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Characterisation of Two Toxic *Gambierdiscus* spp. (Gonyaulacales, Dinophyceae) from the Great Barrier Reef (Australia): *G. lewisii* sp. nov. and *G. holmesii* sp. nov.

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Ciguatera fish poisoning (CFP) is a human illness caused via consumption of seafood contaminated with neurotoxins produced by some species from the epiphytic dinoflagellate genus *Gambierdiscus*. In this study, we describe two new species of *Gambierdiscus* isolated from Heron Island in the Southern Great Barrier Reef, Queensland, Australia. These new species were analysed using light microscopy, scanning electron microscopy, and phylogenetic analyses of nuclear encoded ribosomal ITS, SSU as well as D1-D3 and D8-D10 of the LSU gene regions. *Gambierdiscus lewisii* sp. nov. (Po, 3′, 0a, 7″, 6c,? s, 5‴, 0p, 2⁗) is distinguished by its strong reticulate-foveate ornamentation and is genetically distinct from its sister species, *G. pacificus*. *Gambierdiscus holmesii* sp. nov. (Po, 3′, 0a, 7″, 6c, 6s?, 5‴, 0p, 2⁗) is morphologically distinct from the genetically similar species *G. silvae* because of a strongly ventrally displaced apical pore complex and a characteristic fold at the anterior edge of the sulcus. Both *G. lewisii* and *G. holmesii* produce putative Maitotoxin-(44-Methylgambierone) and compounds

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Abbreviations: CFP, Ciguatera Fish Poisoning; CTX, Ciguatoxin; FLIPR, Fluorescent Imaging Plate Reader; GBR, Great Barrier Reef; ITS, Internal Transcribed Spacer; LSU rDNA, large subunit rDNA; LC-MS/MS, Liquid Chromatography-Tandem Mass Spectrometry; MTX, Maitotoxin; SSU rDNA, small subunit rDNA.

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which show ciguatoxin and maitotoxin-like activities. Identification of two new *Gambierdiscus* species will enable us to more accurately assess the risk of CFP in Australia and internationally.

Key words: Benthic Harmful Algal Bloom (BHAB); Ciguatera Fish Poisoning (CFP); epiphytic dinoflagellate; Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS); Morphology; Phylogeny.

Introduction

Ciguatera fish poisoning (CFP) arises when humans consume marine fish or invertebrates contaminated with potent neurotoxins (Lehane and Lewis 2000). Globally, it is estimated that between 50,000 and 200,000 people are affected each year by CFP (Dickey and Plakas 2010; Lehane and Lewis 2000), making it one of the most prevalent human illnesses associated with seafood consumption (Friedman et al. 2017). Diagnosing CFP is challenging because its symptomology is so complex and varied (Sims 1987). Intoxication can manifest as gastrointestinal (e.g. nausea, vomiting, diarrhoea, abdominal pain), cardiovascular (e.g. arrhythmia, heart block) and/or neurological (e.g. sensory changes, numbness, weakness and fatigue, blurred vision, temperature dysesthesia) symptoms (Dickey and Plakas 2010; Friedman et al. 2017; Gillespie et al. 1985b).

The primary neurotoxins linked with cases of CFP are ciguatoxins (CTXs) (Yasumoto et al. 1977). A recent study suggests maitotoxins (MTXs) can also accumulate in fish tissue (Kohli et al. 2014b), but their role in human illness requires additional investigation. CTXs are highly potent, lipophilic toxins that are known to activate voltage gated sodium channels of mammalian cells (Benoit et al. 1986; Legrand et al. 1989; Lombet et al. 1987). CTXs can form more than 20 structural analogs and the following forms P-CTX-3B, P-CTX-3C, P-CTX-4A, P-CTX-4B, are known to be produced by some species of *Gambierdiscus* (Chinain et al. 2010; Yogi et al 2011, 2014). MTXs are highly potent, hydrophilic toxins which increase the intracellular calcium levels of mammalian cells (Gusovsky and Daly 1990; Murata et al. 1992) and also occur in many structural forms (MTX-1, MTX-2, MTX-3 (44 Methylgambierone), MTX-4) (Holmes and Lewis 1994; Holmes et al. 1990; Pisapia et al. 2017).

CTXs and MTXs are produced by some species of the epibenthic dinoflagellate genus *Gambierdiscus*. These species have therefore been identified as the causative organisms of CFP (Yasumoto et al. 1977). *Gambierdiscus toxicus* (Adachi and Fukuyo) Chinain, Faust, Holmes, Litaker and Tester was the first species of *Gambierdiscus* to be described, from samples collected in the Gambier Islands, French Polynesia (Adachi and Fukuyo 1979). For many years, the genus was thought to be monotypic, however increased research and the application of sequencing approaches to taxonomic identification has led to a significant re-analysis of species within the *Gambierdiscus* genus and the description of many new species (Litaker et al. 2009). Additionally, Gómez et al. (2015) used a DNA sequencing approach to re-classify some morphologically distinct taxa with globular shaped cells (Holmes 1998) into a new genus, *Fukuyoa* F.Gómez, D.X.Qiu, R.M.Lopes & Senjie Lin. Thus, the genus *Gambierdiscus* is now comprised of 16 species (Table 1) and 4 unidentified clades, *G.* ribotype 2 (Litaker et al. 2009), *G.* sp. 3 (Nishimura et al. 2013), *G.* sp. 4 (Xu et al. 2014), *G.* sp. 5 (Dai et al. 2017; Xu et al. 2014), that are genetically distinct and may constitute new undescribed species.

Cases of CFP have been reported in Australia as far back as the 18th century (Lewis 2006). Since then, there have been large outbreaks of the syndrome and two human fatalities (Gillespie et al. 1985b; Hamilton et al. 2010). CFP in Australia is primarily caused by reef fish caught in tropical waters along the east coast (Clark and Whitwell 1968; Gillespie et al. 1985a; Tonge et al. 1967), although cases are now being reported from fish caught in more sub-tropical locations (Farrell et al. 2016a, b, 2017; Kohli et al., 2017). Despite the widespread occurrence of CFP in Australia, very little is known about the causative organisms from this region. Holmes, Lewis and Gillespie were the first to isolate strains of *Gambierdiscus* from Australia (Holmes and Lewis 1994; Holmes et al. 1990, 1991). The identities of the species from these studies remains unconfirmed, as they were isolated prior to our current understanding of the diversity in the genus, and therefore the morphological descriptions do not contain sufficient information for identification by current standards (Litaker et al. 2009). Murray et al. (2014) provided a further glimpse of the diversity of *Gambierdiscus* from tropical eastern Australia. Based on sampling at multiple sites in the central Great Barrier Reef (GBR) region, three species

Table 1. List of species in the epibenthic dinoflagellate genus *Gambierdiscus.*

G. australes Chinain & M.A.Faust (Chinain et al. 1999)
G. balechii S.Fraga, F.Rodríguez & I.Bravo (Fraga et al. 2016)
G. belizeanus M.A.Faust (Faust 1995)
G. caribeaus Vandersea, Litaker, M.A.Faust, Kibler, W.C.Holland & P.A.Tester (Litaker et al. 2009)
G. carolinianus Litaker, Vandersea, M.A.Faust, Kibler, W.C.Holland & P.A.Tester (Litaker et al. 2009)
G. carpenteri Kibler, Litaker, M.A.Faust, W.C.Holland, Vandersea & P.A.Tester (Litaker et al. 2009)
G. cheloniae K.F.Smith, L.Rhodes & S.A.Murray (Smith et al. 2016)
G. excentricus S.Fraga (Fraga et al. 2011)
G. jejuensis S.Jang, H.J.Jeong, Y.D.Yoo (Jang et al. 2018)
G. honu Rhodes, Smith & Murray (Rhodes et al. 2017)
G. lapillus Kretzschmar, Hoppenrath & Murray (Kretzschmar et al. 2017)
G. pacificus Chinain & M.A.Faust (Chinain et al. 1999)
G. polynesiensis Chinain & M.A.Faust (Chinain et al. 1999)
G. silvae S.Fraga & F. Rodríguez (Fraga and Rodríguez 2014)
G. scabrosus T.Nishimura, Shinya Sato & M.Adachi (Nishimura et al. 2014)
G. toxicus Adachi & Fukuyo (Adachi and Fukuyo 1979)

were identified, *G. carpenteri,* a species of similar morphology to *G. belizeanus* (*G.* cf. *belizeanus*), and *G.* cf. *yasumotoi* (genetically closely related to strains identified as *Fukuyoa paulensis*). Since then, *G. carpenteri* has been observed in the GBR by Sparrow et al. (2017), and in Merimbula, a temperate location in southern New South Wales (Kohli et al. 2014a; Larsson et al. 2018; Munday et al. 2017). Kretzschmar et al. (2017) described a new species of *Gambierdiscus, G. lapillus,* isolated from Heron Island in the southern GBR region. In addition, an unidentified strain of *Gambierdiscus* (Richlen et al. 2008) from Heron Island in the GBR, was found to be closely related to a partial SSU sequence of *G. honu* (Rhodes et al. 2017).

Larsson et al. (2018) further contributed to our understanding of *Gambierdiscus* in Australia, providing insight into *Gambierdiscus* toxicology through Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) and the Ca^{2+} influx mammalian SH-SY5Y cell Fluorescent Imaging Plate Reader (FLIPR) bioassay. Four species (*G. carpenteri*, *G. lapillus* and two unidentified strains reported as *Gambierdiscus* sp.) were isolated from the tropical waters of Heron Island in the GBR and one (*G. carpenteri*) from the temperate waters of Merimbula in southern New South Wales. *Gambierdiscus lapillus* and the two unidentified strains were found to produce compounds with CTX-like activity when tested using a combination of LC-MS/MS and a Ca^{2+} influx SH-SY5Y cell FLIPR bioassay.

In this study, we describe two new species of *Gambierdiscus*: *G. holmesii* (strain numbers UTSHI6B1 and HG5) and *G. lewisii* (strain number UTSHI6A6) from Heron Island, Australia. These were identified as *Gambierdiscus* sp, in a previous study (Larsson et al. 2018). These species differ from all currently described species of *Gambierdiscus* by means of a combination of their morphological features, sequences of four regions of rRNA genes, and their toxicity,

Results

Two new species of *Gambierdiscus*, *G. holmesii* sp. nov. and *G. lewisii* sp. nov. were isolated from macroalgal samples collected in the shallow lagoon of Heron Island in the southern GBR region of Queensland, Australia (Fig. 1) The *G. holmesii* strain UTSHI6B1 and the *G. lewisii* strain UTSHI6A6 were isolated from a sample of the brown macroalga *Chnoospora* sp., while the *G. holmesii* strain HG5 was isolated from a sample of *Padina* sp.

Gambierdiscus holmesii sp. nov

Morphology

Cells were anterioposteriorly compressed and round to oval, lenticular (Figs 2A, B, D, 3 A, B, D); very few cells were dorsally pointed in apical view (not shown). Cells were in the size range of most other *Gambierdiscus* species, with a mean depth (ventral to dorsal distance) of 78.8 μm (range 68.8 – 88.9 μm; SD 5.8 μm) and mean width (transdiameter) of 78.9 μm (range 67.1 – 91.4 μm; SD 7.8 μm), with a depth to width ratio of 1.0 (SD 0.06 μm) (n = 20) (Fig. 4A). The mean cell length (apical to antapical distance) was 45.2 μm (38.3 – 53.1 μm; SD 4.4 μm) (n = 20) (Fig. 4B). The epitheca was

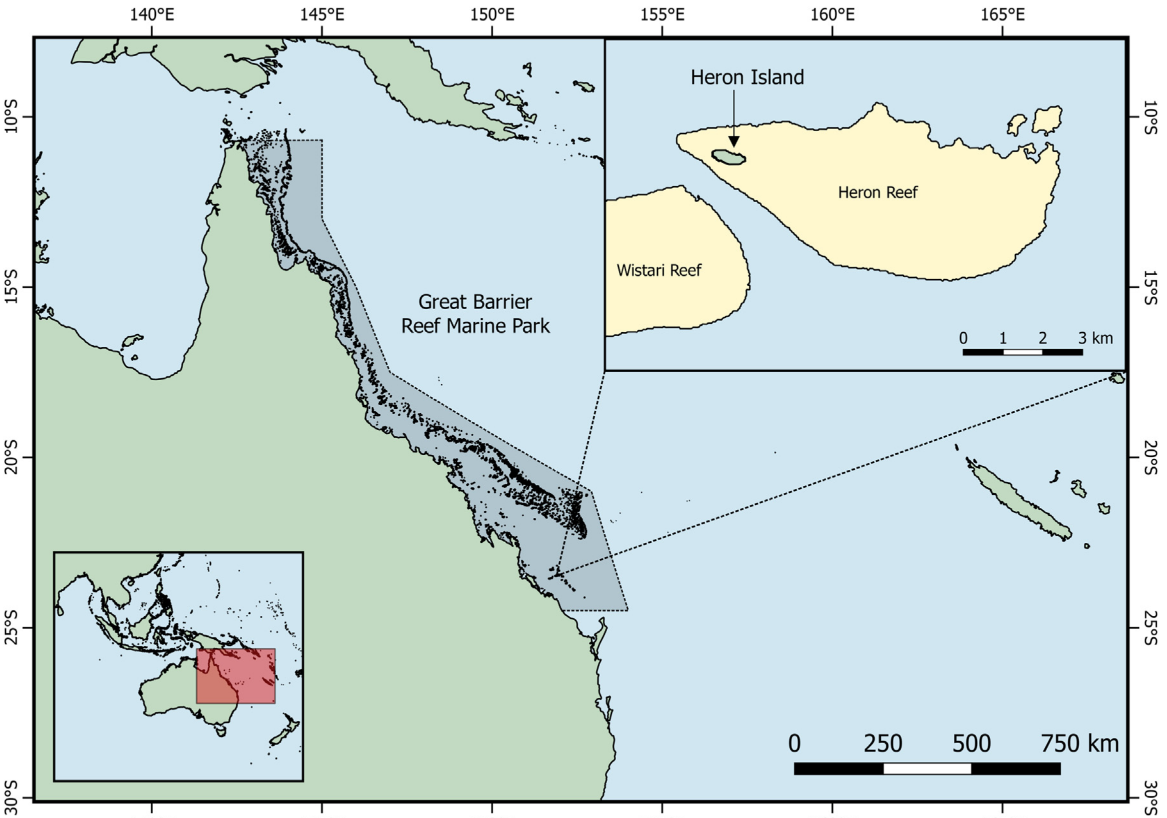

Figure 1. Map indicating the location of Heron Island, Queensland, Australia, the locations from which *Gambierdiscus lewisii* and *Gambierdiscus holmesii* were isolated.

slightly higher than the hypotheca (not shown). The cell surface was smooth or foveate (with shallow depressions) with scattered pores of one size class (Figs 2A-D, 3 A, B, D, 5). Wide intercalary bands were smooth or foveate (Figs 2A-D, 3 A-C, 5 A, B). The plate formula (Kofoid tabulation) was Po, 3′, 0a, 7″, 6c, 6s?, 5‴, 0p, 2⁗ (Figs 2, 3). The apical pore plate (Po) was oval with a fish-hook shaped slit-like apical pore, shifted to the ventral side (Fig. 2A-D), about one third of the epitheca depth from the sulcus. Po was approximately 6.6-7.9 × 3.9-5.0 μm in size (Fig. 2A-F). Twenty-seven to 33 large depressions were distributed over the Po plate and arranged in no constant pattern (Fig. 2E-G); one row of depressions intruded into the hook, associated with additional depressions in the hook center. As seen by an inside view, the depressions contained a net-like membrane with pores or a large pore (Fig. 2G). The largest apical plate was the hatchet-shaped 2′ plate, followed by the pentagonal 3′ plate and the hexagonal 1′ plate (Fig. 2A, B, D). The suture 2′/2″ is about half the length of the 2′/4″ suture. Some shape-variability of plate 2′ (Supplementary Material Fig. S1) was recorded. The shape varied from normal hatchet-shaped with 5 sides (main morphology) to wide hatchet-shaped (Supplementary Material Fig. S1A-G, I, J) and 6-sided (Supplementary Material Fig. S1H, K). The largest precingular plate was plate 3″ (four-sided, covering the left lateral precingular area) and the asymmetrical pentagonal 4″ plate (dorsal) was nearly of the same size (Fig. 2A, B, D). Plate 4″ had a longer 4″/2′ suture compared to the shorter 4″/3′ suture. Plates 5″ and 6″ were on the right lateral to ventral side (Fig. 2A-C). Plates 1″ and 7″ were the smallest (Figs 2C, 3 D) located ventrally. There was a small fold present on the 1″ plate (Figs 2C, 3 D). The largest postcingular plate was the pentagonal 4‴ plate (covering most of the left lateral hypotheca margin), followed by the plates 2‴, 3‴, 5‴ and 1‴ (Fig. 3A-C). The elongated pentagonal first antapical plate (1⁗) was small and the large, pentagonal 2⁗ plate covered the antapex (Fig. 3A-C). Plate 1‴ did not contact plate 2⁗ (Fig. 3A-C). Plate 5‴ did usually not contact plate 2⁗ (Fig. 3A-C), in one case these plates had a suture in common (Supplementary Material Fig. S2C). The sulcus was a deep excavation (Fig. 3C-F), composed of at

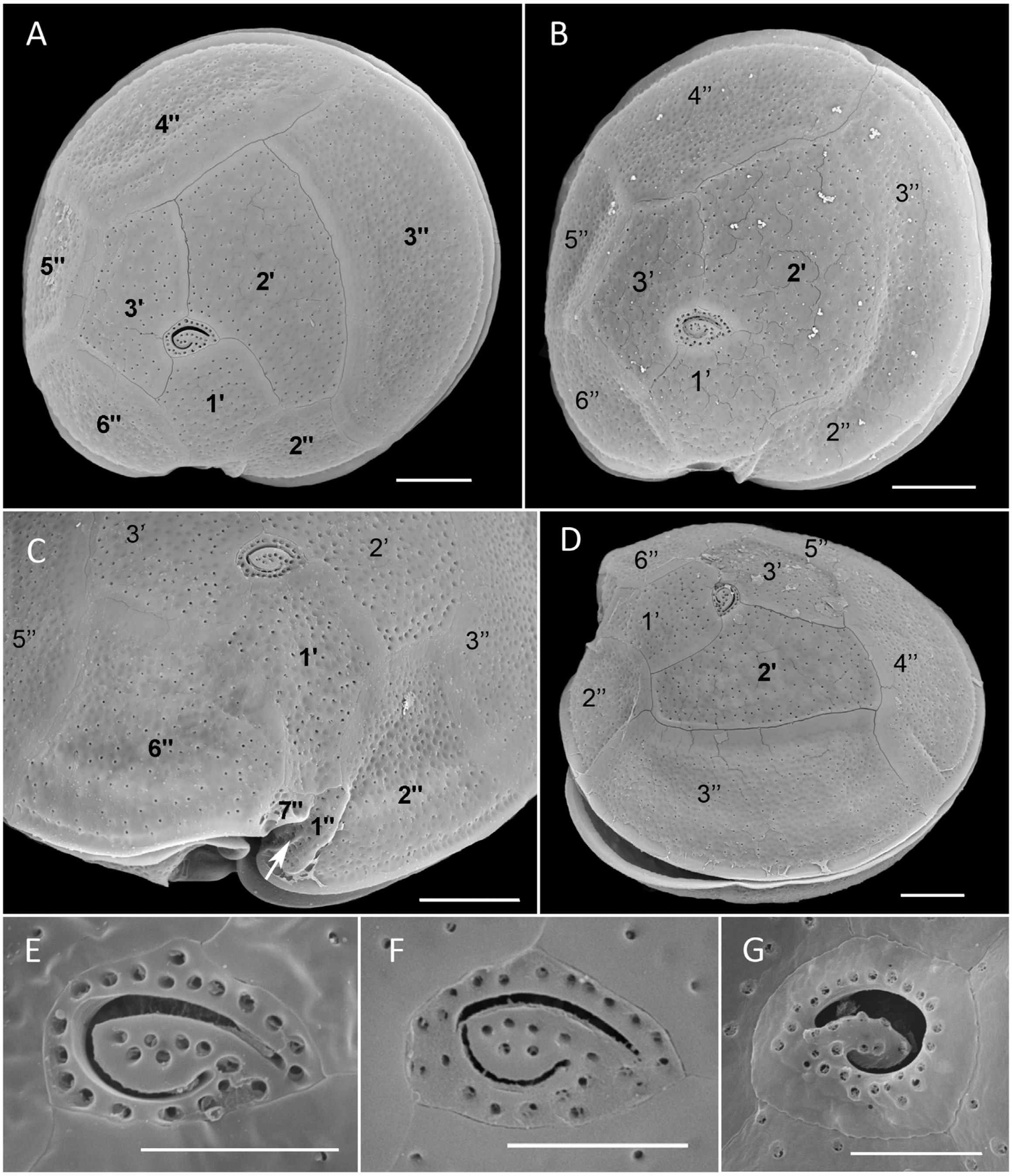

Figure 2. Scanning electron micrographs of *Gambierdiscus* holmesii sp. nov. (isolate UTSHI6B1) of the epitheca. (**A, B**) Apical view. (**C**) Ventral part. (**D**) Left lateral side. (**E-G**) Apical pore complex. (E, F) Outside view of the pore plate (Po). (G) Inside view of Po. Scale bars (A-D) 10 μm, (E-G) 5 μm. Arrow points to fold at anterior edge of the sulcus.

least six plates with the posterior sulcal plate (Sp) located outside the excavation (Fig. 3C-F). The narrow and deep cingulum was difficult to observe and consisted of 6 plates. Some of the (cultured) cells had additional plates as a probable result of plate splits (Supplementary Material Fig. S2).

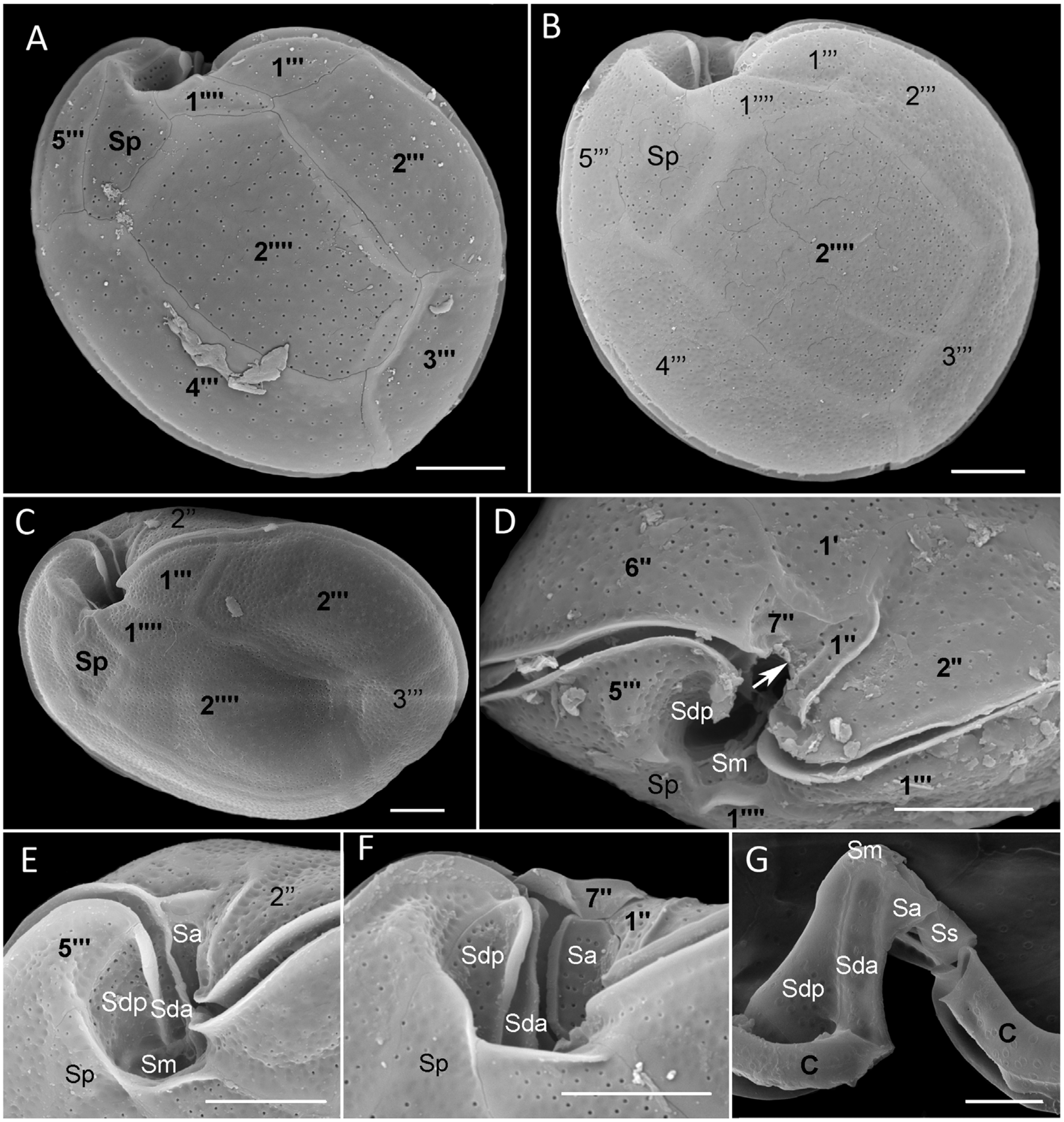

Figure 3. Scanning electron micrographs of *Gambierdiscus* holmesii sp. nov. (isolate UTSHI6B1) of the hypotheca and sulcus. (**A, B**) Hypotheca in antapical view. (**C**) Left lateral to ventral view. (**D**) Ventral view of the sulcal area. (**E-G**) Sulcal details. (G) Inside view from the top. Scale bars (A-F) 10 µm, (G) 5 µm. Arrow points to the fold at the anterior edge of the sulcus.

Phylogenies

In order to compare the sequences of *G. holmesii* sp. nov to the most similar *Gambierdiscus* species, SSU rDNA for *G. silvae* strain VGO1180 was sequenced and deposited in GenBank with the accession ID MH790450.

Both strains of *G. holmesii*, HG5 and UTSHI6B1, were found to cluster together with full support in analyses of each of the four gene regions (Figs 6, 7, 8). Full support is defined as 100% Bootstrap support (BS) in the Maximum Likelihood analysis (ML), and 1.00 support in the Bayesian Inference (BI) Posterior Probability support value (PP). These two strains formed the sister clade to the species *G. silvae*, with full support in analyses of the D1-D3, and with low support (<50%/0.65, ML BS/BI PP) in analyses of the D8-D10 LSU rDNA, respectively (Figs 6, 7). In analyses of the

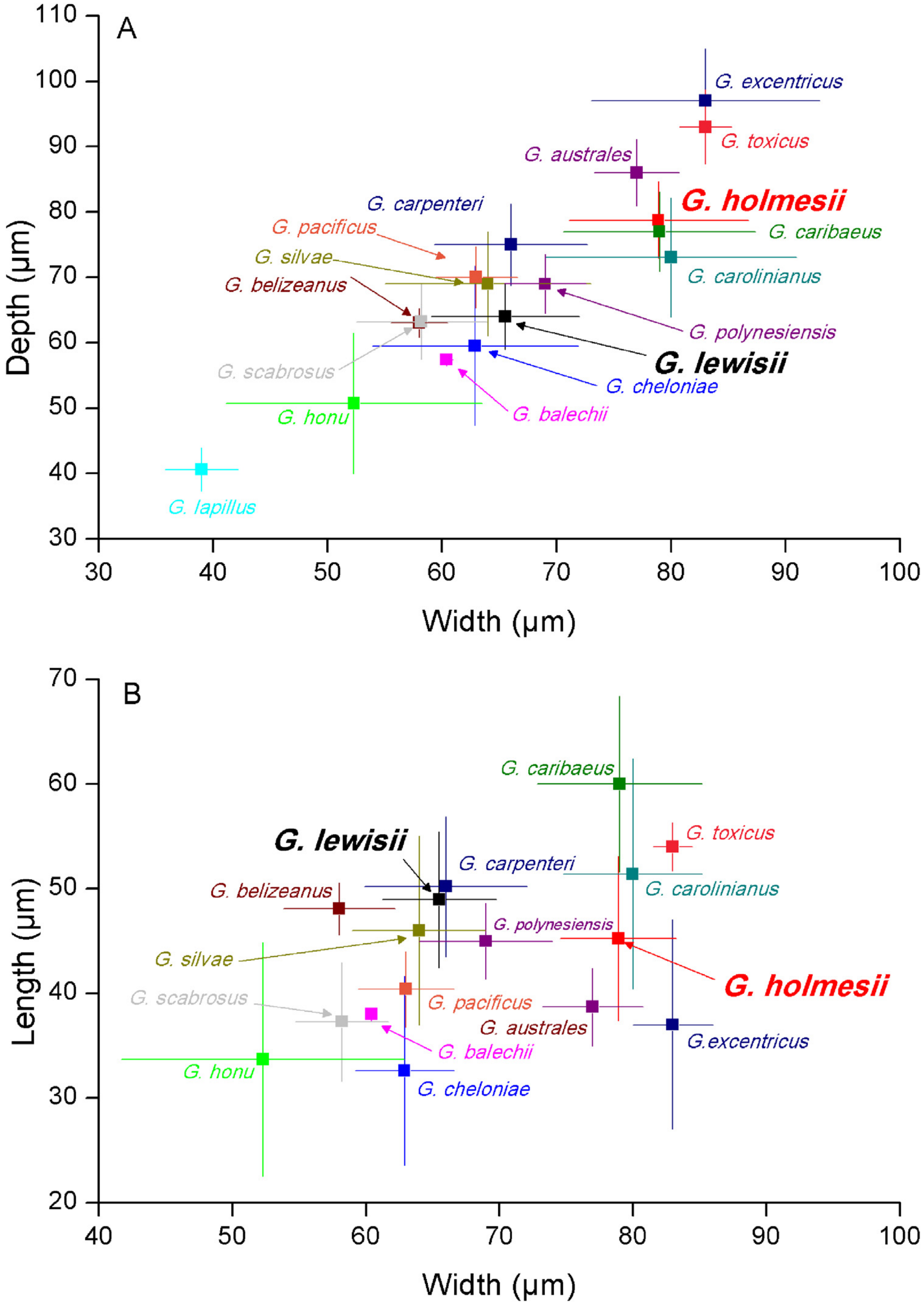

Figure 4. Cell size comparison of type species from the *Gambierdiscus* genus, including *Gambierdiscus holmesii* sp. nov. and *Gambierdiscus lewisii* sp. nov. showing (**A**) depth (ventral to dorsal distance) (µm) versus width (transdiameter) (µm) and (**B**) length (apical to antapical distance) (µm) versus width (µm). Error bars represent SD.

SSU rDNA, the strains formed the sister group to the clade consisting of *G.* ribotype 2, *G. honu, G. belizeanus, G. scabrosus, G. balechii, G. lapillus, G. cheloniae, G. toxicus and G. pacificus* albeit with mixed support (64%/1.00 ML BS/BI PP, respectively) (Fig. 8). In the analysis based on the ITS rDNA (Fig. 9), using both BI and ML, the two strains cluster together are clearly separated from other species of *Gambierdiscus*, with full and high support in the BI and ML analyses, respectively.

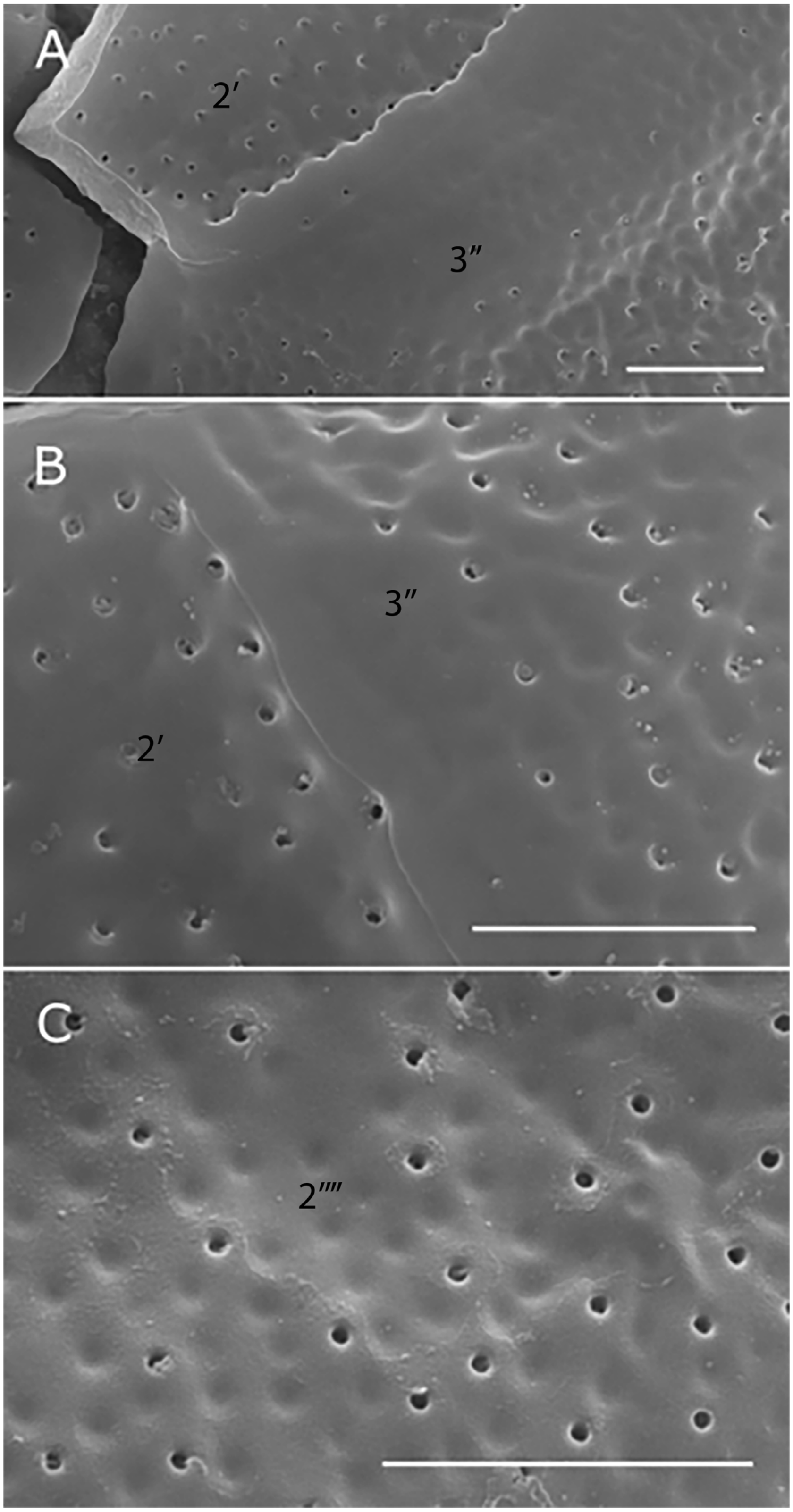

Figure 5. Scanning electron micrographs of *Gambierdiscus* holmesii sp. nov. (isolate UTSHI6B1). (**A-C**) Details of the thecal ornamentation and pores. (A, B) Plate sutures and intercalary band. Scale bars 5 μm.

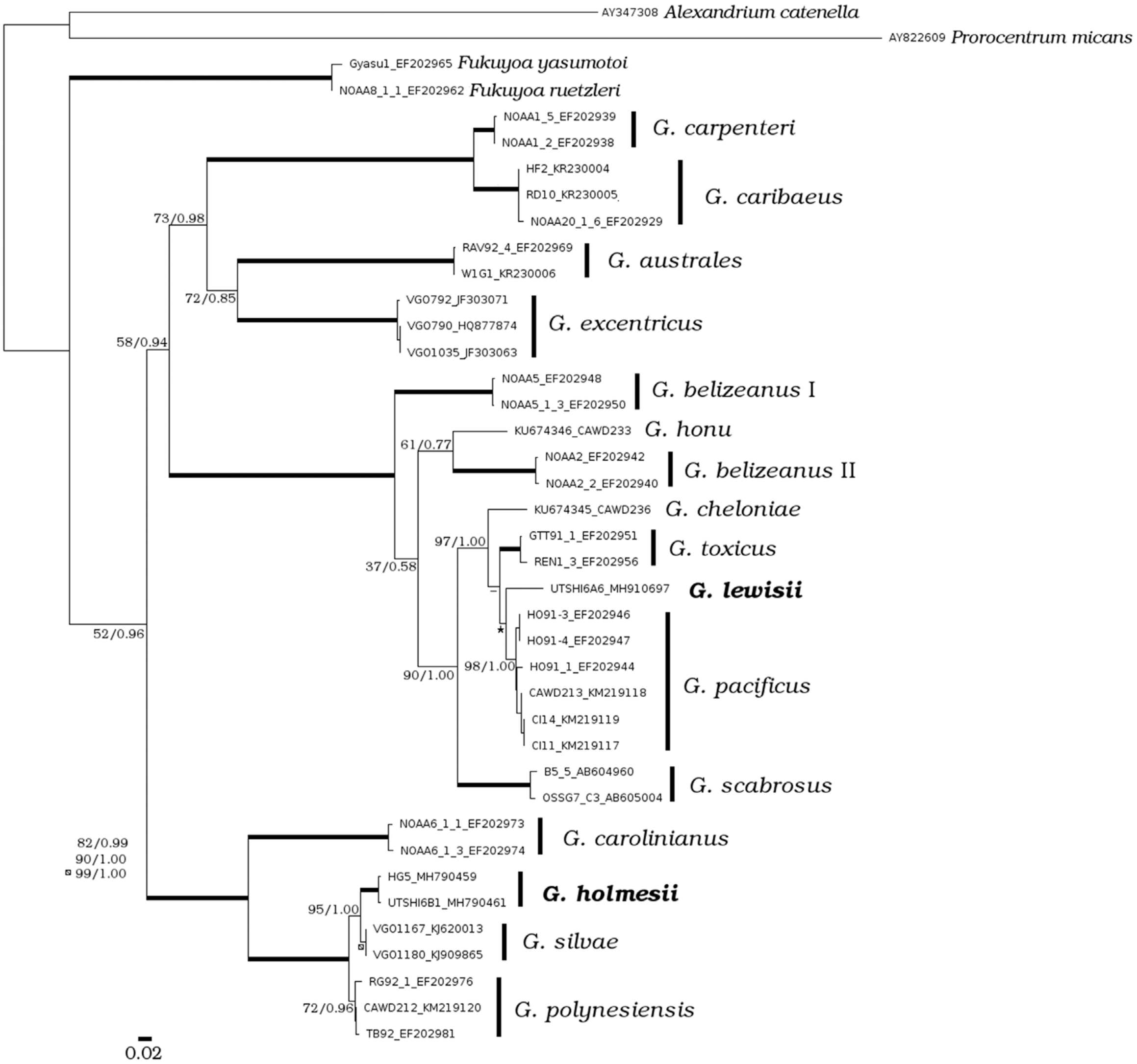

Figure 6. Maximum likelihood phylogenetic analyses of *Gambierdiscus holmesii* sp. nov. and *Gambierdiscus lewisii* sp. nov. from Heron Island, Queensland, Australia, showing alignment of partial LSU rDNA sequences from the D1-D3 region sequences using Bayesian analyses and Maximum Likelihood. Values at the nodes represent Bayesian posterior probability support and ML Bootstrap analyses. The scale bar represents substitutions per site.

The genetic distances of *G. holmesii* from sister groups for all four gene regions are presented (Table 3).

Toxicology

Analysis of the *G. holmesii* strain HG5 using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) did not detect the presence of MTX-1 or any of the CTXs for which there are reference standards (P-CTX-3B, 3C, 4A and 4B). Putative MTX-3 (44-Methylgambierone) was detected, however. The toxicology of the other strain of *G.holmesii*, UTSHI6B1 is described in Larsson et al. (2018).

Gambierdiscus lewisii sp. nov

Morphology

Cells were anterioposteriorly compressed and round lenticular, sometimes dorsally pointed (Figs 10A-C, A, 13, Supplementary Material Fig. S3). Cells were in the size range of most other *Gambierdiscus* spp., with a mean dorsoventral depth of 64.0 μm (range of 56.9 – 78.19 μm; SD 5.1 μm) and mean width of 64.3 μm (range of 57.7 – 69.8 μm;

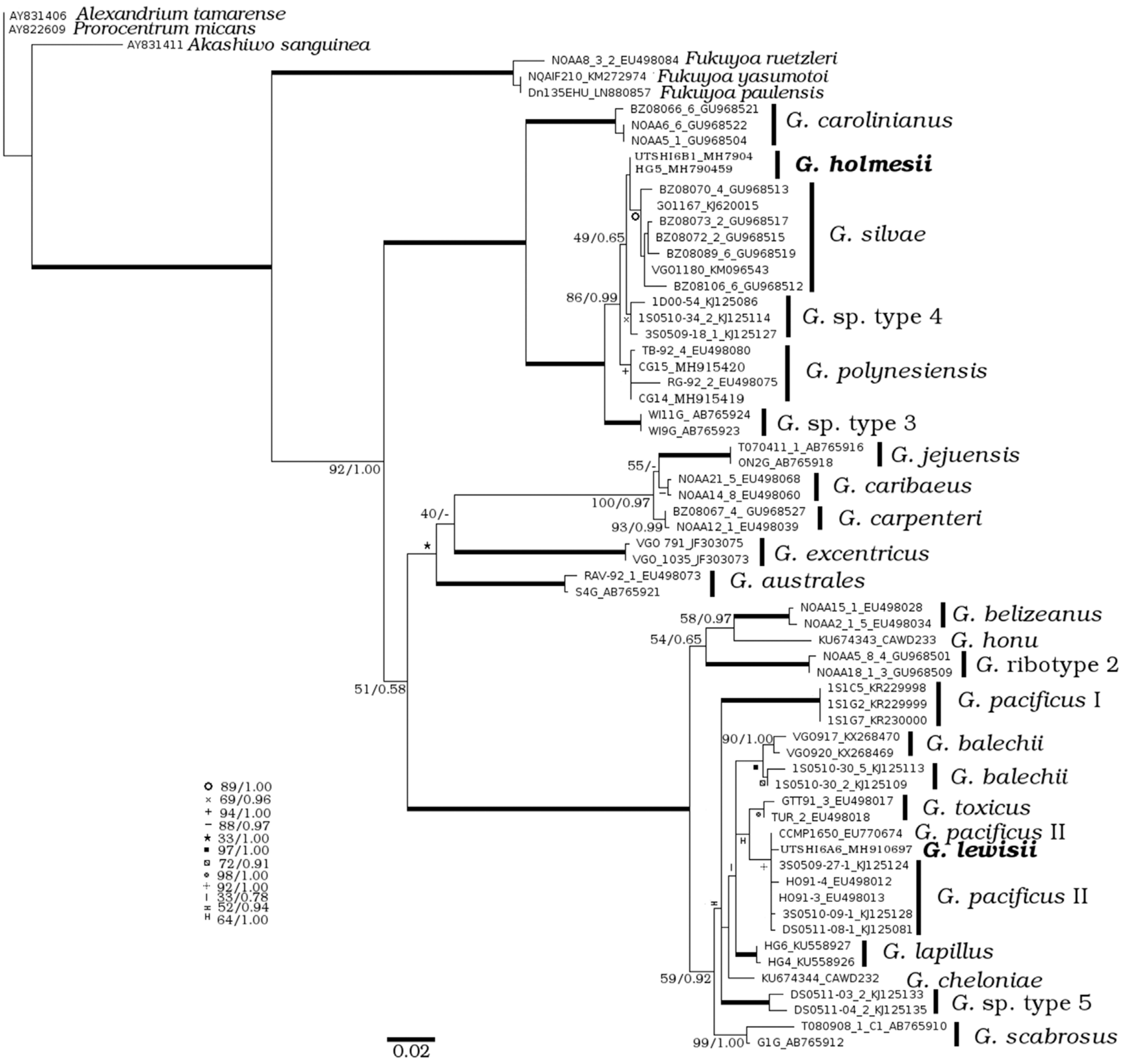

Figure 7. Maximum likelihood phylogenetic analyses of *Gambierdiscus holmesii* sp. nov. and *Gambierdiscus lewisii* sp. nov. from Heron Island, Queensland, Australia the LSU rDNA D8-D10 region sequences using Bayesian Analysis and Maximum Likelihood analysis. Values at the nodes represent Bayesian posterior probability support and ML Bootstrap analyses. The scale bar represents substitutions per site.

SD 3.8 μm), with a depth to width ratio of 0.99 (SD 0.06 μm) (n = 20) (Fig. 4A). The mean cell length was 49.0 μm (43.2 – 57.4 μm; SD 4.3 μm) (n = 20) (Fig. 4B). The hypotheca was higher (longer) than the epitheca, or nearly of equal length (Figs 11E-G, 12 A, Supplementary Material Fig. S3F). The cell surface had a strong reticulate-foveate ornamentation (Figs 10–13), apical and antapical plates with more regularly arranged deep depressions all containing a pore, and pre- and postcingular plates more irregularly reticulated with scattered pores in nearly all reticulation areas (Figs 10A-D,). Wide intercalary bands were ornamented and had a ribbed appearance (Figs 10A-C, A, B). The plate formula (Kofoid tabulation) was Po, 3′, 0a, 7″, 6c,? s, 5‴, 0p, 2⁗ (Figs 10–12). The apical pore plate (Po) is oval with a fish-hook shaped slit-like apical pore, located close to the center of the epitheca, and slightly shifted ventrally (Fig. 10A-C, E-G). Po was about 5.4-6.4 × 2.9-4.5 μm in size (Fig. 10E-G). Twenty-five to 29 large depressions were distributed over the Po plate and arranged in no constant pattern (Fig. 10E-G), rows of depressions intruded into the hook. The largest apical plate was the hatchet-shaped 2′ plate, followed by the pentagonal 3′ plate and the hexagonal 1′ plate

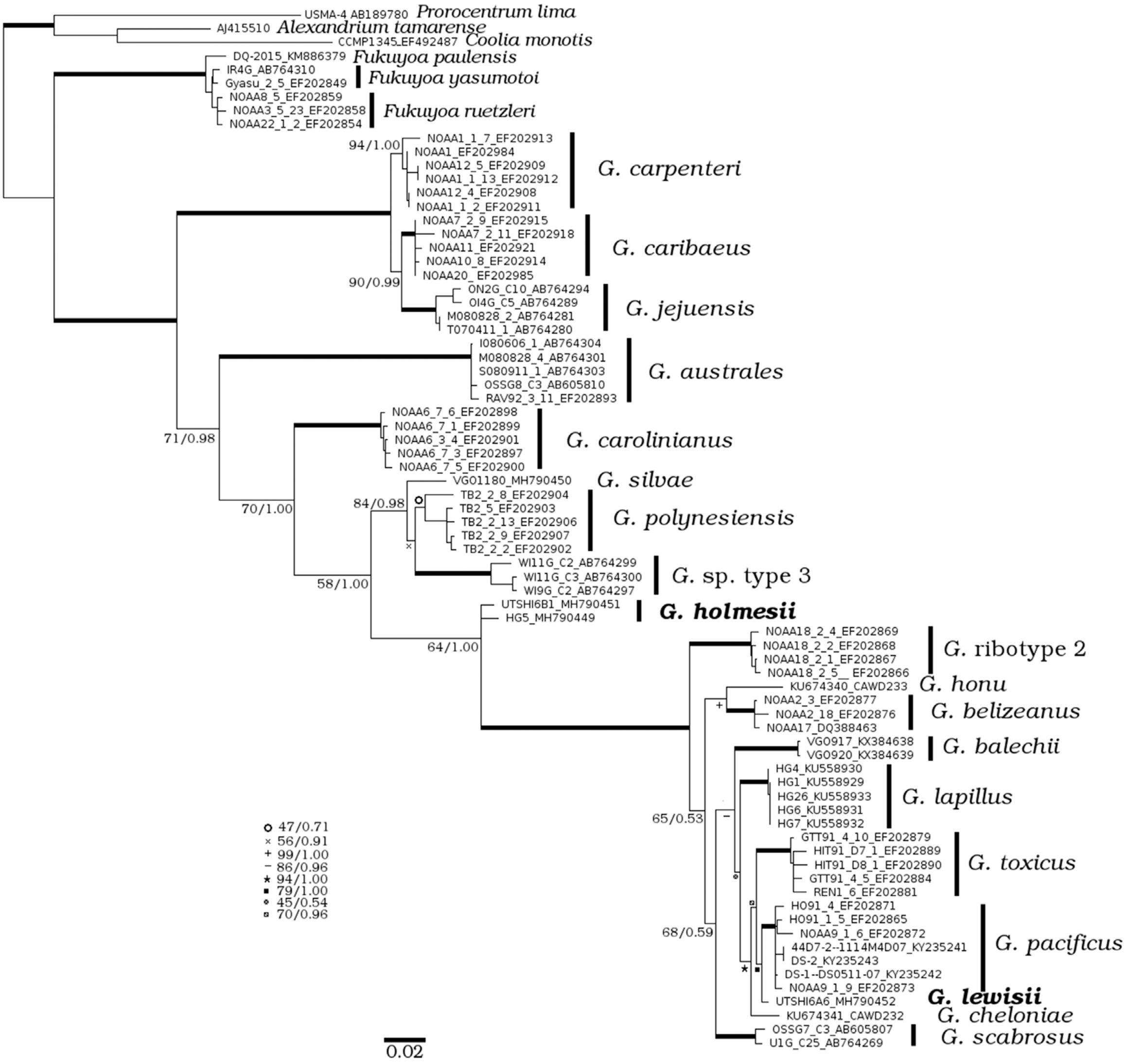

Figure 8. Maximum likelihood phylogenetic analyses of *Gambierdiscus holmesii* sp. nov. and *Gambierdiscus lewisii* sp. nov. from Heron Island, Queensland, Australia the partial SSU rDNA sequences using Bayesian Analysis and Maximum Likelihood analysis. Values at the nodes represent Bayesian posterior probability support and ML Bootstrap analyses. The scale bar represents substitutions per site.

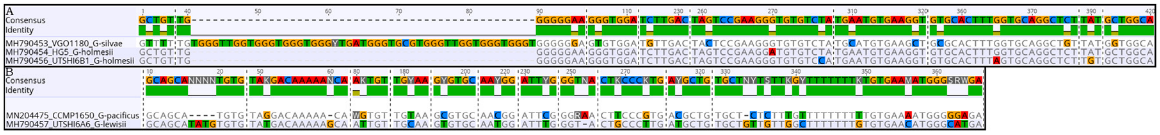

Figure 9. Aligned ITS1/5.8S/ITS2 rDNA sequences for **A**) *G. holmesii* and *G. silvae*; and **B**) *G. lewisii* and *G. pacificus*. Nucleotides which differ between the new species and the closest sister species are shown, while regions with identical base pairs were excluded and denoted with a black dotted vertical line.

(Fig. 10A-C). The suture 2′/2″ is about half the length or more of the 2′/4″ suture. It is rare, but the 2′ plate can be nearly rectangular (Fig. 10C). The largest precingular plate was plate 3″ (four-sided, covering the left lateral precingular area) and the asymmetrical pentagonal 4″ plate (dorsal) was a bit smaller (Fig. 10A-C). Plate 4″ had a much longer 4″/2′ suture compared to the short 4″/3′ suture. Plates 5″ and 6″ were on the right lateral to ventral side (Fig. 10A, B, 11 E, G). Plates 1″

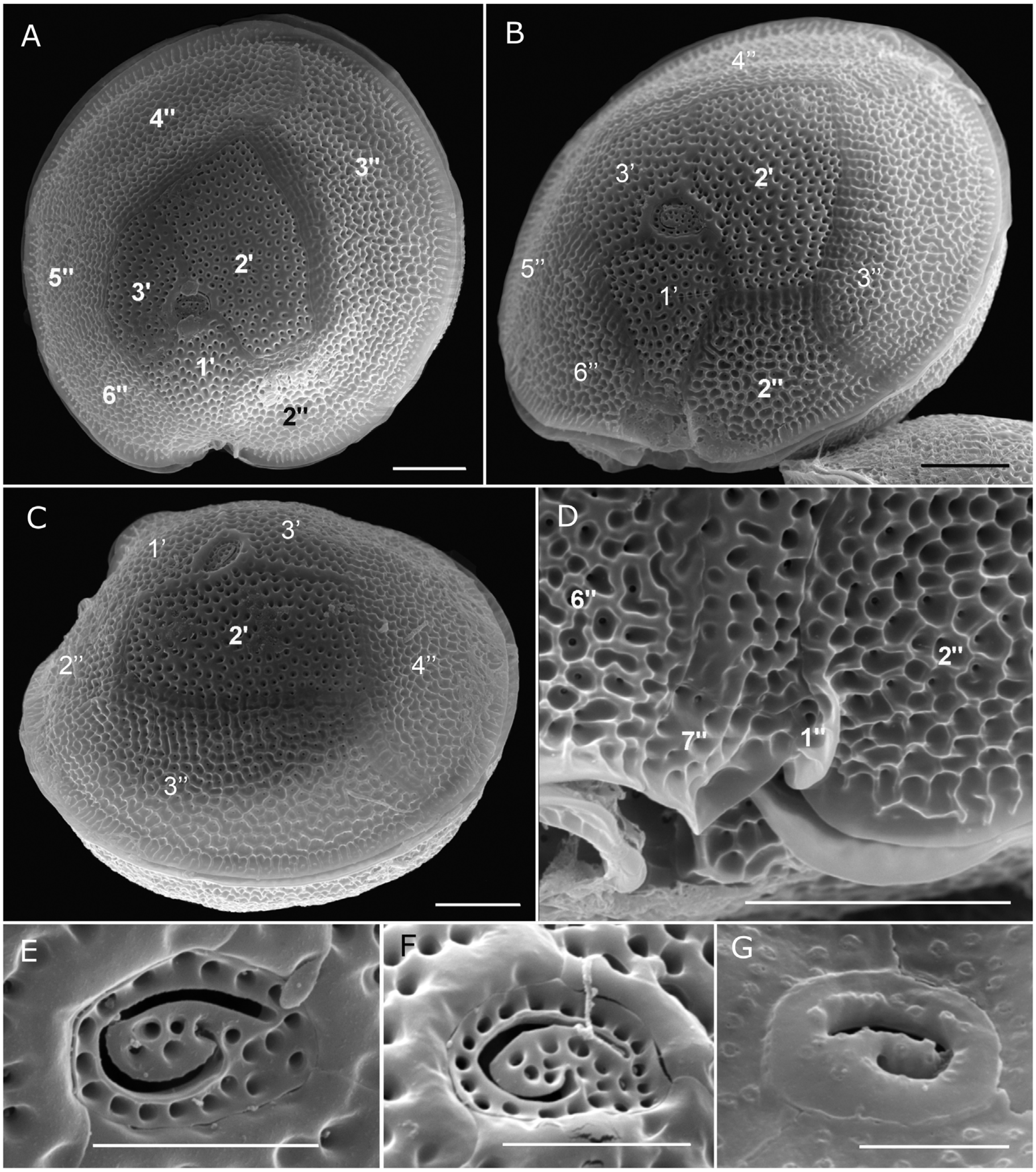

Figure 10. Scanning electron micrographs of *Gambierdiscus* lewisii sp. nov. (isolate UTSHI6A6) of the epitheca. (**A**) Apical view. (**B**) Apical ventral view. (**C**) Left lateral view. (**D**) Ventral part. (**E-G**) Apical pore complex. (E-F) Outside view of the pore plate (Po). (G) Inside view of Po. Scale bars (A-D) 10 μm, (E-G) 5 μm.

and 7″ were the smallest (Fig. 10D) located ventrally. The largest postcingular plate was the 4‴ plate (covering most of the left hypotheca), followed by the plates 2‴ (pentagonal), 3‴ (four-sided), 5‴ (four-sided) and 1‴ (triangular) (Fig. 11A-D). The elongated pentagonal first antapical plate (1⁗) was small and the larger, relatively narrow pentagonal 2⁗ plate covered the antapex (Fig. 11A-D). Plate 1‴ did not contact plate 2⁗ (Fig. 11A-D) and plate 5‴ did not contact plate 2⁗ (Fig. 11). The sulcus was a deep excavation (Figs. 11E, 12) and only four plates could be detected but additional plates were likely hidden in the left sulcal area and the deep sulcal center. The posterior sulcal plate (Sp) was

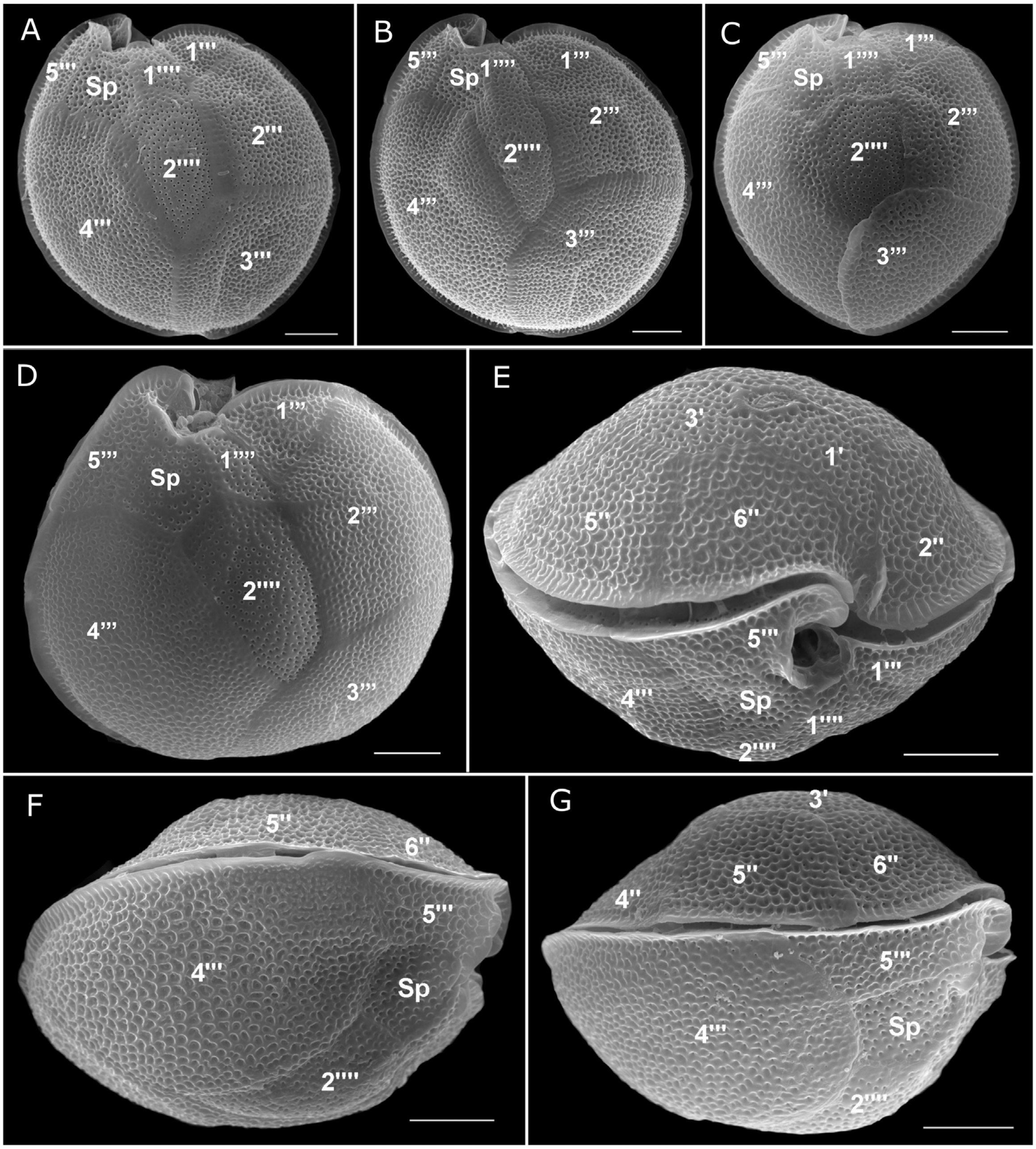

Figure 11. Scanning electron micrographs of *Gambierdiscus* lewisii sp. nov. (isolate UTSHI6A6). (**A-D**) Hypotheca in antapical view. (**E**) Ventral view showing the sulcal area. (**F-G**) Right lateral views. Scale bars 10 μm.

located outside the excavation (Figs. 11E-G, 12 D). The Sda plate was split in a small (Fig. 12D asterisk) and larger part in one case. The narrow and deep cingulum was difficult to observe and consisted of 6 plates.

Phylogenies and Sequence Comparison

The strain of *G. lewisii* UTSHI6B1 clustered as the sister clade to the species *G. pacificus*, with high support (83/1.00, ML BS/BI PP), based on the analyses of the D1-D3 regions of the LSU rDNA (Fig. 6). In the analyses of the D8-D10 region of the LSU rDNA, (Fig. 7), *G. lewisii* clustered within a clade of sequences of *G. pacificus* with relatively high support (92%/1.00 ML BS/BI PP). In the analysis based on SSU rDNA sequences, *G. lewisii* clustered as sister group to a clade comprising *G. pacificus*, with full support (100%/1.00, ML BS/BI PP) (Fig. 8).

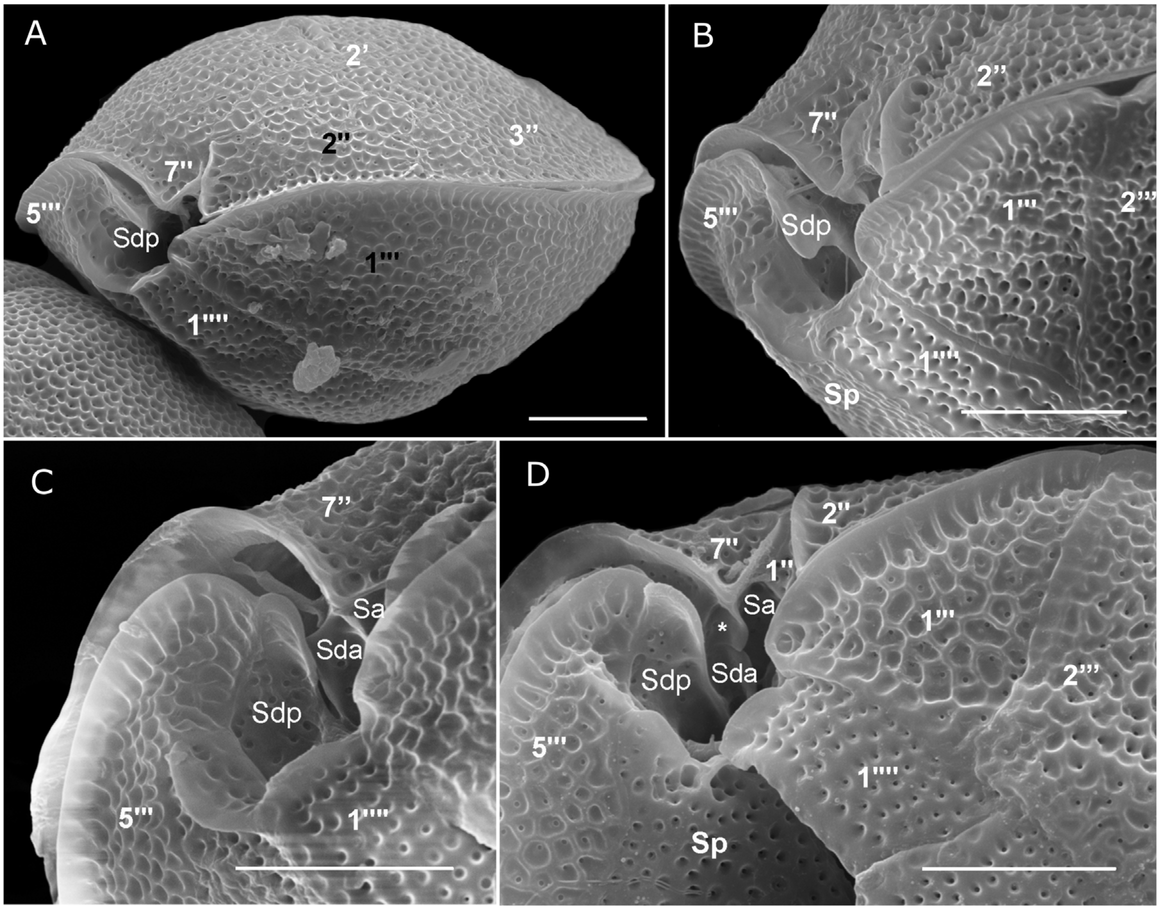

Figure 12. Scanning electron micrographs of *Gambierdiscus* lewisii sp. nov. (isolate UTSHI6A6) of the sulcal area. (**A**) Left lateral to ventral view view of the cell. (**B**) Left lateral view of the sulcal opening. (**C, D**). Sulcal details showing some plates. Scale bars 10 μm.

For the ITS1/5.8S/ITS2 rDNA regions, we aligned these regions from *G.lewisii* with the closest sister species, *G. pacificus* and *G. scabrosus*. We found a similarity of 93.1% in aligned sequences between these two species, which suggests that they are relatively distinct, certainly at species level (Fig. 9, Table 3).

Toxicology

The toxicology of *G. lewisii* strain UTSHI6A6 is described in Larsson et al. (2018).

Discussion

Ciguatera fish poisoning is a human illness associated with the consumption of marine fish and seafood contaminated with CTXs and MTXs produced by species from the epibenthic dinoflagellate genus *Gambierdiscus*. Accurately identifying species in this genus is an essential step toward better understanding the sources of CTXs in the marine food web. Here, we describe two new species of *Gambierdiscus*, *G. holmesii* sp. nov. and *G. lewisii* sp. nov., from the tropical east coast of Australia.

Morphology

Gambierdiscus holmesii sp. nov

Gambierdiscus holmesii is distinct from sister species within the same clade based on several distinctive morphological characters (Table 2). The cell surface of *G. holmesii* was smooth or foveate with scattered pores of one size class (Figs 2A-D, 3 A-D). Thecal plates of *G. silvae* have a foveate ornamentation of different intensity on the apical and antapical (smoother) plates compared to the pre- and postcingular plates, with a pattern of more intense shallow depressions at the cingular margins (Fraga and Rodríguez 2014). Moreover,

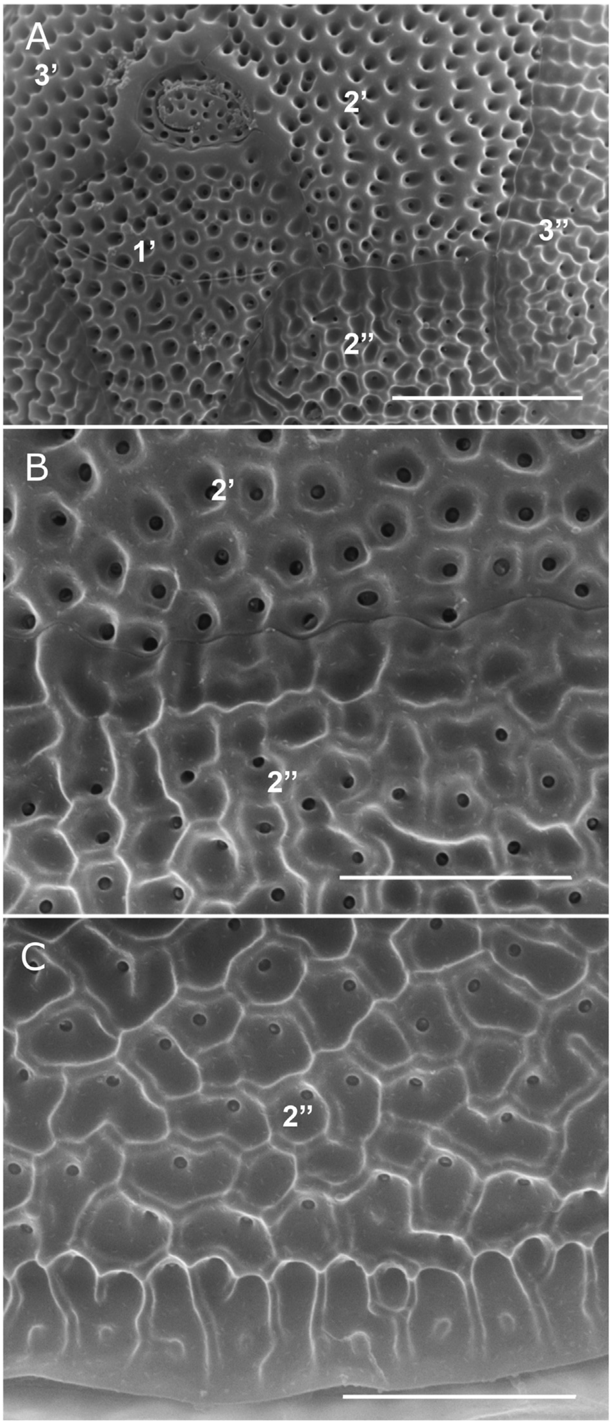

Figure 13. Scanning electron micrographs of *Gambierdiscus* lewisii sp. nov. (isolate UTSHI6A6) showing the reticulate-foveate thecal ornamentation and thecal pores. (**A, B**) Plate sutures and intercalary band. (A) Apical and precingular plates. (B) Plate suture with intercalary band. (**C**) Ornamentation of precingular plate. Scale bars (A) 10 μm, (B, C) 5 μm.

Table 2. Comparison of *Gambierdiscus* species, according to their morphological features. APC = apical pore complex; depth = ventral to dorsal distance; width = transdiameter.

Taxon	Ornamentation	Pores	APC	Epi fold above sulcus	2′ plate	2′′′′ plate	depth [μm]	width [μm]
G. holmesii	smooth to foveate	1 size class	ventrally	Yes	hatchet-shaped wide	large	68.8-88.9	67.1-91.4
G. silvae	Foveate, different intensity	2 size classes	slightly ventrally displaced	No	hatchet-shaped	wide, large	69 ± 8	64 ± 9
G. polynesiensis	smooth	1 size class	ventrally displaced	Yes	hatchet-shaped	wide, large	68.0-85.0	64.0-75.0
G. carolinianus	smooth	1 size class	ventrally displaced	Yes	hatchet-shaped	wide, large	72.2-87.0	75.7-103.3
G. toxicus	smooth	1 size class	ventrally displaced	Yes	hatchet-shaped	wide	94.0-103.0	78.-85.0
G. australes	smooth	1 size class	ventrally displaced	?	rectangular	narrow, long	64.0-93.0	65.0-84.0
G. caribaeus	smooth	1 size class	ventrally displaced	yes	rectangular	wide, large	67.8-97.5	70.2-94.2
G. carpenteri	smooth	1 size class	ventrally displaced	?	rectangular (to hatchet-shaped)	wide, large	66.1-92.2	64.6-84.6
G. jejuensis	smooth	1 size class?	central or ventrally displaced	Yes	hatchet-shaped rarely rectangular	wide, large	63.0-87.9	69.0-93.0

G. pacificus	smooth	1 size class	ventrally displaced	?	rectangular (to hatchet-shaped)	narrow, short	52.0-68.0	60.0-67.0
G. lewisii	reticulate-foveate 2 different types	1 size class	central	Yes	hatchet-shaped rarely rectangular	narrow, short	56.9-78.2	57.8-69.8
G. cheloniae	strong foveate to reticulate-foveate	1 size class	central	Yes	hatchet-shaped	narrow	37.0-80.0	50.0-80.0
G. lapillus	reticulate-foveate 2 different types	1 size class	ventrally displaced	Yes	hatchet-shaped rarely rectangular	narrow	34.4-50.9	32.0-46.9
G. balechii	reticulate-foveate	1 size class	central	Yes	hatchet-shaped	narrow, short	32.0-77.0	36.0-88.0
G. belizeanus	reticulate-foveate strong	1 size class	ventrally displaced	Yes	hatchet-shaped	narrow, short	58.0-98.0	53.0-63.0
G. scabrosus?	reticulate-foveate	1 size class	central or ventrally displaced	?	rectangular	narrow to medium wide	62.5-67.4	58.0-61.6
G. excentricus	smooth	1 size class	strong ventrally displaced	?	~rectangular	medium wide	60.0-115.0	53.0-110.0
G. honu	smooth to foveate	2 size classes	central or ventrally displaced	?	~rectangular	narrow, long	33.6-73.7	35.0-70.2

Table 3. A pairwise comparison of aligned genetic distances of ITS rDNA, D1-D3 rDNA, D8-D10 rDNA, and SSU rDNA between *G. holmesii, G. lewisii*, and their closest sister groups.

Distances	ITS	D1-D3	D8-D10	SSU
G. holmesii- G. silvae	81.7 - 82.0%	96.3 - 96.4%	99.2 - 99.6%	92.3%
G. holmesii- G. sp. Type 4			97.4 - 99.7%	
G. lewisii – G. pacificus	93.1%	91.9 - 92.4%	99.3 - 99.6%	98.4 - 98.1%
G. lewisii – G. scabrosus	43.9 - 50.4%			92.7 - 95.3%

G. silvae has two pore size classes, with larger and less numerous pores on the apical and antapical plates. The general plate pattern of the two species seems to be the same, however the naming of particular plates differs in our current work from that of Fraga and Rodríguez (2014). The first apical plate in *G. silvae* (Fraga and Rodríguez 2014) was interpreted as first precingular plate in our study. Therefore, there is one less apical and one more precingular plate in the present study. The apical pore complex (APC) is only slightly ventrally displaced in *G. silvae* (Fraga and Rodríguez 2014) but more strongly shifted to the ventral side (about one third of the epitheca depth from the sulcus) in *G. holmesii* (Table 2; Fig. 2B). Fraga and Rodríguez (2014) described the second apical plate as usually the largest epithecal plate. It is not larger than plates 3″ and 4″ in *G. holmesii* and also not so in the specimen shown in Fig. 2A for *G. silvae* in Fraga and Rodríguez (2014). There was said to be no distinct fold at the anterior edge of the sulcus formed by plates 1′, 1″, and 2″ in *G. silvae* (Fraga and Rodríguez 2014) but the fold is present in *G. holmesii* (Fig. 3D). *Gambierdiscus polynesiensis* also has a smooth thecal surface, a hatchet-shaped 2′ plate, and a wide and large 2⁗ plate (Chinain et al. 1999). The cell sizes of these two species overlapped, and only minor morphological differences could be found (Table 3): the fold on the 1″ plate and the slightly narrower 2⁗ plate. As differences in CTX analog production between these two species have been found, as *G. polynesiensis* consistently produces detectable quantities of CTX-3B, 3C, 4A, 4B (Chinain et al. 2010; Rhodes et al. 2014), molecular genetic methods may be necessary to confirm the distinction between these two species.

In addition to other morphological features, the cell sizes differ between *G. holmesii* and *G. silvae*, with *G. holmesii* cells being on average significantly larger (Fig. 4). *Gambierdiscus carolinianus* cells are relatively large (72 to 87 μm deep), also have a smooth thecal surface, a hatchet-shaped 2′ plate, and a wide and large 2⁗ plate (Litaker et al. 2009). *Gambierdiscus toxicus* epitype cells are very large (94 to 103 μm deep), have a smooth thecal surface with fine pores, a hatchet-shaped 2′ plate, and a wide pentagonal 2⁗ plate (Litaker et al. 2009). Cells of *G. holmesii* are distinctly smaller. *Gambierdiscus australes* also has a smooth thecal surface with scattered pores, but a rectangular (to only slightly hatchet-shaped) 2′ plate, and a long and narrow 2⁗ plate (Chinain et al. 1999). Cells of *G. australes* were on average larger (64 to 93 μm deep) than *G. holmesii* (Chinain et al. 1999; Litaker et al. 2009) but cell sizes are overlapping (Fig. 10). The narrow 2⁗ plate distinctly separates it from *G. holmesii. Gambierdiscus caribaeus* cells are on average larger (68 to 98 μm deep), also have a smooth thecal surface and a wide and large 2⁗ plate, but a symmetrical rectangular 2′ plate (Litaker et al. 2009). *Gambierdiscus carpenteri* cells are overlapping in size with *G. holmesii* but are larger (66 to 92 μm deep) on average, have a smooth thecal surface and a relative wide and large 2⁗ plate, but a symmetrical rectangular 2′ plate often with a short thecal groove (Litaker et al. 2009). *Gambierdiscus carpenteri* described from Australia differed from the original description (strain from Belize) by a variable shape of the 2′ plate (from hatchet-shaped to rectangular) and the absence of a thecal groove and dorsal rostrum (Kohli et al. 2014a), thus resembling *G. holmesii*. *Gambierdiscus pacificus* has a smooth thecal surface but a relatively short and narrow second antapical plate (Chinain et al. 1999; Litaker et al. 2009) delimiting it from *G. holmesii*.

Gambierdiscus lewisii sp. nov

Gambierdiscus lewisii is distinct from sister species within the same clade based on several morphological features (Table 2). The cell surface of *G. lewisii* had a strong reticulate-foveate ornamentation of two different specifications: (1) apical and antapical plates with more regularly arranged deep depressions, and (2) pre- and postcingular plates more irregularly reticulated. Thecal plates of *G. pacificus* have a smooth thecal surface with pores (Chinain et al. 1999). Cells of *G. pacificus* have a rectangular second apical plate (Chinain et al. 1999) in contrast to *G. lewisii* with a hatchet-shaped 2′ plate. In contrast, Litaker et al. (2009) described an asymmetric hatchet-shaped 2′ plate and cells of a smaller size (52 to 68 μm deep) for *G. pacificus*. Both species have a relatively short and narrow second antapical plate (Chinain et al. 1999).

Recently, a study of *Gambierdiscus* strains in Chinese waters found several strains that were identified as *G. pacificus*, but differed in both morphological features and genetic sequences from those found in previous studies (Zhang et al. 2016). Morphologically, these strains had an areolated cell surface rather than a smooth one (Zhang et al. 2016). Genetically, in comparisons of D8-D10 LSU rDNA sequences, they formed a unique clade not closely related to other strains of *G. pacificus* (Fig. 8), referred to in Figure 8 as *G. pacificus* clade 1. These Chinese strains may therefore represent a novel species.

Gambierdiscus cheloniae has a strong foveate to reticulate-foveate ornamentation and a narrow 2′′′′ plate like *G. lewisii* but showed a very variable tabulation (Smith et al. 2016). *Gambierdiscus lapillus* has the same type of ornamentation and tabulation with a narrow 2′′′′ plate like *G. lewisii* and thus is morphologically similar, but is distinguished based on size (Kretzschmar et al. 2017; Fig. 4A). *Gambierdiscus balechii* has the same type of ornamentation and tabulation with a narrow 2′′′′ plate as *G. lewisii* and spans the size range of *G. lapillus* and *G. lewisii* (Fraga et al. 2016). The 4′′ plate is more symmetrical in *G. balechii* (with sutures 4′′/2′ and 4′′/3′ of nearly equal length) than in *G. lapillus* and *G. lewisii* (Fraga et al. 2016; Kretzschmar et al. 2017). *Gambierdiscus lapillus* was described having a symmetrical 4′′ plate because often it looked nearly rectangular but comparing the 4′′/2′ and 4′′/3′ sutures it was clearly asymmetric. *Gambierdiscus belizeanus* and *G. scabrosus* both have a reticulate-foveate thecal ornamentation and a narrow 2′′′′ plate (Hoppenrath et al. 2014; Litaker et al. 2009; Nishimura et al. 2014). A narrow and medium-wide 2′′′′ plate is also present in *G. australes,* and *G. excentricus,* respectively, but both have a smooth thecal surface with scattered pores (Chinain et al. 1999; Fraga et al. 2011) and *G. excentricus* a characteristically shifted Po plate close to the ventral side. *Gambierdiscus honu* possesses a narrow 2′′′′ type plate as well and has a smooth to foveate thecal ornamentation (Rhodes et al. 2017).

Awareness of the occurrence of intraspecific morphological variability in *Gambierdiscus* species is increasing, and morphological characters considered to be consistently present within a species and useful for species delimitation must be considered with caution. For example, the shape of the second apical plate (hatchet-shaped or rectangular) can show a continuum of variable morphologies within a species (Fraga et al. 2016; Kohli et al. 2014a; Kretzschmar et al. 2017; this study). The same problem appears when judging the symmetry of the fourth (third in some publications) precingular plate (e.g. Fraga et al. 2016). Furthermore, cell length, width and depth has been found to be consistently different for some species of *Gambierdiscus* (e.g. Kretzschmar et al. 2017; Litaker et al. 2009), however other species appear to show very large variability (Bravo et al. 2014; Fraga et al. 2016; Fig. 4). Due to this intraspecific variability, which may not always be included in original descriptions, for best practice identification of *Gambierdiscus* species, the use of multiple approaches is advisable, including molecular genetic data.

Phylogeny and Pairwise Genetic Distance Comparisons

Gambierdiscus holmesii sp. nov

The two strains of the new species *G. holmesii* were closely related with full support, and clearly clustered in their own unique clade in phylogenetic analyses of all three gene regions (Figs. 6, 7, 8). To understand the relationship of the new species, *G. holmesii*, to existing species of *Gambierdiscus*, additional sequencing was undertaken of *G. silvae* strain VGO1180. For three of the four gene regions analysed (ITS, D1-D3 and D8-D10 rDNA), the sister group or most closely related species to *G. holmesii* was *G. silvae* (Figs. 6, 7, 9). However, analyses based on SSU rDNA did not show *G. holmesii* to be closely related to *G. silvae* (Fig. 8), positioning this species as the sister group to a broad clade of species of *Gambierdiscus*. This suggests that this species is genetically distinct and can be readily distinguished using these genetic markers.

In a pairwise comparison of aligned sequences of *G. holmesii* and *G. silvae* (Table 3), we found a divergence of 7.7 % in aligned sequences of SSU rDNA, a divergence of 3.6 - 3.7 % in aligned sequences of D1-D3 LSU rDNA, and 0.4 - 0.8 % in aligned sequences of D8-D10 LSU rDNA. Additionally, we found a divergence of 18 - 18.3 % in aligned ITS rDNA sequences between *G. holmesii* and *G. silvae* (Table 3, Fig. 9).

Gambierdiscus lewisii sp. nov

The strain of *G. lewisii* clearly clustered as a sister species to the clade of *G. pacificus* in analyses based on the D1-D3 region of LSU rDNA, and the SSU rDNA (Figs 6, 8). In analyses of the D8-D10 region of LSU rDNA, *G. lewisii* clustered within the clade of *G. pacificus* (Fig. 7). There was a 0.4 - 0.7 % difference between *G. pacificus* and *G. lewisii* in aligned sequences (782 bp) of the D8-D10 region of LSU rDNA (Table 3). By way of comparison, an alignment of 832 bp in the D8-D10 region of LSU rDNA of three distinct species *Fukuyoa yasumotoi, F. ruetzleri*, and *F. paulensis*, showed differences of 0.1 - 2 %. This is indicative of the conserved nature of the D8-D10 region of LSU rDNA, with a slower evolutionary rate than other nuclear genes, suggesting that differences may not be pronounced even following speciation events.

In a pairwise comparison of aligned sequences of *G. lewisii* and *G. pacificus* (Table 3, Fig. 9), we found a divergence of 1.6 - 1.9 % in aligned sequences of SSU rDNA, a divergence of 7.6 - 8.1 % in aligned sequences of D1-D3 LSU rDNA, a divergence of 6.9 % in aligned sequences of ITS1-5.8S-ITS2 rDNA. Given that several authors, for example Litaker et al. (2007), have suggested that divergences >4% in ITS1-5.8S-ITS2 rDNA in dinoflagellates appear to be a conservative indicator of a species level difference, this indicates substantial divergence between the two species (Table 3). We found a divergence of 49.6 - 56.1% in aligned ITS rDNA sequences between *G. lewisii* and *G. scabrosus*, which was the next most closely related species of dinoflagellate for which ITS data was available (Table 3). As a comparison, there was a difference of 4.7 - 7.3% between *G. lewisii* and *G. scabrosus* in SSU rDNA, which suggests that these two species are at least not distantly related within the genus *Gambierdiscus* (Table 3). Therefore, a difference of ~50% in ITS rDNA between these two species is very large, and suggests that *G. lewisii* is a distinctive species.

Zhang et al. (2016) reported a novel clade of a species with some morphological similarity to *G. lewisii*, which they identified as *G. pacificus* (clade II). These sequences for the D8-D10 region were included in our phylogeny (Fig. 7) and were found to be distinctly different, clustering as a sister group to a clade consisting of 7 different species of *Gambierdiscus* (Fig. 7, *G. pacificus I*). For the D1-D3 phylogenetic inference, the sequence 1S1G7 from Zhang et al. (2016) was excluded, as the final 160bp was divergent from every other *Gambierdiscus* species in a highly conserved region of the D1-D3 LSU gene, suggesting possible sequencing inaccuracies. As this strain appears distantly related to *G. lewisii* in the D8-D10 region, this exclusion is unlikely to have impacted the final tree topology of the D1-D3 phylogeny.

Toxicology

The toxicology of *G. holmesii* strain UTSHI6B1 and *G. lewisii* strain UTSHI6A6 were first investigated in Larsson et al. (2018) (in which they were identified only as *Gambierdiscus* sp.). In this study, we investigated for the first time the toxicology of the strain of *G. holmesii*, HG5. Multiple methods were used to investigate the toxicology of the two new species described in this study. The first was the Ca^{2+} influx mammalian SH-SY5Y cell Fluorescent Imaging Plate Reader (FLIPR) bioassay (Lewis et al. 2016) which can detect broad toxin activity. The method involves an initial separation of the lipophilic toxins (CTXs) from the hydrophilic toxins (MTXs) through a liquid:liquid partition technique. Each phase is then further fractionated into separate compounds by polarity and the toxic effects of each 'fraction' is tested against the SH-SY5Y mammalian cell line. The mode of action of each toxin is used to identify if a CTX-like or MTX toxin is present; whereby MTX-like toxins were identified if calcium influx was evident in the early stages of the assay and CTX-like toxins identified if there was a second peak of activity following addition of an enhancement chemical (veratridine). Using this method, Larsson et al. (2018) showed that cultures of *G. holmesii* strain UTSHI6B1 and *G. lewisii* strain UTSHI6A6 exhibited toxic effects from compounds that had the same mode of activity as CTXs and MTXs (Larsson et al. 2018).

The toxicity of *G. holmesii* and *G. lewisii* was investigated using LC-MS/MS. This is a targeted analytical approach that is able to quantify specific CTXs commonly associated with CFP (P-CTX-3B, 3C, 4A and 4B) and/or specific MTXs, by comparing chromatogram peaks to those of known toxin standards. Neither *G. holmesii* nor *G. lewisii* were found to produce MTX-1 or the CTXs tested for at the level of detection of the assay: P-CTX-3B, 3C, 4A and 4B (this study, and Larsson et al., 2018). Both species produced the putative toxin MTX-3, a compound which has recently been described as 44-Methylgambierone (Murray et al. 2019).

Approximately 23 analogs of CTXs have been described to date (Yogi et al. 2011, Ikehara et al., 2017), and it is likely that other unknown analogs exist. The CTX analog that has shown the most potent toxicity in fish in the Pacific region is P-CTX-1B (Ikehara et al. 2017; Yogi et al. 2011). This analog has never been detected in cultures of *Gambierdiscus* spp, but only in ciguatoxic fish. Other CTX analogs found in ciguatoxic fish in the Pacific region are 52-epi-54-deoxyCTX1B, 54-deoxyCTX1B, 2-hydroxyCTX3C, and 2,3-dihydroxyCTX3. These analogs, along with P-CTX-1B, have been shown to originate from the oxidization by fish liver enzymes during digestion of the *Gambierdiscus*-produced CTX analogs P-CTX4A, 4B and 3C, (Ikehara et al. 2017). These *Gambierdiscus*-produced analogs of CTXs (P-CTX4A, 4B, and 3C) have been detected in cultures of *G. polynesiensis,* in SPATT bags attached to macroalgae in a CFP endemic locations, and in herbivorous fish in areas of endemic CFP toxicity (Chinain et al. 2010; Roué et al. 2018; Yogi et al. 2011).

The species *G. polynesiensis* and *G. excentricus* have been found to produce compounds that are several orders of magnitude higher in CTX-activity than those of other co-occurring *Gambierdiscus* species (Chinain et al. 2010; Litaker et al. 2017; Rhodes et al. 2014). In these studies, other co-occurring *Gambierdiscus* species produced only low level CTX-like activity (Chinain et al. 2010; Litaker et al. 2017; Rhodes et al. 2014). Calibration standards for CTX analogs are necessary to quantify toxins using LC-MS/MS. CTX standards have been purified from large scale mass cultures of *G. polynesiensis* (Chinain et al. 2010; Yogi et al. 2011) or from fish specimens (Yogi et al. 2011, 2014). Standards are not readily available for many of the lesser known CTX analogs. Therefore, it remains unclear whether unknown analogs of CTXs may be contributing to CTX in the food web in Australia, including those that are produced by *G. lewisii* or *G. holmesii*, or whether high CTX-producing species and strains remain to be discovered in this region.

Taxonomic Treatment

Gambierdiscus holmesii Kretzschmar, Larsson, Hoppenrath, Doblin & Murray sp. nov.

Description: Cells anterioposteriorly compressed, round to oval, lenticular. Cell depth 68.75 – 88.87 µm, width 67.05 – 91.36 µm and length 38.25 – 53.10 µm. Smooth or foveate thecal ornamentation with scattered pores of one size class. Intercalary bands smooth or foveate. Thecal tabulation: APC 3′ 0a 7″ 6c 6s? 5‴ 0p 2⁗. APC shifted to the ventral side, about one third of the epitheca depth from the sulcus. Po size: 6.6-7.9 × 3.9-5.0 µm. Hatchet-shaped 2′ plate. 2′/2″ suture about half the length of 2′/4″ suture. Plate 4″ with longer 4″/2′ suture compared to the shorter 4″/3’ suture. Large, pentagonal 2⁗ plate. Plate 1‴ did not contact plate 2⁗.

Etymology: The species was named in honor of Dr. Michael J. Holmes, an Australian researcher who established the foundation of our understanding of the taxonomy and toxicology of *Gambierdiscus* in Australia.

Holotype: Fig. 2A (UTSHI6B1); SEM-stub (designation CEDiT2018H88) deposited at Senckenberg am Meer, German Centre for Marine Biodiversity Research, Centre of Excellence for Dinophyte Taxonomy, Germany.

Isotype: Lugol-fixed subsample of UTSHI6B1 (designation CEDiT2018I89) deposited at the Senckenberg am Meer, German Centre for Marine Biodiversity Research, Centre of Excellence for Dinophyte Taxonomy, Germany.

Type locality: Heron Island (23.4423° S, 151.9148° E), Great Barrier Reef, Australia, South Pacific Ocean.

Molecular diagnosis: Gambierdiscus holmesii can be genetically identified by rDNA sequences deposited in GenBank for strain HG5: ITS (MH790454), SSU (MH790449), LSU D1-D3 (MH790459) and D8-D10 (MH790458) and strain UTSHI6B1 rDNA ITS (MH790456), SSU (MH790451), LSU D1-D3 (MH790461) and D8-D10 (MH790460).

Further information: The strain UTSHI6B1, from which the holotype is derived, has been deposited in the Australian National Algae Culture Collection under strain designation CS-1256.

Gambierdiscus lewisii Larsson, Kretzschmar, Hoppenrath, Doblin & Murray sp. nov.

Description: Cells anterioposteriorly compressed, round, lenticular, sometimes dorsally pointed. Cells 56.89 – 78.15 µm deep, 57.75 – 69.8 µm wide, and 43.24 – 57.41 µm long. Strong reticulate-foveate thecal ornamentation; apical and antapical plates more regularly arranged with deep depressions, pre- and postcingular plates more irregularly reticulated. Intercalary bands ornamented with ribbed appearance. Thecal tabulation: APC 3′ 0a 7″ 6c? s 5‴ 0p 2⁗. APC close to the center of the epitheca, slightly shifted ventrally. Po size: 5.4-6.4 × 2.9-4.5 µm. Hatchet-shaped 2′ plate. 2′/2″ suture about half the length or more of 2′/4″ suture. Plate 4″ with much longer 4″/2′ suture compared to the short 4″/3′ suture. Narrow, pentagonal 2⁗ plate. Plate 1‴ did not contact plate 2⁗.

Etymology: The species was named in honor of Professor Richard J. Lewis, an Australian investigator who pioneered research on *Gambierdiscus* in Australia and continues to contribute to our understanding of the toxins involved in Ciguatera Fish Poisoning.

Holotype: Fig. 9A (UTSHI6A6); SEM-stub (designation CEDiT2018H90) deposited at Senckenberg am Meer, German Centre for Marine Biodiversity Research, Centre of Excellence for Dinophyte Taxonomy, Germany.

Isotype: Lugol-fixed subsample of strain UTSHI6B1 (designation CEDiT2018I91) deposited at the Senckenberg am Meer, German Centre for Marine Biodiversity Research, Centre of Excellence for Dinophyte Taxonomy, Germany.

Type locality: Heron Island (23.4423° S, 151.9148° E), Great Barrier Reef, Australia, South Pacific Ocean.

Molecular diagnosis: Gambierdiscus lewisii can be genetically identified by rDNA sequences deposited in GenBank for strain UTSHI6A6: ITS (MH790457), SSU (MH790452), LSU D1-D3 (MH791069) and D8-10 (MH790462).

Further information: The strain UTSHI6A6, from which the holotype is derived, has been deposited in the Australian National Algae Culture Collection under strain designation CS-1259.

Methods

Sampling, isolation and growth: Strains UTSHI6B1 and UTSHI6A6 were isolated as described in Larsson et al. (2018). Briefly, epiphytic microalgae were removed from the surface of the macroalga *Chnoospora* sp. collected from Heron Island lagoon, Queensland, Australia (23.4423° S, 151.9148° E) (Fig. 1) in July 2014 (austral autumn). Single cells of *Gambierdiscus* were isolated under a light microscope (Nikon Eclipse TS100, Japan) using the micropipette technique (Andersen and Kawachi 2005) and placed in a multi-well microplate with 0.2 μm filter sterilised and autoclaved natural seawater. Cells were incubated in a plant growth chamber (Labec, Sydney, NSW, Australia) at 20 °C under ~100 μmol photons m^{-2} s^{-1} on a 12:12 light:dark cycle. As isolated cells began to divide, modified K-medium (Litaker et al. 2009) was gradually added until monoclonal strains were established. Once isolates reached a concentration of approximately 20 cells per well, each was transferred to 25 cm^2 (50 mL) sterile vented polystyrene tissue culture flasks (Falcon, Corning, NY, USA), oriented horizontally and maintained under the same conditions.

Strain HG5 was isolated as described in Kretzschmar et al. (2017). Briefly, epiphytic microalgae were removed from the surface of a sample of the macroalga *Padina* sp. collected from Heron Island Lagoon, Queensland, Australia (23.4423° S, 151.9148° E) (Fig. 1) in July 2014. Single cells of *Gambierdiscus* were isolated using a micropipette under an inverted light microscope (Nikon Eclipse TS100, Japan) using the micropipette technique (Andersen and Kawachi 2005), washed three times in sterile seawater, and transferred into f/10 medium (Holmes et al. 1991) to establish clonal cultures. Cultures were maintained in f/10 medium at 27 °C under ~60 μmol photons m^{-2} s^{-1} on a 12:12 light:dark cycle. Cultures have been deposited in the Australian National Algae Culture Collection (https://www.csiro.au/en/Research/Collections/ANACC), and strain numbers are given in the species descriptions.

Light microscopy; Cells were initially identified using an inverted light microscope under Bright Field (Nikon Eclipse TS100, Japan). Cells were photographed using an inverted microscope (Nikon Eclipse TS100) fitted with a colour camera (Infinity 1; Lumenera, Ontario, Canada) and cell size dimensions were measured using image analysis software (Infinity Analyze; Lumenera, Ontario, Canada). Measurements of strains UTSHI6B1 (*G. holmesii*) and UTSHI6A6 (*G. lewisii*) were taken for 20 individual cells fixed with Lugol's Iodine solution at a final concentration of 1%.

DNA extraction, PCR amplification and DNA sequencing: DNA for strains UTSHI6B1 and UTSHI6A6 was extracted as described in Larsson et al. (2018). Briefly, approximately 100 mL of each strain was centrifuged at 600x g for 10 min to harvest the cells. DNA was extracted using a MoBio Soil DNA Extraction Kit following the manufacturer's instructions, stored at −80 °C and sent to a commercial service (Australian Genomic Research Facility (AGRF), Queensland, Australia) for further processing and sequencing of the D1-D3 and D8-D10 LSU rDNA regions. Extracts were amplified under conditions specified in Supp. Table 1. Amplification products (~950 bp) were purified and sequenced in both directions using the Sanger sequencing platform.

For strain HG5, DNA was extracted as described in Kretzschmar et al. (2017) using the cetyl trimethylammonium bromide (CTAB) method (Zhou et al. 1999), with the purity of the extracted DNA assessed via Nanodrop (Nanodrop2000; Thermo Scientific, Australia) and the integrity visualized on 1% Agarose gel. PCR amplicons were obtained under the conditions specified in Supp. Table 1 and purified with SureClean Plus (Bioline) before being directly sequenced in both directions using the Sanger sequencing platform by Macrogen Inc. (Seoul, Korea).

In addition, the interspacer (ITS1-5.8S-ITS2) rDNA region was amplified (Supp. Table 1) and sequenced for all strains. PCR amplicons were purified with SureClean Plus (Bioline) and Sanger sequenced by Macrogen Inc. (Seoul, Korea).

The original description of *Gambierdiscus silvae* did not include phylogenetic analyses of the rDNA small subunit (SSU) gene. As one of the *Gambierdiscus* species described in this study is closely related to *G. silvae*, we sought DNA from the type material to sequence for comparison. Extraction of *G. silvae* (VGO1180) was conducted by Francisco Rodríguez Hernández of the Instituto Español de Oceanografía, Spain, using a CTAB protocol adapted from Doyle and Doyle (1987). Briefly, cell pellets were mixed with 700 μL of CTAB buffer solution premixed with 3 mL of mercaptoethanol and 30 mL of proteinase K. Samples were thawed three times in N2 and incubated (65 °C, 1 h) with agitation. Tubes were left at room temperature during 10 min and 700 mL of chlorophorm:isoamyl:alcohol (24:1) were added. Ethanol (1.5 vol of 96%) and sodium acetate (0.1 vol of 3 M, pH 5.2) were added and samples kept overnight at -20 °C. After a centrifugation step (13,000 rpm, 15 min, 4 °C), pellets were rinsed with cold 70% ethanol, centrifuged again in the same conditions and the resultant cell pellet was dried in a vacuum concentrator (miVac, Genevac). PCR amplification was conducted (Supp. Table 1); amplified DNA was then purified with SureClean Plus (Bioline) and Sanger sequenced through Macrogen Inc. (Seoul, Korea).

Phylogenetic analyses: Phylogenetic analyses were performed as described in Kretzschmar et al. (2017) in Geneious v11 (Kearse et al. 2012). Publicly available sequences of *Gambierdiscus* spp. were downloaded from the GenBank reference database (www.ncbi.nlm.nih.gov) and aligned with the sequences from our study using the MUSCLE algorithm with a maximum of 8 iterations (Edgar 2004). Alignments were truncated to 972 bp and 793 bp for the D1-D3, D8-D10 LSU rDNA regions respectively. The SSU region was truncated to 1,527 bp and a 36 bp fragment removed from all species in the alignment as this region of *G. silvae* type strain VG01180 could not be amplified. Full length alignments are available on Zenodo doi:10.5281/zenodo.3377067. Maximum likelihood (ML) phylogenetic trees were generated for all regions with PHYML with 1000 bootstraps (Guindon and Gascuel 2003).

Phylogenetic trees were calculated with Bayesian Inference (BI) and Maximum Likelihood (ML) using a GTR substitution model and an estimated gamma distribution. Bayesian Inference (BI) was also performed for all regions to estimate Posterior Probability (PP) distribution using MrBayes 3.2.6 with Metropolis-Coupled Markov Chain Monte Carlo simulations (Ronquist and Huelsenbeck, 2003). A random starting tree with three heated and one cold chain(s) was used with temperature set at 0.2. Trees were sampled every 100th generation for 2,000,000 generations generated and the first 10% discarded as burn-in.

Scanning electron microscopy (SEM): For the strains UTSHI6B1 and UTSHI6A6, 50 mL of culture was sieved through a 50 μm mesh to concentrate the cells, which were then resuspended in 5 mL of media, preserved with 1% Lugol's Iodine solution and analysed by scanning electron microscopy (SEM) at the Senckenberg am Meer (Wilhelmshaven, Germany). Cells were placed on a 5 μm Millipore filter, rinsed twice in distilled water, and dehydrated in a series of increasing ethanol concentrations (30, 50, 70, 85, 90, 100 %; 10 min each), followed by air drying after addition of hexamethyldisilazane (HMDS) at room temperature. When completely dry, the sample was mounted on a stub and sputter coated with gold-palladium (SCD 050 Bal-Tec). Cells were observed using the SE and/or BSE detector of a Tescan VEGA3 microscope (Elekronen-Optik-Service GmbH, Dortmund, Germany) at 15 kV.

Terminology: Epithecal plates were labelled using the Kofoidian system of plate series (Hoppenrath et al. 2014; Litaker et al. 2009). This is not the same as that proposed by Fraga et al. (2011) who adjusted the system to consider possible plate homologies. The hypothetical interpretation is in light of gonyaulacalean relationships (two asymmetric antapical plates of different size and no posterior intercalary plate) with a posterior sulcal plate outside the depressed longitudinal furrow (Fraga et al. 2011; Hoppenrath et al. 2014), as opposed to Litaker et al. (2009). The description of the thecal ornamentation follows the proposed standard terminology by Hoppenrath et al. (2013) for *Prorocentrum*.

Toxicology: *Gambierdiscus* strain HG5 was grown in a volume 2 L and on day 36, cells (approx. several million) were harvested by centrifugation at 15,000x g for 10 min and the resultant cell pellet freeze dried. Cell pellets were then extracted with 2 mL methanol and screened for the presence of MTXs (MTX-1 and putative MTX-3 (44-Methylgambierone) and CTXs (P-CTX-3B, 3C, 4A, 4B) using a Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) method developed by the Cawthron Institute (Argyle et al. 2016; Kohli et al. 2014a, b; Selwood et al. 2014). The limit of detection of the analysis was approximately 1 ng mL^{-1} for MTX-1 and <1 ng ml^{-1} for the CTX analogues.

Conflict of Interest Statement

The authors declare no conflict of interest in the production of this research.

Author Contributions

A.L.K, M.E.L and S.M. conceived and designed the study. M.E.L isolated cultures and conducted LM measurements, A.L.K and M.E.L maintained microalgal cultures and carried out DNA extraction, A.L.K carried out PCR and phylogenetic analysis; M. H. performed Scanning Electron Microscopy (SEM) and analysed morphological data; A.L.K, M.D, S.M, M.H and M.E.L. drafted, edited and approved the manuscript.

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Appendix A. Supplementary Data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.protis.2019.125699.

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