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Morphology and molecular phylogeny of *Bindiferia* gen. nov. (Dinophyceae), a new marine, sand-dwelling dinoflagellate genus formerly classified within *Amphidinium*

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ABSTRACT

As historically described, the genus *Amphidinium* has long been recognized to be polyphyletic. *Amphidinium sensu lato* is the most diverse of all marine benthic dinoflagellate genera and after the redefinition of the *sensu stricto* genus about 100 species remained of unknown generic affiliation. A species similar to the Australian *sensu lato* species *Amphidinium boggayum* was discovered in France. Morphological and molecular phylogenetic data supported the description of a new species and the classification of the two closely related species within a new athecate, photosynthetic, benthic genus, named *Bindiferia*. Cells were dorsoventrally flattened with smaller asymmetric epicone. The cingulum was descending. The sulcus reached the antapex and had an extension onto the epicone. The apical structure complex formed an anticlockwise loop around the apex. A red stigma was located in the anterior part of the epicone, near the apex. Vegetative division occurred in a non-motile stage within a hyaline sheath. Non-motile cells had dorsal hypocone grooves. The two species differed from each other in epicone size, cingulum displacement, nucleus location and surface groove occurrence. The molecular phylogenetic results showed that *Bindiferia* is a distinct taxon that is only distantly related to *Amphidinium sensu stricto*. The nearest sister group to *Bindiferia* could not be reliably determined, but the apical structure complex suggested a close relationship to the genera of the *Gymnodinium sensu stricto* clade.

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Stigma; Taxonomy

INTRODUCTION

The genus *Amphidinium* Claparède & J. Lachmann was among the largest and most diverse of all marine benthic dinoflagellate genera, containing about 120 species (e.g. Murray & Patterson 2002), but the genus was found to be polyphyletic (Flø Jørgensen *et al.* 2004a). Modern methods have been used to re-investigate the type species of the athecate genera *Gymnodinium* F. Stein and *Gyrodinium* Kofoed & Swezy (Daugbjerg *et al.* 2000; Hansen *et al.* 2000; Hansen & Daugbjerg 2004; Takano & Horiguchi 2004). More precise re-definitions of these genera have caused many of the species formerly assigned to them to be considered ‘*sensu lato* (*s.l.*) taxa’, i.e. taxa without generic affiliation. To distinguish *Amphidinium* from other athecate genera, overly generalized criteria such as epicone dimensions (shorter than 1/3 of the cell length) and cingulum displacement were used in the past. After re-investigations of *A. operculatum* Claparède & J. Lachmann (the type species) and putative relatives, the genus *Amphidinium* was also re-defined as dorsoventrally flattened, athecate dinoflagellates with a minute epicone that

overlays the anterior ventral part of the hypocone and deflects to the left (Murray *et al.* 2004; Flø Jørgensen *et al.* 2004a). The epicone can be irregular triangular-shaped or crescent-shaped. Cells may or may not be photosynthetic. Of the taxa previously classified within *Amphidinium*, only about 20 species fell into the *Amphidinium* Claparède & J. Lachmann *emend.* M.F. Jørgensen, Sh. Murray & Daugbjerg ‘*sensu stricto*’ (*s.s.*) definition, leaving about 100 species of uncertain or unknown generic affiliation (Murray 2003). Recently, six new *Amphidinium s.s.* species were described using a multiple evidence approach (Karafas *et al.* 2017). Six new genera have been characterized already, *Togula* M.F. Jørgensen, Sh. Murray & Daugbjerg, *Prosoaulax* Calado & Moestrup, *Apicoporus* Sparmann, B.S. Leander & Hoppenrath, *Ankistrodinium* Hoppenrath, Sh. Murray, Sparmann & B.S. Leander, *Testudodinium* T. Horiguchi, Maiko Tamura, Katsumata & A. Yamaguchi and *Nusuttodinium* Y. Takano & T. Horiguchi *emend.* R. Onuma & T. Horiguchi (Flø Jørgensen *et al.* 2004b; Calado & Moestrup 2005; Sparmann *et al.* 2008; Hoppenrath *et al.* 2012; Horiguchi *et al.* 2012;

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Takano *et al.* 2014; Onuma *et al.* 2015), of which four are marine benthic genera and one contains benthic species (including its type). *Amphidinium pellucidum* Herdman was transferred into the genus *Gymnodinium* as *G. venator* M.F. Jørgensen & Sh. Murray (Flø Jørgensen *et al.* 2004a, c). More recently, newly discovered taxa with *Amphidinium s.l.* morphology were directly described as type species of new genera, like *Bispinodinium angelaceum* Norico Yamada & T. Horiguchi and *Pellucidodinium psammophilum* R. Onuma & T. Horiguchi (Yamada *et al.* 2013; Onuma *et al.* 2015).

The genus selection for *Amphidinium boggayum* Sh. Murray & D.J. Patterson was an arbitrary choice based on the cingulum placement in relation to the cell apex (Murray & Patterson 2002). The species would have also met criteria for a classification within *Gymnodinium s.s.* (Daugbjerg *et al.* 2000). Later, it has been hypothesized by Flø Jørgensen *et al.* (2004a) that *A. boggayum* belongs to *Gymnodinium* but without formal transfer because of missing statistical support in their cladistic analysis of morphological characters and the lack of molecular phylogenetic data.

During biodiversity studies of benthic dinoflagellates in Brittany, France (Hoppenrath *et al.* 2013) and Broome, NW Australia (Murray *et al.* 2006a, b; Kohli *et al.* 2014; Verma *et al.* 2019), cells similar to *Amphidinium boggayum* were observed and isolated for taxonomical re-investigation of this 'nameless' (without generic identity) *Amphidinium s.l.* taxon and to classify it at genus level. A strain similar to *A. boggayum* isolated from New Zealand (CAWD164, Rhodes *et al.* 2010) was analysed in this study to determine whether specimens from other regions were similar morphologically and genetically to one another.

MATERIAL AND METHODS

Sampling

For *Bindiferia fragilissima sp. nov.*, intertidal sand was collected 8 September 2010 at Le Letty, 'Mer Blanche' (kind of lagoon with small opening to the Atlantic) close to Bénodet, France (47°51.52'N, 4°5.04'W). The samples were taken from the surface (upper 2 cm) and dinoflagellates were extracted by the seawater ice method (Uhlir 1964; Hoppenrath *et al.* 2014).

For *Bindiferia boggaya comb. nov.*, intertidal sand samples were collected at Town Beach, Broome (17°58.08'S, 122°14.16'E), in the tropical north of Western Australia, in September 2003 and in May 2011. The sediment water temperature ranged from 22°C to 29°C and the salinity ranged from 38 to 40 during the sampling period. Samples were also collected from Port Botany, Sydney (33°58.08'S, 151°12.57'E) in June 2003. The first 2 cm or 0.5 cm of sediment were sampled at low tide using a flat spoon (Murray & Patterson 2002).

Culturing

For *Bindiferia fragilissima sp. nov.*, single cells were isolated by micro-pipetting, washed in filtered seawater and then transferred into medium. Clonal cultures were maintained at 19°C under a photon flux density of 6.5 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 12:12 h

(light:dark) cycle in *f/2* medium (Guillard & Ryther 1962) prepared with sterile filtered seawater, salinity 32, in Germany. A cultured strain identified as *A. boggayum*, SM20, was established from a sample collected in Port Botany in June 2003 by picking cells under a Leica DMR light microscope. A single cell was washed in a series of filtered seawater. The strain was maintained in *f/2* medium, with a 12:12 h (light:dark) cycle at 20°C, with a photon flux density of *c.* 60 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

A cultured strain, CAWD164, established from a surface sediment sample collected from Rangaunu Harbour, New Zealand (34°58' S, 173°16' E) in February 2009 was identified as *Amphidinium boggayum* during this study. CAWD164 strain was cultured in *f/2* medium at 25°C, with a 12:12 h (light:dark) cycle under a photo flux density of 40–70 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Two cultured strains of *Togula jolla* M.F. Jørgensen, Sh. Murray & Daugbjerg (CAWD41 and CAWD58) from the Cawthron Collection were additionally sequenced in this study for comparison with *Bindifera*. These strains were originally isolated from New Zealand sand samples, CAWD41 from Awaroa Inlet (Abel Tasman, South Island) and CAWD58 from Napier (North Island).

Microscopy

Living cells of both species were picked using a Leica DMIL inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany), placed on an object slide and observed with a Leica DMRB equipped with differential interference contrast optics at 400× and 640× magnification with oil immersion objectives in Germany. Digital micrographs were taken using a Leica DFC290 and DFC420C camera. A Leica DMI8 inverted microscope with a Leica DFC7000T camera (Leica Microsystems GmbH, Wetzlar, Germany) was used to document cells in the culture Petri dishes.

Bindifera boggaya comb. nov. cells were observed and photographed using a Leica DMR light microscope (Leica Microsystems GmbH, Wetzlar, Germany), a Zeiss Axiophot microscope (Zeiss, München-Halbergmoos, Germany) with differential interference contrast optics and a Zeiss Axiocam digital camera in Australia. An Olympus IX73 inverted microscope with DP27 5 megapixel digital camera (Olympus Pty Ltd, Melbourne, Australia) was used in New Zealand.

For scanning electron microscopy (SEM) of *B. boggaya*, cells were pipetted individually from samples, rinsed three times in filtered seawater and placed on polylysine-coated coverslips. They were fixed in 2% osmium tetroxide in seawater for 20 min, rinsed in distilled water and dehydrated in a series of increasing ethanol concentrations (15, 30, 50, 70, 90, 100%). The cells were critical point dried, sputter coated with gold and observed using a Phillips 505 or a JEOL 840 scanning electron microscope at 5–20 kV.

At least seven fixation protocols were tested for SEM of the French isolate but none was successful. In most cases no cells could be observed because they burst during fixation or at the final dehydration steps. In one case, the few potential cells we found were completely covered by mucus. In a transmission electron microscopic (TEM) preparation only a mess of unordered membranes and vesicles were visible.

DNA extraction, PCR amplification and sequencing

For DNA amplification of *Bindiferia fragilissima* (French culture), and since it was difficult to obtain sequences from extracted DNA, a few cells (10–20) were isolated using a capillary pipette and put directly in a PCR tube. A direct amplification was realized using a nested PCR approach. A first round allowed the amplification of a long amplicon (covering SSU rDNA and partial LSU rDNA) using 18SFW and Dino-ND primers (Hansen & Daugbjerg 2004; Chomérat et al. 2010). It was then possible to obtain sequences for both genes using the internal primers described in Chomérat et al. (2010). The KOD Xtreme hot start DNA polymerase (Novagen) has been used according to the manufacturer's recommendations. The thermal cycles were one initial denaturing step at 94°C for 2 min, 35 cycles of 98°C for 10 s, 52°C for 30 s and 68°C for 3 min.

A volume of 10–80 ml of culture of *B. boggaya* (SM20) was centrifuged for 10 min at 1500–3000 rpm and the pellet transferred to a 1.5-ml Eppendorf tube. DNA was extracted using a modified CTAB method (Flø Jørgensen et al. 2004a). Approximately 1450 bp of the LSU rDNA covering the variable domains D1–D6 were amplified using the primers D1R (Scholin et al. 1994) and 28–1483 R (Daugbjerg et al. 2000); the PCR reaction mixture consisted of 2.5 mM MgCl₂, 1 unit of DNA polymerase (Bioline, Astral Scientific, Caringbah, Australia), 10× NH₄ buffer, 0.2 mM of mixed dNTPs and 10 pmol of primers. The thermal cycles were one initial denaturing step at 94°C for 3 min, 35 cycles of 94°C for 30–60 s, 50–55°C for 30–60 s and 72°C for 1–3 min and a final cycle of 72°C for 5–6 min.

To prepare for extraction, cultures of *B. boggaya* (CAWD164) and *Togula jolla* (CAWD41 and CAWD58) were centrifuged (50 ml, 3000× g, 10 min) and the supernatant decanted, with pelleted cells frozen until further extraction. DNA was extracted using the DNeasy® Powersoil® DNA isolation kit (Qiagen) following the protocol for the automated DNA extraction robot QIAcube® (Qiagen, Carlsbad, USA). The D1–D3 region of the LSU rDNA was amplified using the primer pair D1R and D3B (Nunn et al. 1996), a partial region of the SSU rDNA was amplified using EUKA-EUKB primers (Medlin et al. 1988) and the internal transcribed spacer (ITS) region was amplified with the ITSA and ITSB primer pair (Adachi et al. 1994). Each PCR

reaction master mix included 25 µl MyTaq 2x PCR mix (Bioline, Massachusetts, USA), 2 µl of both forward and reverse primers, 2 µl bovine serum albumin (BSA) and 50–100 ng of template DNA for a total reaction volume of 50 µl. For the primer pair for domains D1–D3 the thermo cycle included an initial denaturing step of 95°C for 4 min, followed by 35 cycles of 95°C for 30 s and 60°C for 2 min, with a final extension of 72°C for 10 min. All other primer pairs thermo cycle conditions included an initial denaturing step of 95°C for 2 min, 35 cycles of 95°C for 30 s, 54°C for 30 s and 72°C for 1 min, with final extension of 72°C for 5 min. Resulting PCR products were purified using the AxyPrep PCR clean-up kits (Axygen, California, USA) then sent to Genomic Analysis services (GAS, University of Otago, New Zealand) for direct sequencing in both directions. Purified PCR product for isolate CAWD164 SSU was sent to Macrogen (Geumcheon-gu, Seoul, South Korea) for commercial cloning as direct sequencing of this region was ineffective. Returned forward and reverse sequences were trimmed using Geneious v9.1.8 (Kearse et al. 2012), aligned using the ClustalW algorithm (Thompson et al. 1994) in Geneious and manually inspected for conflicts.

Alignment and phylogenetic analyses

All new sequences were deposited in GenBank (Table 1). Phylogenetic analyses were performed using initial alignments previously developed (Orr et al. 2012; Murray et al. 2015). Additional dinoflagellate sequences were obtained and added as described above, or downloaded from the GenBank reference database (www.ncbi.nlm.nih.gov). Each rRNA gene (18S, ITS1-5.8S-ITS2 and 28S) was separately aligned using the MUSCLE algorithm (Edgar 2004) as implemented in Geneious Prime. Outgroup taxa were established from previous phylogenies (Murray et al. 2005; Orr et al. 2012). Single gene alignments were checked manually using Geneious Prime, and poorly aligned regions were removed. Alignments were then concatenated, consisting of 114 taxa and 2880 characters, and will be made available under Shauna Murray's profile on ResearchGate.

Phylogenetic trees were calculated with Bayesian Inference (BI) and Maximum Likelihood (ML). The model with the highest Bayesian Information criterion score for the alignment was determined using ModelFinder (Kalyaanamoorthy

Table 1. Accession numbers for newly determined rDNA sequences of *Bindiferia boggaya*, *B. fragilissima* and *Togula jolla* collected at different sites.

accession	latitude, longitude	country: site	species	strain	rRNA region
MW722976	34°58.00'S, 173°16.00'E	New Zealand: Rangaunu	<i>Bindiferia boggaya</i>	CAWD164	ITS
MW722979	34°58.00'S, 173°16.00'E	New Zealand: Rangaunu	<i>Bindiferia boggaya</i>	CAWD164	LSU D1D3
MW720712	34°58.00'S, 173°16.00'E	New Zealand: Rangaunu	<i>Bindiferia boggaya</i>	CAWD164	LSU D8D10
MW720768	34°58.00'S, 173°16.00'E	New Zealand: Rangaunu	<i>Bindiferia boggaya</i>	CAWD164	SSU
MW722980	40°52.12'S, 172°59.24'E	New Zealand: Awaroa	<i>Togula jolla</i>	CAWD41	LSU D1D3
MW722977	40°52.12'S, 172°59.24'E	New Zealand: Awaroa	<i>Togula jolla</i>	CAWD41	ITS
MW720769	40°52.12'S, 172°59.24'E	New Zealand: Awaroa	<i>Togula jolla</i>	CAWD41	SSU
MW722978	39°31.12'S, 176°53.24'E	New Zealand: Napier	<i>Togula jolla</i>	CAWD58	ITS
MW720770	39°31.12'S, 176°53.24'E	New Zealand: Napier	<i>Togula jolla</i>	CAWD58	SSU
MZ188971	33°58.08'S, 151°12.57'E	Australia: Sydney	<i>Bindiferia boggaya</i>	SM20	LSU D1D3
MZ233647	47°51.52'N, 04°05.04'W	France: Letty	<i>Bindiferia fragilissima</i>	CCAP 1100/1	SSU
MZ233677	47°51.52'N, 04°05.04'W	France: Letty	<i>Bindiferia fragilissima</i>	CCAP 1100/1	LSU

et al. 2017), and was found to be a GTR with a proportion of invariant sites and 4 gamma rate categories, and was used for phylogenetic analyses.

Maximum likelihood (ML) phylogenetic trees were generated using IQtree (Nguyen *et al.* 2015) with 1000 bootstraps. Bayesian Inference (BI) was performed to estimate Posterior Probability (PP) distribution using MrBayes 3.2.6 with Metropolis-Coupled Markov chain Monte Carlo simulations (Ronquist & Huelsenbeck 2003). A random starting tree with 4 heated and one cold chain was used with temperature set at 0.2. Trees were sampled every 500 generations for 1,500,000 generations and the first 500,000 generations were discarded as burn-in. Phylogenies were visualized using FigTree (<http://tree.bio.ed.ac.uk/software/figtree/>) and Adobe Illustrator.

RESULTS

Taxonomy

Bindiferia Borchhardt, Chomérat, Sh. Murray & Hoppenrath *gen. nov.*

DESCRIPTION: Athecate, dorsoventrally flattened cells with smaller asymmetric epicone in comparison to the hypocone. Cingulum descending. Sulcus reaching the antapex and extending onto the epicone. Red stigma anterior in the epicone, close to the apex. It is likely that an apical structure complex forming an anticlockwise loop around the apex is also a generic feature but this could not be confirmed for the type yet.

ETYMOLOGY: 'Bindi' is a traditional decoration in India; a red drop, which is applied in the centre of the forehead and symbolizes an eye; 'feria' from Latin verb *fero, ferre*, to carry, to wear. The genus is feminine in gender.

REGISTRATION: <http://phycobank.org/102836>.

TYPE SPECIES: *Bindiferia fragilissima* Borchhardt, Chomérat & Hoppenrath.

Bindiferia fragilissima Borchhardt, Chomérat & Hoppenrath *sp. nov.* Figs 1–14, 33–35

DESCRIPTION: Athecate, dorsoventrally flattened, oval to oblong cells, 30–65 μm (50.9 $\mu\text{m} \pm 2.6$, $n = 44$) long, 25–50 μm (41.4 $\mu\text{m} \pm 1.6$, $n = 44$) wide and approximately 30 μm deep. The smaller asymmetric epicone was 0.2 of the total cell length and slightly narrower than the total cell width (Figs 1–6). The cingulum descends about two cingular widths (Figs 1, 3–5). The longitudinal flagellum is about 1.5–2 \times the cell length (Fig. 5). The narrow sulcus reaches the antapex after curving slightly to the right and it extends onto the epicone in a straight line (Figs 1, 3, 4, 6). The apical structure complex could not be observed. Oval to round nucleus located in the lower (sometimes left) hypocone half (Fig. 2). Golden-brown elongated chloroplasts scattered throughout the cell (Figs 1–3, S1). The visible elongated rods may be part of a (single?) chloroplast mesh (Fig. S1). A small (3.3–4.3 μm long, $n = 10$; 2.0–3.8 μm wide, $n = 30$), oval or rod-shaped, red stigma is located in the anterior-ventral part of the epicone, near the apex (Figs 2, 3, 5, 6, 8–12). After cells burst it looked like a separate structure not connected to any chloroplast. One or rarely two or several small, orange putative food bodies can be present, often visible above the nucleus. Many extrusomes can be visible along the cell periphery (Figs 2, 14a, upper image). Free-swimming cells can become stationary and rapidly change into a non-motile stage that is covered by a hyaline layer (under a coverslip). Motile cells are of the described morphology, see above (Figs 1–5, 14a). 'Stationary motile' cells are able to swim but do not swim. These cells

were wider and usually had two or three longitudinal grooves on the dorsal side of the hypocone (Figs 6–9, 14b). Non-motile cells are sheathed by an irregular hyaline layer, are roundish and usually have three longitudinal grooves on the dorsal side of the hypocone (Figs 10, 14c). On average they are larger, 45–63 μm long (55.5 $\mu\text{m} \pm 4.8$, $n = 20$) and 45–55 μm wide (50.4 $\mu\text{m} \pm 3.8$, $n = 20$). Vegetative cell division takes place during the non-motile stage (Figs 11, 12, 14d, 14e). In the hyaline sheath (temporary division cyst) also double divisions can happen, resulting in up to four cells per cyst. During 'normal' (= not multiple) division daughter cells are in the cyst most of the time and have one or two longitudinal grooves on the hypocone (Figs 12, 14d). The cell division starts at the antapex and the last parts to separate are the epicones (Figs 12, 13, 14e, 14f). The final daughter cell separation takes place outside the cyst (Figs 13, 14f). The still connected cells are already able to swim. After division smaller cells start growing (Fig. 14g). The cyst wall can have a double layer appearance (Fig. 11) and can persist for some time (shown on the Petri dish bottom under culture conditions, Fig. S2). The life cycle still needs further investigation.

HOLOTYPE: cultured cells preserved in ethanol (Art. 44.2 of the ICN; Turland *et al.* 2018) deposited at the dinoflagellate type collection in the Centre of Excellence for Dinophyte Taxonomy (CEDiT, Wilhelmshaven, Germany), which is part of the Herbarium Senckenbergianum Frankfurt/M. (FR) with the designation CEDiT2021H135.

REPRESENTATIVE FIGURES: Figs 1, 2 (same cell).

TYPE LOCALITY: Le Letty, 'Mer Blanche' close to Bénodet, France (47° 51.847'N, 4°5.054'W)

ETYMOLOGY: Latin adjective *fragilissimus*, -a, -um, the most fragile, very fragile; because of the difficulty to work with vegetative cells, exploding all the time.

REGISTRATION: <http://phycobank.org/102837>.

MOLECULAR CHARACTERIZATION: MZ233647 (SSU rRNA gene region), MZ233677 (LSU rRNA gene region).

AUTHENTIC STRAIN: Culture deposited at the Culture Collection of Algae and Protozoa (CCAP), strain designation CCAP 1100/1.

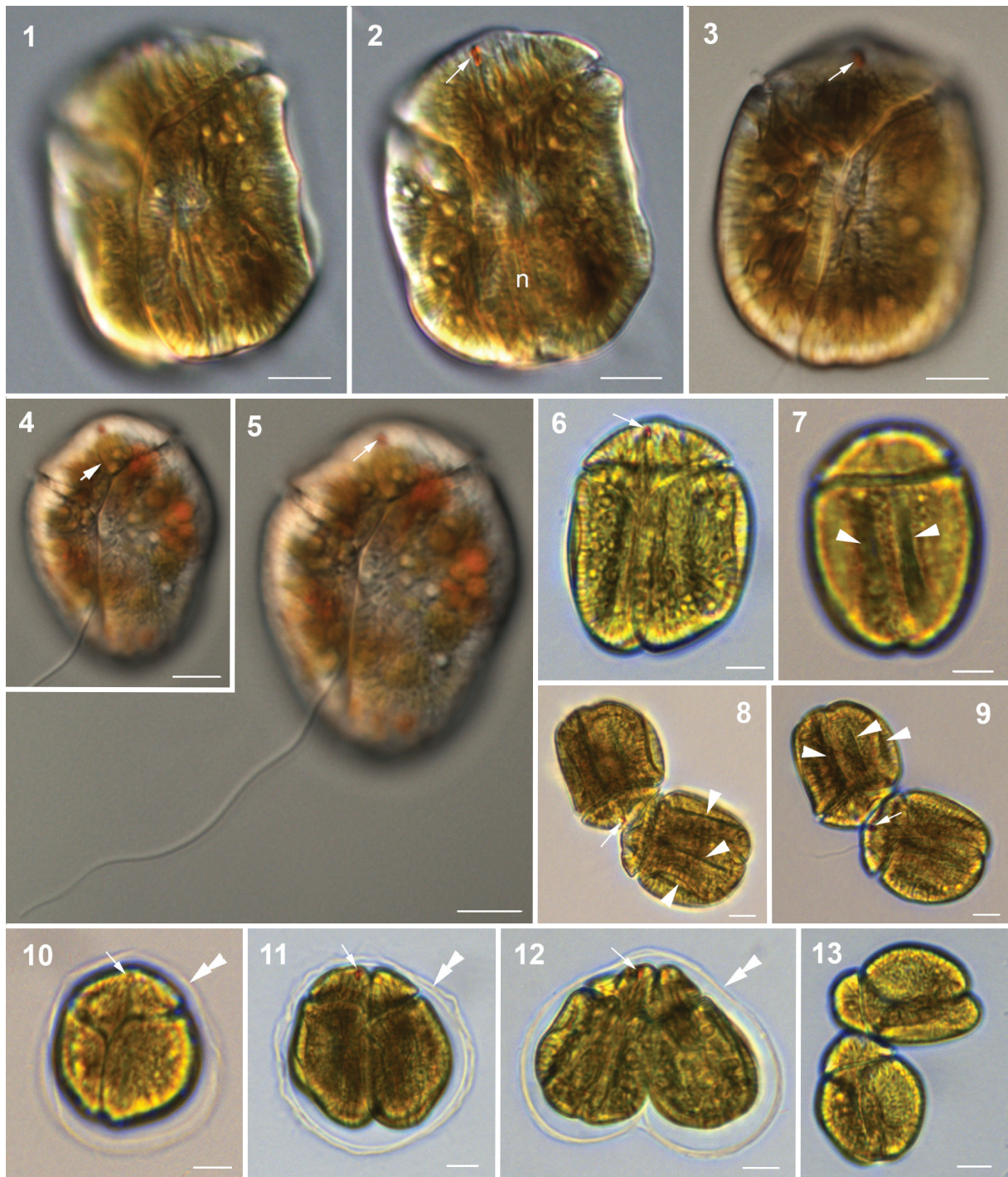
HABITAT: marine sandy sediments.

Bindiferia boggaya (Sh. Murray & D.J. Patterson) Sh. Murray & Hoppenrath *comb. nov.* Figs 15–28

BASIONYM: *Amphidinium boggayum* Sh. Murray & D.J. Patterson in Murray & Patterson 2002, *European Journal of Phycology* 37: 280, figs 2–7.

REGISTRATION: <http://phycobank.org/102841>.

EMENDED DESCRIPTION: Athecate dorsoventrally flattened, oval to oblong cells (Figs 15–17, 19, 20), 39–58 μm long, 25–45 μm wide and approximately 22 μm deep. Smaller epicone asymmetrical, apex slightly to the left cell side (Figs 15–17, 19). It was originally described that the proximal cingulum end originates 0.5–0.6 of the cell length from the apex, rising vertically to 0.2–0.3 of the cell length from the apex, then turning left and continuing at a slightly upward angle on the ventral side (Figs 15, 17, 19). In the present study (after further observations) it is concluded that the cingulum descends about four cingular widths. The furrow becomes narrow where the ends meet (Figs 15, 19). The longitudinal flagellum arises below the proximal cingulum end. The narrow sulcus curves to the right towards the posterior (Figs 17, 19), reaches and sometimes slightly indents the antapex (Fig. 16), and extends onto the epicone (Figs 15, 17). The apical structure complex is connected to the sulcal extension and forms an anticlockwise loop around the apex (Figs 17, 18). Oval nucleus located in the epicone (Figs 16, 20, 24). Numerous golden-brown chloroplasts scattered throughout the cell (Figs 19–25). A small red stigma in the anterior-ventral part of the epicone, near the apex (Figs 19, 23). Small, reddish, putative food bodies



Figs 1–13. Light micrographs of *Bindiferia fragilissima* sp. nov. from France. Scale bars = 10 μ m.

Figs 1, 2. Same actively motile cell in ventral view in different focal planes showing the sulcal path and cingulum; note the red stigma close to the apex (arrow) and the posterior nucleus (n).

Fig. 3. Actively motile cell in ventral view showing the sulcal path and cingulum; note the red stigma close to the apex (arrow).

Figs 4, 5. Same actively motile cell in ventral view showing the sulcal path including the extension onto the epicone (short arrow); note the red stigma close to the apex (arrow) and the length of the longitudinal flagellum.

Fig. 6. 'Stationary motile' cell; note the red stigma (arrow).

Fig. 7. 'Stationary motile' cell with two dorsal longitudinal grooves on the hypocone (arrowheads).

Figs 8, 9. Two 'stationary motile' cells with three dorsal longitudinal grooves on their hypocone (arrowheads); note the red stigma (arrow).

Fig. 10. Non-motile cell in ventral view covered by a hyaline layer (double arrowhead); note the red stigma (arrow).

Fig. 11. Non-motile division stage covered by a hyaline double-layer (double arrowhead); note the red stigma (arrow).

Fig. 12. Non-motile division stage with daughter cells starting to separate posteriorly, covered by a hyaline double-layer (double arrowhead); note the red stigma (arrow).

Fig. 13. Already motile, nearly divided, but still apically connected daughter cells outside the division cyst.

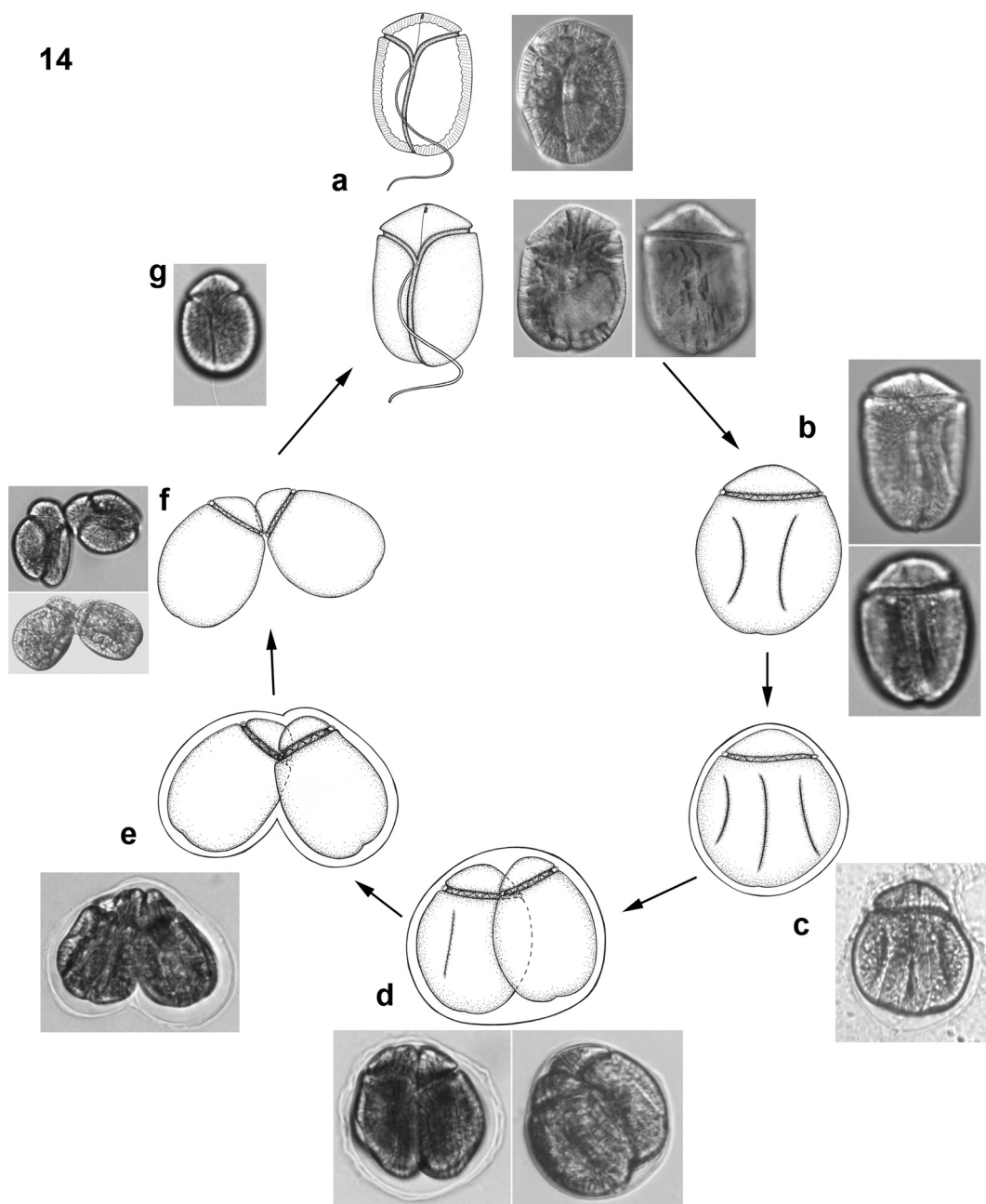


Fig. 14. Hypothetical vegetative life cycle (cell division) of *Bindiferia fragilissima* sp. nov. Free-swimming cells (a) have a smooth cell surface (lower illustrations) and sometimes many extrusomes can be visible along the cell periphery (upper illustrations). Stationary motile cells develop dorsal grooves (b) and get enclosed by a hyaline sheath (c). Vegetative binary fission takes place in the division cyst, with a hyaline sheath (d, e) and motile nearly divided but still apically connected daughter cells without surface grooves get released from the sheath (f). Smaller free-swimming cells with smooth surface (g) start growing.

can be present (Figs 19, 22, 24, 25). Many extrusomes can be visible along the cell periphery (Fig. 24). Free-swimming cells can become stationary and rapidly change into a non-motile stage that is covered by a hyaline layer (Figs 23–25). Non-motile cells are more rounded in shape than swimming cells (Figs 23–26, 29). Vegetative cell division takes place during the non-motile stage (Figs 21, 22, 27, 30). Two or four daughter cells per cyst can develop (Figs 27, 28, 30, 31). Surface grooves were not observed.

MOLECULAR CHARACTERIZATION: MW720768 (SSU rRNA gene region), MW722979, MW720712 (LSU rRNA gene region).

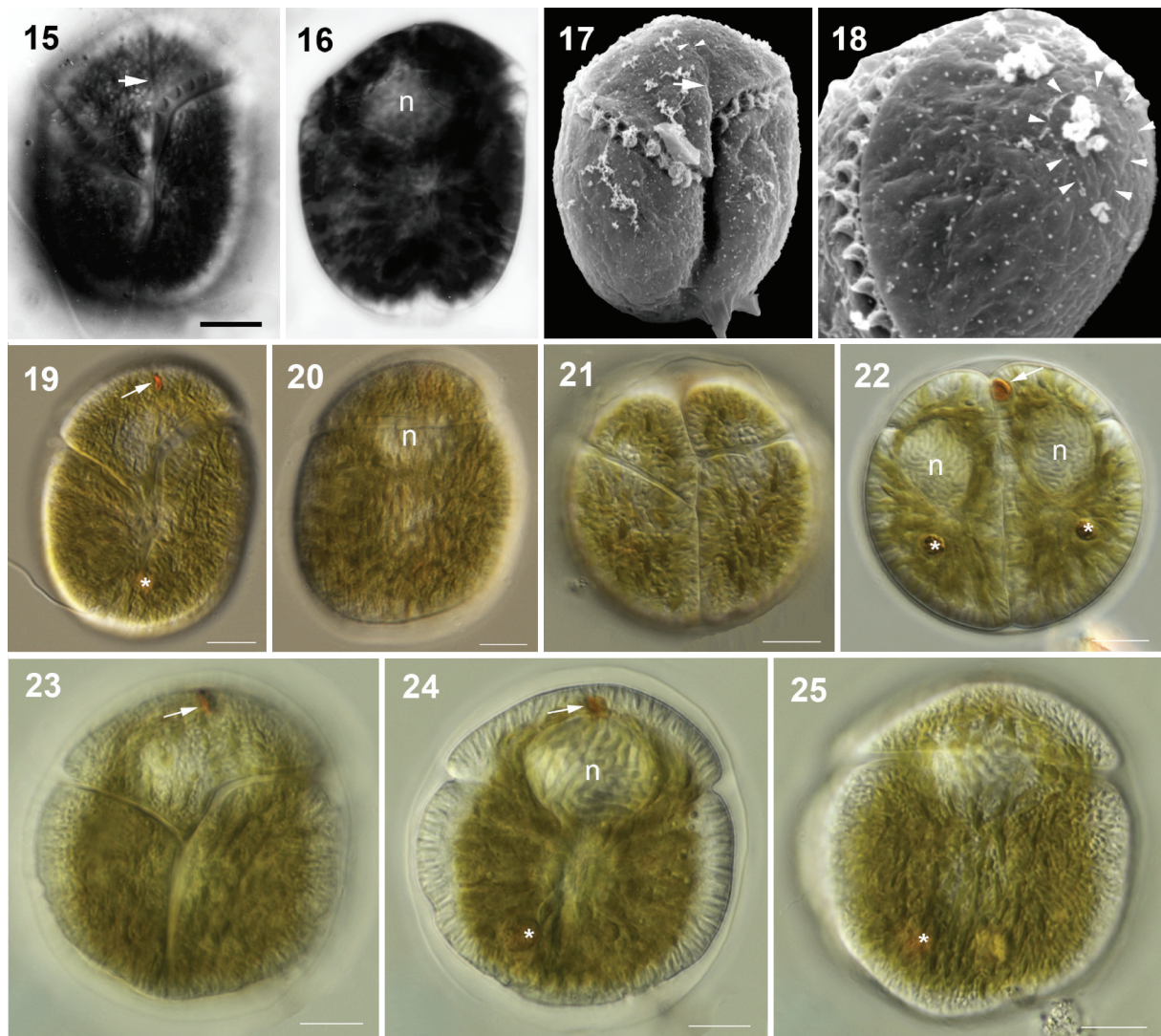
STRAIN: Cawthron Institute Culture Collection of Microalgae (CICCM), accession CAWD164.

HABITAT: marine sandy sediments.

DISTRIBUTION: Sydney (Quibray Bay, Port Botany), Durras Lake, Broome (Town Beach), Australia (Murray & Patterson 2002; Murray & Hoppenrath, unpublished observations); Rangaunu Harbour, New Zealand.

Phylogenetic inferences

The genus *Bindiferia*, including the two new species, was found to be monophyletic with very high support (97/0.98 in ML BS/BI PP, respectively; Fig. 32). The two strains of



Figs 15–25. Light (15, 16, 19–25) and scanning electron (17, 18) micrographs of *Bindiferia boggaya* comb. nov. from Australia. Scale bars = 10 µm.

Figs 15–18. Cells from Sydney, Australia, from the original description (Murray & Patterson 2002).

Fig. 15. Cell in ventral view showing the cingulum and sulcus paths, note the sulcal extension (arrow).

Fig. 16. Cell in mid-focus showing the anteriorly located nucleus (n).

Fig. 17. Cell in ventral view showing the cingulum and sulcus paths; note the sulcal extension (arrow). The small arrowheads indicate the beginning and end points of the apical structure complex.

Fig. 18. Apical view of the epicone showing the loop-shaped apical structure complex (arrowheads).

Figs 19–25. Cells from Broome, Australia.

Fig. 19. Motile cell in ventral view; note the red stigma close to the apex (arrow) and possibly a food body (asterisk).

Fig. 20. Motile cell in dorsal view; note the anterior nucleus (n).

Figs 21, 22. Vegetative division stage in a hyaline sheath in different focal planes; note the red stigma (arrow), the nuclei (n) and possible food bodies (asterisk).

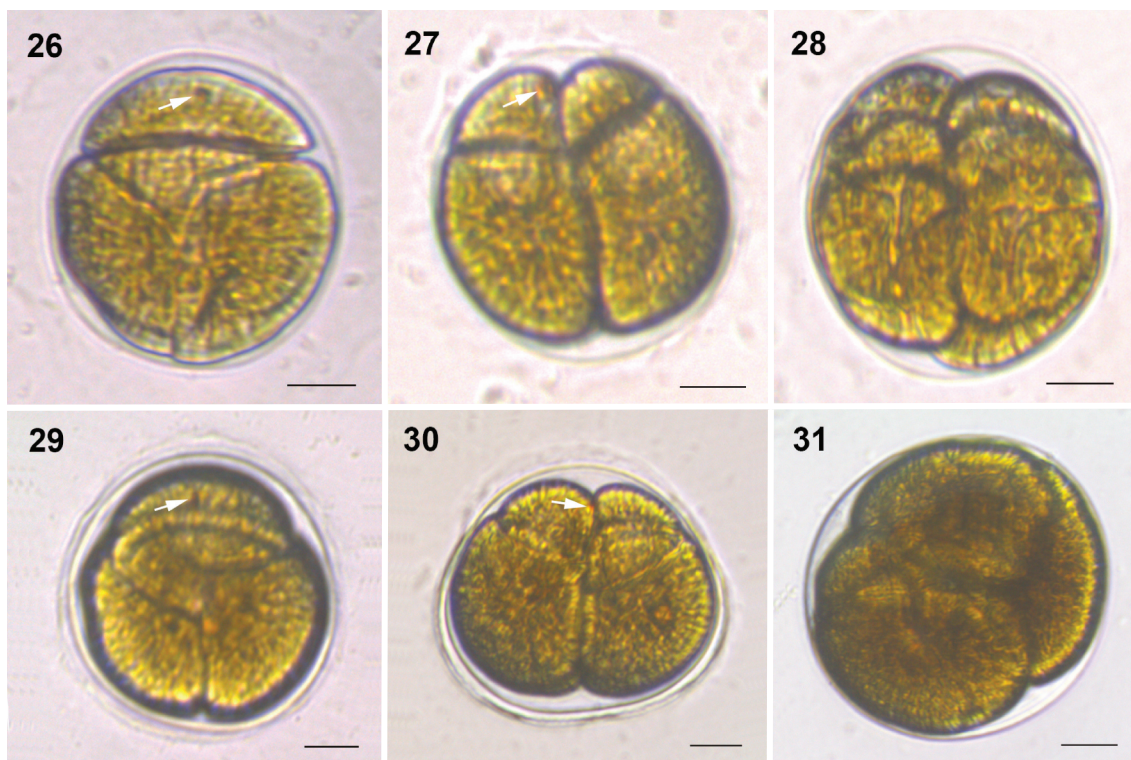
Figs 23–25. Non-motile cell in a hyaline sheath in different focal planes (23, ventral; 24 mid-focus; 25, dorsal); note the red stigma (arrow), the anterior nucleus (n) and a possible food body in the posterior area of the hypocone (asterisk). The dorsal hypocone surface is smooth, without grooves.

B. boggaya from Australia and New Zealand clustered together with full support. The species *B. boggaya* was found to be on a very long branch in the phylogeny, of a similar length to that of species of Gonyaulacales (Fig. 32).

The genus *Bindiferia* was found to be the sister group to the clade of the genus *Gymnodinium* s.s., including the type species *G. fuscum* (Ehrenberg) F. Stein, and closely related genera such as *Lepidodinium* M.M. Watanabe, S. Suda, I. Inouye, Sawaguchi & Chihara, *Gyrodiniellum* N.S. Kang, H.J. Jeong & Moestrup, *Nematodinium* Kofoid & Swezy, *Warnowia* Er. Lindemann,

Polykrikos Buetschli and *Spiniferodinium* T. Horiguchi & Chihara with moderate/low support in the ML BS analysis (70%). However, this topology was not supported by more than 50% in the BI PP analysis (Fig. 32).

In general, the phylogenetic analysis found that dinoflagellates are strongly supported as monophyletic, and that unarmoured dinoflagellates, particularly the genera *Amphidinium* s.s. and *Gyrodinium*, were the most basal of the ‘core’ dinoflagellates. The major orders such as the Prorocentrales, Suessiales, Dinophysales, Gonyaulacales and Peridinales were all supported.



Figs 26–31. Division stages within a hyaline sheath of *Bindiferia boggaya* *comb. nov.* from Broome, Australia (26–28) and New Zealand (29–31). Scale bars = 10 μ m.
Figs 26, 29. Non-motile, rounded cell; note the stigma (arrow).
Figs 27, 30. Two daughter cells in the division cyst; note a stigma (arrow).
Figs 28, 31. Four daughter cells in the division cyst.

DISCUSSION

When describing *Amphidinium boggayum*, Murray & Patterson (2002) discussed that its placement in the genus was an arbitrary choice based on the cingulum placement in relation to the cell apex. Similar *Amphidinium* species had already been described, e.g. *A. asymmetricum* Kofoid & Swezy, *A. corpulentum* Kofoid & Swezy, *A. britannicum* (Herdman) M. Lebour, or *A. ovum* Herdman (Kofoid & Swezy 1921; Herdman 1922, 1924). The species would have also met criteria for a classification within the redefined genus *Gymnodinium* *s.s.* (Daugbjerg *et al.* 2000), like the type of the apical structure complex.

In the original description *A. boggayum* (which was based on cells isolated from field samples that were not cultured) the small apical stigma was not recognized (Murray & Patterson 2002; Figs 15, 16). Later, in cells from samples taken in Broome, Australia, the stigma was observed and documented (Hoppenrath *et al.* 2014; Figs 19–25) and also specimens from the Sydney area show this feature (S.A. Murray, unpublished observations). Division stages were not described before the present study.

The newly described species *B. fragilissima* is morphologically very similar to '*A.* *boggayum*' (Murray & Patterson 2002). Both species are atecate, dorsoventrally flattened, with smaller and narrower asymmetric epicone. The cingulum is descending. The sulcus is narrow, not covered by a flap and curving to the right towards the posterior, reaching the antapex. A sulcal extension runs straight onto the epicone towards the apex. A red stigma is visible at an anterior-ventral location in the epicone, close to the

apex. Both species divide as non-motile cells in hyaline temporary cyst stages. *Bindiferia fragilissima* can be distinguished from '*A.* *boggayum*' by its relatively shorter epicone, its less displaced cingulum and the nucleus located in the hypocone instead of the epicone (Figs 33–38). Furthermore, division stages of *B. fragilissima* showed longitudinal surface grooves on their hypocone that were not recorded for '*A.* *boggayum*'.

The phylogenetic analysis supported the sister group relationship of the two species and thus '*A.* *boggayum*' has been transferred to the new genus *Bindiferia* (Fig. 32). The long branch of the *B. boggaya* clade suggests either that this might be an ancient group of dinoflagellates, or that it has exhibited much more rapid evolutionary rates than other dinoflagellate genera in ribosomal RNA genes. Strong rate heterogeneity in rRNA genes has been observed in multiple dinoflagellate lineages, particularly the Gonyaulacales (John *et al.* 2003; Murray *et al.* 2005; Kretzschmar *et al.* 2019; Žerdoner Čalasan *et al.* 2019). The order level relationships of the genus *Bindiferia* could not be resolved with certainty in this analysis, as the sister group relationship with *Gymnodinium* lacked support (Fig. 32). This suggests that more molecular sequence data, particularly from other genes, would be needed to resolve this topology with certainty.

Both species can be distinguished from similar photosynthetic *Amphidinium* *s.l.* species described from the same habitat. '*Amphidinium*' *asymmetricum* cells were described with a similar cell shape, similar size range and with a longer left side of the hypocone and of the whole cell (Kofoid & Swezy 1921). However, '*A.* *asymmetricum*' was described with

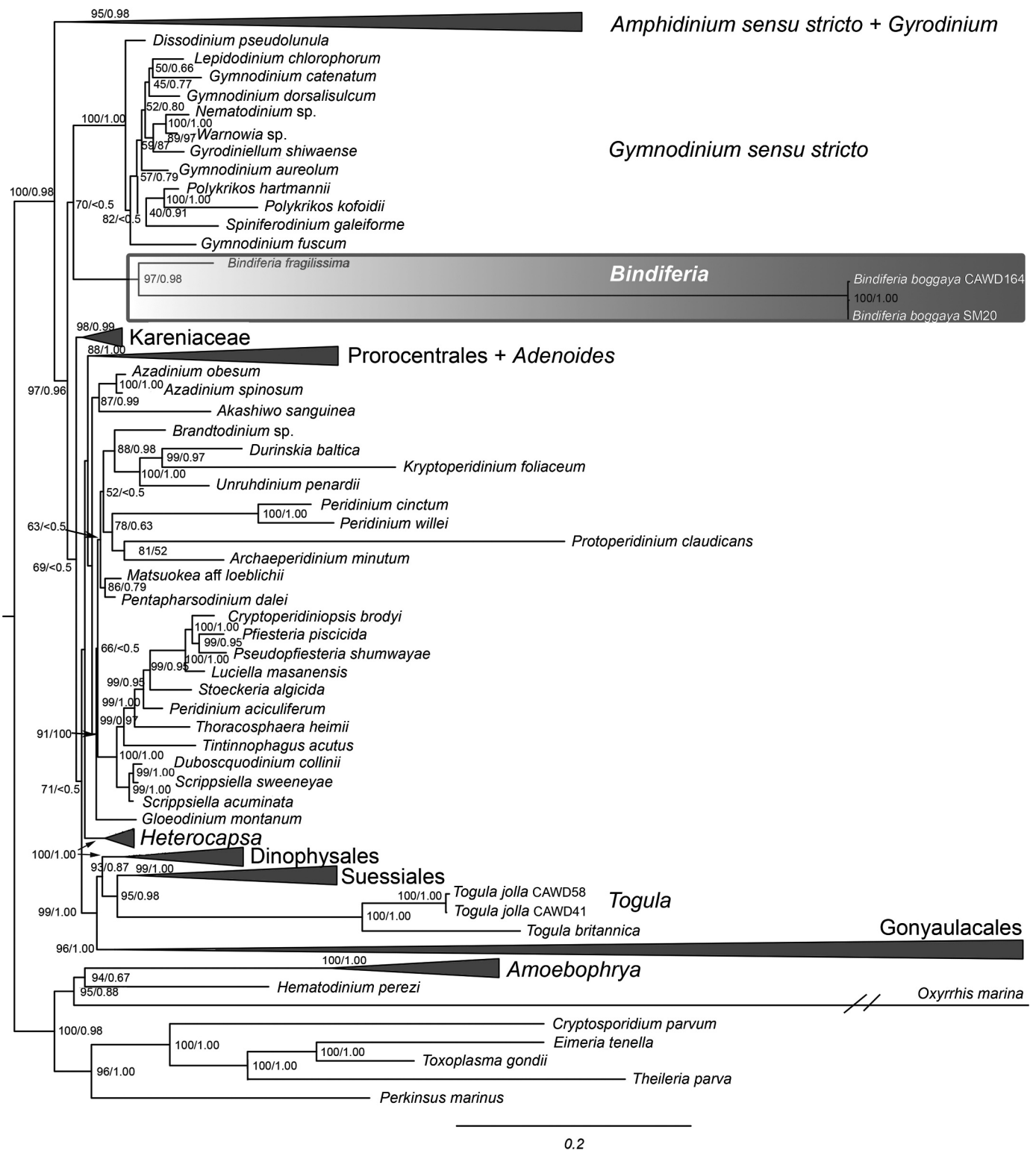
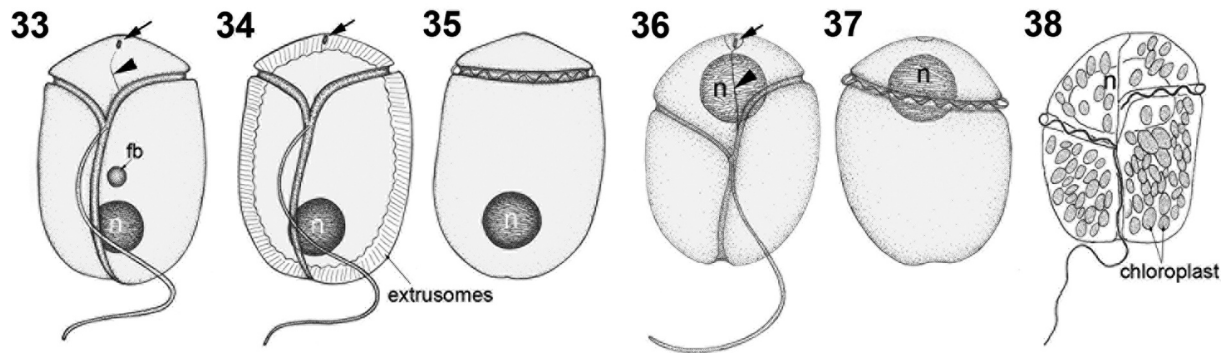


Fig. 32. Molecular phylogeny of the dinoflagellates, including the new sequences of *Bindiferia* and *Togula*, based on a concatenated alignment of 18S, ITS1, 5.8S, ITS2 and 28S rRNA gene regions. Most likely tree shown, with support values based on maximum likelihood bootstrap analysis (ML BS) and Bayesian inference posterior probabilities (BI PP).

surface striations, no sulcal extension and a sulcal flap overhanging to the cell's right. Also '*A. corpulentum*' and '*A. ovum*' were described with a similar cell shape, a smaller epicone, no surface striations and a sulcal extension onto the epicone, but with a less displaced cingulum, a sulcal flap

overhanging to the cell's right and a lateral position of the nucleus (Kofoid & Swezy 1921; Herdman 1924; Larsen 1985; Larsen & Patterson 1990). The sulcal extension in '*A. corpulentum*' is similar to that of *Bindiferia* (Kofoid & Swezy 1921), but it is shorter in '*A. ovum*' (Larsen 1985; Larsen & Patterson



Figs 33–38. Line drawings of *Bindiferia fragilissima* (33–35) and *B. boggaya* (36–38). fb, food body; n, nucleus; arrows indicate stigma; arrowheads indicate sulcal extension.

Figs 33, 34, 36, 38. Ventral views.

Figs 35, 37. Dorsal views.

Fig. 38. Drawing from the original description (Murray & Patterson 2002).

1990; Al-Yamani & Saburova 2010). All these taxa were described without stigma.

Al-Yamani & Saburova (2010) described a photosynthetic species as *Gyrodinium* sp. that had some similarities to *Bindiferia*: a descending cingulum, a narrow sulcus without flap and with a long and straight extension onto the epicone up to the apex. It was shown as non-motile cell within a hyaline sheath and had a coloured spot near the apex. However, the colour of this spot was not as red and its size was larger than in *Bindiferia*, and it is therefore uncertain whether it represented a stigma. The specimens were smaller than the two *Bindiferia* species. This species needs further investigation to clarify its affiliation.

The phototrophic genus *Togula* (also a former *Amphidinium* s.l. taxon), with species having asymmetric, dorsoventrally flattened cells, differs from *Bindiferia* by having no sulcal extension, no apical structure complex and no stigma (Flø Jørgensen *et al.* 2004b). Interestingly, the species divide in a non-motile stage inside hyaline mucous sheaths and the division stages can have longitudinal grooves on their hypocones (Flø Jørgensen *et al.* 2004b). *Togula britannica* (Herdman) M.F. Jørgensen, Sh. Murray & Daugbjerg showed a longitudinal groove on the left ventral hypocone prior to cell division. In *Togula jolla* cell division was preceded by the formation of six to eight longitudinal grooves on the hypocone (first one left-ventral and two dorsal in close succession, followed by additional dorsal). Cell division of *Togula* species began at the antapex and the last parts to split were the epicones (Flø Jørgensen *et al.* 2004b), as observed in *Bindiferia* species. The fact that *Bindiferia* was not closely related to the genus *Togula* in the phylogenetic tree, and that *Togula* appeared as a sister group to the clade Suessiales with high support (95/0.98; Fig. 32) suggests that these two genera are very distinct from one another. Previously, *Togula* had been resolved with full support as the sister group to the species *Gymnodinium catenatum* H.W. Graham, and therefore as a member of the Gymnodiniaceae s.s., in phylogenetic analyses based on

large multi-gene datasets (Janouškovec *et al.* 2017; Price & Bhattacharya 2017). Recent studies of dinoflagellate phylogeny based on large amounts of sequence data have indicated a monophyletic origin of dinoflagellate thecal plates (Orr *et al.* 2012; Bachvaroff *et al.* 2014; Janouškovec *et al.* 2017; Price & Bhattacharya 2017). This suggests that this placement of *Togula* as separate to the Gymnodiniaceae may be an artefact of the use of rRNA genes, or else it may indicate a previously unrecognized relationship with the Suessiales. Future detailed TEM examination of the amphiesmal alveoli of the species of *Togula*, with the determination of the presence or absence of thin thecal plates, may help clarify the matter.

Surface grooves on hypocones of athecate species are a feature rarely described (Fig. S3). Klebs (1884) described a species he named *A. operculatum* with ‘furrows’ or corrugations on the cell surface. This observation has not been verified since the original description but this characteristic was the basis for renaming the taxon as a new species, *A. klebsii* by Kofoid & Swezy (1921). Dividing motile cells of *Amphidinium tomasii* Karafas showed grooves on the hypocone, but non-dividing cells had a smooth surface (Karafas *et al.* 2017). The heterotrophic *Amphidinium pellucidum sensu* Dragesco (1965) was described with longitudinal grooves, a morphological character not originally described for *A. pellucidum* by Herdman (1922) – now re-classified as *Gymnodinium venator* (Flø Jørgensen *et al.* 2004a, c). The species *Testudodinium corrugatum* (J. Larsen & D.J. Patterson) T. Horiguchi, Maiko Tamura & A. Yamaguchi has been described with longitudinal dorsal ribs on the hypocone (Larsen & Patterson 1990; Horiguchi *et al.* 2012). This structure can be interpreted as longitudinal grooves when focussing the description not on the elevations but the depressions between them. The dorsal corrugations were only described for one of the three *Testudodinium* species (Horiguchi *et al.* 2012).

The redefinition of *Amphidinium* s.s. (species with a minute, left-deflected epicone) by Flø Jørgensen *et al.* (2004a), as a first step to resolve the morphologically diverse and polyphyletic assemblage of taxa previously assembled

within *Amphidinium* s.l., highlighted the need to reinvestigate the taxa excluded from the genus in the restricted sense. This has already been accomplished for several taxa, like *Togula* (Flø Jørgensen et al. 2004b; see above). Also the genera *Apicoporos* and *Ankistrodinium* showed novel epicone characteristics that were different from *Amphidinium* s.s. or any other athecate genus (Sparmann et al. 2008; Hoppenrath et al. 2012). Apart from the different epicone size and shape, the sulcal extension onto the epicone and the presence of an apical structure complex, the most striking feature of *Bindiferia* is the stigma close to the apex. A stigma is not known from *Amphidinium* species, with one exception: *A. cupulatisquama* Maiko Tamura & T. Horiguchi has a kidney-shaped stigma, located on the dorsal side of cingulum (Tamura et al. 2009). The molecular phylogenetic results are consistent with the morphological interpretation and clearly show that *Bindiferia* is a distinct genus and only distantly related to the genus *Amphidinium*.

One morphological feature, the anticlockwise loop-shaped apical structure complex, suggests that the genus may be related to genera of the *Gymnodinium* s.s. clade, e.g. *Gymnodinium*, *Lepidodinium*, *Polykrikos*, *Spiniferodinium* and *Warnowia* (Daugbjerg et al. 2000; Hoppenrath & Leander 2007a, b; Hoppenrath et al. 2009; Horiguchi et al. 2011). A sister group relationship of this clade to *Bindiferia* could not be reliably determined with the present molecular phylogenetic analyses. It has been hypothesized by Flø Jørgensen et al. (2004a) that *A. boggayum* belongs to *Gymnodinium*. Their cladistic analysis of morphological and ultrastructural characters grouped *Bindiferia boggaya* (as *A. boggayum*) within the *Gymnodinium* clade with no support for this grouping (Flø Jørgensen et al. 2004a). Thus, the closest relative of *Bindiferia* remains unknown.

Moestrup & Daugbjerg (2007) distinguished five stigma types, all of them situated in the sulcal area close to the flagellar roots. A stigma similar to type A (pigment globules inside the peridinin dino-chloroplast) but located on the dorsal side of the cingulum was described for an *Amphidinium* species (Tamura et al. 2009). Another dorsal stigma can be observed in *Margalefidinium polykrikoides* (Margalef) F. Gómez, Richlen & D.M. Anderson (Iwataki et al. 2010, as *Cochlodinium*). Currently eight different stigma types are known, three of them outside of the chloroplasts (Hoppenrath 2017). To the best of our knowledge this is the first report of a stigma located ventrally close to the apex. There are no ultrastructural data available yet (because of technical problems), only the hint from broken cells that the pigments of the stigma stay together as one unit that seems not to be associated with a chloroplast.

Dinoflagellates whose principle life cycle stage is non-motile, with their cell shapes different from the typical motile stages, were collectively called ‘coccoid dinoflagellates’ by Hoppenrath et al. (2014). Most of the marine benthic coccoid dinoflagellates possess a similar type of life cycle, i.e. non-motile vegetative cells divide to form motile cells within a cell wall (thecate taxa) or sheath (athecate taxa) and the (usually motile) daughter cells are released, leaving behind the parental cell wall or sheath. The duration of the motile phase is often much shorter than that of the non-motile phase. After swimming, the motile cells become

quiescent and return to the non-motile cell stage by developing the characteristic morphology (summarized in Hoppenrath et al. 2014). Some athecate taxa were described: e.g. *Dinothrix rugata* (Maiko Tamura & T. Horiguchi) Norico Yamada & T. Horiguchi (as *Galeidinium* Maiko Tamura & T. Horiguchi), *Pyramidodinium* T. Horiguchi & Sukigara and *Spiniferodinium* produce dome-shaped or pyramid-shaped cell coverings (Horiguchi & Chihara 1987; Horiguchi & Sukigara 2005; Tamura et al. 2005). *Bindiferia* species have a simple hyaline sheath around their dividing cells and the duration of the non-motile life cycle stage is not known. In culture, only very few of the cells are actively motile during the light phase.

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DISCLOSURE STATEMENT

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