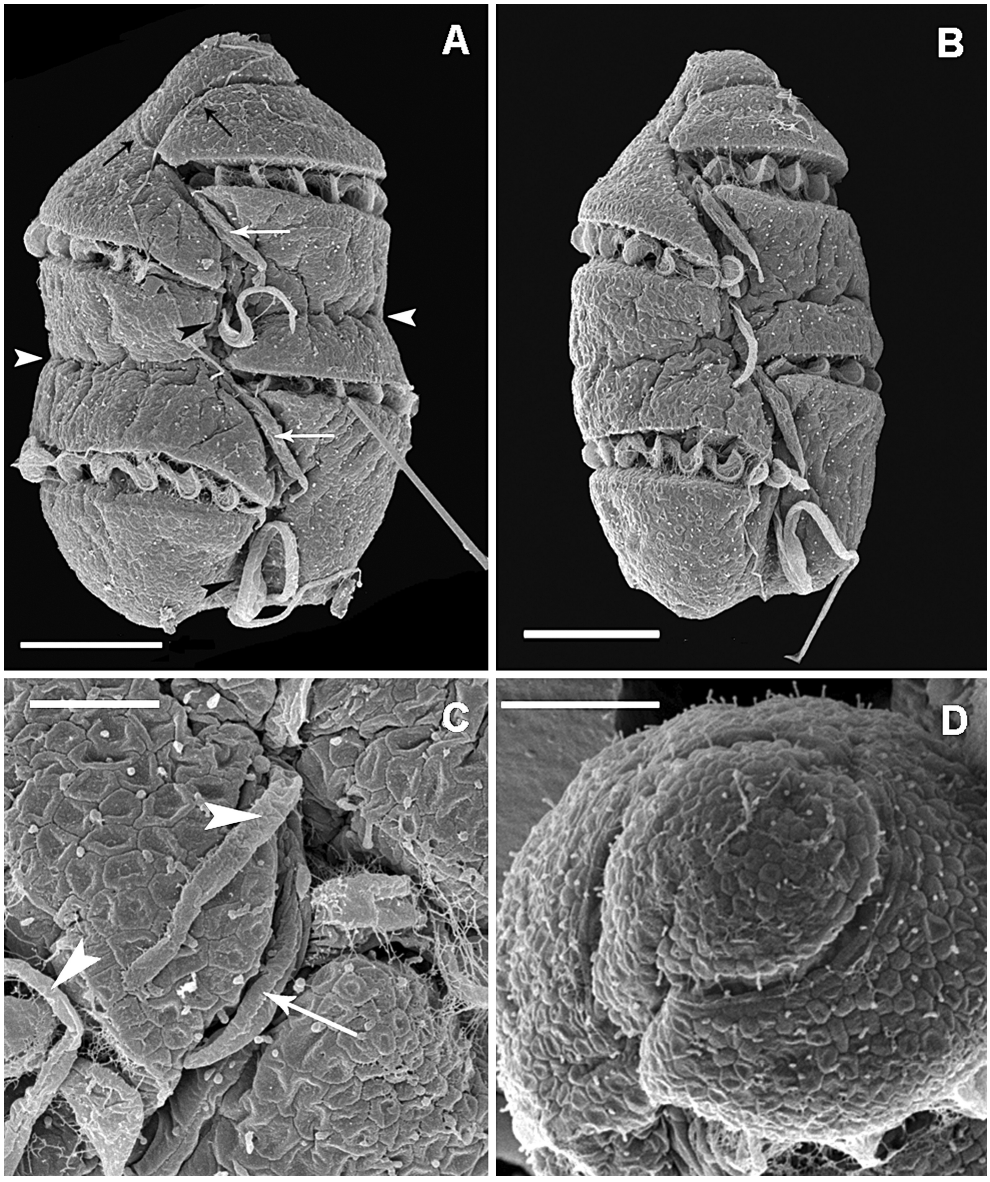

Figure 2. Scanning electron microscopy images of *P. tanit* sp. nov. **A**) and **B**) Ventral view of pseudocolonies, showing the acrobase (black arrows), the stepped junction of the two zooids (white arrowheads), and the presence of peduncles in both zooids (white arrows). Black arrowheads point to the longitudinal flagella. **C**) Detail of the intercingular area of the posterior zooid of a pseudocolony. The peduncle (arrow) is inserted in the proximal end of the cingulum. Arrowheads point to both longitudinal flagella. **D**) Apical view of the apex, showing the detail of the acrobase. Scale bars = A) and B) 10 μm; C) 5 μm; D) 2.5 μm.

well plates for a few weeks and exhibiting a loss of pigmentation tended to be narrower. Single zooids were only observed once, when an isolated pseudocolony dissociated. These were 29–32 μm long and 26–27 μm wide (Fig. 1I), almost round but with a blunted apex. The cingulum was median, displaced three times its width, with overhanging ends. The sulcus was sigmoid, widening in the antapex.

Phylogeny

The sequences obtained for the partial LSU rDNA region were ~690 bp (KF806602 was ~585 bp) and ~1340 bp for the partial SSU rDNA region (Table 1). All the sequences of each region were identical, except KF806602, which differed in 1 position with respect to the other LSU rDNA sequences obtained. The ML phylogenetic trees were constructed with representatives of the Gymnodiniales

Table 1. GenBank accession numbers, locations, and dates of isolation of *P. tanit* and the region targeted for SC-PCR for each DNA sequence obtained in this study.

Accession number	Species	Location	Isolation Date	Target region
KF806598	*Polykrikos tanit*	Arenys Harbour	May-2012	SSU rDNA
KF806599	*Polykrikos tanit*	Arenys Harbour	April- 2013	SSU rDNA
KF806600	*Polykrikos tanit*	Vilanova Harbour	May-2012	LSU rDNA
KF806601	*Polykrikos tanit*	Arenys Harbour	May-2012	LSU rDNA
KF806602	*Polykrikos tanit*	Offshore Barcelona	June-2012	LSU rDNA

sensu stricto clade and other unarmoured species not included in it. *Polarella glacialis* was used as outgroup for both SSU rDNA and LSU rDNA phylogenetic trees.

The tree obtained for SSU rDNA sequences showed that all polykrikoid species were included within the Gymnodiniales sensu stricto clade (100% bootstrap / 1 BPP) (Fig. 3). All *Polykrikos* species clustered together (94%/1) and only *Ph. beauchampii* clustered independently. *P. tanit* occupied a basal position in the clade, as did *P. hartmannii*, although its position was not fully resolved. The remaining species were included in a subclade (87%/0.99) containing on the one hand *P. kofoidii* and *P. schwartzii* (100%/1) and on the other *P. lebourae* and *P. herdmanae* (100%/1).

The phylogenetic position of *P. tanit* based on its LSU rDNA sequences (Fig. 4) agreed with that obtained for the SSU region. All polykrikoid species were included within the Gymnodiniales sensu stricto clade (95%/1). In this case though, the *Polykrikos* clade was not obtained. *Pheopolykrikos beauchampii* (sequence named in GenBank as *Polykrikos beauchampi*) clustered independently, as well as *Polykrikos lebourae*. The clade containing the remaining species was not consistently supported. Species were grouped in two subclades, one containing *P. kofoidii* and *P. schwartzii*

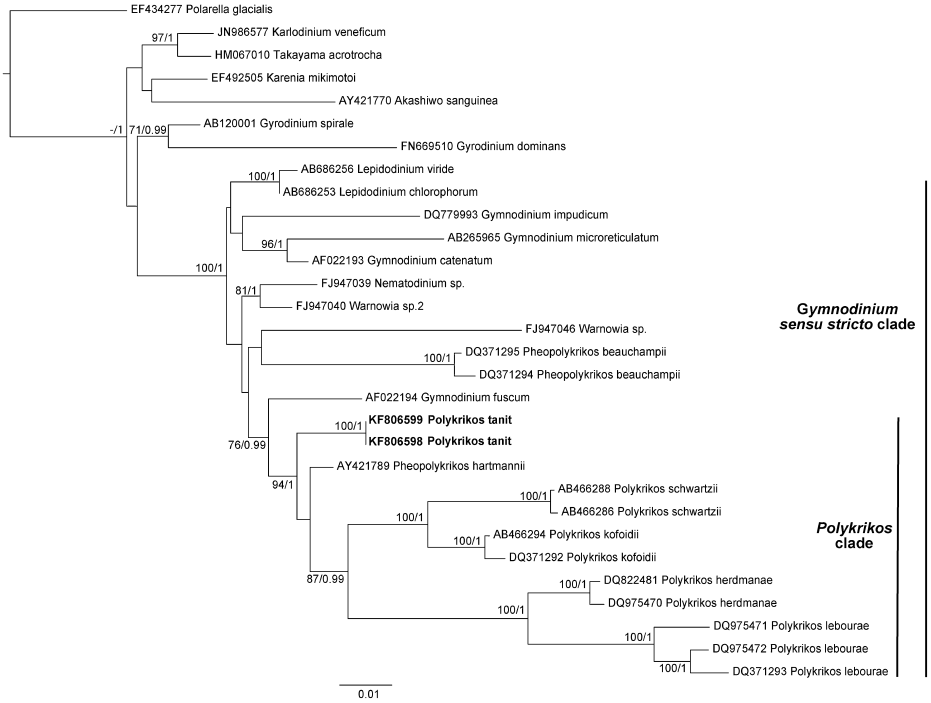

Figure 3. Maximum-likelihood phylogenetic tree of selected species based on the partial SSU rRNA. Numbers on the nodes are the bootstrap values (%) followed by the Bayesian Posterior Probabilities (BPP). Only bootstrap values >70 and BPP >0.9 are shown. *Polarella glacialis* sequence was used as outgroup. Organisms sequenced in this study are shown in bold.

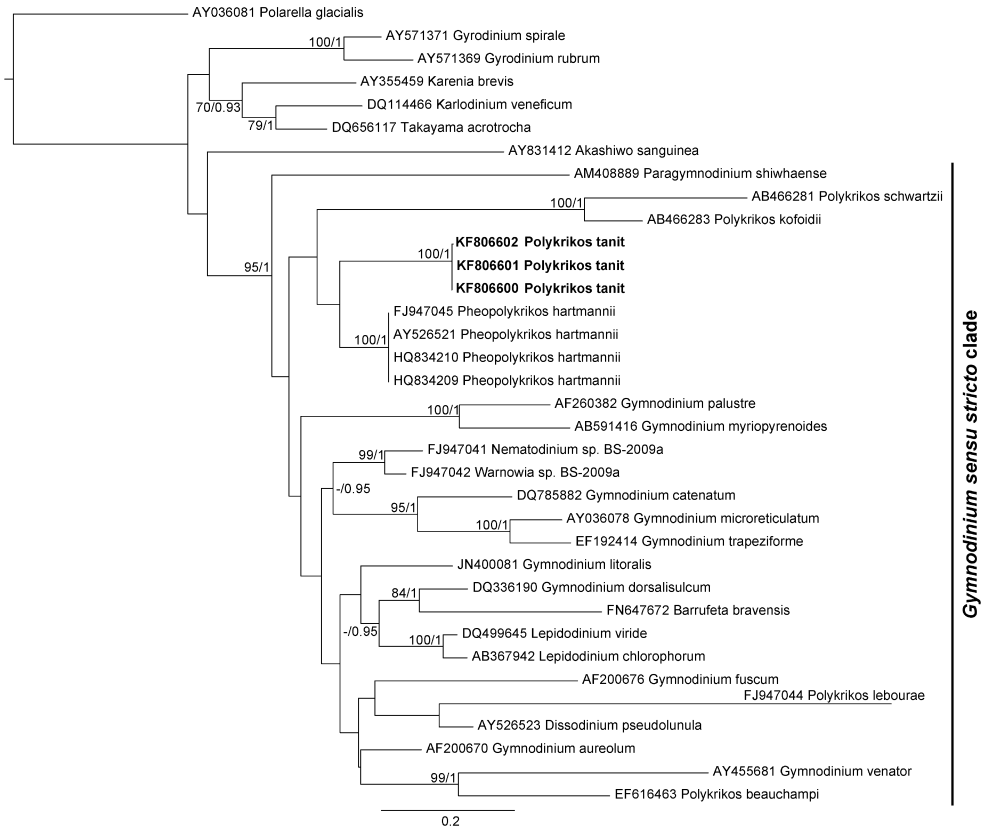

Figure 4. Maximum-likelihood phylogenetic tree of selected species based on the D1–D2 domain of LSU rRNA. Numbers on the nodes are the bootstrap values (%) followed by the Bayesian Posterior Probabilities (BPP). Only bootstrap values >70 and BPP >0.9 are shown. *Polarella glacialis* sequence was used as outgroup. Organisms sequenced in this study are shown in bold.

(100%/1) and the other containing *P. hartmannii* (sequence named in GenBank as *Pheopolykrikos hartmannii*) and the sequences obtained during this study.

Occurrence

Specimens primarily identified as *P. hartmannii* were detected occasionally at abundances <100 cells L^{-1} during the spring and summer months in samplings carried out along the Catalan coast as part of the Monitoring of Harmful Phytoplankton Programme. However, the fact that they deformed when fixed prevented the confirmation of their identity. In this study, further observations of live specimens allowed the unequivocal detection of *P. tanit* during 2012 and 2013. In 2012, the species was detected at Arenys and Vilanova harbours as well as at L'Estartit beach and 1.5 km offshore of Barcelona during May–June. In 2013, we only sampled Arenys Harbour but detected *P. tanit* from the beginning of April well into May. Abundances at all locations never reached 10^3 cells·L^{-1}. The water temperature from all localities ranged from 14 to 22 °C, with a salinity of 31.2–37.8.

Discussion

Morphological Comparison

A detailed comparison of similar pigmented polykrikoid species is provided in Table 2. Studies of a large number of pseudocolonies of *P. tanit* distinguished two morphologies, probably related to "fed" vs. "starved" states, but only pseudocolonies consisting of two zooids were observed, albeit they were able to dissociate. Consequently, our newly isolated specimens resembled *P. hartmannii* or *P. barnegatensis* in the number of zooids.

P. tanit differs from *P. hartmannii* in several characters. The pseudocolonies of these species are different in shape. The left torsion of *P. tanit* pseudocolonies creates an oblique outline of its sulci (Fig. 5A). This curvature is a common feature of most *Polykrikos* species but according to

Table 2. Morphological traits of *Polykrikos tanit* sp. nov. and related species, as provided by available studies.

	P. tanit sp. nov.[a]	*P. barnegatensis*[b]	*P. barnegatensis*[c]	*P. hartmannii*[d,e,f]	*Ph. beauchampii*[g,h]	*P. lebourae*[i]
Pseudocolony length (μm)	46-76	46	-	60-100	100-120	37.5-90
Pseudocolony width (μm)	26-50	31.5	-	42-59	60-75	20-50
Pseudocolony shape	ovate	ovate	ovate	barrel-shaped	barrel-shaped	ovate
Pseudocolony compression	no	no	-	dorsoventral, sometimes longitudinal[j]	dorsoventral	obliquely flattened
Number of zooids per pseudocolony	2	2	2	2	4	8
Number of nuclei per pseudocolony	usually 2	1	2	2	4	2
Dissociation into single zooids	yes	-	-	yes	yes	no
Displacement of cingula	2-3 times its width	2 times its width	2-3 times its width	1-2 times its width	1-2 times its width	1-2 times its width
Overhanging cingula	yes	no	yes	no, sometimes yes[f,k]	no	no
Apical groove	loop-shaped	-	loop-shaped	loop-shaped	loop-shaped[l]	loop-shaped
Fusion border of zooids	stepped	straight	stepped	straight	slightly stepped	fused
Fusion of sulci	no	yes (?)	no	no	no	yes
Plastids	yes	yes	yes	yes	yes	yes
Mixotrophic	yes	no	yes	probably yes[k]	no	yes
Peduncle	yes	-	-	no[j,l]	no	no
Taeniocyst-nematocyst complexes	-	-	-	yes	yes, but not confirmed	sometimes
Cysts	-	-	-	yes	-	hyaline vegetative

[a]This study.
[b]Martin (1929).
[c]Chatton (1952).
[d]Hulburt (1957).
[e]Matsuoka and Fukuyo (1986).
[f]Hoppenrath et al. (2010).
[g]Chatton (1933).
[h]Hoppenrath and Leander (2007a).
[i]Hoppenrath and Leander (2007b).
[j]Tang et al. (2013).
[k]Omura et al. (2012).
[l]Takayama (Personal website).
– not observed or not detailed.

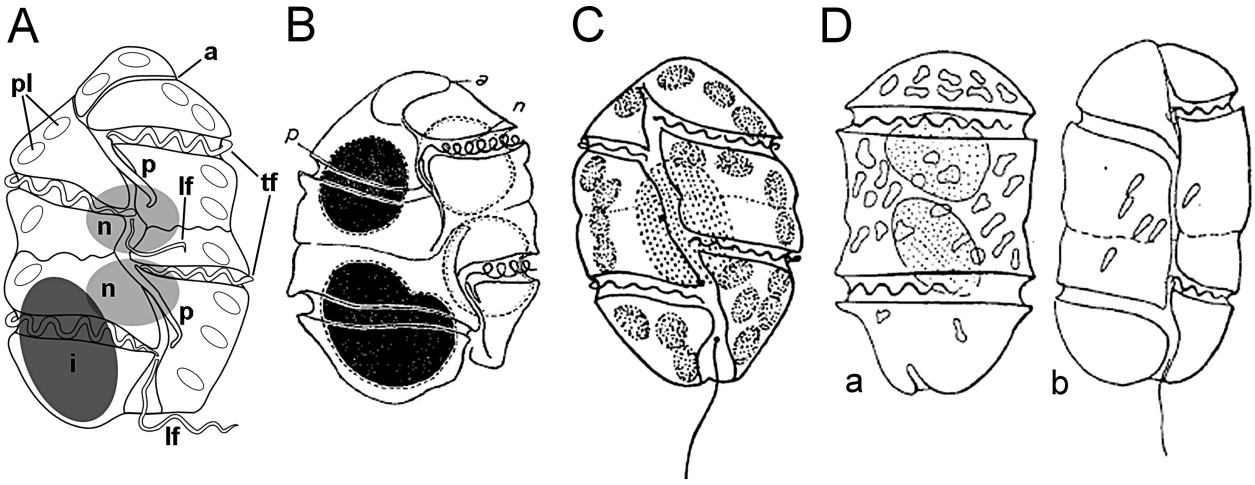

Figure 5. Schematic drawings of **A**) *Polykrikos tanit* sp. nov. Note the acrobase (a), nucleus (n), chloroplasts (pl), peduncle (p), ingestion body (i), longitudinal flagellum (lf) and transverse flagellum (tf). **B**) *P. barnegatensis* [from Chatton (1952)]. Note the acrobase (a), nucleus (n) and ingestion body (p). **C**) *P. barnegatensis* [from Martin (1929)]. **D**) Dorsal (a) and ventral (b) views of *P. hartmannii* [from Zimmermann (1930)]. Drawings are not to scale.

morphological descriptions of *P. hartmannii* (Hoppenrath et al. 2010; Hulburt 1957; Matsuoka and Fukuyo 1986; Zimmermann 1930) the outline of the sulci is commonly straight and there is no torsion (Fig. 5D), although some images from the literature show an outline slightly sigmoid (fig. 1C in Kim et al. (2008), fig. 2A in Hoppenrath et al. (2010), fig. 2B in Tang et al. (2013)). *Ph. beauchampii* (Chatton 1933), which is characterized by four zooids and four nuclei, also show a straight outline of the sulci, with no torsion. Furthermore, for *P. tanit*, overhanging of the ends of the cingula by a variable degree was common, especially in pseudocolonies exhibiting greater torsion, and resulted in the sigmoid outline of the sulci. None of the *P. hartmannii* depictions show overhanging cingula. The presence of food vacuoles has been observed in *P. hartmannii* (Omura et al. 2012), as well as the presence of nematocyst-taeniocyst complexes (Hoppenrath et al. 2010). However, the presence of peduncles has never been observed, while its presence in the intercingular area of zooids was confirmed in scanning electron microscopy (SEM) observations of all *P. tanit* pseudocolonies. Ingestion bodies were also frequently seen in these specimens. Furthermore, our inability to obtain cultures from newly isolated pigmented specimens suggested that it might be an obligate mixotroph organism, as also assumed for the pigmented *P. lebourae* (Hoppenrath and Leander 2007b). Peduncles are feeding appendages (Gaines and Elbrächter 1987), and they have been described in pigmented species such as *Amphidinium cryophilum* (Wedemayer et al. 1982), *Akashiwo sanguinea* or *Gyrodinium instriatum* (Gaines and Elbrächter 1987), in heterotrophic species such as *Gyrodinium lebourae* (Lee 1977) or *Gyrodiniellum shiwhaense* (Kang et al. 2011), and in parasitic species such as *Amyloodinium* spp. (Landsberg et al. 1994). The constant presence of the peduncles supports the mixotrophy of this newly established species. A finger-like structure was observed in the flagellar area of each *P. kofoidii* zooid, but only in gametes; it was therefore assumed to be a “copulation globule”, involved in supporting the contact and fusion of gametes (Tillmann and Hoppenrath 2013).

P. barnegatensis was described after the observation of only one specimen but it significantly differs from our specimens (Fig. 5C). There was no torsion in the pseudocolony described by Martin (1929), although the sulci had an oblique outline. A slightly overhanging cingulum was depicted albeit only for the posterior zooid. All specimens of *P. tanit* showed a stepped fusion border of the zooids, whereas in *P. barnegatensis* the fusion border was not stepped. Other polykrikoid species showed a slight degree of stepped borders, as *Ph. beauchampii* (Chatton 1933) or *P. kofoidii* and *P. schwartzii* (Matsuoka et al. 2009). *P. barnegatensis* was described as having one large beaded nucleus and two zooids. Pseudocolonies of *P. tanit* possess two nuclei, but specimens with only one nucleus were occasionally seen. The life cycles of

polykrikoid species are largely unknown, but the complex life cycle of *P. kofoidii*, including stages with only one nucleus, was recently described (Tillmann and Hoppenrath 2013). Thus, in *P. tanit* pseudocolonies the different number of nuclei could represent different life cycle stages. Nevertheless, the two nuclei were nearly in contact with one another and overlapped with the fusion border of the two zooids. In specimens with only one nucleus, it was never vertically elongated but spherical and centrally located. Correct nuclear discrimination is problematic using common light microscopy methods; consequently, the original description of *P. barnegatensis* may be incorrect. Nonetheless, the depicted shape of the nucleus clearly differs from the nuclei of *P. tanit*. Finally, *P. barnegatensis* is thought to be autotrophic and ingestion bodies are absent. Thus, considering the differences between *P. tanit* and *P. barnegatensis*, i.e., the stepped junction of the zooids, the number and shape of the nuclei, the torsion of the cell body, and the more pronounced overhanging of *P. tanit* cingula, they cannot be considered as the same species.

P. barnegatensis was also reported by Chatton (1952) (Fig. 5B), who did not provide a description of this species, but only a depiction. Although the species was originally described as having one nucleus and two zooids, his drawing showed an organism with two nuclei and two zooids, based on his assumption that the original description was incorrect. Erroneously *P. barnegatensis* was considered to be synonymous with *P. hartmannii* (Chatton 1952). The depicted pseudocolony showed torsion of the cell body, slightly overhanging cingula and a stepped fusion border of zooids. The sulci of the anterior and posterior zooids of the specimen depicted by Chatton (1952) were not connected one another, as demonstrated for *P. hartmannii* (Tang et al. 2013). Based on light microscopy observations, it was our initial impression of *P. tanit* (Fig. 1A) and SEM images confirmed that the sulci were not fused, in contrast to other *Polykrikos* species such as *P. kofoidii*, *P. schwartzii* and *P. lebourae*. The presence and shape of the acrobase was also shown in the depiction provided by Chatton (1952) but it was not shown in that published by Martin (1929). In *P. barnegatensis* sensu Chatton, the acrobase forms a horizontally elongated closed loop around the apex, as observed for *P. kofoidii* and *P. schwartzii* (Nagai et al. 2002), *P. hartmannii* (Takayama 1985), and *P. tanit*. However, the acrobase of *P. lebourae* (Hoppenrath and Leander 2007b) and *Ph. beauchampii* (Omura et al. 2012) is droplet-shaped. According to Chatton (1952), *P. barnegatensis* contains two large ingestion bodies and its nuclei are displaced to one side of the cell. The position of the food vacuoles and the possibility to displace the nucleus was in agreement with our observations of *P. tanit*. He also showed an invagination in the intercingular area, possibly misinterpreting the structure of the peduncles. Thus, although Chatton (1952) considered his organism as *P. barnegatensis*, its morphology better suggests *P. tanit*.

The studied specimens showed too many discrepancies with *P. barnegatensis* to consider *P. barnegatensis* and *P. tanit* the same species. However, the similarities of both species and their differing characters with *P. hartmannii* suggest that *P. barnegatensis* represent a different species and it should not be considered as a synonym of *P. hartmannii*.

Distribution

Chatton (1952) did not provide any information about where the specimen of *P. barnegatensis* was obtained, but we can assume that it was near Thau Lagoon (France, NW Mediterranean Sea), located about 200 km north of Arenys Harbour. The type locality of *P. hartmannii* is Naples Bay (Mediterranean Sea) and that of *Ph. beauchampii* is Thau Lagoon. To the best of our knowledge, the original descriptions of both species refer to unique detections from the Mediterranean Sea reported in the literature (Gómez 2003), although resting cysts similar to those of *P. hartmannii* have been also reported in Southeastern Italy (Moscatello et al. 2004). Nevertheless, our observations of *P. tanit* agree with the fact that small, pigmented polykrikoid species are well represented in the NW Mediterranean Sea. The relatively wide ranges of salinity and temperature recorded during *P. tanit* sample collection are the characteristics of the spring and summer along the Catalan coast and are in contrast with the restricted period of detection. Consequently, and given the different characteristics of the locations where *P. tanit* has been reported, this species may also be present along the Catalan coast during the summer months, but at very low abundances.

Evolutionary characters

The phylogenies obtained in this study are in agreement with those of previous studies that focused on the phylogenetic relationship of polykrikoid organisms (Hoppenrath and Leander 2007a,b; Matsuoka et al. 2009). In this study, both the LSU and the SSU rDNA phylogenies unequivocally support our

specimens as a different species of those previously sequenced. It is phylogenetically distant from *Ph. beauchampii*, the unique current representative of the genus *Pheopolykrikos*, and therefore belongs to the genus *Polykrikos*, as previously demonstrated for *P. hartmannii*. However, a common character of *Polykrikos* species is the presence of taeniocyst-nematocyst complexes. Although these have yet to be observed for *P. tanit* their presence cannot be ruled out because their detection in pigmented species is challenging (Hoppenrath et al. 2010).

P. tanit is phylogenetically and morphologically close to *P. hartmannii* and both species occupy basal positions, conforming to an early sister clade within the *Polykrikos* clade (Hoppenrath et al. 2010). While the LSU rDNA phylogeny does not fully resolve this clade, the SSU rDNA sequences place *P. tanit* in a basal position. Like *P. hartmannii*, the sulci of the two *P. tanit* zooids are not fused and the dinoflagellate contains chloroplasts, which were lost in subsequent species along with the fusion process of the sulci of zooids. Previous studies suggest that photosynthesis was regained in *P. lebourae* (Hoppenrath and Leander 2007b). Furthermore, *P. tanit* has the same number of nuclei and zooids, although specimens with only one nucleus were occasionally observed, in agreement with the hypothesis of zooid doubling during evolution (Hoppenrath and Leander 2007a). The lack of nematocyst-taeniocyst complexes, which as noted above could not be confirmed for *P. tanit*, can also be considered as an early evolutionary character. The mixotrophy of *P. tanit* supports the assumption suggested by Hoppenrath and Leander (2007a) that during evolution the development of heterotrophy was accompanied by a loss of photosynthetic capability in polykrikoid organisms.

Provided that studied specimens differ from previously described *Polykrikos* species both morphologically and phylogenetically, we describe the studied specimens as a new species.

***Polykrikos tanit* sp. nov. Reñé**

(= *Polykrikos barnegatensis sensu* Chatton 1952, fig. 243b)

Description: Unarmoured pseudocolonies (46–76 μm long; 26–50 μm wide) consisting of two zooids and usually two (sometimes one) nuclei located centrally in the pseudocolonies, which are ovate, almost circular in cross-section, and exhibit torsion to the left. The fusion border of the two zooids is visible and stepped. Closed loop-shaped acrobase. Sulci not fused, with sigmoid outline. Descending and overhanging cingula, displaced two–three times their width. Each zooid has its own longitudinal and transverse flagellum and a peduncle in the intercingular area. Mixotrophic.

Etymology: named after Tanit, a Punic goddess worshiped in the Western Mediterranean until the 2nd century A.D., in reference to both the early evolutionary position of the species within the genus and its type locality.

Holotype: Figure 5A. A SEM-stub was deposited in the Electronic Microscopy Laboratory of the Institut of Ciències del Mar (ICM-CSIC) from Barcelona, under the code 20130423-AR.

Isotype: Figure 2A

Type habitat: Marine planktonic

Type locality: Arenys Harbour, Catalonia, NW Mediterranean Sea (41°51′29″ N; 2°33′20.5″ E).

Distribution: NW Mediterranean Sea.

Gene sequences: Sequences have been deposited in GenBank under the accession numbers KF806598-KF806599 for SSU rDNA and KF806600 to KF806602 for LSU rDNA.

Methods

Detection locations, isolation, and morphological observations: ***Observations.*** The target specimens were detected in sub-surface live samples collected from Arenys (41°51′29″ N; 2°33′20.5″ E) and Vilanova (41°12′55″ N; 1° 43′ 50″ E) Harbours as well as L'Estartit beach (42° 2' 47″ N; 3° 11′ 53″ E) and offshore of Barcelona (41° 22′ 35″ N; 2° 12′ 41″ E) (Catalan Coast, NW Mediterranean Sea) at the end of May–June 2012 and from Arenys Harbour in April–May 2013. Random volumes of live samples were concentrated using a 10-μm mesh. The organisms in these filtered samples were observed in a settling chamber under a Leica-Leitz DM-II inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany) equipped with a Sony NEX-5 digital camera (Sony, Tokyo, Japan) and under a phase-contrast Leica DM IRB inverted microscope connected to a ProgRes C10 (JENOPTIK Laser, Optik, Systeme GmbH, Jena, Germany) digital camera. ***Culturing.*** Several attempts were made to culture the organisms. Specimens were isolated and placed in culture wells filled with either L1 medium at a salinity of 37 or filtered seawater from the same sample, or non-filtered seawater to provide potential prey. However, all of these attempts were unsuccessful. A set of prey ranging from 1 to 20 μm was also added to the isolated specimens, with same unsuccessful results. ***Epifluorescence microscopy.*** To determine the number of nuclei in each pseudocolony, live specimens were placed in a slide, stained with 1:100 Sybr Green (Molecular Probes, Eugene, OR, USA) in 0.01 M PBS, pH 7.4, for 20 min, and observed in an epifluorescence Leica-Leitz DM-II inverted microscope through a blue filter. Chloroplast autofluorescence was observed directly on unstained live specimens through the same blue filter. ***Scanning electron microscopy.*** Concentrated natural samples (5–10 ml) were fixed for 15 min at room temperature with an adequate volume of 4% osmium tetroxide to reach a final concentration of 2%. The sample was gravity-filtered through a Nucleopore (Pleasanton, CA, USA) polycarbonate filter (13 mm diameter, pore size 8 μm). The filtered cells were then washed in distilled water,

dehydrated for 10 min each in a 25, 50, 75, 95, and 100% ethanol series, and critical-point dried. The filters were mounted on stubs, sputter-coated with gold, and examined with a JEOL JSM-6500F scanning electron microscope (JEOL-USA Inc., Peabody, MA, USA).

Single-cell PCR amplification, sequencing, and phylogenetic analyses: Each target pseudocolony was transferred several times into filtered seawater drops using Pasteur pipettes, then transferred to 200-µl PCR tubes, adding the minimum volume of seawater, subjected to several rounds of freezing/thawing, and finally stored at -80 °C until processed. Single-cell PCR was conducted with a PCR mixture containing 5 ml of 10× buffer (Qiagen), 1.25 U of Taq DNA polymerase (Qiagen), 0.2 mM of each dNTP, and 0.8 mM of the primers D1R and D2C (Scholin et al. 1994) for the partial LSU region and the primers EUK A (Medlin et al. 1988) and 1209R (Giovannoni et al. 1988) for the partial SSU region. The PCR conditions for LSU were as follows: initial denaturation for 5 min at 95 °C, 40 cycles of 20 s at 95 °C, 30 s at 55 °C, and 1 min at 72 °C, followed by a final extension step for 7 min at 72 °C. The PCR conditions for SSU were: initial denaturation for 5 min at 95 °C, 30 cycles of 45 s at 95 °C, 1 min at 55 °C, and 3 min at 72 °C, followed by a final extension step for 10 min at 72 °C. Ten µl of the PCR products were electrophoresed for 20–30 min at 120 V in a 1.2% agarose gel and then visualized under UV illumination. The remainder of the sample was frozen at -20 °C and later used for sequencing. Purification and sequencing were carried out by an external service (Genoscreen, France). Sequencing was done using both forward and reverse primers and a 3730XL DNA sequencer.

The obtained sequences were aligned with those from GenBank using the MAFFT v.6 program (Katoh et al. 2002) under G-INS-i and manually checked with BioEdit v. 7.0.5 (Hall 1999), obtaining a final alignment of about 830 positions for LSU sequences and 1760 positions for SSU sequences. In both cases, phylogenetic relationships were determined using maximum-likelihood (ML) and Bayesian inference methods. For the former, the GTRGAMMA evolution model was used on RAxML (Randomized Axelerated Maximum Likelihood) v. 7.0.4 (Stamatakis 2006). All model parameters were estimated by RAxML. Repeated runs on distinct starting trees were carried out to select the tree with the best topology (the one with the greatest likelihood of 1000 alternative trees). Bootstrap ML analysis was done with 1000 pseudo-replicates and the consensus tree was computed with the RAxML software. The Bayesian inference was performed with MrBayes v.3.2 (Ronquist et al. 2012), run with a GTR model in which the rates were set to gamma. Each analysis was performed using four Markov chains (MCMC), with one million cycles for each chain. The consensus tree was created from post-burn-in trees and the Bayesian posterior probabilities (BPP) of each clade were examined.

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References

Bütschli O (1873) Einiges über Infusorien. Arch Mikrosk Anat **9**:657–678

Chatton É (1933) *Pheopolykrikos beauchampi* nov. gen., nov. sp., dinoflagellé polydinide autotrophe, dans l'étang de Thau. Bull Soc Zool Fr **58**:251–254

Chatton É (1952) Classe des dinoflagellés ou péridiniens. In Traité de Zoologie Anatonie, Systématique, Biologie Tome I: Phylogénie Protozoaires: Généralités Flagellés (Premier fascicule) (ed) Masson. Paris, pp 309–406

Dodge JD (1982) Marine Dinoflagellates of the British Isles. Her Majesty's Stationery Office, London, 303 p

Gaines G, Elbrächter M (1987) Heterotrophic Nutrition. In The biology of Dinoflagellates (ed) Taylor FJR (ed), Blackwell Sci Publ, Oxford, pp 224–268

Giovannoni SJ, deLong EF, Olsen GJ, Pace NR (1988) Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial cells. J Bacteriol **170**:720–726

Gómez F (2003) Checklist of Mediterranean free-living dinoflagellates. Bot Mar **46**:215–242

Gómez F (2012) A checklist and classification of living dinoflagellates (Dinoflagellata, Alveolata). CICIMAR Oceanides **27**:65–140

Guiry MD, Guiry GM (2013) AlgaeBase. World-wide Electronic Publication, National University of Ireland, Galway. http://www.algaebase.org

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser **41**:95–98

Hoppenrath M, Leander BS (2007a) Character evolution in polykrikoid dinoflagellates. J Phycol **43**:366–377

Hoppenrath M, Leander BS (2007b) Morphology and phylogeny of the pseudocolonial dinoflagellates *Polykrikos lebourae* and *Polykrikos herdmanae* n. sp. Protist **158**:209–227

Hoppenrath M, Yubuki N, Bachvaroff TR, Leander BS (2010) Re-classification of *Pheopolykrikos hartmannii* as *Polykrikos* (Dinophyceae) based partly on the ultrastructure of complex extrusomes. Eur J Protistol **46**:29–37

Hulburt EM (1957) The taxonomy of unarmored Dinophyceae of shallow embayments on Cape Cod, Massachusetts. Biol Bull **112**:196–219

Kang NS, Jeong HJ, Moestrup Ø, Park TG (2011) *Gyrodiniellum shiwhaense* n. gen., n. sp., a new planktonic heterotrophic dinoflagellate from the coastal waters of Western Korea: Morphology and ribosomal DNA gene sequence. J Eukaryot Microbiol **58**:284–309

Katoh K, Misawa K, Kuma K, Miyata T (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res **30**:3059–3066

Kim KY, Iwataki M, Kim CH (2008) Molecular phylogenetic affiliations of *Dissodinium pseudolunula*, *Pheopolykrikos hartmannii, Polykrikos* cf. *schwartzii* and *Polykrikos kofoidii* to *Gymnodinium sensu stricto* species (Dinophyceae). Phycol Res **56**:89–92

Landsberg JH, Steidinger KA, Blakesley BA, Zondervan RL (1994) Scanning electron microscope study of dinospores of *Amyloodinium* cf. *ocellatum*, a pathogenic dinoflagellate parasite of marine fish, and comments on its relationship to the Peridiniales. Dis Aquat Org **20**:23–32

Lee RE (1977) Saprophytic and phagocytic isolates of the colorless heterotrophic dinoflagellate *Gyrodinium lebouriae* Herdman. J Mar Biol Assoc UK **57**:303–315

Loeblich AR III (1980) Dinoflagellate nomenclature. Taxon **29**:321–324

Martin GW (1929) Three new dinoflagellates from New Jersey. Botanical Gazette **87**:556–558

Matsuoka K, Fukuyo Y (1986) Cyst and motile morphology of a colonial dinoflagellate *Pheopolykrikos hartmannii* (Zimmermann) comb. nov. J Plankton Res **8**:811–818

Matsuoka K, Kawami H, Nagai S, Iwataki M, Takayama H (2009) Re-examination of cyst–motile relationships of *Polykrikos kofoidii* Chatton and *Polykrikos schwartzii* Bütschli (Gymnodiniales, Dinophyceae). Rev Palaeobot Palynol **154**:79–90

Medlin L, Elwood HJ, Stickel S, Sogin ML (1988) The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. Gene **71**:491–499

Moscatello S, Rubino F, Saracino OD, Fanelli G, Belmonte G, Boero F (2004) Plankton biodiversity around the Salento Peninsula (South East Italy): an integrated water/sediment approach. Sci Mar **68**:85–102

Nagai S, Matsuyama Y, Takayama H, Kotani Y (2002) Morphology of *Polykrikos kofoidii* and *P. schwartzii* (Dinophyceae, Polykrikaceae) cysts obtained in culture. Phycologia **41**:319–327

Omura T, Iwataki M, Borja VM, Takayama H, Fukuyo Y (2012) Marine Phytoplankton of the Western Pacific. Kouseisha Kouseikaku, Tokyo, 160 p

Ronquist F, Teslenko M, Van Der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP (2012) MrBayes 3. 2: Efficient Bayesian phylogenetic inference and model choice across a large model space. Syst Biol **61**:539–542

Scholin CA, Herzog M, Sogin M, Anderson DM (1994) Identification of group- and strain-specific genetic markers for globally distributed *Alexandrium* (Dinophyceae). 2. Sequence analysis of a fragment of the LSU rRNA gene. J Phycol **30**:999–1011

Sournia A (1986) Atlas du phytoplancton marin: Introduction, Cyanophycées, Dictyochophycées, Dinophycées et Raphidophycées, vol. 1. Editions du Centre National de la Recherche Scientifique, Paris, 219 p

Stamatakis A (2006) RAxML-VI-HPC: Maximum Likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics **22**:2688–2690

Takayama H (1985) Apical grooves of unarmored dinoflagellates. Bull Plankton Soc Japan **32**:29–140

Takayama H (Personal website) http://www.geocities.jp/takayama_haruyoshi/HAB/Welcome.html

Tang YZ, Harke MJ, Gobler CJ (2013) Morphology, phylogeny, dynamics, and ichthyotoxicity of *Pheopolykrikos hartmannii* (Dinophyceae) isolates and blooms from New York, USA. J Phycol http://dx.doi.org/10.1111/jpy.12114

Tillmann U, Hoppenrath M (2013) Life cycle of the pseudocolonial dinoflagellate *Polykrikos kofoidii* (Gymnodiniales, Dinoflagellata). J Phycol **49**:298–317

Wedemayer GJ, Wilcox LW, Graham LE (1982) *Amphidinium cryophilum* sp. nov. (Dinophyceae) a new freshwater dinoflagellate. I. Species description using light and scanning electron microscopy. J Phycol **18**:13–17

Zimmermann W (1930) Neue und wenig bekannte Kleinalgen von Neapel I-V. Zeitschr Bot **23**:419–442