Huia caspica gen. & comb. nov., a dinoflagellate species that recently crossed the marine-freshwater boundary

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Abstract :

The dinoflagellate subfamily Diplopsalidoideae encompasses 11 genera whose plate patterns show a large diversity. In a recently published molecular phylogeny (Liu *et al.* 2015) some of these genera (e.g. *Diplopsalis, Diplopelta*) are polyphyletic, suggesting that further subdivision of these genera is needed. Here we established the cyst-theca relationship of *Diplopsalis caspica* by incubating cysts collected from the East China Sea. Cells of *D. caspica* display a plate formula of Po, X, 3′, 1a, 6″, 3c+t, ?4s, 5‴, 1⁗, characterized by a small, parallelogrammic anterior intercalary plate (1a) located in the middle of the dorsal part of the epitheca. The cysts are spherical and smooth-walled with a theropylic archeopyle. In addition, we obtained four large subunit ribosomal DNA (LSU rDNA) sequences from the germinated motile cells by single-cell polymerase chain reaction. Strains of *D. caspica* from the marine environment of the East China Sea differ at 0–2 positions of LSU rDNA sequences from that of lacustrine strains from NE China. In the molecular phylogeny, *D. caspica* was close to *Lebouraia pusilla* but distant from *D. lenticula*, the type species of *Diplopsalis*. Our results support the systematic importance of plate 1a, and therefore *D. caspica* was transferred to a new genus, *Huia*. The conservative LSU rDNA sequences in *H. caspica* suggest that the marine-freshwater transition occurred recently.

Keywords : cyst-theca relationship, diplopsalioideans, *Diplopsalis caspica*, *Glenodinium caspica*, LSU rDNA

INTRODUCTION

The dinoflagellate subfamily Diplopsalidoideae Abé, informally called the diplopsalioideans, includes 11 genera, characterized by four or five climactal plates, one antapical (fundital) plate or either of these features (Matsuoka 1988; Fensome *et al.* 1993). The type genus of this subfamily, *Diplopsalis* Bergh, was characterized by the plate pattern of Po, X, 3′, 1a, 6″, 3c+t, 5s?, 5‴, 1⁗ (Bergh & Daday 1881; Nie 1943). Other genera (e.g. *Boreadinium* J. D. Dodge & H. B. Hermes, *Diplopelta* Stein, *Diplopsalopsis* Meunier, *Oblea* Balech *ex* Loeblich Jr. & Loeblich III, *Preperidinium* Mangin) differ from *Diplopsalis* only in the number of anterior intercalary or precingular /antapical plates (Abé 1941; Abé 1981; Dodge & Hermes 1981; Dodge & Toriumi 1993).

   Diplopsalis caspica was originally described by Ostenfeld (1902) as possessing three apical plates, and five - what he called - "praemedian" plates, which corresponds to four precingular plates and a small diamond shaped anterior intercalary plate. Later, Ostenfeld (1908) re-observed this species from the Aral Sea, did not comment on his previous description, but redrew the species as having six precingular plates, sharing an identical plate pattern with *D. lenticula*. Lemmermann (1910, p. 674) considered it to belong to the genus *Peridinium* Ehrenberg, and transferred it to *Peridinium caspicum* (Ostenfeld) Lemmermann. Lindemann (1927, p. 419-420) restudied samples from the Caspian Sea and did not agree with Lemmermann's reassignment of the species and considered Ostenfeld's specimen published in 1908 a typical *Diplopsalis*; his own specimens however showed variation in the shape of second

intercalary plate (being quadra or penta) and number of precingular plates. It is doubtful whether these correspond to the same species. Schiller (1935, p. 110) however, considered it to belong to the genus *Glenodinium* Ehrenberg, and named it *Glenodinium caspicum* (Ostenfeld) Schiller. The species has not been reported subsequently although it is easily recognized by the small, diamond shaped anterior intercalary plate. In 2015 it was again reported from Jihongtan reservoir at Qingdao, Shandong Province, China, and its detailed morphology was documented for the first time (Zhang *et al.* 2015). Zhang *et al.* (2015) provided a complete tabulation of Po, X, 3′, 1a, 6″, 4c, 4s, 5‴, 1⁗. This tabulation and the phylogenetic position reported by Zhang *et al*. (2015) suggested a warranted attribution of this species to the genus *Diplopsalis*.

Another molecular phylogeny published later, based on large subunit ribosomal DNA (LSU rDNA) sequences largely supported the validity of the diplopsalid genera, but two new genera (*Niea* T. Liu, K.N. Mertens & H. Gu and *Qia* T. Liu, K.N. Mertens & H. Gu) were established based on the shape of the first apical plate or the anterior intercalary plate, although they share an identical plate pattern with *Oblea* and *Diplopsalis* (Liu *et al.* 2015). As a consequence, the genera *Diplopsalis* and *Oblea* were emended by Liu *et al.* (2015). However, *Diplopsalis caspica* did not fit this emended description, and in the molecular phylogeny reported by Liu *et al.* (2015) the genus *Diplopsalis* is polyphyletic, suggesting that nomenclatural changes are needed. These systematic changes are suggested here.

A cyst stage has not been reported for *Diplopsalis caspica,* although cyst stages are commonly found in the diplopsalioideans. In contrast with the closely related *Protoperidinium* species, the cyst morphology of diplopsalioideans is rather conservative. The latter are generally spherical and smooth with a theropylic archeopyle, corresponding to suturing along predetermined plate boundaries (Matsuoka 1988; Lewis 1990; Dale *et al.* 1993). Only three species, *Niea acanthocysta* (Kawami, Iwataki & Matsuoka) T. Liu, K.N. Mertens & H. Gu, *Niea chinensis* T. Liu, K.N. Mertens & H. Gu and *Diplopelta symmetrica* Pavillard, produce cysts with processes (Dale *et al.* 1993; Kawami *et al.* 2006; Liu *et al.* 2015), which is helpful to differentiate species with similar thecate morphology. Here the cyst-theca relationship of *Diplopsalis caspica* is established for the first time.

In terms of ecological preferences *D. caspica* has previously been reported from brackish waters by Ostenfeld (1902, 1908) and from fresh water by Zhang *et al.* (2015). There are approximately 1,700 extant marine species and 220 freshwater species of dinoflagellates (Taylor *et al.* 2008). The first marine dinoflagellates are suggested to have originated around 250 million years ago based on the fossil record (Fensome *et al.* 1996), however, only a fraction of dinoflagellates have succeeded in colonizing fresh water habitat (Logares *et al.* 2007b). The earliest colonization extended back to 150 million years ago, based on the fossil cyst record produced by freshwater dinoflagellates (Batten 1989). More recent invasions were reported for the lacustrine species "*Peridinium*" *aciculiferum*, which occurred around 30 million years ago (Logares *et al.* 2007b). Historical colonization inevitably led to ecological

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speciation, but species splitting might have not finished yet, such as *Kolkwitziella acuta* (Apstein) Elbrächter and *Diplopsalis caspica*, two species inhabiting brackish as well as fresh water (Ostenfeld 1902; Mertens *et al.* 2015; Zhang *et al.* 2015). Here we report the presence of *D. caspica* from fully marine waters for the first time, report its cyst-theca relationship and phylogenetic position using a newly obtained LSU rDNA sequence, and explore when the marine-freshwater transition occurred.

MATERIALS AND METHODS

Surface sediment samples were collected with a small boat using a Van Veen grab nearby Fuzhou (East China Sea, 25.8275°N, 119.7307°E, salinity=28) on November 28, 2010 (Fig. S1). All samples were stored in the dark at 4°C until further treatment. Approximately 5 g wet weight of sediment material was diluted in a 20 mL beaker with filtered seawater with a salinity of 30 and sonicated for 1 min in an ultrasonic bath (JY96 Xinzhi, Ningbo, China). The water slurry was rinsed with filtered seawater whilst sieving through a 20 µm nylon mesh. The fraction retained by the sieve was resuspended with 1 mL filtered seawater, and used for cyst isolation. Using a BX51 microscope (Olympus Corp., Tokyo, Japan), nine spherical and brown cysts were isolated with a micropipette into 96 well plates, with each well containing 300 µL f/2-Si medium (Guillard & Ryther 1962). The plates were incubated at 20°C, 90 µmol photons $\cdot$ m^{-2} $\cdot$ s^{-1} with a light: dark cycle of 12:12 h, and examined daily with an inverted microscope AE31 (Motic, Xiamen, China).

After germination, both cells and empty cysts were individually transferred onto cleaned glass slides and examined at × 400 magnification with the light microscope

with a digital camera (Qimaging, Burnaby, Canada). No mounting medium was used. Calcofluor white was used to discern plates in vegetative cells (Fritz & Triemer 1985). The Kofoidian system was used for designating the thecal plates (Fensome *et al.* 1993). All cyst and motile cell measurements in the species descriptions cite the minimum, average (in parentheses) and maximum values, in that order.

Identified cells were rinsed several times in sterilized distilled water, broken by squeezing the coverslip above, and then transferred into a PCR tube. Four germinated cells were used as the template to amplify about 1430 bp of the LSU rRNA gene (D1-D6 domains), using the primers D1R (forward, 5′-ACCCGCTGAATTTAAGCATA-3′) (Scholin *et al.* 1994) and 28-1483R (reverse, 5′-GCTACTACCACCAAGATCTGC-3′) (Daugbjerg *et al.* 2000). A 50 μL PCR cocktail containing 0.2 μM forward and reverse primer, PCR buffer, 50 μM dNTP, 1U of Taq DNA polymerase (Takara, Dalian, China) was subjected to 35 cycles using a Mastercycler PCR (Eppendorf, Hamburg, Germany). The PCR protocol was identical to that of Liu *et al.* (2015). Sequences were deposited in GenBank (KX056274 − KX056276).

Sequences newly obtained were first aligned with those of related species available in GenBank using BioEdit v7.0.0 (Hall 1999) and Mafft (Katoh *et al.* 2005). The apicomplexan *Neospora caninum* Dubey, Carpenter, Speer, Topper & Ugglawas selected as an outgroup. A general time reversible model (GTR +G) was selected with Akaike information criterion based on the result of JmodelTest (Posada 2008). Maximum likelihood (ML) analyses were conducted with „RAxML" v7.2.6

(Stamatakis 2006) using the best-fitting substitution model on the T-REX web server

(Boc *et al.* 2012). Bootstrap analysis was based on 1000 replicates. A Bayesian

inference (BI) analysis was performed with MrBayes 3.0b4 (Ronquist & Huelsenbeck

2003) using the best-fitting substitution model. Four Markov chain Monte Carlo

chains ran for ten million generations, sampling every 1000 generations with a burnin

of 10%. A majority rule consensus tree was created in order to examine the posterior

probabilities of each clade.

RESULTS

Huia H. Gu, K.N. Mertens, T. Liu gen. nov.

Description. Armoured dinoflagellate with a plate formula of Po, X, 3′, 1a, 6″,

3c+t, ?4s, 5‴, 1⁗. A small, parallelogrammic anterior intercalary plate (1a) located in

the middle of the dorsal part of the epitheca. Cysts spherical and smooth, with a

theropylic archeopyle.

Type species: *Huia caspica* (Ostenfeld) H. Gu, K.N. Mertens, T. Liu comb. nov.

Basionym: *Diplopsalis caspica* Ostenfeld (1902, Phytoplankton fra det Kaspiske

Hav. Videnskabelige Meddelelser fra Dansk Naturhistorisk Forening 1901: pp. 132,

133, fig. 1).

Synonyms: *Peridinium caspicum* (Ostenfeld) Lemmermann 1910, p. 674, figs 1–5;

Glenodinium caspicum (Ostenfeld) Schiller 1935, p. 110, fig. 105a–d.

Etymology: the genus is named in honor of professor Hongjun Hu who has carried

out pioneering taxonomic work on freshwater microalgae in China.

A total of six cysts germinated which yielded motile cells after between one to

seven days of incubation. The vegetative cells are subspherical, contain lipid bodies and are pale green in color (Fig. 1). The plate formula is Po, X, 3′, 1a, 6″, 3c+t,?4s, 5‴, 1⁗. The first apical plate (1′) is rhombic (ortho-type), in touch with the canal plate and anterior part of the anterior sulcal plate (Sa) (Figs 2, 4). The apical pore complex consists of a round pore plate (Po) raising to form a pronounced horn and a short canal plate (X) (Fig.3). Plates 2′ and 3′ are six-sided with the latter relatively larger (Fig. 5). There is only one small, parallelogrammic anterior intercalary plate (1a) located in the middle of the dorsal part of the epitheca (Fig. 5). Among the six precingular plates, plates 3″, 4″ and 6″ are five-sided whereas the others are four-sided (Fig. 5). The cingulum consists of three cingular plates plus a transitional plate and descends slightly with a displacement less than half of its width (Figs 2, 4, 6). The cingulum has short cingular list with ribs (Figs 2, 4). We were unable to verify the exact number of sulcal plates, but it comprises at least a Sa plate, a left sulcal plate (Ss), a right sulcal plate (Sd) and a posterior plate (Sp) (Figs 2, 7). The cells have a C type of anterior sulcal (Sa) plate sensu Dodge & Hermes (1981). The left sulcal plate (Ss) is sheltered by a large list on the left of the sulcus (Fig. 2); The posterior sulcus plate (Sp) is small and of the Caspica–type (Fig. 7). The five postcingular plates are four-sided and there is only one large antapical plate (Fig. 7). The thecal plates are ornamented with numerous pores, which are often arranged in rows in marginal areas (Figs 5, 7). The living cyst is spherical and brown, full of pale granules and has a thick wall (Fig. 8). The surface of the cyst wall is smooth and devoid of ornamentation (Fig. 9). The archeopyle is theropylic, following the sutures between

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plates 2′, 1a, 3′ and 2″, 3″, 4″, 5″ (Figs 10–12).

The incubated motile cells are 30.0 (35.8) 38.0 μm long and 32.0 (38.2) 40.0 μm wide (n=6), and the cysts germinated to give identifiable thecae are 35.0 (39.7) 43.0 μm in diameter (n=6).

Molecular phylogenetics

Four cells of *Huia caspica* from the East China Sea share nearly identical LSU rDNA sequences, differing only in 1 or 2 positions. One of these sequences is identical to that of *Diplopsalis caspica* (KJ995959) isolated from freshwater (Zhang *et al.* 2015).

The best phylogenetic tree constructed by BI analyses (Fig. 13) was similar to the ML tree differing only in a few internal nodes, such as the *Peridinium cinctum* (O. F. Müller) Ehrenberg and *Akashiwo sanguinea* (K. Hirasaka) G. Hansen & Ø. Moestrup groups. The subfamily Diplopsalidoideae was polyphyletic and consisted of three well-resolved clades (I, II, III) intermingled by clades *Protoperidinium* sensu stricto, Oceanica and Monovela. *Huia caspica* and *Lebouraia pusilla* (Balech & R.Akselman) J. D. Dodge & S. Toriumi form a clade with strong support (ML bootstrap: 100/ BI posterior probability: 0.95). They were nested within clade Diplopsalidoideae I together with *Diplopsalis lenticula* Bergh, *Oblea rotunda* and *Boreadinium breve* (Abé) Sournia.

DISCUSSION

Here we present evidence that *H. caspica* can inhabit fully marine environments for the first time. The marine cells of *H. caspica* (32–40 μm) are more slender than those

from brackish (56–72 μm) and fresh water (48–60 μm) (Ostenfeld 1902; Zhang *et al.* 2015). We consider them conspecific since the size of cells can be variable in many species (e.g. Mertens *et al.* 2015).

Like other diplopsalioideans, *Huia caspica* has a complex taxonomic history. It has been transferred to *Peridinium* (Lemmermann 1910) and *Glenodinium* (Schiller 1935). *Huia caspica* does not belong to the genus *Peridinium*, since this is now considered to be characterized by five cingular plates, as observed on the type species, *Peridinium cinctum* (Boltovskoy 1975), which contrasts with four cingular plates (3c+t) in *H. caspica*. The attribution to the genus *Glenodinium* is difficult to assess, since the genus *Glenodinium* has been too vaguely described by Ehrenberg (1836) (see e.g. Loeblich 1980). The plates of its type species *G. cinctum* Ehrenberg were not described, but it possesses plastids and a red horseshoe-shaped eyespot (Ehrenberg 1836). Several *Glenodinium* species have been reclassified, for instance, *G. ambiguum* Thompson and *G. balticum* Levander were transferred to *Kansodinium* Carty et Cox and *Durinskia* Carty et Cox respectively (Carty & Cox 1986), and more recently, *G. edax* was transferred to *Tyrrannodinium* (Calado 2011) and *G. acuminatum* (Ehrenb.) E. Jørgensen was transferred to *Scrippsiella* (Kretschmann *et al.* 2015). Wołoszyńska (1917) even transferred *G. cinctum* (the type species of *Glenodinium*) to *Sphaerodinium* (characterized by a plate pattern of 4′, 4a, 7″, ?c, ?s, 6‴, 2⁗ by Wołoszyńska 1917; four sulcal plates and possibly eight cingular plates in *Sphaerodinium cracoviense* as observed by Craveiro *et al.* 2010) because both genera have a distinctive horseshoe shaped eyespot, but if that was true, *Sphaerodinium*

became a junior synonym of *Glenodinium* (Loeblich 1980). Either way, it is unlikely

that *D. caspica* would belong to *Glenodinium*.

Very recently, the genus *Diplopsalis* has been emended to incorporate only those

species with a narrow, five-sided anterior intercalary plate located in the lower part of

the dorsal epitheca (Liu *et al.* 2015). As a consequence, *D. lebouriae* was transferred

to a new genus *Qia* because it possesses a six-sided anterior intercalary plate. In this

emended description, *Diplopsalis* is not suitable to incorporate *D. caspica* because of

its small anterior intercalary plate, thus we transfer it to a new genus *Huia*.

Kansodinium ambiguum (R.H.Thompson) S. Carty & Elenor R. Cox, a freshwater

species from Kansas, USA, shares a small, diamond-shaped anterior intercalary plate

with *H. caspica*, but differs in having only five precingular plates (Carty & Cox 1986).

The specimen called *D. caspica* by Lindemann (1927, p. 419-420) from the Caspian

Sea with five precingular plates resembles *Kansodinium ambiguum* in its tabulation*,*

but differs in its more rounded cell shape, smoother plate ornamentation, and different

plate shapes; our specimens of *H. caspica* never showed five precingular plates. The

phylogenetic relationship between *Kansodinium* and *Huia* will be the focus of future

work.

Here we present the cyst-theca relationship of *Huia caspica* and thus added to the

species diversity of diplopsalioideans with a cyst stage. The cyst morphology of *H.

caspica* is superficially indistinguishable from the cysts of *Niea chinensis*, *Diplopelta

globula* (Abé) Balech, *Diplopsalis lenticula*, *Diplopsalopsis ovata* (Abé) J.D. Dodge

& Toriumi, and *Gotoius abei* Matsuoka regarding the spherical shape and smooth

surface. Furthermore, all of these species possess   a theropylic archeopyle, although

the suturing differs and there are differences in size and color (Matsuoka 1988; Liu *et*

al. 2015). The cyst of *Diplopsalopsis ovata* has two flagellar scars and can be

separated from the other species (Liu *et al.* 2015). So far, nearly half of the

diplopsalioideans are known to produce cysts (Matsuoka 1988; Liu *et al.* 2015), in

contrast to about 13-16% of all living dinoflagellates (Head 1996). From an

evolutionary view, the cyst stages may help the heterotrophic species to survive

periods when food sources are scarce. Massive encystment was reported for

Preperidinium meunierii (Pavillard) Elbrächter to retain the population and prevent

washing out from the coastal environments (Joyce & Pitcher 2004).

Our molecular phylogeny largely conforms to the previous report regarding the

subdivisions of the diplopsalioideans into three clades (Liu *et al.* 2015). *Huia caspica*

is phylogenetically distant from *D. lenticula*, consistent with the fact that they differ

in the cell shape (spherical versus lenticular) and the shape of plate 1a

(parallelogrammic versus narrow and five-sided). It is surprising that the closest

relative of *Huia* is *Lebouraia* as they differ in the shape of the first apical plate (an

ortho 1′ versus a meta 1′) and the number of anterior intercalary plates (one versus

two). Either difference will justify their classification into various genera. The genera

Oblea and *Boreadinium* share a meta 1′ with *Lebouraia*, but differ in the number of

anterior intercalary plates (Liu *et al.* 2015). They are not grouped together in the

molecular phylogeny, supporting the systematic significance of the number and shape

of anterior intercalary plates in this group of dinoflagellates. This is in contrast with

the genus *Protoperidinium*, which incorporates species with one, two or three anterior intercalary plates, but remains a monophyletic group (Gu *et al.* 2015). The Diplopsalioideans might have experienced a rapid diversification during their evolution. Unfortunately, other molecular markers such as small subunit ribosomal (SSU) and internal transcribed spacer (ITS) are available only for a few species of the diplopsalioideans, thus molecular phylogeny study with concatenated data will be the focus of future work.

Species of the subfamily Diplopsalidoideae are predominantly marine (Abé 1941; Nie 1943; Abé 1981; Dodge & Hermes 1981; Matsuoka 1988; Dodge & Toriumi 1993), although many of them are able to inhabit brackish waters too, such as diplopsalioideans recorded in the Black Sea (Gómez & Boicenco 2004). Some of them, e.g. *Oblea rotunda* (Lebour) Balech *ex* Sournia, can even tolerate salinities as low as 1.1 (Chomérat *et al.* 2004). The inland migration of marine dinoflagellates has been reported for the autotrophic species *Scrippsiella trochoidea* (Hameed & Saburova 2015). The Diplopsalidoideae are exclusively heterotrophic and many of them (e.g. *Diplopsalis*, *Oblea*, *Preperidinium*) are generalists feeding on a variety of diatoms and other dinoflagellates (Strom & Buskey 1993; Naustvoll 1998, 2000), thus it is not surprising that some of them have followed their prey and resided in waters with much lower salinity, as suggested for *Oblea rotunda* (Chomérat *et al.* 2004).

Only a few dinoflagellate species have succeeded in crossing the marine-freshwater boundary. So far, *Kolkwitziella acuta* and *Huia caspica* are known to be present in both brackish and fresh waters (Ostenfeld 1902; Mertens *et al.* 2015;

Zhang *et al.* 2015). Other dinoflagellates known to inhabit both marine and fresh waters are limited to "*Scrippsiella*" *hangoei* and *Durinskia baltica* (K. M. Levander) S. Carty *&* E. R. Cox (Imanian & Keeling 2007; Annenkova *et al.* 2015). It is worth noting that all of them have a known cyst stage except *D. baltica* (Kremp & Parrow 2006; Mertens *et al.* 2015), suggesting that cysts might be helpful to make the transition. To our knowledge, no species of other unicellular algae can inhabit both marine and fresh water ecosystems. The limited number of species that have crossed the marine-freshwater boundary suggest that it is difficult to make such a transition. The extant freshwater species might be the outcome of invasions long time ago and subsequent diversification, as marine and freshwater species usually do not group together in the molecular phylogeny (Logares *et al.* 2007b). The adaption to freshwater of a marine dinoflagellate might be accompanied by changes in genome structure and gene expression. This has occurred in the marine–freshwater evolutionof stickleback fish species flock (Jones *et al.* 2012).

The variation of those neutral molecular markers might not reflect the rapid diversification process (Orr & Smith 1998), as demonstrated in "*Scrippsiella*" *hangoei* and "*Peridinium*" *aciculiferum* which have evolved into different morphospecies but retained identical ribosomal DNA sequences (Logares *et al.* 2007a). The brackish "*S.*" *hangoei* differs from freshwater "*S.*" *hangoei* at only one position in ITS region and the latter even shares identical ITS sequence with freshwater "*Peridinium*" *baicalense* and "*P.*" *euryceps*, possibly due to rapid diversification of this species flock (Annenkova *et al.* 2015). Sharing of an identical

LSU rDNA sequence between marine and freshwater *Huia caspica* suggest that its marine-freshwater transition might be a recent event. In fact, the artificial reservoir where *H. caspica* was recovered is connected to the Yellow River (Zhang *et al.* 2015), which was separated from the Bohai Sea until around 0.15 MaBP (Wang *et al.* 2001). A single species from different habitats can show pronounced variation in ribosomal DNA sequences, for instance, marine *Durinskia baltica* (AF231803) differs from its freshwater counterpart (GU999528) at 32 positions (98.33% similarity) in SSU sequence. *Durinskia baltica* was classified within a diatom harbouring clade and most species within the clade inhabit fresh waters (You *et al.* 2015), suggesting that *D. baltica* might have re-colonized the marine waters.

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FIGURE CAPTIONS

Figs 1–7. LM of live (Fig. 1) and calcoflour stained (Figs 2–7) vegetative cells of

Huia caspica.

1. Cell showing pale green granules.

2. Cell in ventral view, showing the first apical plates (1′), an anterior sulcal plate (Sa), a left sulcal plate (Ss) and a right sulcal plate (Sd).

3. Cell in apical view, showing a round pore plate (Po) and a short canal plate (X).

4. Cell in ventral view, showing the first apical plates (1′).

5. Cell in apical view, showing three apical plates (1′–3′), six precingular plates (1″–6″) and one anterior intercalary plate (1a).

6. Cell in ventral view showing three cingular plates (1c–3c), the transitional plate (t).

7. Cell in antapical view showing five postcingular plates (1‴–5‴), one antapical plate (1‴′) and a posterior sulcal plate (Sp).
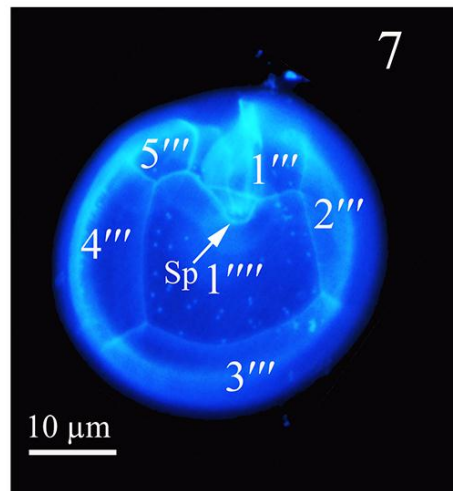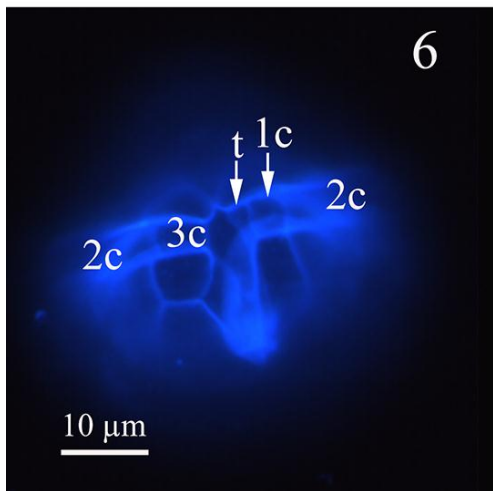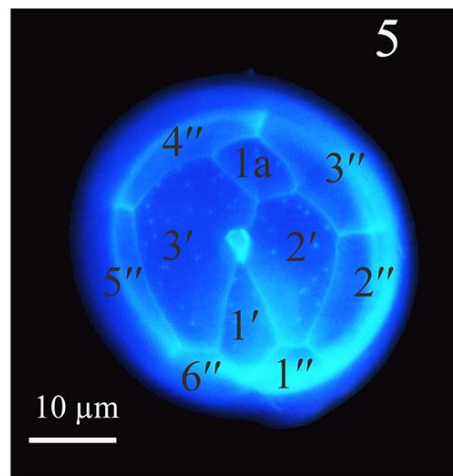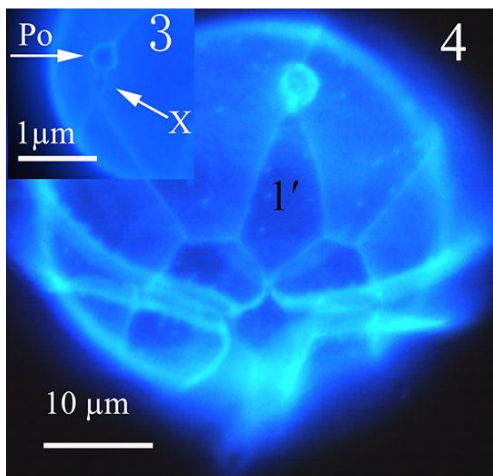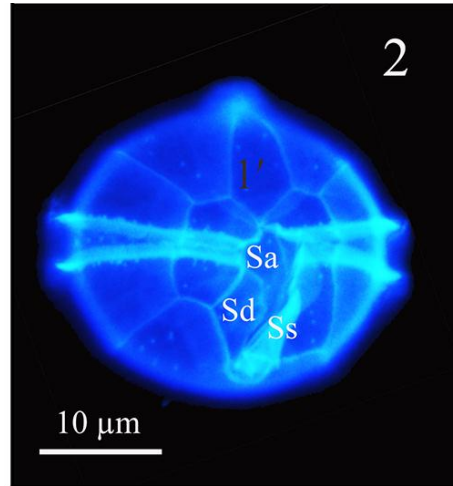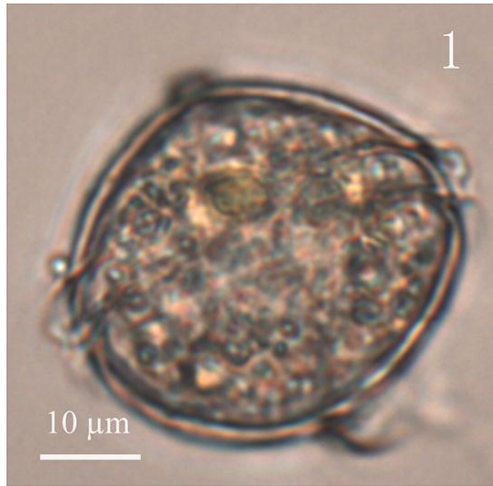
Figs 8–11. LM of cysts of *Huia caspica.*

8. A living cyst showing the thick walls and full of granules.

9. An empty cyst showing the smooth wall.

10, 11. Empty cysts showing the theropylic archeopyle (arrows).
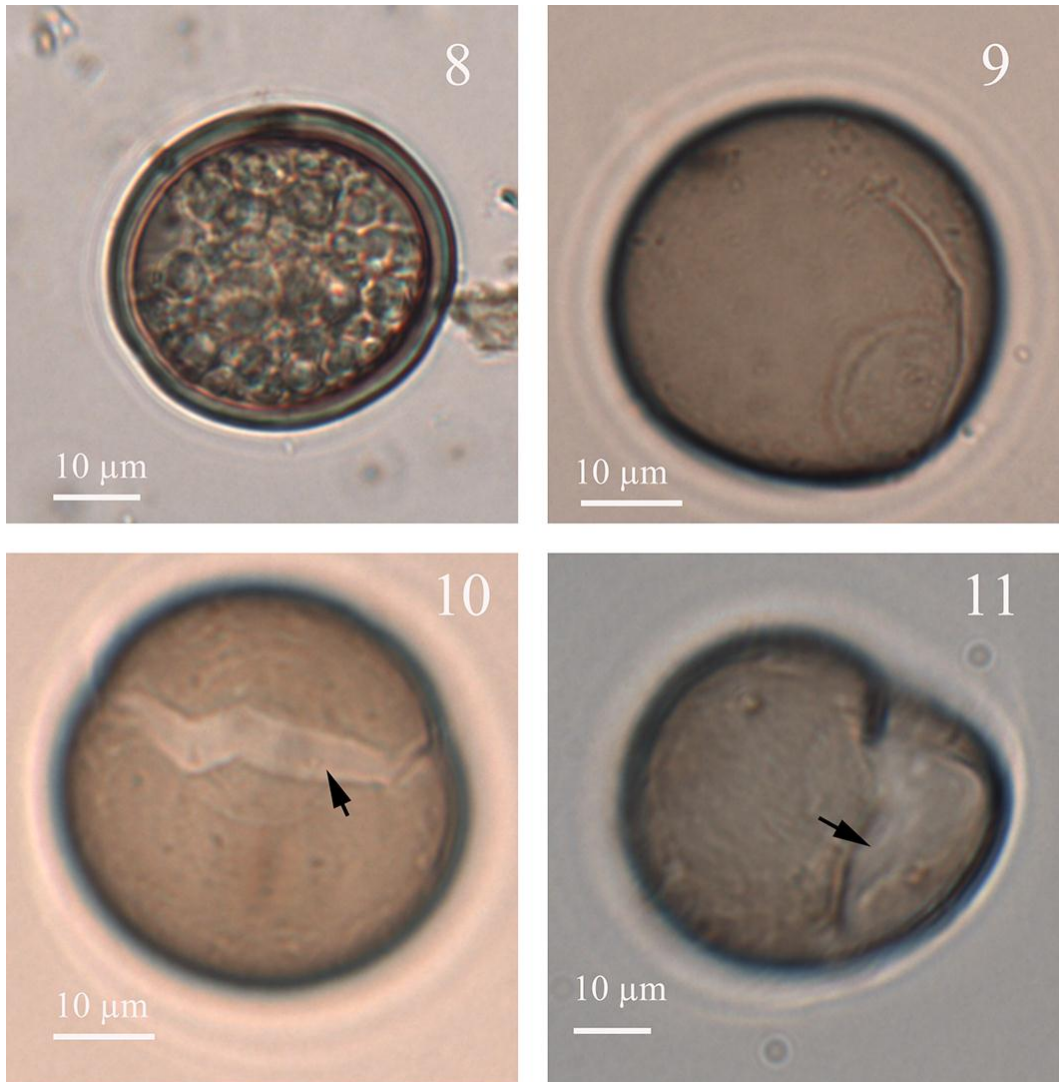
Fig. 12.   Drawing of the archeopyle. The anterior intercalary plate is shaded, and the archeopyle sutures are indicated with heavy black lines.
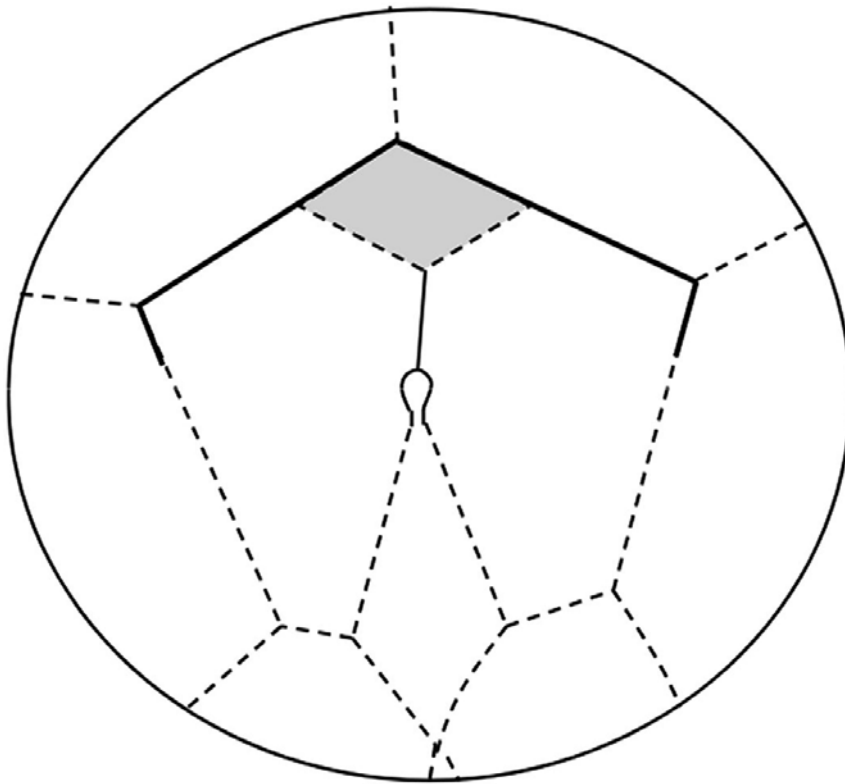
Fig. 13. A phylogenetic tree of the diplopsalioideans inferred from partial large subunit ribosomal DNA sequences using Bayesian inference (BI). The apicomplexan *Neospora caninum* was selected as an outgroup. The scale bar indicates the number of the substitutions per site. Bootstrap value (>50%) for maximum likelihood (ML) / Posterior probability (>0.9) for BI support are shown. * indicates maximal support (ML bootstrap support=100 / BI posterior probability=1.00). Dashed lines indicate a half length. Clades are labeled and marked with vertical lines on the right. Sequences obtained in this study are indicated in bold.

Neospora caninum AF001946
Amphidinium carterae AF260380
Amphidinium klebsii AF260381
Akashiwo sanguinea AF260396
Warnowia sp. FJ947042
Spiniferodinium galeiforme GU295206
Gymnodinium palustre AF260382
Scrippsiella trochoidea var. *aciculifera* AF260393
Protoceratium reticulatum FJ155822
Diplopsalis lenticula KP702707
Diplopsalis lenticula EF152783
Diplopsalis lenticula KP702708
Oblea rotunda KP702719
Boreadinium breve KP702702
Boreadinium breve KP702703
Huia caspica KJ995959
Huia caspica KX056274
Huia caspica KX056275
Huia caspica KX056276
Lebouraia pusilla KP702713
Lebouraia pusilla KP702714

Clade *Diplopsalioideae* I

Preperidinium meunieri DQ444232
Preperidinium cf. *meunieri* KP702709

Clade *Diplopsalioideae* II

Diplopelta globula KP702712
Diplopelta bomba AB716930
Gotoius excentricus AB716922
Diplopsalopsis ovata KP702710
Diplopsalopsis ovata KP702711
Niea torta AB716924
Niea chinensis KP702720
Niea acanthocysta KP702715
Qia lebouriae AB716921
Qia lebouriae KP702704

Clade *Diplopsalioideae* III

Protoperidinium claudicans AB255842
Protoperidinium paraoblongum AB255858
Protoperidnium steidingerae EF152991
Protoperidinium depressum EF152889
Protoperidinium oblongum EF152894

Clade Oceanica

Archaeperidinium bailongense KM042418
Archaeperidinium minutum AB781001
Archaeperidinium saanichi AB702990
Archaeperidinium constrictum KM042419
Protoperidinium haizhouense KF651019
Protoperidinium parthenopes KF651026
Herdmania litoralis AB564307
Protoperidinium monovelum AB716927

Clade Monovela

Protoperidinium conicoides EF152811
Protoperidinium bipes AB284160
Protoperidinium pellucidum DQ444233
Protoperidinium angustum DQ444237
Protoperidinium divergens DQ444236
Protoperidinium crassipes EF152846
Protoperidinium thulesense AB716929
P. denticulatum AB255849
Protoperidinium leonis EF613364
Protoperidinium shanghaiense KM591212
Protoperidinium punctulatum EF613365
Protoperidinium humile KM591206
Kolkwitziella acuta LC075592
Protoperidinium excentricum DQ444229
Protoperidinium stellatum KM042425

Clade *Protoperidinium* sensu stricto

Peridinium willei AF260384
Peridinium cinctum AF260385
Heterocapsa arctica AY571372
Heterocapsa triquetra AF260401
Alexandrium pseudogonyaulax AY154958
Alexandrium margalefii AY154957

100/-
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100/-
100/0.98
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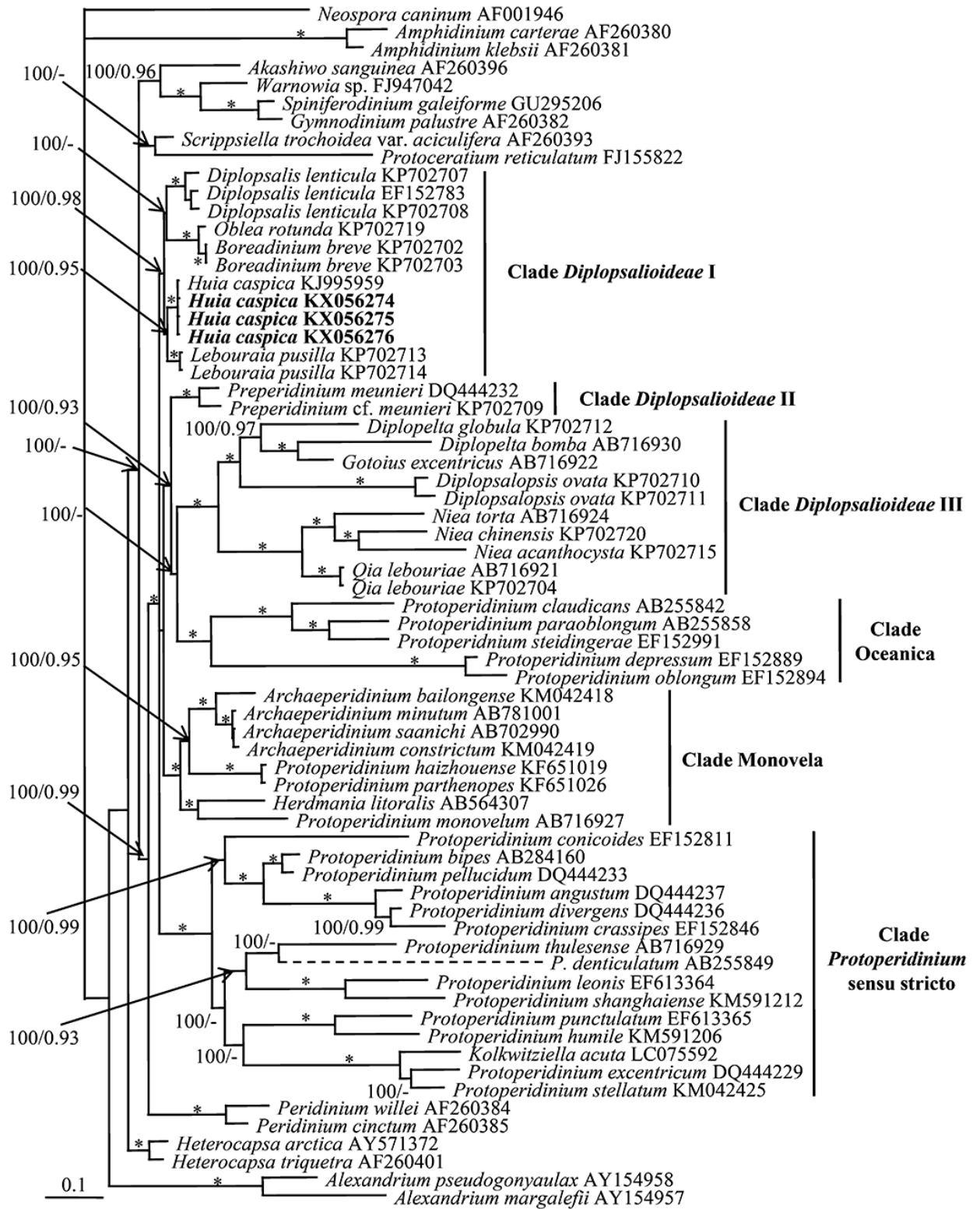100/-
100/-
100/-
100/0.99
100/0.93

0.1

Fig. S1 Map showing the distribution of *Huia caspica*: cysts (this study, shown in red circle), motile cells in plankton samples (literature, shown in blue circle).