***Psammodinium inclinatum* gen. nov. et comb. nov. (=Thecadinium inclinatum Balech)**
is the closest relative to the toxic dinoflagellate genera *Gambierdiscus* and *Fukuyoa*

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ABSTRACT

The heterotrophic sand-dwelling dinoflagellate *Thecadinium inclinatum* has been re-examined by light and scanning electron microscopy in order to resolve the discrepancies on its plate pattern from the literature, and to obtain its phylogenetic information single-cell PCR technique has been used. The comparison of morphological and molecular information available for other *Thecadinium* species confirms the genus is polyphyletic and *T. inclinatum* seems not related to other representatives of the genus *sensu lato*. Thus, a new genus and combination for the species, *Psammodinium inclinatum* gen. nov., comb. nov. is proposed. Cells are heterotrophic and strongly laterally flattened, with sulcal pocket. The revised tabulation is: APC 3' 7'' 7c 7s? 5''' 1p 2'''' with a long-shank fishhook-shaped apical pore and descending cingulum. The cingulum inclines ventrally and declines on the right lateral side producing an asymmetrical epitheca. The epitheca is much smaller than the hypotheca. The phylogenetic results showed a strong relationship with the autotrophic epiphytic genera *Gambierdiscus* and *Fukuyoa*, being closely related with the latter. The *Gambierdiscus* species typically have a tropical and sub-tropical distribution and produce ciguatoxins, causing thousands of intoxications every year by consumption of contaminated fish. *Fukuyoa* representatives have a wider distribution including warm and temperate waters, and it has been demonstrated that they are also able to produce ciguatoxins, even though at lower amounts. *P. inclinatum*, which potential toxicity remains to be determined, represents an interesting independent evolutionary branch that resulted in the loss of chloroplasts, the strong lateral compression and the adaptation to sandy habitats in temperate and cold waters.

Keywords: benthic; sand-dwelling; dinoflagellate; phylogeny; taxonomy

1. INTRODUCTION

The genus *Thecadinium* Kofoid et Skogsberg comprises laterally flattened thecate dinoflagellates, with a reduced epitheca and a large hypotheca with two large lateral hypothecal plates (Hoppenrath et al., 2014). They inhabit marine sandy sediments and at date, there are 10 species described of *Thecadinium*, including autotrophic species like the type species *T. kofoidii* (E.C. Herdman) Larsen, and *T. pseudokofoidii* Selina, Efimova et Hoppenrath, *T. yashimaense* Yoshimatsu, Toriumi et Dodge or *T. arenarium* Yoshimatsu, Toriumi et Dodge, and heterotrophic species such as *T. neopetasatum* Saunders et Dodge, *T. acanthium* Hoppenrath, *T. ornatum* Hoppenrath, *T. ovatum* Yoshimatsu, Toriumi et Dodge, *T. striatum* Yoshimatsu, Toriumi et Dodge and *T. inclinatum* Balech (Hoppenrath, 2000; Hoppenrath et al., 2014; Hoppenrath et al., 2004; Saunders and Dodge, 1984; Selina et al., 2019; Yoshimatsu et al., 2004). All of them agreed with the general diagnosis of the genus *sensu lato*. Nevertheless, some species can be grouped based on morphological characteristics to each subgroup. *Thecadinium kofoidii* and *T. pseudokofoidii* represent the redefined *sensu stricto* genus *Thecadinium* Kofoid et Skogsberg emend. Selina et Hoppenrath with the tabulation APC 4' 1a 3" 5c 8s 4''' 1'''' (Selina et al., 2019). The unique traits of the genus are the right sulcal plate contacting the Po plate and an incomplete precingular plate series. *Thecadinium neopetasatum* shows high resemblance with *Thecadinium sensu stricto* species, but by contrast, it is not pigmented, the precingular plate series is complete and the apical pore has a different shape (Hoppenrath, 2000; Saunders and Dodge, 1984). This species is also strongly laterally flattened, with a small cap-like epitheca, and has smooth plates with scattered pores and the tabulation APC 3' 1a 6" 6c ?s 5''' 1'''' (Hoppenrath, 2000). The heterotrophic species *T. ornatum*, *T. acanthium*, *T. ovatum*, and *T. striatum* have a very similar plate pattern (3/4' 6" 6c ?s 5''' 1''''), no apical pore plate,

a ventral pore on the first apical plate and a third precingular plate of special morphology with several pores and pimples (“multi-pimple-plate”) or a large depression (“dimple-plate”) (Hoppenrath, 2000; Yoshimatsu et al., 2004). The autotrophic *T. yashimaense* (Po 3' 1a 6" 5-7/8c 5s 6" 2"") and *T. arenarium* (Po 3' 1a 6" 5c 4s 5" 1"") have a strongly asymmetrical epitheca and hypotheca caused by the extreme path of the cingulum. The apical pore plate is horseshoe shaped, and the thecal plates are thick, ornamented and having large pore-like depressions with small pores inside (Bolch and Campbell, 2004; Hoppenrath et al., 2004; Yoshimatsu et al., 2004). Finally, the heterotrophic *Thecadinium inclinatum* (Balech, 1956) has an asymmetrical epitheca, a sulcus deeply indented and a hook-shaped apical pore (Hoppenrath et al., 2004; Saunders and Dodge, 1984). The thecal plates are smooth with scattered pores. The thecal plate pattern is not clear, APC 3' 6" 7c 7s 5" 1p 1" or APC 3' 7" ?c ?s 5" 1" (Hoppenrath et al., 2004; Yoshimatsu et al., 2004). A former member of the *Thecadinium* genus, *T. dragescoi* Balech was transferred to the genus *Amphidiniopsis* by Hoppenrath et al. (2012).

Even though first studies on sand-dwelling dinoflagellates were conducted in the early twentieth century, those organisms were not studied in depth until the 2000s (Hoppenrath et al., 2014). By contrast, epiphytic species have received more attention by the scientific community, mainly because many of them are toxin-producers and cause intoxications in humans (Berdalet et al., 2017). The main agents of intoxications caused by benthic (epiphytic) dinoflagellates are species of the autotrophic genus *Gambierdiscus* Adachi et Fukuyo, responsible of ciguatera intoxications (Litaker et al., 2010). Up to 25,000-500,000 intoxications are reported yearly by consumption of fish contaminated with ciguatoxins, mostly affecting tropical areas (Parsons et al., 2012). Recently, some species of *Gambierdiscus* have been transferred to the new genus *Fukuyoa* F.Gómez, D.Qiu,

R.M.Lopes et S.Lin, based on morphological and phylogenetic differences (Gómez et al., 2015). While *Gambierdiscus* comprises anteriorposteriorly compressed, lenticular species, *Fukuyoa* species are globular with some degree of lateral compression. At present, there are 16 species described of *Gambierdiscus*, and 3 named species of *Fukuyoa* (Berdalet et al., 2017; Laza-Martínez et al., 2016; Rhodes et al., 2017).

The molecular information obtained for the sand-dwelling species *T. inclinatum* from samplings carried out in the German Wadden Sea confirmed that this species was not related to some other *Thecadinium* species, including *T. kofoidii*, the type species of the genus. Instead, its phylogenetic position showed a strong relationship with the autotrophic, epiphytic and toxin-producing genera *Fukuyoa* and *Gambierdiscus*. It results in the proposal of the new genus and combination *Psammodinium inclinatum*.

2. MATERIAL AND METHODS

2.1 Sampling and cell separation:

Sediments were obtained during March 2009 in List (Sylt), Germany (55° 01'58"N; 8° 43'94"E), and during June 2010, April 2012, and August - September 2018 in Wilhelmshaven, Germany (53° 30'36"N; 8° 07'43"E). Samples were taken during low tide from intertidal areas using a spoon and were immediately taken to the laboratory. Around 100 g of sediment were placed in a PVC tube with a mesh of 55 µm attached to the bottom and the sand-dwelling dinoflagellates were separated from the sediment using the seawater ice method (Uhlir, 1964) and accumulated in a Petri dish beneath the filter.

2.2 Morphological identification:

The Petri dishes containing the separated cells were examined under a Leica DMIL inverted light microscope (Leica Microsystems GmbH, Wetzlar, Germany). Living cells identified as *P. inclinatum* were individually picked and separated for a detailed observation under light microscopy (LM) using a Leica DMRB microscope (Leica Microsystems GmbH, Wetzlar, Germany) equipped with differential interference contrast at 400 and 630 times magnification with oil immersion objectives. Digital photos were taken using a Leica DFC420C camera (Leica Microsystems GmbH, Wetzlar, Germany). Other cells were used for scanning electron microscopy (SEM) observation. Those isolated cells were fixed with Lugol's iodine and after some days, preserved cells were accumulated into a 5.0 µm pore size 13-mm filter, dehydrated with an ethanol series, chemically dried with hexamethyldisilazane (HMDS), sputter coated with gold-palladium (SCD 050 Bal-Tec) and investigated using a Tescan VEGA3 microscope (Elektro-Optik-Service GmbH, Dortmund, Germany) at 15 kV. Finally, some living cells obtained from each sampling location in 2009, 2010, 2012 and 2018 (Supplementary Table 2) were individually isolated, washed several times in autoclaved seawater, and 1 to 10 cells were placed together in a 0.2 mL PCR tube and stored at -20°C until further molecular analyses.

2.3 PCR amplification, sequencing, and phylogenetic analysis:

Total DNA from MH2009, MH2010 and MH2012 isolates (Supplementary Table 2) was extracted using MasterPure complete DNA & RNA Purification kit (Epicentre, USA) following manufacturer's instructions, and used as template for subsequent PCR reactions. Isolate AR39 from 2018 was subjected to several freeze-thaw rounds and directly used as template for single-cell PCRs. Details about the PCR procedures carried out to obtain their partial SSU and D1-D3 LSU rDNA sequences are available in Supplementary Material 1.

Briefly, a first PCR was conducted in order to amplify the whole region of interest, and subsequent (semi-)nested PCRs were performed to obtain different fragments of the target regions. The list of primers used is detailed in Supplementary Table 1 and all sequences obtained were deposited in GenBank under the Accession Numbers MK575197-MK575200 for SSU rDNA and MK575499-MK575502 for LSU rDNA sequences (Supplementary Table 2). Sequences obtained were aligned with a selection of sequences from GenBank covering the genera *Gambierdiscus* and *Fukuyoa*, as well as other representatives of the order Gonyaulacales, using the MAFFT v.7 online server (Kato et al., 2002) under auto option. The alignments were manually checked with BioEdit v. 7.0.9 (Hall, 1999), obtaining 1751 positions for SSU rDNA sequences and 840 positions for LSU rDNA sequences. Subsequently, the alignments were trimmed using Gblocks using the less stringent options (Castresana, 2000) to remove poorly aligned regions, resulting in a final alignment of 1703 and 551 positions respectively. Phylogenetic relationships were determined as described in Reñé et al. (2017) using analyses of maximum-likelihood and Bayesian inference.

3. RESULTS

3.1 Morphological observations:

The oval cells (Fig. 1) examined were 43.0-60.0 μm long and 30.0-47.5 μm deep ($n = 19$). Cells were laterally compressed, with a small asymmetrical epitheca and a large hypotheca (Fig. 1). The descending cingulum first ascends in a smooth arc from left ventral, has its highest position dorsally and then descends more steeply on the right lateral side (Figs. 1A-C, large arrowheads). The sulcus was deeply indented (sulcal pocket; Figs. 1A, B, E, small arrowhead) and 1-2 large pusules were usually present (Figs. 1B, F). The organisms were

not pigmented, commonly showing food vacuoles in the hypotheca (Fig. 1A). The elongated nucleus ran horizontally in the middle of the cell (Figs. 1D, E). The thecal plates were smooth with scattered small pores (Fig. 2). The tabulation was: APC 3' 7" ?c ?s 5" 1p 2" (Figs. 2-5). The hypotheca was composed of five postcingular plates (Figs. 2A-G). The lateral postcingular plates 2" and 4" occupied most of the hypotheca (Figs. 2A-D). The third postcingular plate (3") was located dorsal and the two ventral plates were relatively small (Figs. 2A-E). The end of the cingulum is running into the sulcal pocket (Fig. 2E). The newly discovered first antapical plate (1") was located left of the sulcus below the first postcingular plate (Figs. 2E-G). The posterior intercalary plate was located at the lower dorsal cell end (Figs. 2A, B, D, G-I). The apical pore plate (Po) had a long-shank fishhook-shaped apical pore, surrounded by a single row of pores (Fig. 3B, 4G). The APC was slightly orientated to the left side of the epitheca (Fig. 3). There were three apical plates and seven precingular plates could be confirmed (Figs. 3, 4).

3.2 Phylogenetic relationships:

The SSU rDNA phylogeny (Fig. 5) shows the sequences obtained for *Psammodinium inclinatum* cluster together with the sequences of *Gambierdiscus* and *Fukuyoa* species (100 % bootstrap support / 1 Bayesian posterior probability), forming a sister clade with *Fukuyoa* representatives (100%/1). All sequences belonging to *Gambierdiscus* species clustered together (100%/1), as did those belonging to *Fukuyoa* (100%/1). The sequence of *Triadinium polyedricum* clustered at the base of the clade containing the three genera (99%/1). All those organisms belonged to the order Gonyaulacales (84%/1). The sequences available for *Thecadinium kofoidii*, *T. pseudokofoidii* and *Thecadinium yashimaense* do not show a phylogenetic relationship with *P. inclinatum*. Additionally, *T. kofoidii* and *T.*

pseudokofoidii sequences clustered together (100%/1), while *T. yashimaense* sequences clustered independently as a sister group, but with low support. The LSU rDNA phylogeny (Fig. 6) shows the same phylogenetic relationships than SSU rDNA phylogeny. The sequences of *P. inclinatum* clustered together with the sequences of *Gambierdiscus* and *Fukuyoa* species (99%/1), forming as a sister group with *Fukuyoa* representatives (100%/1). All sequences belonging to *Fukuyoa* (100%/1) and *Gambierdiscus* (100%/1) cluster together. Sequences of *Triadinium polyedricum* also clustered at the base of the so-called *Gambierdiscus* lineage with high support (97%/1). Sequences available of other *Thecadinium sensu lato* representatives (*T. yashimaense*, *T. ovatum*, *T. kofoidii* and *T. pseudokofoidii*) clustered independently and did not show a phylogenetic relationship with *P. inclinatum*, with the exception of *T. kofoidii* and *T. pseudokofoidii*, which cluster together with high support (96%/0.99).

3.3 Nomenclatural change:

***Psammodinium* gen. nov. Hoppenrath & Reñé**

Diagnosis: Cell ovoid, laterally flattened, thecate and heterotrophic. Epitheca smaller than hypotheca. Cingulum descending. Sulcal pocket. APC with long-shank hook shaped apical pore. Tabulation: APC 3' 7" 7c 7s? 5''' 1p 2'''.

Type species: *Psammodinium inclinatum* (Balech) Hoppenrath et Reñé comb. nov.

Etymology: *psammos* = sand in Greek, sand as habitat and *-dinium* suffix for dinoflagellates; refers to the sandy interstitial habitat, in contrast to the epiphytic living style of its close relatives.

***Psammodinium inclinatum* (Balech) Hoppenrath & Reñé comb. nov.**

216 Basionym: *Thecadinium inclinatum* Balech 1956, Étude des dinoflagellés du sable de
217 Roscoff. Revue Algologique 2, p.40
218 Synonym: *Sabulodinium inclinatum* (Balech) Saunders et Dodge 1984
219 Non: *Thecadinium inclinatum* sensu v. Bernuth 1967 (CCMP 1890, CCCM 682)
220 *Thecadinium inclinatum* sensu Vogel et Meeuse 1968
221 *Thecadinium inclinatum* sensu Baillie 1971
222 *Thecadinium inclinatum* sensu Loeblich III 1984
223

224 4. DISCUSSION

225 Given the discrepancies present in the literature about the plate formula of *Psammmodinium*
226 *inclinatum* (Hoppenrath et al., 2004; Yoshimatsu et al., 2004), a detailed morphological
227 characterization of the specimens collected from field samples was performed under SEM.
228 Morphological examination of specimens under LM agreed with the observations
229 performed by Hoppenrath et al. (2004) for specimens obtained in the North German
230 Wadden Sea. The newly reported cell sizes are smaller as documented before by
231 Hoppenrath et al. (2004) and Yoshimatsu et al. (2004), see Table 1.
232

233 4.1 Plate tabulation of *P. inclinatum* and relationship with *Thecadinium* species:

234 There are some discrepancies on *P. inclinatum* plate tabulation in the literature (Fig. 7).
235 Balech (1956) only provided details for some sulcal plates in the original description of the
236 species (Fig. 7D). Saunders and Dodge (1984) showed first epithecal and hypothecal plates
237 of the right lateral cell side (as *Sabulodinium inclinatum*). Hoppenrath et al. (2004) and
238 Yoshimatsu et al. (2004) provided a detailed plate pattern examination (Figs. 7A, 7C), but
239 the plate formula differed, being Po 3' 6" 7c 7s 5''' 1p 1'''' (Hoppenrath et al., 2004) or Po 3'

7" ?c ?s 5" 1" (Yoshimatsu et al., 2004). Besides the different number of precingular plates observed (Figs. 7Aa, 7Ca), Yoshimatsu et al. (2004) interpreted the antapical plate (1") of Hoppenrath et al. (2004) as posterior sulcal and the posterior intercalary as antapical plate (Figs. 7Ab, 7Cb). The shape of the antapical or posterior sulcal plate also differed among the studies, being elongated and narrow in Yoshimatsu et al. (2004) and wider and hexagonal in Hoppenrath et al. (2004). The present study demonstrated clearly that the shape of this plate (now interpreted as second antapical plate) is wide and pentagonal but located asymmetrically on the antapex more to the right lateral side below the longitudinal furrow (sulcus) (Fig. 7Bb). A new first antapical plate was located left of the sulcus below the first postcingular plate and described here for the first time (Fig. 7Bb). Judged from the published SEM images by Yoshimatsu et al. (2004; Figs. 7, 14) this plate was also present in their isolates (indicated in our Figs. 7Cb, 7Cc as dotted line). The different number of precingular plates was originated by the observations of the ventral area. Re-examination of the original SEM images of Hoppenrath et al. (2004) revealed that the sixth precingular plate in fact was two plates (see their figure 8f, reproduced herein as Fig. 4D) and this could be confirmed in the present study but not nicely demonstrated (Fig. 7Ba). There are still differences between the emended tabulation of *P. inclinatum* and the species documented by Yoshimatsu et al. (2004) (Figs. 7B, 7C). Shapes, sizes, and relative locations of the epithecal plates and the number of hypothecal plates need verification for the Japanese taxon. For example, the second precingular plate as depicted by Yoshimatsu et al. (2004, fig. 47c; redrawn as Fig. 7Ca herein) is relatively large and should be in contact with the Po plate, but is not in their fig. 47a (redrawn as Fig. 7Cc herein). In addition the density of thecal pores seems to be higher on the plates of the cells from Japan. Molecular data for both taxa will help to decide whether or not they are conspecific.

The polyphyly of the genus *Thecadinium* has been suggested in the literature (Hoppenrath, 2000; Hoppenrath et al., 2004). The phylogenetic information available is still scarce but none of the known *Thecadinium* species seems related to *P. inclinatum*. The redefined genus *sensu stricto* (*T. kofoidii* and *T. pseudokofoidii*) is unique with the tabulation APC 4' 1a 3" 5c 8s 4" 1"', the traits of the right sulcal plate contacting the Po plate and an incomplete precingular plate series (Selina et al., 2019). The morphological features of the group formed by *T. ornatum*, *T. acanthium*, *T. ovatum*, and *T. striatum* suggest they belong to the same genus, but their phylogenetic position and relationships are still unknown. *Thecadinium arenarium* and *T. yashimaense* share nearly identical morphological characters and the latter is not related phylogenetically with *P. inclinatum* (Figs. 5, 6). It is not clear whether *T. neopetasatum* could be related with the type species *T. kofoidii*. In any case, the sequences available of *T. kofoidii* and *T. pseudokofoidii*, *T. yashimaense*, *T. ovatum* and *P. inclinatum* do not cluster together. Thus, it suggests that current members of the *Thecadinium sensu lato* genus should be separated into four different genera, *Psammodinium* being the first.

4.2 Evolutionary patterns of *P. inclinatum*, *Gambierdiscus* spp. and *Fukuyoa* spp.:

The phylogenetic position of *P. inclinatum* entails interesting insights on its evolutionary history, given the evident differences to closely related species. Under a morphological perspective, some characters are shared by members of the three genera *Gambierdiscus*, *Fukuyoa* and *Psammodinium*, while others are only shared with *Fukuyoa* species, its closest relatives (Fig. 8, Table 1). All share the same (fish)hook-shape of the apical pore, even though having a long “shank” in *Fukuyoa* and *Psammodinium*, and a short in *Gambierdiscus*. The cingulum is descending in *Fukuyoa* and *Psammodinium*, while

ascending in *Gambierdiscus*. All three genera have three apical plates, seven precingular plates, five postcingular and two antapical plates. The largest apical plate is 1' in *Fukuyoa* and 2' in *Gambierdiscus*. In *P. inclinatum* they are of similar size (Fig. 8). In Yoshimatsu et al. (2004) plates 2' and 3' are of similar size (Fig. 7Ca). Some *Gambierdiscus* species have a wide 1p plate (Fraga and Rodríguez, 2014; Jang et al., 2018; Litaker et al., 2009), similar to planktonic relatives like *Alexandrium* (Balech, 1995) and *Triadinium* (Dodge, 1981), while it is narrow in other *Gambierdiscus* (Kretzschmar et al., 2017; Rhodes et al., 2017) and *Fukuyoa* species (Gómez et al., 2015; Holmes, 1998; Litaker et al., 2009). *Psammodinium* shows a narrow to medium wide 1p plate with lateral flattening ('cup-like'). The first antapical plate is narrow and running up the left sulcal side, similar to *F. yasumotoi* but different to *F. paulensis* and *Gambierdiscus* species (Figs. 8 Ca-d). In *Psammodinium* and *Fukuyoa* species, the precingular 4" and postcingular 3"" plates are aligned and occupy a dorsal position (Gómez et al., 2015; Litaker et al., 2009). By contrast, plates 3" – 3"" and 4" – 4"" are aligned in *Gambierdiscus* species, and the dorsal position is occupied by the suture between 3" / 4" and 3"" / 4"" (Fig. 8). In any case, some variability exist in the position of the sutures, and precingular and postcingular plates are nearly aligned in some *Gambierdiscus* species like *G. carolinianus* (Litaker et al., 2009), while clearly shifted in others like *G. excentricus* (Fraga et al., 2011). *Triadinium polyedricum* shows intermediate features. The suture 3" / 4" occupies the dorsal position, like in *Gambierdiscus* species, but 3"" is in dorsal position, like in *Fukuyoa* and *Psammodinium*, thus precingular and postcingular plates being completely shifted (Shin et al., 2016). While *Gambierdiscus* representatives showed an anterior-posterior compression, resulting in lenticular cells, *Fukuyoa* members showed a slight lateral compression, even though remaining globular. In the case of *P. inclinatum*, it showed a strong lateral compression, in

agreement with sand-dwelling dinoflagellate features, which commonly show strong lateral or dorso-ventral cell compression that facilitates their attachment and movement in interstitial habitats (Hoppenrath et al., 2014).

In contrast to all closest planktonic and epiphytic relatives, *P. inclinatum* lacks chloroplasts. Although the loss of autotrophy is not rare in the evolutionary history of dinoflagellates, e.g. *Polykrikos* representatives (Hoppenrath and Leander, 2007), *P. inclinatum* represents the only known heterotrophic species among all close relatives.

Gambierdiscus and *Fukuyoa* genera have a special interest due to their capability of producing ciguatera fish poisoning (CFP), causing intoxications in humans by consumption of contaminated fish. Toxins related with CFP are ciguatoxins and maitotoxins. All *Gambierdiscus* representatives are considered as toxic, but important differences in the toxin composition and production exist between species, strains, and their growth-phase (Holland et al., 2013; Litaker et al., 2017). It has been demonstrated that *Fukuyoa* representatives are also capable to produce CFP-toxins, even though their toxicity varies across species and strains (Gómez et al., 2015; Holland et al., 2013; Larsson et al., 2019; Laza-Martínez et al., 2016; Leung et al., 2018; Litaker et al., 2017). Although it is uncommon in heterotrophic dinoflagellates, it should be tested whether *P. inclinatum* has genes related to toxin production and whether it is capable to produce ciguatoxins.

However, it relies in a future availability of cultured strains or obtaining high numbers of cells from natural samples, which is unlikely. Maybe single cell genomics or transcriptomics will elucidate this issue in future.

Gambierdiscus species present a tropical and sub-tropical distribution, although an expansion to more temperate areas, like the Mediterranean Sea (Aligizaki and Nikolaidis, 2008) or temperate areas of Japan (Nishimura et al., 2013) is being reported. *Fukuyoa*

species show a wider distribution, including the tropical Pacific, the North Atlantic, New Zealand, Australia, China, Japan, and the Mediterranean Sea (Gómez et al., 2015; Laza-Martínez et al., 2016; Leung et al., 2018; Litaker et al., 2009; Murray et al., 2014; Nishimura et al., 2013; Rhodes et al., 2014). By contrast, *P. inclinatum* shows a completely different distribution, with records in higher latitudes, like the North Sea, the Atlantic coast of France, the Seto Inland Sea of Japan (Hoppenrath et al. (2014) and references therein) or the temperate waters of the Mediterranean Sea (Hoppenrath et al. 2014 (unpublished obs., Fig. 1F herein), Reñé (unpublished obs.)).

All information available suggests that an ancestor likely planktonic, globular, and autotrophic (i.e. *Triadinium*-like) evolved into an epiphytic, autotrophic, warm-water distributed and with some degree of cell compression organism (i.e. *Gambierdiscus* / *Fukuyoa*-like), and then evolving independently into the sand-dwelling, laterally flattened, heterotrophic and temperate / cold-water species *P. inclinatum*.

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Figure 1: Light microscopy micrographs of *Psammodinium inclinatum* using differential interference contrast (DIC). A-C) Same cell in lateral view in different focal planes, German isolate (Wilhelmshaven 2018). A) Showing the apical pore complex (arrow), the sulcal area (s) with the sulcal pocket (arrowhead) and colored food bodies (fb). B) Note the path of the cingulum (large arrowheads) and two pusules (p) of different size. C) Focus on the right lateral side with descending cingulum (large arrowhead). Note the apical pore complex (arrow). D, E) Same cell in lateral view showing the nucleus (n), German isolate. D) Focus on the left lateral side with ascending cingulum (large arrowhead). E) Note the sulcal area (s) with the sulcal pocket (arrowhead). F) Cell in lateral view, Mediterranean isolate from Elba, Italy (St. Andrea sediment, sampled 2010). Note the sulcal pocket (arrowhead) and two pusules (p) of different size. Scale bars = 10 μ m.

Figure 2: Scanning electron microscopy (SEM) of *Psammodinium inclinatum*

(Wilhelmshaven, sample 2018). A) Left lateral view. B, C) Right lateral view. D) Dorsal to right lateral view. E) Ventral to left lateral view. F) Sulcal area in ventral view. G) Antapical to ventral view showing the two antapical (''') and the posterior intercalary (1p) plates. E-G) Note the suture between the first precingular (1''') and first antapical (1''') plates (arrow). H) Antapex showing the shape of the second antapical plate (2'''). I) Posterior intercalary plate with scattered pores. Scale bars = 10 μ m.

Figure 3: Scanning electron microscopy (SEM) of *Psammodinium inclinatum*, epitheca details (Wilhelmshaven, sample 2018). A) Left lateral to apical view. B) Apical pore complex; apical pore plate with long-shank fishhook-shaped apical pore surrounded by a single row of pores. C) Ventral epitheca, right lateral view. D) Dorsal epitheca, right lateral view. Scale bars = 10 μm , B) 5 μm .

Figure 4: Scanning electron microscopy (SEM) of *Psammodinium inclinatum*. Epithec
 tabulation details from the study partly published by Hoppenrath et al. (2004). A-C) Right
 lateral views. D, E) Ventral view showing the first and seventh precingular plates, arrows
 indicating the suture. F) Sutures between apical plates (arrows). G) Apical pore complex;
 apical pore plate with long-shank fishhook-shaped apical pore surrounded by a single row
 of pores. Scale bars = 10 μ m, G) 5 μ m.

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534 **Figure 5:** Maximum likelihood phylogenetic tree of partial SSU rDNA gene sequences of
 535 selected Gonyaulacales representatives. Members of other dinoflagellate orders were used
 536 as outgroups. Sequences obtained in this study are highlighted in bold. Numbers on the
 537 nodes represent the maximum-likelihood bootstrap values (%) and Bayesian posterior
 538 probabilities. Only values >80% and 0.95 respectively are shown. Black dots represent
 539 nodes with maximum support. The “*Gambierdiscus*” lineage and other *Thecadinium sensu*
 540 *lato* sequences are shaded.

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548 **Figure 6:** Maximum likelihood phylogenetic tree of partial (D1-D3) LSU rDNA gene

549 sequences of selected Gonyaulacales representatives. Members of other dinoflagellate

550 orders were used as outgroups. Sequences obtained in this study are highlighted in bold.

551 Numbers on the nodes represent the maximum-likelihood bootstrap values (%) and

552 Bayesian posterior probabilities. Only values >80% and 0.95 respectively are shown. Black

553 dots represent nodes with maximum support. The “*Gambierdiscus*” lineage and other

554 *Thecadinium sensu lato* sequences are shaded.

Figure 7: Tabulation of *Psammodinium inclinatum*. Comparison with Hoppenrath et al. (2004) and Yoshimatsu et al. (2004). A) *Thecadinium inclinatum*, redrawn from Hoppenrath et al. (2004). B) *Psammodinium inclinatum*, present study. C) *Thecadinium inclinatum*, redrawn from Yoshimatsu et al. (2004). D) Sulcal plates, redrawn from Balech (1956). a) Epitheca. b) Hypotheca. c) Ventral view. d) Dorsal view. e) Left lateral. f) Right lateral. Arrows in B indicate newly observed plate borders compared to the earlier study by Hoppenrath. Large arrows with questionmark in C indicate discrepancies between the drawings and also the SEM images in Yoshimatsu et al. (2004). Dotted lines mark plate sutures visible on their SEMs.

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571 **Figure 8:** Tabulation comparison between A) *Gambierdiscus toxicus*, B) *Psammodinium*

572 *inclinatum*, C) *Fukuyoa paulensis*, and D) *F. yasumotoi*. a) Epitheca. b) Hypotheca. c)

573 Ventral view. d) Dorsal view.

Table 1: Comparison of observed morphological characters of *Psammodinium inclinatum* with existing literature and other phylogenetically close genera. n.d. : not determined.

Species	<i>P. inclinatum</i>	<i>P. inclinatum</i>	<i>P. inclinatum</i>	<i>P. inclinatum</i>	<i>P. inclinatum</i>	<i>Fukuyoa</i> spp.	<i>Gambierdiscus</i> spp.	<i>Triadinium polyedricum</i>
Location	Sylt / Wilhelmshaven (Germany)	Sylt (Germany)	Seto inland Sea (Japan)	Holy Island (United Kingdom)	Roscoff (France)	-	-	Korea
Length / Depth	43.0 - 60.0 μ m	55.0 - 79.0 μ m	55.0 - 75.0 μ m	52 μ m	54 - 64 μ m	45 - 63 μ m	41 - 97 μ m	53 $\pm$ 5.1 μ m
Depth / Width	30.0 - 47.5 μ m	44.0 - 62.0 μ m	43.0 - 59.0 μ m	50 μ m	40 - 48 μ m	38 - 50 μ m	39 - 87 μ m	55 $\pm$ 5.3 μ m
Cell shape	oval	oval to slightly rectangular	elliptical	irregularly ovoid	-	globular	lenticular	globular
Flattening	lateral	lateral	lateral	lateral	lateral	slightly lateral	anterior-posterior	not compressed
Chloroplasts	absent	absent	absent	absent	absent	present	present	present
Nucleus location	central	central	central	n.d.	n.d.	hypothecal	central or hypothecal	central
Thecal surface	smooth with scattered pores	smooth with scattered pores	smooth with small pores	n.d.	n.d.	smooth with small pores	smoothly ornamentated ⁶ , 8, 10, 11, 13, 17, 18 or reticulate-foveate ^{9, 12, 14, 15, 16}	prominent pores
Plate formula	Po 3' 7" ?c ?s 5" 1p 2"	Po 3' 6" 7c 7s 5" 1p 1"	Po 3' 7" 6c ?s 5" 1"	n. d.	n.d.	Po 3' 7" 6c 7s 5" 1p 2"	Po 3' 7" 5" 1p 2"	Po 3' 7" 5" 1p 2"
Cingulum position	epitheca 1/3 - 1/4 of cell length	epitheca 1/3 - 1/4 of cell length	n.d.	epitheca 1/3 of cell length	epitheca 1/3 of cell length	epitheca smaller than hypotheca	central	central
Cingulum	descending	descending	n.d.	n.d.	descending	descending	ascending	descending
Sulcus morphology	pocket-like	pocket-like	n.d.	n.d.	n.d.	not pouch-like	pouch-like	not pouch-like
Apical pore	long-shank hook shaped surrounded by a perforated pore plate	hook shaped surrounded by a perforated pore plate	fishhook shaped with pore plate with small pores	U-shaped surrounded by trichocyst pores	n.d.	long-shank fishhook	short-shank fishhook	short-shank fishhook
Apical plates	1' ~ 2'	1' ~ 2'	1' < 2'	n.d.	n.d.	1' > 2'	1' < 2'	similar
Shape of 1p plate (as 2" in some <i>Gambierdiscus</i> species)	narrow to medium wide	narrow to medium wide	narrow to medium wide (as 1")	n.d.	n.d.	narrow and elongated	wide ^{6, 8, 10, 13, 18} or narrow and elongated ^{9, 10, 11, 12, 14, 15, 16, 17}	wide
Position of 4" plate	dorsal	dorsal (as 3")	dorsal	n.d.	n.d.	dorsal	lateral	lateral
Antapical plates	2" > 1 "	n.d.	n.d.	n. d.	n.d.	2" ~ 1"	2" > 1 "	2" ~ 1"
References	this study	1	2	3	4	5, 6, 7	6, 8-18	19

¹ Hoppenrath et al. (2004); ² Yoshimatsu et al. (2004); ³ Saunders and Dodge (1984); ⁴ Balech (1956); ⁵ Holmes (1998); ⁶ Litaker et al. (2009); ⁷ Gómez et al. (2015); ⁸ Adachi and Fukuyo (1979); ⁹ Faust (1995); ¹⁰ Chinain et al. (1999); ¹¹ Fraga et al. (2011); ¹² Fraga et al. (2016); ¹³ Fraga and Rodríguez (2014); ¹⁴ Nishimura et al. (2014); ¹⁵ Smith et al. (2016); ¹⁶ Kretzschmar et al. (2017); ¹⁷ Rhodes et al. (2017); ¹⁸ Jang et al. (2018); ¹⁹ Shin et al. (2016).

581 **Supplementary Table 1:** List of primers used in the PCRs in order to obtain the partial SSU rDNA and LSU rDNA sequences.

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Primer	Direction	Region	Sequence
PF1	Forward	18S	5'-GCGCTACCTGGTTGATCCTGCC-3'
EK-82F	Forward	18S	5'-GAAACTGCGAATGGCTC-3'
DIN-464F	Forward	18S	5'-TAACAATACAGGGCATCCAT-3'
1050F	Forward	18S	5'-GGGGGAGTATGGTCGCAAG-3'
1250R	Reverse	18S	5'-TAACGGAATTAACCAGACA-3'
EK-1520R	Reverse	18S	5'-CYGCAGGTTACCTAC-3'
R4	Reverse	18S	5'-GATCCTTCTGCAGGTTACCTAC-3'
DinFi	Forward	28S	5'-GCATATAAGTAMGYGGWGG-3'
DinRi	Reverse	28S	5'-CCGTGTTTCAAGACGGGTC-3'
28S-1611R	Reverse	28S	5'-CTTGGASACCTGMTGCGG-3'

597 **Supplementary Table 2:** Relation of molecular sequences obtained, corresponding isolation details and primers used for Sanger
598 sequencing. The positions of the sequenced region are provided with reference to the SSU rDNA sequence of *Alexandrium minutum*
599 (GenBank accession number U27499) and *Fukuyoa yasumotoi* (EF202967) respectively.

Organism	Identifier	Isolation date	Location	Isolated Cells	GenBank accession number	Gene	Sequence Length	Sequenced positions	Primer pairs
<i>Psammodinium inclinatum</i>	MH2009	30/03/2009	Fähranlager, List, Sylt (Germany)	5	MK575197	SSU rDNA	1701	50 - 1781	DIN-464F; EK-1520R; PF1; 1250R; 1050F; R4
					MK575499	LSU rDNA	667	77 - 744	DinFi; DinRi
<i>Psammodinium inclinatum</i>	MH2010	10/06/2010	Fliegerdeich, Wilhelmshaven (Germany)	8	MK575198	SSU rDNA	1489	50 - 1539	EK-82F; DIN-464F; EK-1520R
					MK575500	LSU rDNA	714	34 - 748	DinFi; DinRi
<i>Psammodinium inclinatum</i>	MH2012	20/04/2012	Fliegerdeich, Wilhelmshaven (Germany)	10	MK575199	SSU rDNA	1081	50 - 1131	EK-82F; DIN-464F; EK-1520R
					MK575501	LSU rDNA	671	77 – 748	DinFi; DinRi
<i>Psammodinium inclinatum</i>	AR39	20/08/2018	Fliegerdeich, Wilhelmshaven (Germany)	1	MK575200	SSU rDNA	1108	539 - 1647	DIN-464F; EK-1520R
					MK575502	LSU rDNA	717	28 - 745	DinFi; DinRi

SUPPLEMENTARY MATERIAL 1:

- Details of PCR amplifications:

The three DNA extracts and single-cell isolate were subjected to a first 25 µL PCR using EK-82F – 28S-1611R primers with a PCR mixture containing 2 µL of 10X buffer (TaKaRa Bio), 1.5 mM MgCl₂, 1 U of TaKaRa Taq DNA polymerase (TaKaRa Bio), 0.2mM of each dNTP, and 0.4 mM of each primer. PCR conditions were as follows: initial denaturation for 3 min at 95°C, followed by 6 cycles of 15 s at 95°C, 30 s at 58-53 °C, decreasing 1°C each cycle, and 2 min at 72°C, and 34 additional cycles at annealing temperature of 52°C, followed by a final extension step for 5 min at 72°C. The resulting product was used as template for semi-nested PCRs to amplify the 18S and 28S regions independently, using primers EK-82F and EK-1520R, and DinFi – 28S-1611R respectively. The PCR mixture contained 1 µL of template, 2.5 µL of 10X buffer (Invitrogen, Thermo Fisher Scientific Corp.) containing 15 mM MgCl₂, 1.25 U of Platinum Taq DNA polymerase (Invitrogen, Thermo Fisher Scientific Corp.), 0.2 mM of each dNTP, and 0.4 mM of each primer. PCR conditions were as follows: initial denaturation for 2 min at 94°C, 35 cycles of 15 s at 94°C, 30 s at 55°C, and 1 min at 72°C, followed by a final extension step for 5 min at 72°C. 4 µL of PCR products were electrophoresed in an agarose gel and then visualized under UV illumination. In the case of single-cell isolate AR39, the bands were too faint, and a second semi-nested PCR was conducted using DIN-464F (dinoflagellates specific) and EK-1520R primers for 18S rDNA region, and DinFi-DinRi primers for 28S rDNA region, directly using 1 µL of previous PCR product as template, and under the same conditions than above. PCR products were purified using ExoSAP-IT (Applied Biosystems, Thermo Fisher Scientific Corp.).

Additionally, the DNA extracts corresponding to *P. inclinatum* MH2009 isolates were also amplified using puReTaq Ready-to-go PCR beads (GE Healthcare Bio-Sciences, Inc.) and 0.2 mM of PF1- R4 primers to amplify the complete 18S rDNA region. PCR conditions were as follows: initial denaturation for 4 min at 94°C, followed by 5 cycles of 30 s at 94°C, 1 min at 45°C, and 1 min 45 s at 72°C, and 35 additional cycles at annealing temperature of 55°C, followed by a final extension step for 10 min at 72°C. PCR product was electrophoresed in an agarose gel and then visualized under UV illumination. The band was excised from the gel, purified with QIAquick Gel Extraction Kit (Qiagen Inc.) and 1 µL of purified PCR product was used as template for successive semi-nested PCRs using 0.2 mM of PF1–1250R and 1050F–R4 primer pairs to amplify the different fragments of 18S rDNA. PCR conditions were as follows: initial denaturation for 3 min at 95°C, 44 cycles of 45 s at 95°C, 45 s at 50°C, and 3 min at 72°C, followed by a final extension step for 7 min at 72°C. PCR product was again electrophoresed in an agarose gel, the band was excised from the gel, and purified with QIAquick Gel Extraction Kit (Qiagen Inc.).

644 Sanger sequencing was carried out by external services (Macrogen, Netherlands, and Genoscreen,
645 France) using primers specified in Supplementary Table 2. Fragments were merged using BioEdit
646 v.7.0.9 (Hall, 1999) and in the case of sequence MK575197, forward and reverse fragments did not
647 completely overlap. All 18S rRNA sequences obtained of *P. inclinatum* were aligned, and the missing
648 positions of that sequence was determined and manually added as ambiguities (Ns).

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