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Cyst-Theca Relationship and Phylogenetic Position of *Impagidinium caspienense* Incubated from Caspian Sea Surface Sediments: Relation to *Gonyaulax baltica* and Evidence for Heterospory within Gonyaulacoid Dinoflagellates

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Abstract :

We investigate the cyst-theca relationship of *Impagidinium caspienense*. Through an incubation experiment, we succeeded in examining the motile stage. Additional molecular analysis of single-cyst PCR (LSU and SSU rDNA) reveal that the cyst is related to the species *Gonyaulax baltica* Ellegaard et al. (2002). The ability of this species to belong to two types of cyst-based genera (spiniferate and impagidinioid) suggests that environmental (particularly salinity) and not genetic factors explain the formation of both morphotypes by *Gonyaulax baltica*, which provides evidence for heterospory in this species. The affiliation to *Gonyaulax baltica* demonstrates that *Impagidinium caspienense* is not endemic to the Caspian Sea. The phylogenetic position of several other gonyaulacoid species is also documented: *Impagidinium pallidum, Ataxiodinium choane, Pyxidinopsis psilata, Spiniferites belerius,* and *Spiniferites ramosus*. The LSU and SSU rDNA based phylogenies suggest that the genera *Impagidinium* and *Spiniferites* are not monophyletic, and that *Pyxidinopsis psilata* and *Ataxiodinium choane* are close to *Gonyaulax verior* and *Gonyaulax polygramma*, respectively. In addition, this study accentuates the importance of cyst morphology in the classification of the Gonyaulacales.

Keywords : *Ataxiodinium choane*, Baltic Sea, *Impagidinium pallidum*, *Pyxidinopsis psilata*, salinity, Spiniferites belerius, Spiniferites ramosus

DINOFLAGELLATES form a group of mostly unicellular planktonic organisms, some of which produce fossilizable resting cysts as part of their life cycle. The existence of two life stages, respectively called the motile stage and the cyst stage, has caused the erection of separate biological and paleontological classification systems, and the most recent version of the International Code of Nomenclature for algae, fungi and plants (ICN) stipulates that separate names are allowed for the fossil and living dinoflagellates (effectively cyst and motile stage) (McNeill et al. 2012). In the order Gonyaulacales, several genera have been erected to describe the cyst stages. For some of these genera, the corresponding motile stage has been established, but not for others. One of the most common gonyaulacoid cyst genera is the genus *Spiniferites*, with at least 13 extant cyst-based species [12 reported by Zonneveld et al. (2013) and the recently described *Spiniferites multisphaerus* Price et Pospelova 2014]. Only four of the 13 extant *Spiniferites* species (*S. elongatus* Reid 1974, *S. pachydermus* (M.R.Rossignol 1964) Reid 1974, *S. membranaceus* (M.R.Rossignol 1964) Sarjeant 1970, *S. ramosus* (Ehrenberg 1837) Mantell 1854) have been related to four out of the 21 known motile-based species of *Gonyaulax* (*G. elongata* (Reid 1974) Ellegaard, Daugbjerg, Rochon, J.Lewis et Harding 2003, *G. ellegaardiae* Mertens, Aydin, Takano, Yamaguchi et Matsuoka in Mertens et al. 2015, *G. membranacea* (M.R.Rossignol 1964) Ellegaard, Daugbjerg, Rochon, J.Lewis et Harding 2003, *G. spinifera* (Claparède et Lachmann 1859) Diesing 1866).

One of the most enigmatic gonyaulacoid cyst genera is the genus *Impagidinium* Stover et Evitt 1978, which can be described as *Spiniferites* cysts consistently lacking furcated processes, with *Impagidinium dispertitum* as the type species. The tabulation in *Impagidinium* and *Spiniferites* is considered identical (e.g., Matsuoka and Head 2013). Until now, none of the 12 described extant *Impagidinium* species (e.g., Zonneveld et al. 2013) has been correlated with a motile stage (e.g., Head 1996). In addition, 52 extinct species of *Impagidinium* have been described (Fensome et al. 2008). *Impagidinium* species are important dinoflagellate cyst species in paleoceanographic reconstructions and biostratigraphy because, together with species belonging to the genus *Nematosphaeropsis* Deflandre et Cookson 1955, their increased abundance denotes the presence of oceanic vs. coastal waters (e.g., Dale and Dale 2002; Wall et al. 1977).

The motile-based genus *Gonyaulax*, on the other hand, has been related to at least 12 different cyst morphologies belonging to six different cyst-based genera (Rochon et al. 2009). The motile stages have often been identified as belonging to the *Gonyaulax spinifera* "complex" (Dale 1983), but recent work has shown that this "complex" consists of several pseudocryptic species differing in subtle morphological and genetic features (e.g., Mertens et al. 2015a).

The Caspian Sea is the world's largest inland sea in terms of both area and volume, stretching from 36°N to 62°N (Fig. S1). The catchment area is ~3.5 million km^2 (UNEP 2006). Water inputs comprise river discharges, including the Volga (contributing up to 80–85% of the total), Emba, Ural, and Terek rivers (Rodionov 1994). A north-south gradient in water salinity is generated (Marret et al. 2004, their Fig. 2C, D), with mostly freshwater in the northern end of the basin to almost homogeneous water-column salinity (12.5–13.5 psu) in the central and southern basins (Kosarev and Yablonskaya 1994). In the southern basin, seasonal salinity changes are less than ~0.2–0.4 psu. Mean annual salinity increases from the surface to the bottom waters only by 0.1–0.3 psu (Kosarev and Yablonskaya 1994; Zenkevitch 1963). These specific environmental conditions in the Caspian Sea are considered to have resulted in endemic species of all kinds of organisms, also in endemic dinoflagellate cysts (Marret et al. 2004).

The species *Impagidinium caspienense* was described from recent sediments of the Southern Caspian Sea by Marret et al. (2004), where it is one of the most abundant taxa. Marret et al. (2004) also noted its occurrence in the late Holocene of the Aral Sea, later confirmed by Sorrel et al. (2006). Additionally, the species has been recorded in (1) Late Pleistocene to earliest Holocene sediments of core MAR02-89P from the Marmara Sea shelf (Roberts 2012); (2) Late Holocene sediments of Lake Sapanca, near the southeastern Marmara Sea (Leroy and Albay 2010; Leroy et al. 2009); (3) the early Holocene of the southwestern Black Sea core MAR 02-45P (Marret et al. 2009); (4) the Holocene of the Black Sea (Mudie et al. 2007); (5) Holocene of the Caspian Sea (Leroy et al. 2007, 2013a); (6) the late Pleistocene to Holocene of the centre of the middle and south Caspian Sea basins (Leroy et al. 2013b, 2014); and (7) the northern Caspian Sea (Richards et al. 2014). The irregular height of the septa, the occurrence of a high septum at the junction of the paraplate 1′′′′ and the sulcus, and the low intratabular relief separate *Impagidinium caspienense* from all other extant *Impagidinium* species (Marret et al. 2004, p. 13).

Here, through incubation experiments and molecular analysis, we clarify the cyst-theca relation of *Impagidinium caspiense*, and document its phylogenetic relationships through SSU and LSU rDNA sequencing. In addition, the SSU and LSU rDNA sequences of several further gonyaulacoid cyst-based species are documented to gain further insight in the gonyaulacoid phylogeny: *Impagidinium pallidum* J.P. Bujak 1984, *Ataxiodinium choane* Reid 1974, *Spiniferites belerius* Reid 1974, and *Spiniferites ramosus*.

MATERIALS AND METHODS

Sediment sampling was done by S.B., S.L., A.P., V.P., and K.M. Incubation studies were performed by K.N.M. at the Institute for East China Sea Research at the Nagasaki University (Japan) and at the LER BO, Concarneau (France). Molecular work was done by Y.T. at the same institution in Japan and by H.G. at the Third Institute of Oceanography in China.

Sample collection for cyst incubation

One surface sediment sample was collected from the southwestern (SW) Caspian Sea on the 3rd of September 2011, using a Van Veen grab (37.51°N, 49.91°E; 25 m water depth) for cyst incubation of *Impagidinium caspienense*. This sediment sample was stored in a refrigerator at 4 °C.

Germination experiment

About 0.5–1 cm^3 of wet sediment was immersed in filtered seawater and after one minute of sonication, using an As One^{TM} US-2R sonic bath, the immersion was rinsed through a 20 µm metallic-meshed calibrated Sanpo^{TM} sieve, using filtered seawater. From this residue, the cyst

fraction was separated, using the density method (sodiumpolytungstate (SPT) at a density of 1.3 g cm^{-1} (Bolch 1997)).

Subsequently, single cysts were hand-picked and transferred to Nunclon 0.5-ml microwells at 20 °C filled with EMS medium (salinity 12 psu) (Watanabe et al. 2000), at a light intensity of 100 µmol photons m^{-2} s^{-1} and 24 hours light; temperature and salinity were comparable to the SW Caspian Sea environment. The incubation experiment was initiated on the 9th of November 2011 and followed up until the 6th of December 2011.

Cysts were regularly checked for germination, and observations of the cells were performed under an Olympus IX70 inverted light microscope (Olympus, Tokyo, Japan). Encysted and excysted cysts and vegetative stages were photographed, using an Olympus BX51 light microscope with a Nikon digital sight DS-1L 1 module, all with 100x oil immersion objectives at 1000x magnification.

Kofoidean nomenclature is used to designate the plate numbers. We follow the interpretation of Ellegaard et al. (2002) of the first postcingular homolog as the small elongate plate bordering the sulcus. For simplicity, we chose not to use the 'para' terminology to distinguish features of cysts from the motile counterparts.

Molecular analysis

One cyst and one motile, that germinated from another cyst of *Impagidinium caspienense*, were isolated from the same SW Caspian Sea surface sediment sample for molecular analyses; other gonyaulacoid cyst-based species were also isolated from surface sediment samples from the Caspian Sea or other regions in the world: one cyst of *Impagidinium pallidum*, two cysts of *Ataxiodinium choane*, one cyst of *Spiniferites belerius*, three cysts of *Spiniferites ramosus*, and three cysts of *Pyxidinopsis psilata* (Wall et Dale in Wall et al. 1973) Head 1994 (Fig. S1, Table 1). For all these species, except for *P. psilata*, the following procedure was applied. The cysts or motiles were sonicated in a 200 µL polymerase chain reaction (PCR) tube with sterilized seawater in order to remove extraneous matters. The cysts were individually transferred on a glass slide with a frame of vinyl tape, and then observed and photographed, using an Olympus BX51 microscope equipped with Nomarski differential interference contrast optics (Olympus, Tokyo, Japan) and with an Olympus DP71 digital camera. After taking photographs, the cover slip was carefully removed. Under an inverted microscope (Olympus CKX41), the cell was crushed with a fine glass needle. The whole crushed cell was transferred into a 200 µL PCR tube containing 3 µL of Milli-Q water. This technique and the following PCR protocol are modifications of the methods in Takano and Horiguchi (2005). We determined the sequences of ITS regions (internal transcribed spacer 1 - 5.8 rDNA - ITS2) and partial sequences of LSU rDNA from single cysts and 5–10 cultured cells. In the first round of PCR, the external primers (SR1 and LSU R2; Takano and Horiguchi 2005) were used with PCR mixtures of KOD-Plus-Ver. 2 Kit (Toyobo, Osaka, Japan) and the following PCR conditions: one initial cycle of denaturation at 94 ˚C for 2 min, followed by 35 cycles of denaturation at 94 ˚C for 30 s, annealing at 50 ˚C for 30 s, and extension at 72 ˚C for 2 min and final extension at 72 ˚C for 5 min. In the second round of PCR, two sets of primers (SR12cF and 25R1, LSU D1R and LSU R2; Takano and Horiguchi 2005) were used with PCR mixtures of KOD-Plus-Ver. 2 Kit (Toyobo, Osaka, Japan), 0.5 µL of the first round PCR product as DNA template, and the same PCR conditions, except for an extension at 72 ˚C for 1 min. In the third round of PCR, three sets of primers (SR12cF and 25F1R, LSU D1R and 25R1, LSU D3A and LSU R2; Takano and Horiguchi 2005) were used with PCR mixtures of the TaKaRa EX taq system (Takara Bio Inc., Shiga, Japan), 0.5 µL of

the second round PCR products as DNA template, and the same PCR conditions, except for an extension at 72 ˚C for 30 s. The PCR products were sequenced directly, using the ABI PRISM BigDye Terminator Cycle Sequencing Kit (Perkin-Elmer, Foster City, CA, USA). We sequenced both the forward and reverse strands.

The cysts of *Pyxidinopsis psilata* were isolated and rinsed several times in sterilized water, broken by pressing the cover slip and microscope slide together, and then transferred into a PCR tube. Partial SSU rDNA, partial LSU rDNA, and the total ITS1–5.8S–ITS2 were amplified, using the same procedure mentioned above. The PCR product was purified, using a DNA purification kit (Shangong, Shanghai, China) and sequenced directly in both directions on an ABI PRISM 3730XL (Applied Biosystems, Foster City, CA, USA), following the manufacturer's instructions.

Sequence alignments and phylogenetic analyses

Newly obtained SSU and LSU rDNA sequences were aligned with related sequences downloaded from the GenBank, using the MAFFT v7.110 (Katoh and Standley 2013) online program (http://mafft.cbrc.jp/alignment/server/). The aligned sequences were manually checked with BioEdit v. 7.2.5 (Hall 1999). *Gymnodinium fuscum* and *Prorocentrum micans* were used as outgroup in the SSU and LSU rDNA analyses, respectively. The program jModelTest (Posada 2008) was used to select the most appropriate model of molecular evolution with Akaike Information Criterion (AIC). The Bayesian reconstruction of the data matrix was performed, using MrBayes 3.1 (Ronquist and Huelsenbeck 2003) with the best-fitting substitution model (GTR+G for SSU, TIM3+G for LSU). Four Markov chain Monte Carlo (MCMC) chains ran for 1,000,000 generations, sampling every 100 generations. Convergence diagnostics were graphically estimated, using AWTY (http://ceb.scs.fsu.edu/awty) (Nylander et al. 2008) and the first 10% of burn-in trees were discarded. A majority rule consensus tree was created to examine the posterior probabilities of each clade. Maximum likelihood (ML) analyses were conducted with RAxML v7.2.6 (Stamatakis 2006) on the T-REX web server (Boc et al. 2012), using the GTR+G model. Node support was assessed with 1,000 bootstrap replicates.

Scanning electron microscopy (SEM)

For SEM of motile stages belonging to the cyst-based species *Impagidinium caspienense*, a plankton sample was taken from the SW Caspian Sea at 20 m water depth (37.30 °N, 49.29 °E; on 15th May 2016), using a plankton net. The sample was fixed by ethanol (96%), and later rinsed with distilled water on a metal 20-µm-sieve. Gonyaulacoid motile stages were isolated, using a micropipette from the sieved sample on a polycarbonate membrane filter (GTTP Isopore, 0.22 µm pore size; Millipore, Billerica, MA, USA), and afterwards air-dried for several days. The filters were glued to a stub and sputter-coated, using a Cressington 108 Auto Sputter coater (Cressington, Watford, UK). The cells were observed, using a Zeiss Gemini Sigma 300 field emission SEM (Zeiss, Oberkochen Germany).

For SEM observation of the cyst-based species *Impagidinium caspienense*, the palynological residue from sample US01 (SR9403, 0–3 cm, 38.74 °N, 53.19 °E, 13 m water depth, SE Caspian Sea; Leroy et al. 2013b) was washed with distilled water and dehydrated in a graded ethanol series (30 to 100% in six steps), critical-point dried with CO_2 (Bal-Tec CPD 030; Bal-Tec, L.A., CA, U.S.A.), glued onto a stub, sputter coated with platinum/palladium for 90 s (JEOL JFC-2300 HR; JEOL, Tokyo, Japan) and examined, using a JEOL 6330F scanning electron microscope.

RESULTS

Incubation experiment

A total of 51 cysts of *Impagidinium caspienense* were isolated on 3rd October 2011, of which 15 cysts germinated (29.4%) after one to three weeks of the experiment, but only three cysts (5.9%) germinated into motile cells and formed motile cells that could be studied, although they were often immature. The description below is based on these three cells. All other cells did not succeed in forming complete thecae. We were unable to grow the cells into a culture.

Description of the motile stage of *Impagidinium caspienense*

The robust cell is ellipsoidal in dorso-ventral view (Fig. 1E), while slightly dorso-ventrally flattened in polar view (Fig. 1I). The epitheca is conical with a short apical boss, and the hypotheca is rounded (Fig. 1D, E, H). Germinated specimens had few trichocyst pores and plates with few reticulations (Fig. 1G). The apical pore complex is smooth (Fig. 1C), and surrounded by a low ridge, which is smooth or scalloped (Fig. 1B, C). The tabulation is sexiform gonyaulacoid, with an S-type ventral organization, including the contact between the anterior sulcal plate and the fourth apical homolog. The Q-plate is small and difficult to distinguish from the third and fourth apical plates The ventral pore is large and located between the 4′ and 1′ ('a' in Fig. 1C). Both the sixth precingular and the second postcingular are elongate and subtriangular in shape (Fig. 1D, F). The cingulum in our newly formed cells is levigate (Fig. 1D, F–H). In the germinated specimens, the ends of the cingulum overlap by 1.0–1.1 widths, form an angle of 20 degrees with the main axis of the cells and are displaced by 1.7–2.5 widths (Fig. 1D, F). The sulcus is levigate, and six sulcal plates could be distinguished: the anterior sulcal plate (as), which is hook shaped (Fig. 1D); the median sulcal plate (ms); the broad posterior sulcal plate (ps), which is not as high as wide (Fig. 1F); the right accessory sulcal plate (ras); the right sulcal plate (rs); and the left sulcal plate (ls) (Fig. 1F). A number of short acuminate spines is recognizable on the hypotheca, particularly on the antapical plate (Fig. 1F).

Size: length: 34.4–37.2 µm, width: 30.0–33.1 µm, depth: 27.9–31.8 µm (mean: 35.8 x 31.6 x 29.9 µm; n = 2).

Tabulation formula: 2 pr, 4′, 6′′, 6c, 6s, 6′′′, 1p, 1′′′′.

Description of cyst stage of *Impagidinium caspienense*

The cyst is proximate with subspherical to ellipsoidal ambitus (Fig. 2I). The epicyst has a smooth rounded apex with an apical boss of 0.5–1.1 µm in height (mean: 1.1 µm, n = 7) formed by the apical pore complex (apc) (Fig. 2F, L). Tegillum and luxuria are closely appressed, except along the sutural septa where they are separated (Fig. 2C). The height of the sutural septa is low (1.3–2.4 µm, mean: 1.9 µm, n = 27), except around the antapical plate 1′′′′ where the septa are higher (2.3–4.3 µm, mean: 3.4 µm, n = 6x3) (Fig. 2I, K). The tabulation expressed by the sutural septa delineates a gonyaulacacean tabulation, and is identical to the tabulation expressed by the theca. The cingulum does not overlap, and the displacement is by 2.5–2.7 widths. The sulcal tabulation is expressed, showing five sulcal plates as mentioned for the theca (Fig. 2D, E). These are the large anterior sulcal plate (as), which is hook-shaped (Fig. 2D), the broad posterior sulcal plate (ps), which is not as high as wide (Fig. 2E), the right accessory sulcal plate (ras), the right sulcal plate (rs), and the left sulcal plate (ls) (Fig. 2D). The presence of the pore on 4′ and the flagellar pore could also be observed (resp. Fig. 2A, D). The archeopyle is precingular; in the culture, the plate often remained attached (Fig. 2A, G). The torsion is neutral (Fig. 2G). The wall texture is finely granulate, sometimes with scattered granules and has low intratabular suturo-cavate relief on the plates. When cell contents are still present, a red body is recognizable (Fig. 2J–L).

Size: length (including apical boss): 34.0–39.3 µm, width: 26.8–31.7 µm, depth: 27.0–30.8 µm (mean: 36.8 (n = 9) x 31.7 (n = 9) x 30.8 µm (n = 6)).

Tabulation formula: 2 pr, 4′, 6′′, 6c, 6s, 6′′′, 1p, 1′′′′.

Study of cells from a plankton sample and cysts from palynological residue, using SEM

Several cells were found in a plankton sample from the Anzali wetland (Fig. 3A–D) whose tabulation was identical under the scanning electron microscope (SEM) to the cells germinated from *Impagidinium caspienense* (see above) and also showed the distinct acuminate spines on the antapical plate. However, these planktonic cells had a more pronounced ornamentation of the theca as well as a larger overhang and displacement of the cingulum (four times the width of the cingulum).

Several cysts were studied, using SEM in the palynologically treated surface sediment sample (US01), and again these cysts showed the same tabulation as visible on the cysts identified as *Impagidinium caspienense* (Fig. 3E, F). *Impagidinium caspienense* dominated our sediment samples along with *Lingulodinium machaerophorum*, which were also the most common cysts identified by Marret et al. (2004) in surface sediments of the Caspian Sea.

Molecular analysis of *Impagidinium caspienense*

We determined sequences of the SSU (1,737 bp), ITS regions (534 bp), and partial LSU rDNA (1,240 bp) from one cyst and from one motile cell germinated through incubation of another cyst of *Impagidinium caspienense* collected in the southwestern Caspian Sea (SSU; LC222300, ITS; LC222301, LSU; LC222302). The sequenced cyst is shown in Fig. 2L. The sequences of cyst and motile cell were identical.

Molecular analysis of further species

We determined sequences of the SSU (1,743 bp) and partial LSU rDNA (1,270 bp) of one cyst of *Impagidinium pallidum* (SSU: LC222303, LSU: LC222304; Fig. 4A–C), the SSU (1,731 bp) and partial LSU rDNA (1,221 bp) of two cysts of *Ataxiodinium choane* (SSU: LC222305, LSU; LC222306: Fig. 4D–F), the SSU (1,729 bp) and partial LSU rDNA (1,254 bp) of one cyst of *Spiniferites belerius* (SSU: LC222309, LSU: LC222310; Fig. 4G–I), the SSU (1,743 bp) and partial LSU rDNA (1,254 bp) of three cysts of *Spiniferites ramosus* (SSU: LC222307, LSU: LC222308; Fig. 4J–L), and the SSU (1,635 bp) and partial LSU rDNA (1,293 bp) of three cysts of *Pyxidinopsis psilata* (SSU: KY681700, LSU: KY681701; Fig. 4M–O) (Table 1).

Phylogenies based on LSU rDNA and SSU rDNA

The LSU rDNA based phylogenies (Fig. 5, 6) show that the sequence of *Impagidinium caspienense* is identical with that of *Gonyaulax baltica* (a SSU rDNA sequence is not available for this species). *Impagidinium caspienense/Gonyaulax baltica* forms a well-supported clade with *Spiniferites belerius* in both phylogenies (Bayesian posterior probabilities: 1.0; ML bootstrap support values: 100). Likewise, *Ataxiodinium choane* forms a clade with *Gonyaulax polygramma* in both phylogenies with maximal support. *Impagidinium pallidum* is closest to the clade composed of *Spiniferites belerius* and *Impagidinium caspienense/Gonyaulax baltica* in both phylogenies. *Spiniferites ramosus* is the adelphotaxon to other species previously sequenced as *Spiniferites ramosus* or as *Gonyaulax spinifera*. *Pyxidinopsis psilata* forms a clade with *Gonyaulax verior* with maximal support.

DISCUSSION

Identification of motile stage morphology as *Gonyaulax baltica*

There are currently 71 extant species that belong to the genus *Gonyaulax* Diesing (Gómez 2012), of which only 21 are assigned to this genus, using the *Gonyaulax* generic concept of Dodge (1989), i.e., the 17 species listed by Dodge (1989), plus the four recently established ones: *G. baltica* Ellegaard, Lewis et Harding 2002, *G. ellegaardiae* Mertens, Aydin, Takano, Yamaguchi et Matsuoka 2015, *G. membranacea* (Rossignol) Ellegaard, Daugbjerg, Rochon, Lewis et Harding 2003, *G. elongata* (Reid) Ellegaard, Daugbjerg, Rochon, Lewis et Harding 2003). The germinated cells of *Impagidinium caspienense* match only *Gonyaulax baltica* as described by Ellegaard et al. (2002), particularly in the long triangular sixth precingular plate, the torsion, and the many small spines on the antapical plates that are characteristic for the motile cell of *Gonyaulax baltica* (see Table 6 in Ellegaard et al. 2002). There are some differences, more particularly in the overhang and displacement of the cingulum (resp. 1.0–1.1 widths and 1.7–2.5 widths), which are less distinct than in the specimens of Ellegaard et al. (2002) (resp. 2–3.3 widths and 2.5–4.5 widths). However, planktonic cells also show such a large displacement and overhang (Fig. 3A–D). The more pronounced ornamentation of the theca of the planktonic cells is not unusual, since these show more mature features than freshly incubated thecae; this is consistent with the developmental series observed in plankton (e.g., Taylor 1962).

Identification of cysts as *Impagidinium caspienense*

The cysts correspond to *Impagidinium caspienense* as described from recent surface sediments by Marret et al. (2004), except for some differences. The enlarged septum is located within the antapical plate, and not on the junction between plate 1′′′′ and the sulcus. Another difference in interpretation is the first postcingular homolog as the elongate small plate bordering the sulcus (Fig. 2E), which is illustrated differently in the interpretation of Marret et al. (2004) of the tabulation (their Fig. 3E), which is probably a mistake in their drawing. The ventral pore and the complete sulcal tabulation were not observed by Marret et al. (2004). The SEM investigation of sample US01, shows the fine granulate texture and a sulcal tabulation in these cysts matching *I. caspienense* (Fig. 3E).

Comparison of cyst morphology of *Impagidinium caspienense* from the Caspian sea with the cyst of *Gonyaulax baltica* from the Baltic Sea

Impagidinium caspienense described here from the Caspian Sea, differs from the cyst of *Gonyaulax baltica* as depicted by Ellegaard et al. (2002) from the Baltic Sea, in that the latter either bear furcate processes or lack the sutural septa that are consistently present on *Impagidinium caspienense* fossilized in sediments. However, here we consider that cysts that can be identified as *Impagidinium caspienense* in the Caspian Sea, are also present in the Kattegat-Baltic, and that it is an end member in cysts produced by *Gonyaulax baltica*.. In fact, Dale (1996, p. 1262) previously reported the presence of *Spiniferites* species with extremely reduced processes in the Baltic Sea that he considered could be included in the generic description of *Impagidinium*, but no illustrations of these specimens were provided. In addition, Ellegaard (2000, p. 73) noted that many *Spiniferites* with reduced processes she observed in a fjord in Northern Denmark (Limfjord) could be attributed to *Impagidinium*, and specimens shown in that publication as *Spiniferites* cf. *bulloideus* (her plate II, Fig. 1–4) and *Spiniferites membranaceus* (her Plate II, Fig. 7) can be assigned to *Impagidinium caspienense*, in our opinion. In addition, the gradient in morphology between the *Spiniferites* morphologies associated with *Gonyaulax baltica* with long processes, and reduction in process length towards morphologies that can be attributed to *Impagidinium caspienense*, can be related to lowering of sea surface salinity. Such a morphological gradient is observed in

culture experiments of *Gonyaulax baltica* (Ellegaard et al. 2002) and across the salinity gradient in the Baltic Sea (Dale 1996; Gundersen 1988).

Molecular identification of *Impagidinium caspienense*

Comparison of the obtained sequences shows that they are identical to the LSU rDNA sequence of *Gonyaulax baltica* in the original description by Ellegaard et al. (2002). Unfortunately, no SSU rDNA sequence is available for *Gonyaulax baltica*.

Resulting identification of motile stage of *Impagidinium caspienense*

The identification of the species was based on four datasets, namely the morphology and the molecular analysis of the motile stage and cyst. The observed motile stage and the molecular analysis of the cyst support the identification as cyst and motile stage of *Gonyaulax baltica*. *Gonyaulax baltica* which had been described by Ellegaard et al. (2002) through germination of *Spiniferites* cysts isolated from the Kattegat (salinities of upper layer typically between 18–26 psu; Leppäranta and Myrberg 2009). The cysts studied here correspond to *Impagidinium caspienense* as described by Marret et al. (2004). The present study is the first in which a cyst-theca relation is established for an *Impagidinium* species, confirming earlier suggestions that *Impagidinium* cysts are produced by members of the *Gonyaulax spinifera* group (Dale 1983). Dale (1976) applied the informal term "*Gonyaulax spinifera* group" to those cysts that apparently correlate to either *G. spinifera*, *G. digitale*, or *G. scrippsae*. *Gonyaulax baltica* is considered similar to *G. scrippsae* (Ellegaard et al. 2002, p. 783). In conclusion, *G. baltica* and *I. caspienense* form a single species.

Is *Impagidinium caspienense* a true *Impagidinium* species?

In terms of cyst morphology, there is no problem to classify *Impagidinium caspienense* as an *Impagidinium* species, following the original description of *Impagidinium* by Stover and Evitt (1978, p. 165) which allows for some variation in the tabulation and morphology. However, *Impagidinium caspienense* bears an apical boss, which Stover and Evitt (1978) consider atypical for *Impagidinium* (they use the term "horn").

Impagidinium caspienense has an exceptional autecology as it is the only *Impagidinium* species that is abundant in coastal waters, while its congeners are dominant in oceanic environments (e.g., Dale and Dale 2002; Zonneveld et al. 2013). Also, the cysts do not seem to show the same sturdiness as other common *Impagidinium* species; *Impagidinium caspienense* cysts are often folded, compressed or torn after palynological preparation.

In addition, to its exceptional ecological niche, the phylogenetic position of *Impagidinium caspienense* suggests that it does not belong to the genus *Impagidinium*, which is not monophyletic in the trees because of the separate position of *Impagidinium pallidum*. *Impagidinium caspienense* is an even more unique *Impagidinium* species because its motile stage, *Gonyaulax baltica,* is known to also produce *Spiniferites* type cysts (Ellegaard et al. 2002). This is thus the first time that it is shown that one organism can produce both types of cysts (spiniferate and impagidinioid), and can be considered the first concrete evidence for so-called "heterospory". Wall and Dale (1968) coined this term to describe their observations of gonyaulacoid species, which already suggested that one thecate stage may produce two or more types of cyst-based species. This should not be confused with a large gradient in cyst morphology, which does not necessarily include different cyst-based species (e.g., as documented for *Lingulodinium machaerophorum* by Mertens et al. 2009). Later, culture experiments using strains of *Gonyaulax spinifera* suggested the formation of cysts assigned to *Spiniferites ramosus* and *Nematosphaeropsis labyrinthus* at different salinity and temperature conditions (Rochon et al. 2009), which further pointed towards the possibility of heterospory

in *Gonyaulax*. The discovery of heterospory can have profound taxonomic implications. If one species (*Gonyaulax baltica*) can produce two cyst-based species belonging to two cyst-based genera (*Spiniferites* and *Impagidinium*), this would suggest that both genera have to be lumped. An obvious solution would be to reassign *Impagidinium caspienense* to a new cyst-based genus that would also allow spiniferate morphologies; but, this would be premature because we lack too much phylogenetic information about other *Impagidinium* species. Fortunately, presently there is no nomenclatural conflict: because there is no cyst-based name for the *Spiniferites* species associated with *Gonyaulax baltica* (Ellegaard et al. 2002), and for such specimens, the informal name "*Spiniferites bulloideus* sensu Gundersen 1988" can be used. The names *Gonyaulax baltica* and *Impagidinium caspienense* can coexist for now because the ICN stipulates that separate names are allowed for fossil and living dinoflagellates (McNeill et al. 2012). When the correct name for the *Spiniferites* species associated with *Gonyaulax baltica* is identified, in combination with further cyst-theca experiments and sequencing of other *Impagidinium* species, a solution will need to be found for these complicated issues.

Are there endemic species in the Caspian Sea?

The identification of *Impagidinium caspienense* as belonging to *Gonyaulax baltica* suggests that *Gonyaulax baltica* is distributed in both the Caspian Sea and the Baltic Sea. Other Caspian Sea species have also been shown to be more widely distributed, such as *Lingulodinium polyedra* (Mertens et al. 2012), *Kolkwitziella acuta* (Mertens et al. 2015b), and *Scrippsiella plana* (Luo et al. 2016). However, the sequence of *Pyxidinopsis psilata* documented here is unique and it might be a species endemic to the Caspian Sea, although this cyst type had originally been described from early Holocene sediments of the Black Sea (Wall et al. 1973; as *Tectatodinium psilatum*) and it is occasionally recorded in more recent sediments of the Black Sea (e.g., Mudie et al. 2004); so, this species could have a more widespread distribution. The remaining potentially endemic species are *Spiniferites cruciformis* and *Caspidinium rugosum* (Marret et al. 2004). Future studies should investigate their affiliation and distribution, to establish whether they are truly endemic.

Implications for cyst-based and motile-based classification

The phylogenies demonstrate that both *Impagidinium* and *Spiniferites* are not monophyletic, and *Ataxiodinium* and *Bitectatodinium* are nested within *Spiniferites* (Fig. 5, 6). This problem is currently difficult to resolve without making radical changes to the cyst-based taxonomy. Accordingly, we choose to be conservative and await more information from cyst-theca experiments in combination with sequencing in other genera, such as *Achomosphaera, Nematosphaeropsis, Tectatodinium,* etc. to better understand the phylogenies.

In addition, the present phylogenies show that *Ataxiodinium choane* is close to *Gonyaulax polygramma* and that *Pyxidinopsis psilata* is close to *Gonyaulax verior* (Fig. 5, 6). These results imply that the motile-based genus *Gonyaulax* will need to be split into different genera. It is particularly of note that *Gonyaulax* is not monophyletic because *Gonyaulax verior* is nested in a separate clade. This is suggestive for the fact that *Gonyaulax verior* will need to be transferred into a different genus, as supported by its unique tabulation that is different from *Gonyaulax, Amylax,* and *Lingulodinium*; however, this is complicated because two different tabulations have been recorded for *Gonyaulax verior* (Matsuoka et al. 1988; Zonneveld and Dale 1994) and will thus need further study.

Conclusions

Here, we show through incubation experiments and single-cell PCR that the motile-based species *Gonyaulax baltica* can form two types of cyst morphologies belonging to two cyst-based genera: *Spiniferites* and *Impagidinium*. This is the first definite proof for heterospory within gonyaulacoid dinoflagellates. Also, this shows that *Impagidinium caspienense* is not endemic to the Caspian Sea and that *Gonyaulax baltica* is more widely distributed as well. The LSU and SSU rDNA based phylogenies suggest that the genera *Impagidinium* and *Spiniferites* are not monophyletic and that *Pyxidinopsis psilata* and *Ataxiodinium choane* are close to respectively *Gonyaulax verior* and *Gonyaulax polygramma*. *Ataxiodinium* and *Pyxidinopsis* belong to different clades in the phylogenies, stressing the importance of cyst morphologies for future reclassification of the genus *Gonyaulax.*

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FIGURE LEGEND

Figure 1 Light micrographs of a thecate stage, germinated from the *Impagidinium caspienense* cyst shown in Fig. 2A–C, E–H. (**A**) Antapical view of hypotheca (low focus). (**B**) Apical view of epitheca (high focus). **C.** Apical view of epitheca, showing apical pore complex and arrow shows pore on 4´ plate. (**D**) Ventral side showing extensive torsion and long 6´´ plate, both characteristic for *Gonyaulax baltica*, and hookshaped anterior sulcal plate (as). (**E**) Longitudinal optical section. (**F**) View of hypotheca showing sulcal area, arrows mark short antapical spines typical for *Gonyaulax baltica*. The posterior sulcal plate (ps),

right accessory sulcal plate (ras), right sulcal plate (rs), and left sulcal plate (ls) are shown. (**G**) Lateral view showing 1´´ plate with trichocyst pores. (**H**) Dorsal view. (**I**) Polar view of optical cross section. Scale bars = 10 µm.

Figure 2 Light micrographs of *Impagidinium caspienense*. Cyst stage related to the motile stage shown in Fig. 1 (A–C, E–H; other specimens are shown in D, I and J, K, L) (**A**) Apical view of epicyst showing opened operculum (corresponding to 3´´), arrow marks ventral pore on 4´ (vp). (**B**) Antapical view of hypocyst. (**C**) Dorsal view showing opened archeopyle. (**D**) Ventral view. (**E, F**) Ventral view showing some of the sulcal plates and the cingular displacement. (**G**) Dorsal view. (**H**) Lateral right view. (**D, I**) Other specimen. (**I**) Optical section showing ambitus. (**J, K**) Optical section of cysts will cell contents isolated from sediment showing orange inclusion and lipid droplets. (**L**) Longitudinal optical section of cyst with cell content used for single cyst-PCR. Scale bars = 10 µm.

Figure 3 Scanning electron microscope images of life stages of *Impagidinium caspienense*. (**A–D**) Motile stage of *Impagidinium caspienense* as observed in the southwestern Caspian Sea. (**A**) Ventral view showing the tabulation and the elongated 6´´. (**B**) Detail of cell shown in A. (**C**) Hypotheca showing the full tabulation. (**D**) Detail of cell shown in A, showing the distinct presence of spines on the hypotheca. (**E, F**) *Impagidinium caspienense* as observed in surface sediment from the SE Caspian Sea (US01). (**E**) Ventral view showing sulcal tabulation: anterior sulcal plate (as), posterior sulcal plate (ps), right accessory sulcal plate (ras), median sulcal plate (m), right sulcal plate (rs), and left sulcal plate (ls). (**F**) Finely granulate surface of the cysts and the precingular archeopyle. Scale bars = 10 µm.

Figure 4 Light micrographs of further species that were sequenced, specimens shown are those that were used for molecular analysis, except for O. (**A–C**) Different focal planes of same specimen of *Impagidinium pallidum* Bujak 1984 from surface sediment in Gibbs Fjord (NE Baffin Island). (**D–F**) Different focal planes of single specimen of *Ataxiodinium choane* Reid 1974 isolated from surface sediment in the Kattegat, Denmark. (**G–I**) Different focal plates of same specimen of *Spiniferites belerius* Reid 1974 isolated from surface sediment in the Saroma Lake (Hokkaido, Japan). (**J–L**) Different focal planes of same specimen of *Spiniferites ramosus* (Ehrenberg) Mantell 1854 isolated from surface sediment in the Strait of Georgia. (**M–O**) Three different specimens of *Pyxidinopsis psilata* (Wall and Dale in Wall et al. 1973) Head 1994 isolated from surface sediment in the SW Caspian Sea. Scale bars = 10 µm.

Figure 5 Molecular phylogeny of *Impagidinium caspienense* inferred from partial large subunit rDNA (LSrDNA) sequences based on Bayesian inference (BI). *Prorocentrum micans* was used as outgroup. Numbers at nodes represent Bayesian posterior probabilities and the ML bootstrap values; asterisks indicate the maximal support in BA and ML (1.0 and 100%, respectively). Bootstrap values >50% and posterior probabilities above 0.7 are shown. Newly obtained sequences were indicated as bold. Scale bar = nucleotide substitutions per site.

Figure 6 Molecular phylogeny of *Impagidinium caspienense* inferred from partial small subunit rDNA (SSrDNA) sequences based on Bayesian inference (BI). *Gymnodinium fuscum* was used as outgroup. Numbers at nodes represent Bayesian posterior probabilities and the ML bootstrap values; asterisks indicate the maximal support in BA and ML (1.0 and 100%, respectively). Bootstrap values >50% and posterior probabilities above 0.7 are shown. Newly obtained sequences were indicated as bold. Scale bar = nucleotide substitutions per site.

SUPPORTING INFORMATION

Figure S1 Map showing the sampling locations mentioned in the text.

Table 1. Species isolated for single-cell PCR.

Species name	Locality	Latitude	Longitude	Water depth (m)	Sampling date	Sampling device	LSU	SSU	Sampled by
Ataxiodinium choane	Kattegat, Denmark	57.50 °N	11.80 °E	28	01.05.2010	Boxcore	XXXXX	XXXXX	Anna Godhe
Pyxidinopsis psilata	SW Caspian Sea, Iran	37.51 °N	49.91 °E	25	03.09.2011	Van Veen grab	XXXXX	XXXXX	Siamak Bagheri
Impagidinium pallidum	Gibbs Fjord, NE Baffin Island, Canada	70.76 °N	72.25 °W	447	21.10.2011	Boxcore	XXXXX	XXXXX	Anna Pienkowski
Spiniferites belerius	Saroma Lake, Hokkaido, Japan	44.12 °N	143.87 °E	18.3	22.07.2011	TFO corer	XXXXX	XXXXX	Yoshihito Takano & Kenneth Mertens
Spiniferites ramosus	Strait of Georgia, Canada	49.04 °N	123.43 °W	300	02.10.2011	ROPOS	XXXXX	XXXXX	Vera Pospelova