Morphology, ultrastructure and molecular phylogeny of cyst-producing *Caladoa arcachonensis* gen. et sp. nov. (Peridinales, Dinophyceae) from France and Indonesia

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Abstract :

The dinoflagellate order Peridinales encompasses several well circumscribed families. However, the family level of some genera, such as *Bysmatrum* and *Vulcanodinium*, has remained elusive for many years. Four *Peridinium*-like strains were established from the Atlantic coast of France and North Sulawesi, Indonesia through cyst germination or isolation of single cells. The cyst-theca relationship was established on specimens from the French Atlantic. Their morphologies were examined using light, scanning and transmission electron microscopy. The cells were characterized by a much larger epitheca relative to the hypotheca, a large anterior sulcal (Sa) plate deeply intruding the epitheca and a small first anterior intercalary plate. The plate formula was identified as Po, cp, X, 4', 3a, 7'', 6C, 5S, 5''', 2''', shared by *Apocalathium*, *Chimonodinium*, *Fusiperidinium* and *Scrippsiella* of the family Thoracosphaeraceae but the configuration of Sa plate and anterior intercalary plates is different. Transmission electron microscopy showed that the eyespot was located within a chloroplast comprising two rows of lipid globules and thus belongs to type A. All four strains were classified within a new genus *Caladoa* as *C. arcachonensis* gen. et sp. nov. Small subunit ribosomal DNA (SSU rDNA), partial large subunit ribosomal DNA (LSU rDNA) and internal transcribed spacer ribosomal DNA (ITS rDNA) sequences were obtained from all strains. Genetic distance based on ITS rDNA sequences between French and Indonesian strains reached 0.17, suggesting cryptic speciation in *C. arcachonensis*. The maximum likelihood and Bayesian inference analysis based on concatenated data from SSU and LSU rDNA sequences revealed that *Caladoa* is monophyletic and closest to *Bysmatrum*. Our results supported that *Caladoa* and *Bysmatrum* are members of the order Peridinales but their family level remains to be determined. Our results also support that *Vulcanodinium* is closest to the family Peridiniaceae.

Keywords : *Bysmatrum*, cyst, dinoflagellate, eyespot, Peridinales, plate overlap

45 Introduction

46 The dinoflagellate order Peridiniales is characterized by a symmetrical first apical plate
47 and two more or less symmetrical antapical plates (Fensome *et al.*, 1993). Peridiniales
48 currently encompasses several families including Blastodiniaceae, Heterocapsaceae,
49 Kryptoperidiniaceae, Peridiniaceae, Peridiniopsidaceae, Protoperidiniaceae,
50 Thoracosphaeraceae and Zooxanthellaceae (Gottschling *et al.*, 2017). The morphological
51 features in some of these families are well circumscribed, e.g. Peridiniopsidaceae has six
52 cingular plates and less than two anterior intercalary plates (Gottschling *et al.*, 2017);
53 Protoperidiniaceae has three cingular plates and one transitional plate (Fensome *et al.*,
54 1993). In other families, however, morphological features are not well defined. For
55 instance, Kryptoperidiniaceae includes species with one or two anterior intercalary plates
56 (Pienaar *et al.*, 2007; Saburova *et al.*, 2012; You *et al.*, 2015), but some genera (e.g.
57 *Galeidinium* M. Tamura & T. Horiguchi) do not show any thecal plate pattern (Tamura *et*
58 *al.*, 2005). The family Thoracosphaeraceae has unified the subfamily Calciodinelloideae
59 and the order Thoracosphaerales, but its emended description was not provided by
60 Elbrächter *et al.*, (2008).

61 Information on the evolutionary history and cell ultrastructure are important for
62 systematics, even at the family level. For instance, Kryptoperidiniaceae includes a small
63 group of dinoflagellates hosting a tertiary endosymbiont derived from a diatom
64 (Horiguchi & Takano, 2006; Kretschmann *et al.*, 2018). Kryptoperidiniaceae currently
65 includes *Durinskia* S. Carty & E.R. Cox, *Blixaea* M. Gottschling, *Galeidinium*,

66 *Kryptoperidinium* Lindemann, and *Unruhadinium* M. Gottschling. All these genera share a
67 type D eyespot (Tamura *et al.*, 2005; You *et al.*, 2015; Yamada *et al.*, 2017) that was
68 likely derived from the original dinoflagellate chloroplast (Moestrup & Daugbjerg, 2007).
69 The woloszynskioid dinoflagellates families Tovelliaceae and Borghiellaceae are
70 characterized by a type C eyespot (composed of pigment globules not bound by
71 membranes outside of the chloroplast) (Lindberg *et al.*, 2005) and by a type B eyespot
72 (intraplastidic with bricklike crystals overlying the chloroplast), respectively (Moestrup *et al.*, 2009). In addition to the eyespot, peduncle microtubules were also suggested to be
73 one of the diagnostic features to separate Peridiniopsidaceae from Peridiniaceae
74 (Gottschling *et al.*, 2017).

77 Among 2000 extant dinoflagellate species, only 13% to 16% of the motile stages
78 have been related to resting cysts (Head, 1996). Cyst morphologies are often considered
79 to be useful at interspecific level, e.g. *Protoperidinium* Bergh produces a number of cysts
80 classified within different cyst genera (Gu *et al.*, 2015), but also at the generic level, e.g.
81 **cysts of *Biecheleria* Ø. Moestrup, K. Lindberg & N. Daugbjerg are spherical and**
82 **bear numerous short processes (Moestrup *et al.*, 2009),** or even at the subfamily level, e.g.
83 Calciadinelloideae is characterized by possessing calcareous cysts (Elbrächter *et al.*,
84 2008).

85 Molecularly, more than 90% of peridinialean species can be reliably placed into one
86 of the families of Peridiniales (Gottschling *et al.*, 2017), but some genera (e.g.

Bysmatrum M.A. Faust & K.A. Steidinger, *Vulcanodinium* E. Nézan & N. Chomérat) have not been identified to the family level yet, although they display a peridinialean tabulation. *Vulcanodinium* was reported to be closely related with *Peridinium cinctum* (O.F. Müller) C.G. Ehrenberg based on LSU sequences (Nézan & Chomérat, 2011) and concatenated data from SSU, ITS and LSU sequences (Luo *et al.*, 2018), but whether *Vulcanodinium* is also a member of Peridiniaceae is not clear. The phylogenetic position of *Bysmatrum* is elusive too: it is close to *Peridinium* C.G. Ehrenberg with low support in the phylogeny based on concatenated data from SSU, ITS and LSU sequences (Anglès *et al.*, 2017; Luo *et al.*, 2018), but sometimes it is closer to *Prorocentrum* C.G. Ehrenberg (Gottschling *et al.*, 2012; Dawut *et al.*, 2018).

To clarify the phylogenetic positions of *Vulcanodinium* and *Bysmatrum*, sequences from closely related species of these two genera are essential. In the present study, four strains of *Peridinium*-like species were established from the Atlantic coast of France and North Sulawesi, Indonesia by incubating cysts or isolating single cells. The four cultured strains were examined morphologically and ultrastructurally, the latter with an emphasis on the eyespot. In addition, small subunit ribosomal DNA (SSU rDNA), partial large subunit ribosomal DNA (LSU rDNA) and internal transcribed spacer ribosomal DNA (ITS rDNA) sequences were determined for the cultured strains and molecular phylogeny was inferred using concatenated SSU and LSU rDNA sequences.

Material and methods

Sample collection and treatment

Seagrass and sand samples were collected from the seabed by scuba divers near Manado and Bitung, North Sulawesi of Indonesia on July 31 and August 1, 2016 and placed into bottles containing seawater from the same location. The samples were stirred vigorously to detach the epibenthic cells and the suspension settled in a composite settling chamber. The settled material was rinsed with filtered seawater and transferred into a polycarbonate bottle. Single *Peridinium*-like cells were isolated from this material and the strains TIO339 and TIO340 were established (Table 1).

Sediment sampling was done using an Ekman grab on April 15 and 19, 2016 in a shallow natural reservoir used for oyster storage before commercialization (water depth 1.2–1.5 m). The small basin is located in Arcachon Bay in the Gironde region of southwestern France. The top 2 cm of sediment were sliced off and stored in the dark at 4 °C until further treatment. Approximately 5 g of wet sediment was mixed with 20 mL of filtered seawater and stirred vigorously to dislodge detrital particles. The settled material was subsequently sieved through 120 µm and 10 µm filters. Single cysts were isolated with a micropipette using an inverted Eclipse TS100 (Nikon, Tokyo, Japan) microscope, alternatively small quantity of sediment was directly incubated in small containers with f/2-Si medium (Guillard & Ryther, 1962) at 20°C, 90 µE·m⁻²·s⁻¹ under a 12:12 h light:

126 dark cycle. The strains TIO278 and TIO282 were established using direct incubation
127 (Table 1).

128 *Morphological study of motile cells with microscopy*

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130 Live cells were examined and photographed using a Zeiss Axio Imager light microscope
131 (Carl Zeiss, Göttingen, Germany) equipped with a Zeiss AxioCam HRC digital camera.
132 The size of 30 cells was measured using Axiovision (4.8.2 version) software at $\times 1000$
133 magnification. To observe the shape and location of the nucleus, cells were stained with
134 1:100,000 SYBR Green (Sigma Aldrich, St. Louis, USA) for 1 min, and photographed
135 using the Zeiss fluorescence microscope with a Zeiss-38 filter set (excitation BP 470/40,
136 beam splitter FT 495, emission BP 525/50). Chloroplast autofluorescence microscopy
137 was carried out on live cells using the above mentioned microscope equipped with a
138 Chroma filter cube (emission filter ET480/20x, dichromatic mirror AT505dc, suppression
139 filter AT515lp), and digitally photographed using a Zeiss AxioCam HRC digital camera.

140 For scanning electron microscopy (SEM), mid-exponential batch cultures of four
141 strains were concentrated by a Universal 320 R centrifuge (Hettich-Zentrifugen,
142 Tuttlingen, Germany) at 850 g for 10 min at room temperature. Cells were fixed with
143 2.5% glutaraldehyde for 3 h at 8°C, rinsed with Milli-Q water twice and post-fixed with
144 1% OsO₄ overnight at 8°C in a tube. The supernatant was removed and the settled cells
145 were transferred to a coverslip coated with poly-L-lysine (molecular weight

70,000–150,000). The cells attached to the cover slip were rinsed in Milli-Q water twice.

The samples were then dehydrated in a graded ethanol series (10, 30, 50, 70, 90 and 3× in 100%, 10 min at each step), critical point dried (K850 Critical Point Dryer, Quorum/Emitech, West Sussex, UK), sputter-coated with gold, and examined with a Zeiss Sigma FE (Carl Zeiss Inc., Oberkochen, Germany) scanning electron microscope.

Labelling of tabulation follows a modified Kofoed system (Fensome *et al.*, 1993), and the sulcal plate labelling is according to Balech (1980).

Transmission electron microscopy (TEM)

Mid-exponential batch cultures of strain TIO278 (France) were fixed in 2.5% glutaraldehyde in phosphate buffer saline (PBS, 0.1 M at pH 7.4) for 1 h, concentrated by centrifugation and then washed three times with the same PBS for 10 min each. They were post-fixed in 1% OsO₄ overnight at 4°C and washed three times with the same PBS for 10 min each. The cells were then dehydrated through a graded ethanol series (10, 30, 50, 70, 95, 3× in 100%, 10 min at each step). The pellet was embedded in Spurr's resin (Spurr, 1969) and sectioned with a Reichert Ultracut E microtome (Leica, Vienna, Austria), mounted on Formvar-coated grids, stained with uranyl acetate and lead citrate, and observed in a JEOL JEM-100 transmission electron microscope (JEOL, Tokyo, Japan).

164 *PCR amplifications and sequencing*

165 The total algal DNA was extracted from 10 mL of exponentially growing cultures using a
166 MiniBEST Universal DNA Extraction Kit (Takara, Tokyo, Japan) according to the
167 manufacturer's protocol. PCR amplifications were carried out using 1×PCR buffer, 50
168 µM dNTP mixture, 0.2 µM of each primer, 10 ng of template genomic DNA, and 1 U of
169 ExTaq DNA Polymerase (Takara, Tokyo, Japan) in 50 µL reactions. The SSU rDNA was
170 amplified using the primers of PRIMER A/PRIMER B (Medlin *et al.* 1988). The LSU
171 rDNA was amplified using the primers of D1R/28-1483R (Scholin *et al.*, 1994;
172 Daugbjerg *et al.*, 2000). The total ITS1–5.8S–ITS2 was amplified using ITSA/ITSB
173 primers (Adachi *et al.*, 1996). The thermal cycle procedure was 4 min at 94°C, followed
174 by 30 cycles of 1 min at 94°C, 1 min at 45°C, 1 min at 72°C, and final extension of 7 min
175 at 72°C with a Mastercycler (Eppendorf, Hamburg, Germany). The PCR product was
176 purified using a SanPrep Column DNA Gel Extraction Kit (Sangon Biotech, Shanghai, China)
177 and sequenced directly in both directions on an ABI PRISM 3730XL (Applied
178 Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Newly
179 obtained sequences were deposited in GenBank with accession numbers MK012070 to
180 MK012084.

181 *Sequence alignment and phylogenetic analysis*

182 Newly obtained sequences (SSU and partial LSU rDNA) were incorporated into a

systematically representative set of dinoflagellates available in GenBank. Sequences were aligned using MAFFT v7.110 (Katoh & Standley, 2013) online program (<http://mafft.cbrc.jp/alignment/server/>) with default settings. Alignments were manually checked with BioEdit v. 7.0.5 (Hall, 1999). For Bayesian inference (BI), the program jModelTest (Posada, 2008) was used to select the most appropriate model of molecular evolution with Akaike Information Criterion (AIC). Bayesian reconstruction of the data matrix was performed using MrBayes 3.2 (Ronquist & Huelsenbeck, 2003) with the best-fitting substitution model (GTR+G). Four Markov chain Monte Carlo (MCMC) chains ran for 2,000,000 generations, sampling every 100 generations. The first 10% of burn-in trees were discarded. A majority rule consensus tree was created in order 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 model GTR+G. Node support was assessed with 1000 bootstrap replicates.

Multiple ITS1-5.8S-ITS2 sequences of new strains were aligned using MAFFT v7.110 (Katoh & Standley, 2013) online program with default settings. Completed alignments were saved as NEXUS files and imported into PAUP*4b10 software (Swofford, 2002) to estimate divergence rates using simple uncorrected pairwise (p) distance matrices.

Results

Caladoa Z. Luo, K.N. Mertens & H.F. Gu *gen. nov.*

DIAGNOSIS: Cells with a plate formula of Po, cp, X, 4', 3a, 7'', 6C, 5S, 5''', 2'''. A large anterior sulcal plate deeply intrudes the epitheca. Chloroplasts with a stalked pyrenoid and a type A eyespot is present. Cells differ from *Chimonodinium*, *Fusiperidinium*, *Apocalathium*, *Scrippsiella*, *Vulcanodinium* and *Bysmatrum* in the size and location of Sa plate and from *Peridinium* by the presence of an additional cingular plate.

ETYMOLOGY: The epithet *Caladoa* is named after António José Calado, who carried out outstanding work on freshwater dinoflagellate taxonomy.

TYPE SPECIES: *Caladoa arcachonensis* Z. Luo, K.N. Mertens & H.F. Gu

Caladoa arcachonensis Z. Luo, K.N. Mertens & H.F. Gu *sp. nov.* (Figs. 1–26)

DIAGNOSIS: Cells are 16.7–23.5 µm long and 13.1–19.4 µm wide. The cells have a rounded epitheca and hypotheca with an epitheca: hypotheca length ratio of 1.5–1.8. The thecae display a plate formula of Po, cp, X, 4', 3a, 7'', 6C, 5S, 5''', 2'''. A large anterior sulcal plate deeply intrudes in the epitheca. Plate 1a is about half the size of 3a and one quarter the size of 2a. Each cell has a single reticulated chloroplast with one stalked pyrenoid. An eyespot comprising two rows of lipid globules is situated within a chloroplast and belongs to type A. The nucleus is spherical and located posteriorly. Cysts are proximate with a chasmic archeopyle.

HOLOTYPE: SEM stub of strain TIO278 designated as TIO201803 ([illustrated in Fig. 9](#)) and deposited at Third Institute of Oceanography, State Oceanic Administration,

Xiamen 361005, China.

TYPE LOCALITY: Arcachon Bay, France (1°4'0.0" W, 44°38'11.0"N); Collection date: 15 ~~and 19~~ April 2016.

ETYMOLOGY: The epithet *arcachonensis* is named after the type locality, Arcachon Bay.

HABITAT: Benthic sand-dwelling or epiphytic on seagrass.

DISTRIBUTION: Brackish and marine coastal water of French Atlantic (21.3–23 psu), North Sulawesi, Indonesia.

GENBANK ACCESSION NUMBER SEQUENCES: MK012071 (SSU rDNA), MK012076 (LSU rDNA) and MK012081 (ITS rDNA) of strain TIO278.

Morphology

Cysts of *Caladoa arcachonensis*, isolated from Arcachon Bay, France surface sediment samples are spherical or ovoid in shape, proximate and with a well-defined posteriorly located cingulum (Fig. 1). They are 23.9–28.1 µm long (mean = 26.6 ± 2.3 µm, n = 3) and 19.9–24.4 µm wide (mean = 22.0 ± 2.3µm, n = 3). Each cyst contains an endospore, green to brown granules, a pyrenoid and the red eyespot. Two cysts were isolated, but only one germinated, displaying a chasmic archeopyle on the epitheca (Fig. 2). The cyst wall is transparent and it appears to be smooth (Fig. 3). Sometimes, small detrital particles can be attached to the cyst wall surface. The germinated cell had a pronounced

245 eyespot in the sulcal area (Fig. 4). The cysts withstand palynological treatment.

246 Cells of the French strain TIO278 are 16.7–23.5 μm long (mean = $19.8 \pm 2.2 \mu\text{m}$, n
247 = 30) and 13.1–19.4 μm wide (mean = $16.4 \pm 1.8 \mu\text{m}$, n = 30). The cells have a rounded
248 epitheca and hypotheca with a much larger epitheca (Fig. 5). There is a ring-like pyrenoid
249 located in the epicone and one pronounced orange eyespot in the sulcal area (Fig. 6).
250 There is a single chloroplast forming a network in the periphery of the cell ('c' in Fig. 7).
251 The nucleus is rounded and located posteriorly ('N' in Fig. 8).

252 The thecae have a plate formula of Po, cp, X, 4', 3a, 7'', 6C, 5S, 5''', 2'''' (Figs. 9–16).

253 The epitheca: hypotheca length ratio in the dorsal side is 1.5–1.8 (mean = 1.6 ± 0.1 , n =
254 7). Thecal pores with a diameter of 0.1–0.2 μm are randomly scattered throughout the
255 thecal plates (Figs. 10, 11, 15). The epithecal plates are symmetrical in arrangement
256 (bipesoid). There are four apical plates. Plate 1' and 3' are six-sided and are nearly
257 symmetrical (Figs. 13, 14). Plate 2' and 4' are seven-sided and similar in size, much
258 larger than the other apical plates (Figs. 13, 14). There are three anterior intercalary plates
259 (1a, 2a and 3a), of which 1a and 3a are pentagonal and 2a is hexagonal. Plate 1a is about
260 half the size of 3a and one quarter the size of 2a (Fig. 14). There are seven precingular
261 plates among which the fourth is four-sided and smallest, whereas the others are
262 five-sided (Figs. 9, 10, 13, 14). The cingulum is 1.6–2.8 μm wide (mean = $2.3 \pm 0.4 \mu\text{m}$,
263 n = 12), situated in the low part of the cell and descended ca. half its width (Fig. 9). The
264 cingulum comprises six plates. The cingular plates are similar in size except C1 is
265 relatively smaller (Figs. 9, 10, 15). The apical pore complex is tear-shaped comprising a

round pore plate (Po), a round apical pore covered by a cover plate (cp) and an elongated canal plate (X) (Figs. 13, 14). There are five postcingular plates, among them the third and fifth postcingular plates (3''', 5''') are five-sided whereas the other plates are four-sided (Fig. 12). The antapical plates (1''', 2''') are five-sided and similar in size (Fig. 12).

There are five sulcal plates. The large anterior sulcal plate (Sa) intrudes the epitheca (Fig. 16). The left sulcal (Ss) is elongated and narrow. The median sulcal (Sm) is small and always masked by the sulcal list emerging from the right sulcal plate (Sd). The posterior sulcal plate (Sp) is seven-sided and has no contact with the cingulum (Figs. 15, 16). A peduncle was not observed. Schematic drawings showing the plate patterns of *Caladoa arcachonensis* are provided in Figs. 17–20. Plate overlap (Figs. 19, 20) was identified individually for each suture by inspecting cells with visible growth bands or by internal theca views (Figs. 9–16). The third precingular and postcingular plates and the fourth cingular plate were identified as the keystone plates (i.e., plates overlapping all their neighbors).

Longitudinal sections through the cell of TIO278 show a large spherical nucleus in the hypotheca, a stalked pyrenoid in the epitheca, a single chloroplast in the periphery and an eyespot in the sulcal region (Figs. 21, 22). The nucleus consists of condensed chromosomes with a smooth nuclear envelope. The chloroplast is enveloped by three membranes and the thylakoids are grouped in threes to form lamellae (Fig. 23). The stalked pyrenoid is surrounded by a starch sheath without intrusion of lamellae (Fig. 24).

Many diamond-shaped trichocysts were observed beneath the thecate wall (Fig. 23). The eyespot is located within a chloroplast comprising two rows of ~ 20 lipid globules in each row (Figs. 25, 26). The lipid globules are ~ 100–150 nm in diameter. A microtubular strand of the peduncle and a pusular system were not observed but an intensive search was not carried out.

Cells of the Indonesian strain TIO339 are 13.2–18.9 μm long (mean = $16.5 \pm 1.5 \mu\text{m}$, $n = 24$) and 10.7–15.2 μm wide (mean = $12.9 \pm 1.1 \mu\text{m}$, $n = 29$). The cells show a spherical nucleus in the hypotheca, an orange eyespot in the sulcal region and a single reticulated chloroplast with a doughnut-shaped pyrenoid in the epitheca (Figs. 27, 28). Cell morphology of strain TIO339 is indistinguishable from strain TIO278 (Figs. 29–34).

Molecular analysis and phylogeny

The French strains TIO278 and TIO282 shared identical SSU, LSU and ITS rDNA sequences, and the Indonesian strains TIO339 and TIO340 shared identical SSU, LSU and ITS rDNA sequences as well. Strain TIO278 differed from TIO339 at 13 positions (SSU rDNA, 99.2% similarity out of 1724 bp), 61 positions (LSU rDNA, 95.5% similarity out of 1331 bp), and 131 positions (ITS rDNA, 79.8% similarity out of 648 bp). The genetic distance based on ITS rDNA sequences was 0.17 between French and Indonesian strains.

The maximum likelihood (ML) and Bayesian inference (BI) analysis based on combined SSU rDNA and partial LSU rDNA sequences yielded similar phylogenetic

trees. The BI tree was illustrated in Fig. 35. The order Peridiniales is well resolved (0.9 BPP/55 BS) encompassing the families Peridiniaceae, Peridiniopsidaceae, Thoracosphaeraceae, Kryptoperidiniaceae, and the genera *Caladoa*, *Bysmatrum* and *Vulcanodinium* as well. The genus *Caladoa* was monophyletic with maximal support (1.0 BPP/100 BS), which formed a sister clade of *Bysmatrum* with maximal support. They were closest to a well resolved clade (0.90 BPP/93 BS) formed by Peridiniaceae and *Vulcanodinium rugosum* E. Nézan & N. Chomérat with a low Bayesian posterior probability (<0.7 BPP) and moderate ML bootstrap support (58 BS).

Discussion

Morphology

To determinate plate homologies, the plate overlap pattern was investigated. Plate overlap pattern of strain TIO278 (Figs. 19, 20) is generally consistent with other dinoflagellates, following a trend from dorsal to ventral and from equatorial to poles (Netzel & Dürr, 1984). The third precingular plate is identified as the keystone plate in our strain, also previously reported for *Parvodinium marciniakii* J. Kretschmann, P. Owsianny, A.Z. Zerdoner & M. Gottschling by Kretschmann *et al.* (2018) and *Parvodinium umbonatum* (F. Stein) S. Carty by Luo *et al.* (2018). In contrast, the fourth precingular plate was the keystone plate in many other species of Peridiniales including *Bysmatrum subsalsum* (C.H. Ostenfeld) M.A. Faust & K.A. Steidinger and

- 327 *Vulcanodinium rugosum* (Luo *et al.*, 2018), *Scrippsiella acuminata* (C.G. Ehrenberg) J.
 328 Kretschmann, M. Elbrächter, C. Zinssmeister, S. Soehner, M. Kirsch, W.-H. Kusber & M.
 329 Gottschling (Kretschmann *et al.*, 2015), *Peridinium cinctum* (Dürr, 1979),
 330 *Fusiperidinium wisconsinense* (S. Eddy) F.M.G. McCarthy, H. Gu, K.N. Mertens & C.
 331 Carbonell-Moore (McCarthy *et al.*, 2018), *Heterocapsa triquetra* (C.G. Ehrenberg) F.
 332 Stein (Tillmann *et al.*, 2017), *Parvodium travinskii* J. Kretschmann, P. Owsianny, A.Z.
 333 Zerdoner & M. Gottschling, *Parvodium mixtum* Wołoszyńska ex J. Kretschmann, P.
 334 Owsianny, A.Z. Zerdoner & M. Gottschling (Kretschmann *et al.*, 2018) and *Parvodium*
 335 *umbonatum* (Elbrächter & Meyer, 2001). In addition, *Caladoa* has a primategulate series
 336 that touches the terttegulate series, which can also be found in *Scrippsiella* Balech ex
 337 A.R.Loeblich III, *Fusiperidinium* F.M.G. McCarthy, H. Gu, K.N. Mertens & C.
 338 Carbonell-Moore, *Parvodium*, and *Heterocapsa* F. Stein, but is different from
 339 *Bysmatrum*, *Vulcanodinium*, *Peridinium* and *Protoperidinium*, where both series are
 340 separated (partly) by the sectegulate series.
- 341 The central ventral plate of strain TIO278 (denoted here by Sa) is overlapped by all
 342 adjacent epithelial plates including the first apical plate 1'. This ventral plate is identified
 343 as homologous with the anterior sulcal plate (Sa) instead of a precingular plate because
 344 plate 1' is generally overlapped by precingular plates (Dürr & Netzel, 1974; Fensome *et*
 345 *al.*, 1993). The plate pattern of strain TIO278 was thus determined as Po, cp, X, 4', 3a, 7",
 346 6C, 5S, 5"', 2'''. This plate formula is reminiscent of several previously described genera,
 347 including the freshwater genera *Chimonodinium* S.C. Craveiro, A.J. Calado, N.

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4 348 Daugbjerg, Gert Hansen & Ø. Moestrup and *Fusiperidinium* (Craveiro *et al.*, 2011;
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6 349 McCarthy *et al.*, 2018), freshwater-brackish genus *Apocalathium* S.C. Craveiro, N.
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9 350 Daugbjerg, Ø. Moestrup & A.J. Calado (Craveiro *et al.*, 2016), and brackish-marine
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11 351 genera *Bysmatrum*, *Scrippsiella* and *Vulcanodinium* (Nézan & Chomérat, 2011; Luo *et*
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14 352 *al.*, 2016; Luo *et al.*, 2018). The strain TIO278 can be separated from these genera
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17 353 primarily based on the size and location of Sa plate, which is large and intrudes deeply in
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19 354 the epitheca. Such kind of Sa plate was only reported in marine *Heterocapsa* (Tillmann *et*
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22 355 *al.*, 2017) and *Laciniporus* Saburova & N. Chomérat (Saburova & Chomérat 2018),
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24 356 freshwater *Parvodinium* (Carty, 2008) and *Palatinus* S. Craveiro, A. Calado, N.
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27 357 Daugbjerg & Ø. Moestrup (Craveiro *et al.*, 2009). A similar plate was observed in
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29 358 *Sphaerodinium cracoviense* J. Wołoszyńska (Craveiro *et al.*, 2010), but the authors
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32 359 expressed some doubts whether it should be considered as a Sa plate. Interestingly, such a
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35 360 type of deeply intruding Sa with similar overlap with the 1' has also been recorded for the
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38 361 fossil cyst *Susadinium? pinna* (Below) Lentin & Williams by Below (1987, as *Dodekovia*
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40 362 *pinna* Below) described from the Lower Jurassic (Toarcian), a species assigned to the
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43 363 Heterocapsaceae (Fensome *et al.*, 1993); this suggests that this type of Sa is a primitive
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46 364 evolutionary trait, not present in genera that have presumably evolved later, such as
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48 365 *Protoperidinium*.
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54 368 Strain TIO278 differs from *Chimonodinium* in the presence of a pyrenoid and lack of a

peduncle (Craveiro *et al.*, 2011), from *Scrippsiella* in the size of the first cingular plate (Luo *et al.*, 2016) and it differs from *Fusiperidinium*, *Apocalathium* and *Vulcanodinium* in the presence of an eyespot (Nézan & Chomérat, 2011; Craveiro *et al.*, 2016; McCarthy *et al.*, 2018), as well as it differs from *Bysmatrum* in the configuration of anterior intercalary plates. Plates 2a and 3a are separated in *Bysmatrum*, but they touch in strain TIO278. Strain TIO278 shares a similar Sa plate with *Heterocapsa* and *Laciniporus*, but it differs in the plate pattern. Only two anterior intercalary plates are present in *Heterocapsa* and *Laciniporus* and an eyespot is not observed (Tillmann *et al.*, 2017, Saburova & Chomérat 2018). Strain TIO278 shares a type A eyespot with *Peridinium* but differs in the number of cingular plates (six versus five), therefore, a new genus *Caladoa* is established to incorporate TIO278 and related strains.

Caladoa arcachonensis is morphologically close to the freshwater “*Peridinium subatranum*” (invalidly described), *Apocalathium aciculiferum* var. *inermis* (J. Wołoszyńska) Ø. Moestrup & A.J. Calado and *Chimonodinium lomnickii* var. *minus* (J. Wołoszyńska) Ø. Moestrup based on the plate pattern and a large Sa plate, but it differs in the relative size of anterior intercalary plates and the size of the apical pore (Wołoszyńska, 1916, Wołoszyńska, 1937, Wołoszyńska, 1952, Moestrup & Calado, 2018). *Caladoa arcachonensis* is also morphologically close to *Chimonodinium lomnickii* (J. Wołoszyńska) S.C. Craveiro, A.J. Calado, N. Daugbjerg, Gert Hansen & Ø. Moestrup and *Chimonodinium lomnickii* var. *wierzejskii* (J. Wołoszyńska) S.C. Craveiro, A.J. Calado, N. Daugbjerg, Gert Hansen & Ø. Moestrup but differs in the size and location of

the Sa and relative size of anterior intercalary plates (Craveiro *et al.*, 2011, Moestrup & Calado, 2018). *Caladoa arcachonensis* differs from *Peridinium gutwinski*J. Wołoszyńska and *Peridinium bipes* F. Stein in the number of cingular plates and relative size of anterior intercalary plates (Boltovskoy, 1989; Lee *et al.*, 2006), and differs from *Peridinium allorgei* M. Lefèvre in the cell shape and relative size of anterior intercalary plates (Moestrup & Calado 2018). *Caladoa arcachonensis* differs from *Apocalathium malmogiense* (G. Sjöstedt) S.C. Craveiro, N. Daugbjerg, Ø. Moestrup & A.J. Calado in a much larger epitheca and relative size of anterior intercalary plates (Craveiro *et al.*, 2016).

Phylogeny and genetic differentiation

The only morphological difference between French strain TIO278 and Indonesian strain TIO339 is the cell size, therefore, they are considered to be the same morphospecies. These strains however inhabit temperate and tropical waters, respectively, and are likely to differ in their eco-physiological traits. These two strains also have a genetic distance, based on ITS sequences, as much as 0.17, and that is much larger than the threshold value (0.04) for differentiating dinoflagellate species at interspecific level (Litaker *et al.*, 2007), therefore suggesting the existence of cryptic species. The French strain has a cyst stage, but whether the Indonesian strain produces cysts is yet to be determined.

Our molecular phylogeny supports the classification of *Caladoa* within the order Peridiniales, consistent with the morphological features characteristic of Peridiniales, such as a symmetrical 1' plate and two symmetrical antapical plates. The close

relationship between *Caladoa* and *Bysmatrum*, however, is surprising. *Bysmatrum* shows an asymmetrical 1' plate and two somewhat asymmetrical antapical plates that even do not fit to the definition of Peridiniales (Fensome *et al.*, 1993). However, *Bysmatrum* and *Caladoa* share identical plate pattern and most importantly, they share a type A eyespot (located within the chloroplast and consisted of ~~several~~two rows of opaque globules) (Moestrup & Daugbjerg, 2007; Dawut *et al.*, 2018). A type A eyespot appears to be a common feature of Peridiniales, reported also in *Scrippsiella acuminata* and *Chimonodinium lomnickii* (Craveiro *et al.*, 2011), *Palatinus apiculatus* (C.G. Ehrenberg) S. Craveiro, A. Calado, N. Daugbjerg & Ø. Moestrup (Craveiro *et al.*, 2009), *Naiadinium polonicum* (J. Wołoszyńska) S. Carty (Craveiro *et al.*, 2015), and *Peridinium willei* H. Huitfeldt-Kaas (Moestrup & Daugbjerg, 2007). *Bysmatrum* can also have a type B eyespot but this is a modified type A eyespot, with the difference only in the presence of an overlying brick like crystals (Luo *et al.*, 2018). *Caladoa* and *Bysmatrum* both have proximate cysts that are similar to the motile cells (Anglès *et al.*, 2017; Luo *et al.*, 2018). A new family is possible for *Caladoa* and *Bysmatrum* in view of their morphological similarity, but evidence from more closely related species is needed.

The close relationship between *Vulcanodinium* and *Peridinium* sensu stricto has been reported before based on the LSU rDNA sequences (Nézan & Chomérat, 2011), concatenated data from SSU, ITS and LSU rDNA sequences (Luo *et al.*, 2018), and supported here again based on concatenated data from SSU and LSU rDNA sequences. It is worth noting that the closest relative of *Peridinium* is a brackish species, in contrast

with the suggestion that marine and freshwater species are usually not closely related (Logares *et al.*, 2007). However, *Vulcanodinium* has six cingular plates compared to five in *Peridinium* sensu stricto (Boltovskoy, 1975; Nézan & Chomérat, 2011), and *Vulcanodinium* does not have a type A eyespot whereas *Peridinium cinctum* and *P. willei* have one (Spector & Triemer, 1979; Moestrup & Daugbjerg, 2007). The assignment of *Vulcanodinium* to Peridiniaceae is possible depending on the emendation of Peridiniaceae. Multi-gene phylogenies or phylotranscriptomics should provide more evidence for a definite assignment.

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Author contributions

Z. Luo: light microscopy, electron microscopy and drafting manuscript; K.N. Mertens: original concept, drafting and editing manuscript, incubation experiment, light

microscopy; E. Nézan: drafting and editing manuscript; Li Gu: electron microscopy; Vera Pospelova: palynological treatment, drafting and editing manuscript; H. Thoha: sampling and editing manuscript; H. Gu: original concept and editing of manuscript.

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657 **Figure captions**

658 **Figs. 1–4.** Light micrographs of cysts and cells of *Caladoa arcachonensis* from France.

659 **Fig. 1.** Ventral view showing the outline and a post-equatorial cingulum of a living

660 cyst with a pyrenoid (p) and eyespot (arrow). **Fig. 2.** The cyst in Fig. 1 upon

661 germination showing the chasmic archeopyle (arrow). **Fig. 3.** The cyst in Fig. 1

662 upon germination showing granular surface. **Fig. 4.** Ventral view of the germinated

cell from the cyst in Fig. 1, showing a red eyespot in the sulcal area (e). Scale bars
= 5 μ m.

Figs. 5–8. Light micrographs of cells of *Caladoa arcachonensis* strain TIO278 from France. **Fig. 5.** Ventral view showing a rounded epitheca and hypotheca with a post-equatorial cingulum. **Fig. 6.** Lateral view showing a ring like pyrenoid (p) and a red eyespot (arrow). **Fig. 7.** Epifluorescence image of a cell in ventral view showing the single reticulate chloroplast (c) in the periphery of the cell. **Fig. 8.** Epifluorescence image of a SYBR Green-stained cell showing a spherical nucleus (N). Scale bars = 5 μ m.

Figs. 9–12. Scanning electron micrographs of vegetative cells of *Caladoa arcachonensis* strain TIO278 from France. **Fig. 9.** Ventral view showing the first apical plate (1'), anterior sulcal plate (Sa), right sulcal plate (Sd), posterior sulcal plate (Sp), and the first three cingular plates (C1 – C3). **Fig. 10.** Dorsal view showing three anterior intercalary plates (1a – 3a), three precingular plates (3'' – 5''), three cingular plates (C3 – C5) and two postcingular plates (3''', 4'''). **Fig. 11.** Ventral-lateral right view showing two precingular plates (6'', 7''), sixth cingular plate (C6) and fifth postcingular plate (5'''). **Fig. 12.** Dorso-antapical view showing five postcingular plates (1''' – 5''') and two antapical plates (1''', 2''') of similar size. Scale bars = 5 μ m.

Figs. 13–16. Scanning electron micrographs of vegetative cells of *Caladoa arcachonensis* strain TIO278 from France. **Fig. 13.** Ventro-apical view showing four apical plates (1' – 4'), the anterior sulcal plate (Sa), the first anterior intercalary plate (1a) and three precingular plates (1'', 2'', 7''). **Fig. 14.** Dorso-apical view showing three apical plates (2' – 4'), three anterior intercalary plates (1a – 3a) and five precingular plates (2'' – 6''). **Fig. 15.** Internal view of the hypotheca showing six cingular plates (C1 – C6) and right sulcal plate (Sd), left sulcal plate (Ss), median sulcal plate (Sm) and posterior sulcal plate (Sp). **Fig. 16.** Sulcal area showing the first cingular plate (C1) and plates Sa, Sd, Sp and Ss (arrow). Scale bars = 5 µm.

Figs. 17–20. Schematic drawings of thecal plate patterns of *Caladoa arcachonensis*. **Fig. 17.** Ventral view. **Fig. 18.** Dorsal view. **Fig. 19.** Apical view showing plate overlap patterns. **Fig. 20.** Antapical view showing plate overlap patterns.

Figs. 21–26. Transmission electron micrographs of vegetative cells of *Caladoa arcachonensis* strain TIO278 from France. **Figs. 21, 22.** Longitudinal sections through the cell showing a large nucleus (N), a stalked pyrenoid (p), a single chloroplast (c) in the periphery of the cell and an eyespot (arrow). **Fig. 23.** The chloroplast showing the thylakoids grouped in threes to form lamellae and several

trichocysts (arrow). **Fig. 24.** Detail of the stalked pyrenoid (p) with dense matrices and a surrounding starch (s). **Figs. 25, 26.** The eyespot (e) located within a chloroplast (c) comprising two rows of globular lipids. Scale: Figs. 21, 22 = 5 μm , Figs. 23–26 = 1 μm .

Figs. 27–28. Light micrographs of vegetative cells of *Caladoa arcachonensis* strain TIO339 from Indonesia. **Fig. 27.** Lateral view of a living cell showing a pyrenoid (p), a nucleus (N) and an eyespot (arrow). **Fig. 28.** Dorwal view of a living cell showing a single chloroplast forming a network. Scale bars = 5 μm .

Figs. 29–34. Scanning electron micrographs of vegetative cells of *Caladoa arcachonensis* strain TIO339 from Indonesia. **Fig. 29.** Ventro-antapical view showing the anterior sulcal plate (Sa), the right sulcal plate (Sd), posterior sulcal plate (Sp), two postcingular plates (1''', 5''') and two antapical plates (1''', 2'''). **Fig. 30.** Dorsal view showing three precingular plates (4'' – 6''), three cingular plates (C4 – C6) and three postcingular plates (3''' – 5'''). **Fig. 31.** Dorso-apical view showing four precingular plates (2'' – 5''), three anterior intercalary plates (1a – 3a). **Fig. 32.** Antapical view showing four postcingular plates (2''' – 5'''), plate Sp and two antapical plates (1''', 2''') of similar size. **Fig. 33.** Internal view of the hypotheca showing six cingular plates (C1 – C6). **Fig. 34.** The sulcus showing Sa, Sd, Sp and left sulcal plate (arrow). Scale bars = 5 μm .

Fig. 35. Phylogeny of *Caladoa* inferred from concatenated SSU and partial LSU rDNA sequences using maximum likelihood (ML). New sequences are indicated in bold. Branch lengths are drawn to scale, with the scale bar indicating the number of nucleotide substitutions per site. Numbers on branches are statistical support values to clusters on the right of them (left: ML bootstrap support (BS) values; right: Bayesian posterior probabilities (BPP). Bootstrap support values >50% and Bayesian posterior probabilities above 0.7 are shown. Asterisk indicates maximal support (ML BS = 100% and BPP = 1.00).

Table 1 Strains of *Caladoa arcachonensis* examined in the present study, including collection data, locations and sources

Species	Strains	Longitude	Latitude	Collectiton date	Sources	Collection locality
<i>Caladoa arcachonensis</i>	TIO278, TIO282	1°4'0.0" W	44°38'11.0"N	15 and 19 April 2016	Sediment	Arcachon Bay, France
<i>Caladoa arcachonensis</i>	TIO339	124°51'6.4"E	1°35'44.8"N	2016.07.31	Seagrass	Manado, Indonesia
<i>Caladoa arcachonensis</i>	TIO340	125°13'38.6"E	1°32'3.7"N	2016.08.01	Coral <u>sands</u>	Bitung, Indonesia

Figs. 1–4. Light micrographs of cysts and cells of *Caladoa arcachonensis* from France. Fig. 1. Ventral view showing the outline and a post-equatorial cingulum of a living cyst with a pyrenoid (p) and eyespot (arrow). Fig. 2. The cyst in Fig. 1 upon germination showing the chasmic archeopyle (arrow). Fig. 3. The cyst in Fig. 1 upon germination showing granular surface. Fig. 4. Ventral view of the germinated cell from the cyst in Fig. 1, showing a red eyespot in the sulcal area (e). Scale bars = 5 µm.

170x193mm (300 x 300 DPI)

Figs. 5–8. Light micrographs of cells of *Caladoa arcachonensis* strain TIO278 from France. Fig. 5. Ventral view showing a rounded epitheca and hypotheca with a post-equatorial cingulum. Fig. 6. Lateral view showing a ring like pyrenoid (p) and a red eyespot (arrow). Fig. 7. Epifluorescence image of a cell in ventral view showing the single reticulate chloroplast (c) in the periphery of the cell. Fig. 8. Epifluorescence image of a SYBR Green-stained cell showing a spherical nucleus (N). Scale bars = 5 µm.

80x94mm (300 x 300 DPI)

Figs. 9–12. Scanning electron micrographs of vegetative cells of *Caladoa arcachonensis* strain TIO278 from France. Fig. 9. Ventral view showing the first apical plate (1'), anterior sulcal plate (Sa), right sulcal plate (Sd), posterior sulcal plate (Sp), and the first three cingular plates (C1 – C3). Fig. 10. Dorsal view showing three anterior intercalary plates (1a – 3a), three precingular plates (3'' – 5''), three cingular plates (C3 – C5) and two postcingular plates (3''' – 4'''). Fig. 11. Ventral-lateral right view showing two precingular plates (6'', 7''), sixth cingular plate (C6) and fifth postcingular plate (5'''). Fig. 12. Dorso-antapical view showing five postcingular plates (1''' – 5''') and two antapical plates (1''', 2''') of similar size. Scale bars = 5 μm.

170x205mm (300 x 300 DPI)

Figs. 13–16. Scanning electron micrographs of vegetative cells of *Caladoa arcachonensis* strain TIO278 from France. Fig. 13. Ventro-apical view showing four apical plates (1' – 4'), the anterior sulcal plate (Sa), the first anterior intercalary plate (1a) and three precingular plates (1'', 2'', 7''). Fig. 14. Dorso-apical view showing three apical plates (2' – 4'), three anterior intercalary plates (1a – 3a) and five precingular plates (2'' – 6''). Fig. 15. Internal view of the hypotheca showing six cingular plates (C1 – C6) and right sulcal plate (Sd), left sulcal plate (Ss), median sulcal plate (Sm) and posterior sulcal plate (Sp). Fig. 16. Sulcal area showing the first cingular plate (C1) and plates Sa, Sd, Sp and Ss (arrow). Scale bars = 5 µm.

170x173mm (300 x 300 DPI)

Figs. 21–26. Transmission electron micrographs of vegetative cells of *Caladoa arcachonensis* strain TIO278 from France. Figs. 21, 22. Transverse section through the cell showing a large nucleus (N), a stalked pyrenoid (p), a single chloroplast (c) in the periphery of the cell and an eyespot (arrow). Fig. 23. The chloroplast showing the thylakoids grouped in threes to form lamellae and several trichocysts (arrow). Fig. 24. Detail of the stalked pyrenoid (p) with dense matrices and a surrounding starch (s). Figs. 25, 26. The eyespot (e) located within a chloroplast (c) comprising several rows of globular lipids. Scale: Figs. 21, 22 = 5 μ m, Figs. 23–26 = 1 μ m.

170x138mm (300 x 300 DPI)

Figs. 27–28. Light micrographs of vegetative cells of *Caladoa arcachonensis* strain TIO339 from Indonesia. Fig. 27. Lateral view of a living cell showing a pyrenoid (p), a nucleus (N) and an eyespot (arrow). Fig. 28. Dorsal view of a living cell showing a single chloroplast forming a network. Scale bars = 5 µm.

80x47mm (300 x 300 DPI)

Figs. 29–34. Scanning electron micrographs of vegetative cells of *Caladoa arcachonensis* strain TIO339 from Indonesia. Fig. 29. Vento-antapical view showing the anterior sulcal plate (Sa), the right sulcal plate (Sd), posterior sulcal plate (Sp), two postcingular plates (1''', 5''') and two antapical plates (1''''', 2'''''). Fig. 30. Dorsal view showing three precingular plates (4'' – 6''), three cingular plates (C4 – C6) and three postcingular plates (3'''' – 5'''). Fig. 31. Dorso-apical view showing four precingular plates (2'' – 5''), three anterior intercalary plates (1a – 3a). Fig. 32. Antapical view showing four postcingular plates (2'''' – 5'''), plate Sp and two antapical plates (1''''', 2''''') of similar size. Fig. 33. Internal view of the hypotheca showing six cingular plates (C1 – C6). Fig. 34. The sulcus showing Sa, Sd, Sp and left sulcal plate (arrow). Scale bars = 5 µm.

170x118mm (300 x 300 DPI)

Fig. 35. Phylogeny of *Caladoa* inferred from concatenated SSU and partial LSU rDNA sequences using maximum likelihood (ML). New sequences are indicated in bold. Branch lengths are drawn to scale, with the scale bar indicating the number of nucleotide substitutions per site. Numbers on branches are statistical support values to clusters on the right of them (left: ML bootstrap support (BS) values; right: Bayesian posterior probabilities (BPP)). Bootstrap support values >50% and Bayesian posterior probabilities above 0.7 are shown. Asterisk indicates maximal support (ML BS = 100% and BPP = 1.00).

196x254mm (300 x 300 DPI)