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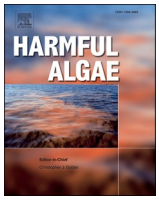

Taxonomy, distribution, and habitat associations of *Gambierdiscus* from coastal waters off Queensland, Australia

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ABSTRACT

Ciguatera poisoning is the most prevalent non-bacterial seafood illness globally, with an estimated 10,000 to 50,000 cases reported annually. It is caused by consumption of seafood (mainly fish) that have accumulated ciguatoxins produced primarily by benthic dinoflagellates of the genus *Gambierdiscus*. Ciguatera poses a significant public health and fisheries challenge in tropical and subtropical regions. Although these dinoflagellates are widespread across tropical and subtropical marine ecosystems, their ecology, taxonomy, and environmental drivers remain poorly understood in many regions, limiting the capacity to monitor and predict ciguatera risk. This study uses DNA metabarcoding (targeting the V8-V9 regions of the 18S rRNA gene) to investigate the diversity, spatial distribution, and ecological associations of *Gambierdiscus* species across multiple coastal sites in Queensland, Australia. Results revealed the distribution of known species (*G. carpenteri, G. holmesii*) and several potential new phylotypes, notably a genetically distinct group (Clade II GBR) that may represent an undescribed lineage. Importantly, this study provides the first confirmed detection of *G. holmesii*, a known ciguatoxin producer, in Hervey Bay and Gladstone, extending its known geographic range from offshore to inshore reefs and coastal habitats of the Great Barrier Reef. This study is also the first documented record of any *Gambierdiscus* taxa in the Gladstone coastal region. Taxa composition was influenced by macroalgal host, and shallow coral reef habitats supported the highest species diversity and detection frequency, suggesting their role as ecological hotspots. Canonical correspondence analysis (CCA) identified associations between species and macroalgal hosts, including a strong link between *G. holmesii* and *Cladophora* sp., while *G. carpenteri* showed no specific host preference. Overall, this study provides ecological characterisation of *Gambierdiscus* communities across diverse tropical and subtropical habitats in Queensland, contributing to regional ciguatera risk assessment and global efforts to understand the ecology and distribution of benthic toxic dinoflagellates.

1. Introduction

Marine benthic dinoflagellates of the genus *Gambierdiscus* Adachi & Fukuyo (Dinophyceae; Gonyaulacales) are considered the causative agents of ciguatera poisoning (CP); the most common non-bacterial seafood-related illness in the world (Argyle et al., 2023; Chinain et al., 2021). The illness is responsible for up to 50,000 global cases annually (Friedman et al., 2017), although these numbers are conservative, as it is thought to be hugely underreported (Bengio et al., 2024; Huff et al., 2024). Species belonging to *Gambierdiscus* produce two major groups of potent polycyclic polyether neurotoxins: maitotoxins (MTXs) and ciguatoxins (CTXs). CTXs are lipophilic compounds, that bioaccumulate in marine animal tissue. Subsequently, when consumed by humans, symptoms manifest in the digestive, joint, muscle, cardiovascular, and nervous systems (Friedman et al., 2017; Wang et al., 2022). Although the illness has existed for centuries (Chinain et al., 2019, 2020; Rongo et al., 2009), its prevention and effective treatment are still not known, posing a serious risk to public health and global fishery economies (Litaker et al., 2017; Liu et al., 2025; Trick et al., 2020).

1.1. *Gambierdiscus* taxonomy

Accurate identification of *Gambierdiscus* species is essential for understanding toxin production and assessing CP risk. Twenty species are

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currently recognised, primarily distributed across tropical and subtropical oceans, with occasional detections in temperate regions (Chinain et al., 2023; Funaki et al., 2022; Murray et al., 2025; Nguyen-Ngoc et al., 2023; Wang et al., 2022). Historically, taxonomy relied on morphological traits such as thecal plate arrangements and pore patterns, but overlapping features made species difficult to distinguish (Beaxls et al., 1979; Holmes, 1998; Nishimura et al., 2013; Richlen et al., 2008). Molecular analyses of ribosomal DNA regions (SSU, LSU, 5.8S, ITS) have revealed high cryptic diversity, enabling recognition of new species and ribotypes and provide a robust framework for species delimitation (Chinain et al., 1999; Litaker et al., 2009; Nishimura et al., 2013; Richlen et al., 2024; Stuart et al., 2022).

Phylogenetic analyses has also facilitated the construction of a clade based classification system for *Gambierdiscus* first suggested by Nishimura et al. (2013). This system groups taxa into evolutionary clades based on shared genetic markers and has become a practical framework for studying species boundaries and ecological traits (Funaki et al., 2022; Nishimura et al., 2013; Richlen et al., 2024; Stuart et al., 2022). Notably, what was historically classified as *Gambierdiscus* spp. within Clade I (Nishimura et al., 2013) has since been reassigned to a distinct genus, *Fukuyoa* F.Gómez, D.Qiu, R.M. Lopes & Senjie Lin, which includes four known species (Li et al., 2021). Molecular delineation has highlighted substantial intra- and interspecific diversity among ciguatoxin-producing species such as *G. polynesiensis* Chinain & M.A. Faust and *G. silvae* S.Fraga & F.Rodríguez, revealing population structuring linked to local environmental conditions (Chinain et al., 2010; Litaker et al., 2009; Malto et al., 2022; Mudge et al., 2022; Murray et al., 2025; Sibat et al., 2025; Stuart et al., 2022; Vacarizas et al., 2018). Continued molecular studies are essential to refine taxonomy, understand biogeography, and assess toxicological potential across the genus.

1.2. Gambierdiscus habitat

Gambierdiscus are predominantly epi-benthic, attaching to a wide range of substrates in mostly tropical to subtropical marine environments. Their primary habitats are coral reef ecosystems, although they have been found in a range of environments, such as seagrass meadows, river mouths, and anthropogenic structures such as petroleum platforms (Argyle et al., 2023; Chinain et al., 2021; Kohli et al., 2014; Parsons et al., 2011; Rains and Parsons, 2015; Schulze et al., 2020). These habitats usually have macroalgal cover, providing increased surface area, nutrient-rich microenvironments, and physical protection; making them ideal microhabitats for these dinoflagellates (Rains and Parsons, 2015; Richlen et al., 2024). Species-specific associations with macroalgal hosts have been reported, and the composition of macroalgal communities can influence both the abundance and diversity of *Gambierdiscus* present at a site (Parsons et al., 2011; Rains and Parsons, 2015; Richlen et al., 2024). Given the epiphytic lifestyle of *Gambierdiscus*, increases in macroalgal abundance and shifts in community composition, driven by climate change, eutrophication, and coral reef degradation, may enhance their available habitat, and increase their distribution, potential enhancing toxin exposure risks (Gingold et al., 2014; Mustapa et al., 2019; Rains and Parsons, 2015; Skinner et al., 2013).

1.3. Ciguatera poisoning, Queensland, Australia

Ciguatera poisoning has been documented in Australia since at least the late 1700s, with early accounts from Captain Cook's expeditions and subsequent reports from colonial settlers. Today, CP remains the most common seafood-related illness in Australia, primarily linked to large reef fish such as Spanish mackerel (*Scomberomorus commerson*), coral trout (*Plectropomus leopardus*), and redthroat emperor (*Lethrinus miniatus*) (Farrell et al., 2017; Gillespie et al., 1986; Stewart et al., 2010). Officially, 236 cases were reported in Queensland between 2014 and 2025 (The State of Queensland, 2025), though actual numbers are likely higher due to underreporting and diagnostic challenges (Farrell et al., 2017; Friedman et al., 2017; Holmes et al., 2021).

Most cases have historically occurred in tropical waters along the Great Barrier Reef (GBR), but outbreaks are increasingly reported in subtropical and temperate regions, including southern Queensland and New South Wales (Edwards et al., 2019; Farrell et al., 2016; Skinner et al., 2011). This southward shift may reflect improved diagnostics, climate-driven changes in *Gambierdiscus* distribution, fish migration, and expanding seafood trade networks (Chinain et al., 2020; Kohli et al., 2014; Perkins et al., 2024; Smith et al., 2017). CP remains a complex public health and biosecurity concern, influenced by environmental, biological, and socio-economic factors. Continued monitoring of toxic species, fish vectors, and community awareness is essential to mitigate risk (Farrell et al., 2016a).

1.4. Gambierdiscus taxonomy and distribution, Queensland, Australia

Early studies first documenting *Gambierdiscus* in Australian waters (Gillespie et al., 1986; Holmes et al., 1991; Lewis and Holmes, 1993), initially assumed to be monotypic (*G. toxicus*; Adachi and Fukuyo, 1979) due to limited taxonomic resolution. Advances in molecular and morphological methods revealed a far greater diversity. Over the past two decades, multiple *Gambierdiscus* species have been identified in Australian waters (Table 1). Notably, *G. carpenteri* Kibler, Litaker, M.A. Faust, W.C.Holland, Vandersea & P.A.Tester and *G. cf. belizeanus* were reported from the GBR and *G. carpenteri* was detected in southern temperate regions such as Merimbula, New South Wales (Sparrow et al., 2017; Kohli et al., 2014a; Larsson et al., 2018) and more recently in Hervey Bay at the mouth of the Burrum River (Perkins et al., 2025).

Subsequent research expanded known *Gambierdiscus* diversity in

Table 1
Summary of *Gambierdiscus* species reported in Australian waters and their authorities, their recorded locations, toxins produced (if known) and corresponding references for detection in Australian waters.

Species	Location	Toxins produced	References
G. carpenteri Kibler et al.	Townsville, Hervey Bay (QLD), Merimbula (NSW)	MTX-like activity***	(Kohli et al., 2014; Perkins et al., 2025; Sparrow et al., 2017)
*G. cf. belizeanus** Faust	Heron Is. (QLD)	Unknown	(Murray et al., 2014)
G. honu Rhodes et al.	Heron Is. (QLD)	MTX-3	(Rhodes et al., 2017; Richlen et al., 2008)
G. lapillus Kretzschmar et al.	Heron Is. (QLD)	CTX-like activity***	(Kretzschmar et al., 2017)
G. lewisii Larsson et al.	Heron Is., Palm Island, Bramble Reef (QLD)	44-methylgambierone	(Kretzschmar et al., 2019)
G. holmesii Kretzschmar et al.	Heron Is., Orpheus Is., Beaver Reef (QLD)	M-seco-CTX4A/B	(Kretzschmar et al., 2019; Murray et al., 2025)
*G. polynesiensis*Chinain & Faust	Heron Is., Orpheus Is., Beaver Reef (QLD)	M-seco-CTX4A/B	(Murray et al., 2025; Sibat et al., 2025)
G. bagnisii Murray et al.	Orpheus Is., Beaver Reef (QLD)	Unknown CTX-like compound	(Murray et al., 2025)
*G. toxicus*** Adachi and Fukuyo	Arlington Reef, Hervey Bay (QLD)	Unconfirmed (historical)	(Holmes et al., 1991)

* Closely resembles but is not definitively identified as the named species.
** Identification of *G. toxicus* is not certain as the study/studies occurred prior to revision of the genus.
*** Maitotoxin-like or ciguatoxin-like activities via bioassays but toxins haven't been characterised.

Queensland and globally, with Kretzschmar et al. (2017) describing new species *G. lapillus* and later identifying two more new species, *G. lewisii* and *G. holmesii*, all from Heron Island (Kretzschmar et al., 2019). Until recently, however, no studies had confirmed the presence of CTX-producing *Gambierdiscus* species in Australian waters. This changed with the work of Murray et al. (2025), who conducted extensive sampling across offshore reefs of the GBR. This study detected known CTX producer *G. polynesiensis* in Australian waters for the first time and describing a new species, *G. bagnisii* sp. nov. They also provided the first confirmed evidence of CTX production in Australian *Gambierdiscus* from *G. holmesii*, which was found to produce M-seco-CTX4A/B. In addition, both *G. bagnisii* and *G. holmesii* were found to produce an unidentified compound previously reported only from *G. polynesiensis*, suggesting a shared toxicological profile among members within Clade III.

Despite these advances, most studies to date have focused on offshore reef environments (Table 1), particularly Heron Island, and comparatively little is known about the presence, diversity, or ecological dynamics of *Gambierdiscus* in coastal and nearshore habitats. This represents a critical knowledge gap, especially considering the potential for human exposure to toxic species in areas closer to shore. This study addresses that gap by applying DNA metabarcoding to assess *Gambierdiscus* diversity and distribution across three coastal regions: Townsville, Gladstone, and Hervey Bay. We also examine key environmental drivers, including habitat type, depth, and host macroalgae, with the goal of improving molecular monitoring tools for ciguatera risk and enhancing our understanding of *Gambierdiscus* biogeography in tropical and subtropical Australia.

2. Methods

2.1. Sample regions, collection, and preservation

Samples were collected from benthic macroalgae across three coastal locations in Queensland, Australia: Townsville, Gladstone, and Hervey Bay (Fig. 1). Multiple sites were sampled within each region (Townsville n = 4, Gladstone n = 5, Hervey Bay n = 5), with five replicate samples and one field control per site. Site selection was based on the presence of diverse benthic habitats and, importantly, these locations have a history of CP incidents (Australian Institute of Food Safety, 2014; Holmes et al., 2021; Krasnova et al., 2023). However, comprehensive studies on the distribution and diversity of *Gambierdiscus* spp. within these areas are lacking. The proximity of each location to large human populations further emphasises the ecological and public health significance of the study. In total, 70 samples and 14 field controls were collected during the wet season, between 9th November 2022 and 11th January 2023, a period characterised by increased rainfall and higher temperatures in tropical and subtropical climates.

2.1.1. Environmental context by region

2.1.1.1. Townsville (Tropical). Located on the central Queensland coast (19.26°S, 146.82°E), Townsville experiences a tropical climate with warm sea temperatures (24–30°C) and seasonal rainfall (Nov–Apr). Sampling was conducted at Cape Pallarenda (n=1), Three Mile Creek (n=1), and bays around Magnetic Island (n=2). These sites span turbid, flood-prone shorelines to clearer fringing reefs. Three Mile Creek is heavily impacted by sediment runoff and shoreline change, while Magnetic Island shows long-term reef degradation (Wolanski and Hopper, 2025).

2.1.1.2. Gladstone (Subtropical). Situated just below the Tropic of Capricorn (23.82°S, 151.23°E), Gladstone has subtropical waters (20–28°C). Sampling occurred around Facing Island at Rat Island (n=2), the northeastern shore (n=2), and Manning Reef's sheltered bay (n=1). These areas range from exposed coasts to protected reefs. The region is ecologically distinct due to major port activity and industrial impacts, contributing to sedimentation and reef degradation (Ghosh et al., 2022; McIntosh et al., 2019). Abundant macroalgae across these sites supports potential dinoflagellate habitats across a gradient of exposures and depths.

2.1.1.3. Hervey Bay (Subtropical). Hervey Bay (25.00°S, 152.85°E)

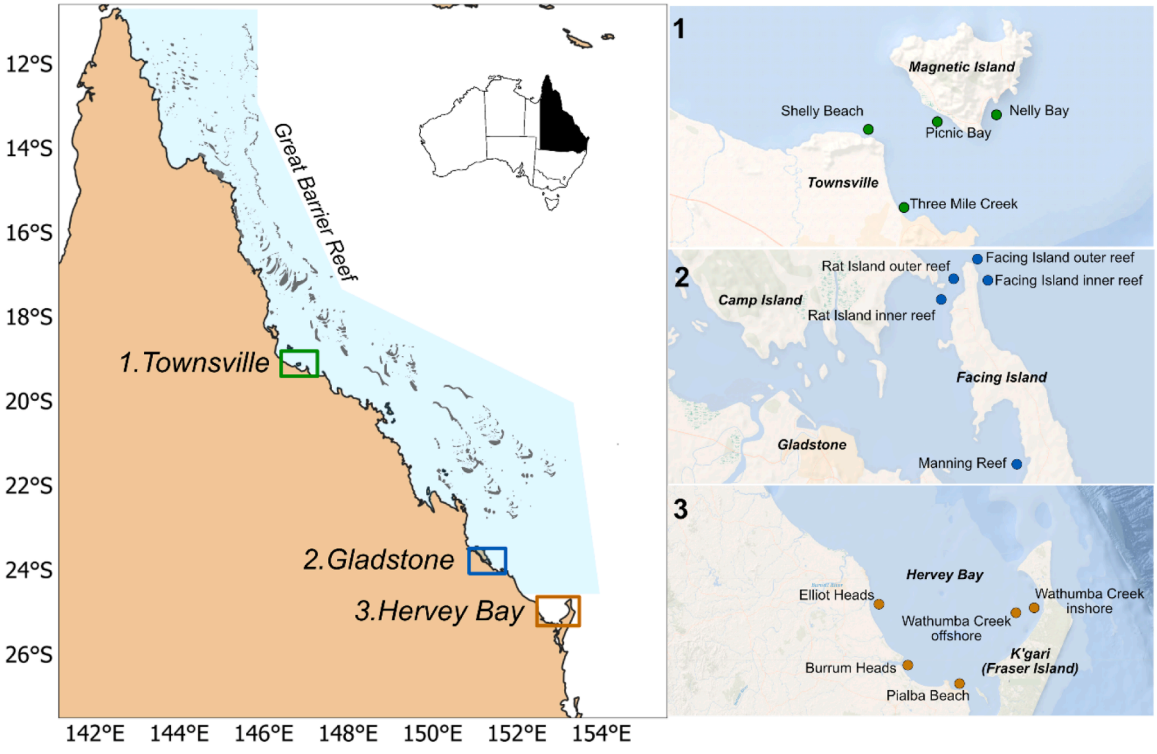

Fig. 1. Sampling locations (left) along the Queensland coast, Australia and sample collection sites for each location (right).

marks a transitional zone between tropical and temperate waters (18–26°C) with high rainfall (~1,000 mm/year). Sites included Burrum Heads (n=1), Elliot Heads (n=1), Pialba Reef (n=1), and Platypus Bay (n=2). These span estuarine margins, coral reefs, and seagrass beds. The area is influenced by river runoff and shelter from K'gari (Fraser Island). Platypus Bay, a known ciguatera hotspot, hosts dense *Cladophora* algal mats (Butler et al., 2013, 2015; Holmes et al., 2021; Milham-Scott, 2021).

2.1.2. Sample collection and preservation

At each site, habitat type for macroalgae sample collection was recorded and categorised: subtidal reef (always submerged), intertidal reef (exposed at low tide), sand (benthic macroalgal assemblages, with no benthic structure), seagrass (macroalgae growing on seagrass), shipwreck (macroalgae attached to artificial structure), or river mouth (macroalgae collected directly from river/creek mouth banks). In addition, the genus and specie (if known) of the macroalgae collected was identified and recorded, along with water depth at the point of sampling (Table S1).

To target *Gambierdiscus* spp., five replicate collections of approximately 500 g of submerged macroalgae were detached from the substrate at each site. Samples were placed in 36 × 48 cm zip-lock bags, sealed, and shaken vigorously to dislodge attached dinoflagellates. The resulting water was filtered through a 150 µm mesh to remove larger algal fragments (Perkins et al., 2025; Smith et al., 2017). The choice of this size of mesh is due to the known size of *Gambierdiscus* cells ranging from 42 µm to 140 µm (Holmes and Lewis, 2022; Wang et al., 2022). Each replicate consisted of 300 mL of the filtered sample combined with 100 mL of Longmire's preservation buffer (Williams et al., 2016). Field controls consisted of 300 mL of MilliQ water mixed with 100 mL of Longmire's buffer, processed alongside the samples. All samples and controls were stored at room temperature until DNA extraction. This preservation method has been demonstrated to maintain DNA integrity for up to three months under tropical conditions (~26.3°C; Cooper et al., 2022).

2.2. Laboratory workflow, sequencing and data processing

DNA extractions were performed at the environmental DNA (eDNA) laboratory at James Cook University, Townsville, Australia, following the methodology outlined by Perkins et al. (2025). In brief, DNA was extracted from 100 mL of water samples and filtered macroalgae using the preserve, precipitate, lyse, precipitate, purify (PPLPP) method described by Edmunds and Burrows (2020). Alongside field samples, two DNA extraction controls were included to monitor for contamination. Extracted DNA was further purified using the DNeasy PowerClean Pro Cleanup Kit (Qiagen®) to remove potential inhibitors.

Prior to DNA sequencing, two commonly used primer sets targeting the 18S rRNA gene were used to determine the most suitable region for *Gambierdiscus* detection. The V4 region was tested using primers SSU556F/SSU911R (Smith et al., 2017), and the V8-V9 regions using primers 18SV8FAlveolata/18SV9RAlveolata3 (Funaki et al., 2022). Primer performance was assessed using qPCR on DNA standards representing two *Gambierdiscus* species: *G. pacificus* (gBlock synthetic template; 390 bp) and *G. carpenteri* (genomic DNA), across a 1:10 dilution series (standards 4 to 7; 0.01 ng/µL to 0.00001 ng/µL) focusing on lower concentrations to better reflect trace DNA levels typically encountered in environmental assays (Klymus et al., 2020). To assess taxonomic specificity, using the same dilution series and standards, both primer sets were also tested on *Gyrodinium aureolum*, as a representative non-target species because of its global distribution and is phylogenetically distinct from *Gambierdiscus*.

Quantitative PCR (qPCR) reactions were carried out in 25 µL volumes containing 11 µL SYBR Green Master Mix (ThermoFisher, AU), 7.7 µL UltraPure H_2O, and 1.65 µL of each primer (10 mM). Thermocycling was performed on a QuantStudio™ 3 Real-Time PCR System (Applied Biosystems, AU) with the following conditions: initial denaturation at 98 °C for 10 seconds, followed by 45 cycles at 68 °C for 30 seconds, and a final annealing step at 69 °C for 5 minutes. Melt curve analysis was conducted from 65 to 95 °C in 0.3 °C increments to assess amplification specificity. Based on this evaluation, the V8–V9 primer set was selected for all downstream sequencing analyses due to better amplification performance and reduced risk of non-target amplification.

The V8–V9 region (~394 bp) of the 18S rRNA gene was amplified using Alveolata-specific primers 18SV8FAlveolata (5′-GCCCTTA-GATGTTCTGGGCT-3′) and 18SV9RAlveolata3 (5′-TGTTAC-GACTTCTCCTTCCTCTAAG-3′) (Funaki et al., 2022), modified with Illumina Nextera overhang adapters. This primer pair was selected based on prior testing that demonstrated improved specificity for *Gambierdiscus* detection (Funaki et al., 2022). PCR reactions (25 µL) consisted of 11 µL SYBR Green Master Mix (ThermoFisher, AU), 7.7 µL UltraPure H_2O, and 1.65 µL of each primer (10 mM). Thermal cycling conditions were initial denaturation at 94°C for 2 minutes; 40 cycles of 68°C for 10 seconds, 58°C for 30 seconds, and 68°C for 25 seconds; with a final extension at 68°C for 5 minutes.

A total of 87 samples, including field samples, field blanks, extraction blanks, and PCR blanks, were processed. Each sample was amplified in triplicate, resulting in 261 PCR products for sequencing. Library preparation and sequencing was conducted at the Australian Genome Research Facility (AGRF) using Illumina MiSeq technology with 500-cycle paired-end chemistry.

Sequencing data were processed using the DADA2 pipeline in RStudio (Callahan et al., 2017; R Core Team, 2018). Reads were filtered and trimmed with the following parameters: maximum expected errors (maxEE) = (2, 2), no ambiguous bases (maxN = 0), a quality score truncation threshold (truncQ) = 2, a minimum sequence length (minLen) = 150, and PhiX sequence removal enabled. Processing was performed in single-threaded mode (multithread = FALSE) with output file compression (compress = TRUE). Paired reads were merged with a minimum 12-base overlap, and chimeric sequences were removed.

Resulting Amplicon Sequence Variants (ASVs) were assigned taxonomy using the SILVA SSU Release 132 database, aligning with the 18S V8–V9 region (Glöckner et al., 2017). Potential contaminants were identified and removed using the 'decontam' package (Davis et al., 2018), applying a prevalence-based method with a threshold of 0.5. Following contaminant removal, all blank and control samples were excluded, and samples with zero read counts were filtered out. For downstream analysis, only ASVs and reads assigned to the genus *Gambierdiscus* at ≥95% identity was retained. This less stringent % identity threshold was chosen to capture a broader diversity of *Gambierdiscus* sequences, allowing exploration of potential new species, phylotypes, or unknown strains through phylogenetic placement.

2.3. Phylogenetic analysis

To assess phylogenetic relationships between the sequences collected in this study along with reference data, 169 small subunit ribosomal RNA (SSU rRNA) sequences of *Gambierdiscus*, representing all known species and phylotypes were retrieved from the National Centre for Biotechnology Information (NCBI, 2021) (Table S2). They were then aligned with the 184 ASVs assigned to the genus *Gambierdiscus* in this study and trimmed to the 18S V8-V9 regions using MUSCLE in Geneious v10.2.6. A maximum likelihood (ML) phylogenetic tree was generated in IQ-TREE (Nguyen et al., 2015) using a TIMe+G4+FO substitution model. Branch support was assessed using 1000 ultrafast bootstrap replicates and 1000 SH-aLRT replicates. The resulting tree was visualised and annotated in iTOL (Letunic and Bork, 2021) using a clade classification system consistent with previous studies (Funaki et al., 2022; Larsson et al., 2018; Nishimura et al., 2013).

To investigate genetic divergence among *Gambierdiscus* clades, pairwise genetic distances were calculated using the Kimura 2-parameter (K2P) model (Kimura, 1980). ASVs were re-aligned with the same

reference sequences used in the phylogenetic tree using MEGA11 (Tamura et al., 2021). A pairwise distance matrix was generated in MEGA using the K2P model with complete deletion of gaps/missing data and exported as a CSV file for further analysis in R studio (R Core Team, 2018).

Average genetic distances within and between clades were calculated using all pairwise comparisons, with the mean, minimum, maximum, and standard deviation (SD) computed for each group. To reduce redundancy and simplify interpretation, within-clade distances were separated from between-clade comparisons, and reversed duplicates (e.g., Clade II vs Clade III and Clade III vs Clade II) were removed. This yielded a cleaned dataset of unique pairwise clade comparisons. The final table included statistics for both intra-clade (within-clade) and inter-clade (between-clade) comparisons.

For a focused assessment of divergence within Clade II, ASVs and reference sequences from *Gambierdiscus* sp. C2 were compared to a subgroup, observed exclusively in the Great Barrier Reef, referred to here as *Gambierdiscus* sp. C2 GBR. This analysis included four comparison categories: intra-clade (Clade II), intra-clade (Clade II GBR), inter-clade (Clade II vs Clade II GBR), and intergeneric (Clade II vs *Fukuyoa* spp. outgroup). These categories were visualised using the ggplot2 package (Wickham, 2016), displaying mean K2P genetic distances and associated ranges (min–max) to facilitate direct comparison of divergence levels.

Next, single-rate and multi-rate Poisson Tree Processes (PTP) analyses were performed using the mPTP web server (https://mptp.h-its.org), with a p-value of 0.001 specified for the single-rate model (Kapli et al., 2017). These analyses aimed to delineate putative species within Clade 2 and assess the distinctiveness of *Gambierdiscus* sp. C2 GBR. To further examine differences between *Gambierdiscus* sp. C2 GBR and other Clade 2 ASVs, a haplotype-level network was constructed using PopART v1.7 (Leigh and Bryant, 2015). Sequences from Clade 2 and Clade 2 GBR were extracted from the aligned dataset and used to generate a network. The analysis was performed using the median-joining (MJ) algorithm (Bandelt et al., 1999), which accounts for reticulate relationships and provides a visual representation of genetic variation within and between these groups. In addition, the traits compared in the network represent the locations the ASVs were detected and the size of the circle for each haplotype is determined by the number of reads.

2.4. Further data analysis

Based on the phylogenetic framework described above, all subsequent analyses were conducted using five *Gambierdiscus* taxa that exhibited clear genetic and ecological differentiation. Although not all ASVs could be resolved to species level, clade-level groupings were used where appropriate to capture ecologically meaningful variation.

All data processing and visualisation were conducted using in RStudio (R Core Team, 2018). First, ASV richness of each taxon per site was visualised through a horizontal bar plot generated using *ggplot2* (Wickham, 2014). To further visualise, sequence read abundance data were converted to presence–absence (detection) of each taxon per replicate, within each site and visualised in a bubble plot using *ggplot2* (Wickham, 2014). The resulting data was overlaid onto maps of sampling locations, where the size of each point represents frequency of detection. To assess the distribution of *Gambierdiscus* taxa across depth categories, sequence read abundance data were converted to presence–absence (detection) per replicate. Samples were classified into "Shallow" (0.2–4.2 m) and "Deep" (12.6–18 m) groups based on recorded depths. Detection frequency for each taxon was calculated as the number of positive replicates within each depth category. To account for differences in sampling effort, detection counts were standardised within each taxon by expressing detections as proportions of total detections across depth categories. Data processing and statistical analyses were performed using the *dplyr* and *ggplot2* packages (Wickham, 2014).

For habitat and macroalgal host analyses, data were grouped by *Gambierdiscus* taxa and either habitat or algal type. Frequency of detection was calculated as the proportion of replicate samples within each group where a taxon was present. Total read abundances were summed per group, and proportional abundances were calculated by dividing each taxon's read count by the total reads within the respective habitat or algal category. Taxa were factorised to maintain consistent ordering in plots. Stacked bar plots of detection frequency and proportional read abundance were generated using *ggplot2* (Wickham, 2016) to visualise taxon distribution patterns. These plots were combined to facilitate direct comparison of detection frequency and read proportion across habitats and macroalgal hosts.

Canonical Correspondence Analysis (CCA) was used to investigate the influence of environmental variables and macroalgal host species on the distribution patterns of *Gambierdiscus* taxa based on frequency of detection. All analyses were conducted using the software PAST v4.13 (Hammer et al., 2001). CCA was performed in two stages. First, environmental factors including site locality, depth, habitat type, and dominant macroalgae were used as explanatory variables to assess their collective influence on *Gambierdiscus* assemblages. A second CCA was run using only macroalgal host genera as environmental variables to explore potential host-specific associations with different *Gambierdiscus* taxa. Eigenvalues and axis significance were evaluated using permutation tests (9,999 permutations).

3. Results

3.1. Primer performance

The V8–V9 region was the most suitable for detection of *Gambierdiscus* in environmental samples in Queensland coastal waters. Quantitative PCR (qPCR) testing revealed that the V8-V9 primers outperformed the V4 primers in both amplification efficiency and specificity for *Gambierdiscus*. For *Gambierdiscus pacificus*, both primer sets amplified across the full dilution range (0.01 ng/μL to 0.00001 ng/μL; Figs. S1 & S2), but the V8–V9 primers produced a sharp, single melt peak (~83–84 °C; Fig. S3), indicating specific amplification. In contrast, the V4 primers generated broader, less defined melt peaks, suggesting non-specific products. The performance difference was more pronounced in genomic DNA from *Gambierdiscus carpenteri*. V8-V9 primers amplified all standards consistently with Ct values between 20 and 40 cycles (Fig. S4), while V4 primers only produced weak amplification at higher concentrations and failed to detect lower dilutions (Fig. S5). Importantly, the V8-V9 primers did not amplify any *Gyrodinium aureolum* DNA, while the V4 primers produced weak, late-cycle amplification at higher concentrations (Fig. S6), indicating potential for non-target detection. Together, these results confirmed that the V8–V9 region was the most suitable for specific, sensitive detection of *Gambierdiscus* in environmental samples in Queensland coastal waters and validated its use for subsequent metabarcoding analyses.

3.2. DNA metabarcoding

Gambierdiscus accounted for 184 unique sequences from the 2,008 ASVs assigned to the phylum Dinoflagellate and accounted for the highest read count of any genus in the dataset, totalling 241,824 reads (Fig. S7).

3.3. Phylogenetic analysis

Six distinct lineages were identified using Maximum likelihood (ML) phylogenetic analyses corresponding to known *Gambierdiscus* species and the outgroup genus: *Fukuyoa* (Fig. 2). Previously established clades (Clades II-VI) (Funaki et al., 2022; Larsson et al., 2018; Nishimura et al., 2013), were strongly supported (Clade II, bootstrap = 100; Clade III,

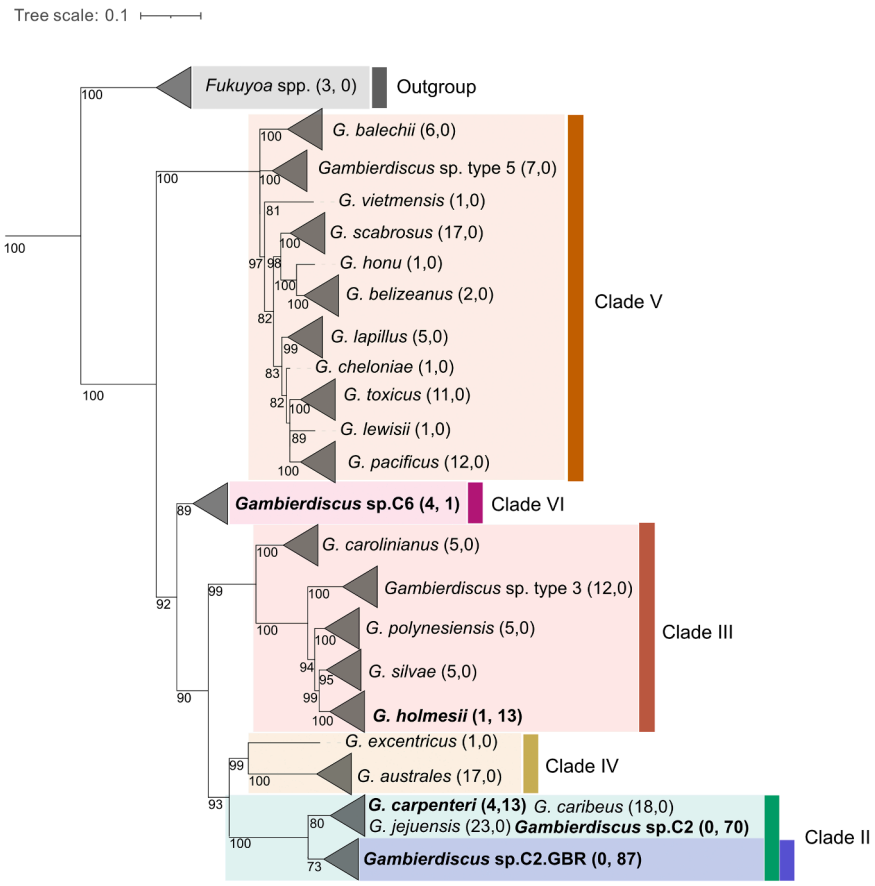

Fig. 2. Maximum likelihood phylogenetic tree of *Gambierdiscus* ASVs from this study and reference sequences based on the 18S rRNA V8–V9 region. The tree was constructed using 184 *Gambierdiscus* ASVs and 169 reference sequences from GenBank representing all described species and phylotypes retrieved from NCBI. Major clades are labelled, and colour coded and ASVs from this study are in bold. Numbers in brackets after each species/phylotype represent firstly sequences obtained from NCBI and, secondarily ASVs sequenced in this study.

bootstrap = 99; Clade IV; Clade V, bootstrap = 100; Clade VI, bootstrap = VI: Fig 2). Clade II contained multiple reference sequences from known species including *G. carpenteri, G. caribaeus*, and *G. jejuensis*. 13 Amplicon Sequence Variants (ASVs) were contained within a monophyletic group with *G. carpenteri*. A further 70 ASVs fell within Clade II but did not form a monophyletic group with any known/described species. In addition, a distinct lineage comprising 87 ASVs was identified within Clade II and is hereafter referred to as *Gambierdiscus* sp. C2 GBR. All 87 ASVs in *Gambierdiscus* sp. C2 GBR were phylogenetically distinct from known species within Clade II and received moderate bootstrap support (bootstrap = 73), suggesting the presence of previously uncharacterised diversity within this lineage. Whereas *G. carpenteri, G. caribaeus, G. jejuensis*, and the 70 ASVs from within this group received higher bootstrap support (bootstrap = 80). Together both groups from within clade II form a strongly supported monophyletic clade (bootstrap = 100) which is the sister-taxon to Clade IV. Thirteen ASVs assigned to *Gambierdiscus holmesii* fell within Clade III, together forming a well-supported monophyletic cluster along with a reference sequence of *G. holmesii* from Heron Island, Queensland (bootstrap = 100). A single ASV fell within Clade VI, here designated *Gambierdiscus* sp. C6, forming a monophyletic group with four sequences from a new clade (bootstrap = 89) detected from Japan (Funaki et al., 2022).

To further investigate genetic differences between ASVs from *Gambierdiscus* sp. C2 and *Gambierdiscus* sp. C2 GBR, Kimura-2-Parameter (K2P) pairwise distances were calculated within and between *Gambierdiscus* sp. C2 (Clade II) and *Gambierdiscus* sp. C2 GBR (Clade II GBR) (Table S3 & Fig. S8). Intra-clade distances were lowest within *Gambierdiscus* sp. C2, followed by *Gambierdiscus* sp. C2 GBR (Clade II). Inter-clade distances between *Gambierdiscus* sp. C2 and *Gambierdiscus* sp. C2 GBR were higher than intra-clade values but showed some overlap, reinforcing *Gambierdiscus* sp. C2 and *Gambierdiscus* sp. C2 GBR close evolutionary relationship.

To validate the distinctiveness of *Gambierdiscus* sp. C2 GBR further, species delimitation models using both Poisson Tree Processes (PTP) and Multi-rate Poisson Tree Processes (MPTP) were applied. The MPTP model identified nine putative species, which strongly correspond to the clades observed in the phylogenetic tree (Fig. 2), including support for *Gambierdiscus* sp. C2 GBR as a distinct entity from other Clade II taxa (Fig. S9). While the PTP model also suggested species boundaries and support for *Gambierdiscus* sp. C2 GBR as a distinct entity from other Clade II taxa, it also suggested a higher number of species, assigning sequences from different strains of the same species as distinct species, and each ASV in Clade II GBR as separate species (Fig. S10). This assignment of species designations is because of its assumption of a single evolutionary rate across all branches (Rabosky et al., 2017; Zheng et al., 2024). In contrast, MPTP accounts for heterogeneity in substitution rates across the phylogenetic tree, allowing for more biologically meaningful species delimitation (Kapli et al., 2017). This approach is particularly suitable to the dataset, as the 87 ASVs within Clade 2 GBR exhibit varying degrees of divergence but remain evolutionarily clustered. By recognising that different lineages evolve at different rates, MPTP minimises artificial species inflation and provides a more realistic classification of *Gambierdiscus* sp. C2 GBR as a distinct group within Clade II lending support to it being a distinct phylotype.

The haplotype network (Fig. 3) further supports the phylogenetic tree and MPTP species delimitation model by revealing distinct groups

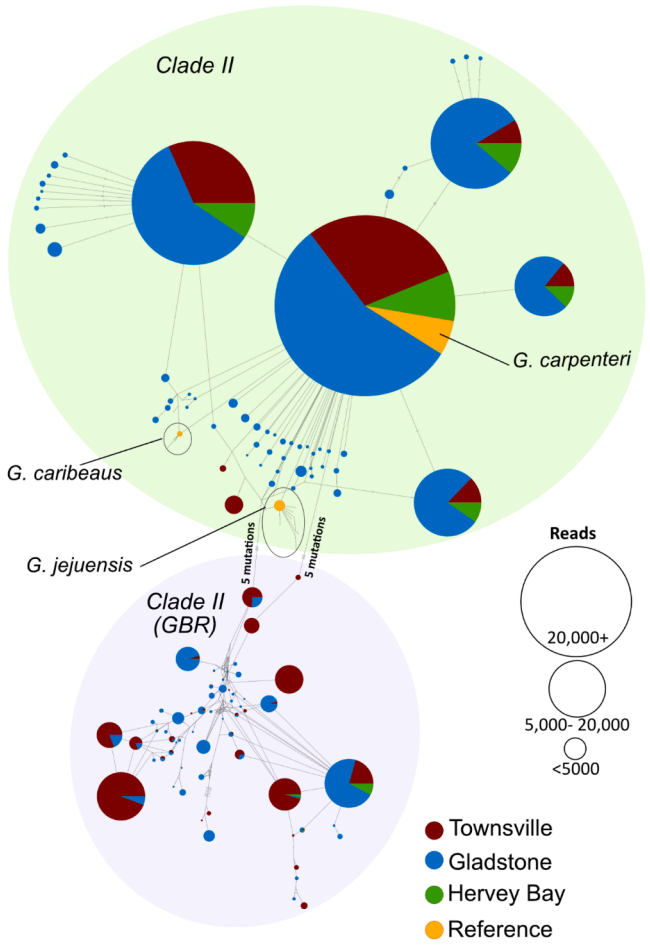

Fig. 3. Haplotype network of *Gambierdiscus* sequences, including reference sequences from NCBI. Each circle represents a unique haplotype, with circle size proportional to total read abundance. Colours within circles indicate the geographic origin of haplotypes. Clusters are shaded to indicate major groups, including Clade II and a distinct GBR-specific Clade II subgroup. Mutational steps between haplotypes are indicated by connecting lines, with labelled distances for branches representing five or more mutations.

within ASVs from Clade II. Within this clade, haplotypes mainly cluster around reference sequences for *G. carpenteri, G. caribaeus*, and *G. jejuensis*, while *Gambierdiscus* sp. C2 GBR forms a genetically distinct, densely connected cluster. Notably, *G. carpenteri* has several dominant central haplotypes, that accounted for the majority of reads and surrounded by multiple singletons. In contrast, the haplotype structure of *Gambierdiscus* sp. C2 GBR is more diffuse, lacking a central dominant haplotype. At least five mutational steps separate *Gambierdiscus* sp. C2 GBR from other Clade II sequences, reinforcing its genetic distinctiveness. Geographically, *Gambierdiscus* sp. C2 GBR was detected more frequently in Townsville and secondly Gladstone, with only two haplotypes detected from Hervey Bay. Whereas *Gambierdiscus* sp. Clade 2 is more dominant in Gladstone, with some of the dominant haplotypes detected also in Townsville and Hervey Bay.

3.4. *Gambierdiscus* distribution across the Queensland coast

Overall, spatial patterns suggest that *G. carpenteri, Gambierdiscus* sp. C2, and *Gambierdiscus* sp. C2 GBR have broad ecological tolerances and geographic ranges, while *G. holmesii* and *Gambierdiscus* sp. C6 are more spatially restricted (Fig. 5).

Across the 14 sampled sites spanning Townsville, Gladstone, and Hervey Bay, both ASV richness and *Gambierdiscus* community composition exhibited strong spatial variability (Fig. 5). Gladstone showed the highest ASV richness, particularly at Rat Island sites where over 175 unique ASVs were detected (Fig. 4). In contrast, sites in Hervey Bay (e.g., Burrum Heads and Pialba Beach) and Townsville (e.g., Three Mile Creek and Shelly Beach) had notably lower richness.

Community composition varied markedly among locations based on detection frequency across replicates. *G. carpenteri, Gambierdiscus* sp. C2 and *Gambierdiscus* sp. C2 GBR were the most widespread and frequently detected taxa across all three locations, often co-occurring and dominating assemblages (Fig. 5). In Townsville (Fig. 5A), these three were consistently detected around Magnetic Island. Three Mile Creek was an exception, where only *G. carpenteri* was detected, and at low frequency. In Gladstone (Fig. 5B), *Gambierdiscus* was recorded for the first time, with all five taxa detected, making it the most taxonomically diverse region in the study. *G. carpenteri, Gambierdiscus* sp. C2 and *Gambierdiscus* sp. C2 GBR were again the most frequently detected, particularly at Rat Island, while *G. holmesii* and *Gambierdiscus* sp. C6 were detected in fewer replicates, suggesting lower prevalence. Hervey Bay sites (Fig. 5C) were generally dominated by *G. carpenteri, Gambierdiscus* sp. C2 and *Gambierdiscus* sp. C2 GBR, although *G. holmesii* was detected at Burrum Heads and was the only taxon detected at Elliot Heads, in four of the five replicates.

3.5. *Gambierdiscus* habitat associations

All five *Gambierdiscus* taxa were detected more frequently and in greater abundance in shallow waters (0–5 m) compared to deeper sites (12–18 m; Fig. 6). *G. carpenteri, Gambierdiscus* sp. C2, and *Gambierdiscus* sp. C2 GBR exhibited the highest read counts in shallow waters. In contrast, samples from deep areas showed reduced read counts across all taxa, though *G. carpenteri* and *Gambierdiscus* sp. C2 were still present at depths of 13–17 m, albeit at lower relative abundance. *Gambierdiscus* sp. C6 and *G. holmesii* were absent from all deep sites and restricted entirely too shallow habitats. The heatmap visualisation clearly illustrates the pronounced depth preference of most taxa, with shallow environments supporting higher overall relative abundance and diversity of *Gambierdiscus*.

Both frequency of detection and proportional read abundance showed similar patterns with dominant taxa across habitats and macroalgal hosts, with some important differences (Fig. 7). For example, *G. holmesii* was most dominant by proportional reads at the river mouth but had a lower frequency of detection there, indicating high read abundance in fewer replicates. Additionally, frequency of detection revealed rarer taxa that were not visible in proportional read plots, providing a fuller picture of taxon presence.

Patterns indicated that *Asparagopsis taxiformis* and *Sargassum* supported the highest taxonomic richness and detection frequencies among macroalgal hosts. Among habitats, reef environments exhibited the highest richness, with shipwreck sites also showing high frequency of detection. *G. carpenteri* and *Gambierdiscus* sp. C2 were broadly dominant across habitats and hosts, while *G. holmesii* and *Gambierdiscus* sp. C6 displayed more restricted distributions. These patterns suggest ecological preferences related to habitat and host type within the *Gambierdiscus* assemblage.

Canonical Correspondence Analysis (CCA) revealed that site locality is the primary driver of *Gambierdiscus* taxa distributions, with macroalgal associations playing a secondary but important role, while depth and habitat exert limited influence (Fig. 8). The first two axes explained most of the variation (axis 1: 83.23%, $p = 0.001$; axis 2: 15.56%, $p = 0.004$), confirming a strong influence of local environmental conditions.

Site had the strongest influence on species composition (axis 1: 0.359), while macroalgae contributed primarily along axis 2 (0.121). *Gambierdiscus* sp. C2 GBR was closely aligned with axis 2 (score = 2.599), positioned opposite depth and toward macroalgae, suggesting that its distribution is more strongly associated with macroalgal hosts than with depth. In contrast, *G. carpenteri* had moderate negative scores

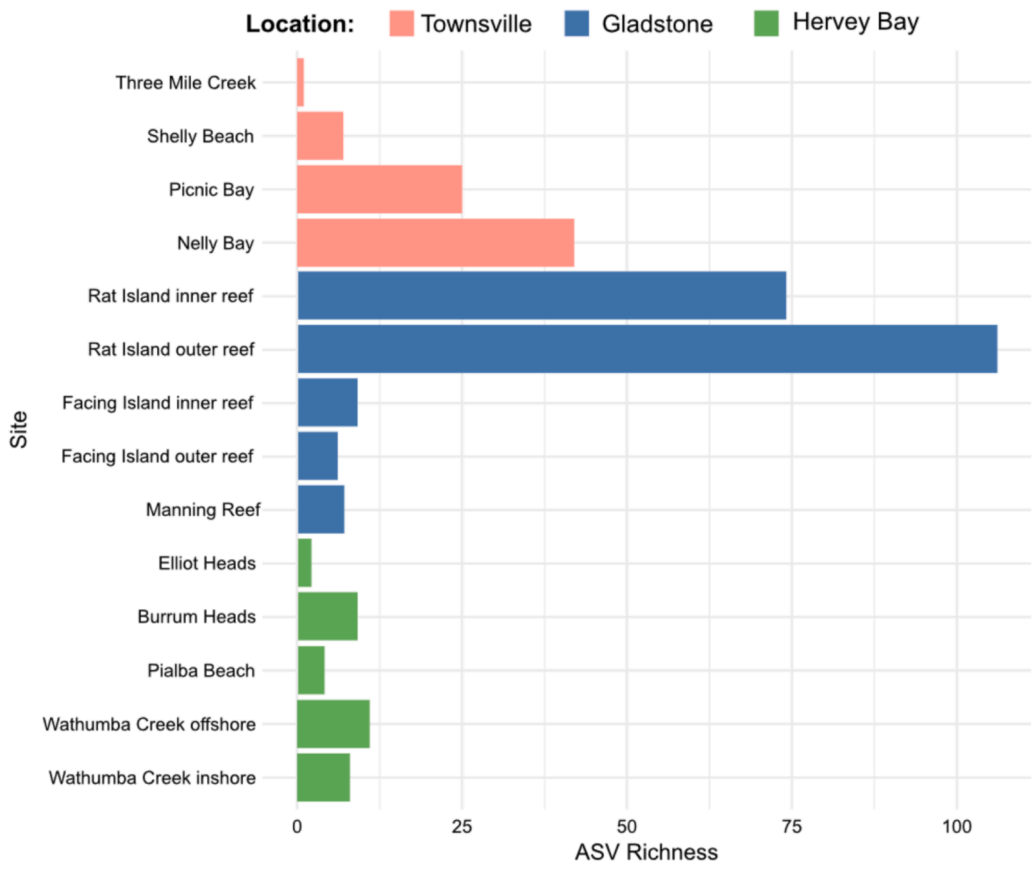

Fig. 4. Amplicon sequence variant (ASV) richness and *Gambierdiscus* community composition across 14 sites in Queensland, Australia. Sites are grouped by location (Townsville = green, Gladstone = blue, Hervey Bay = orange).

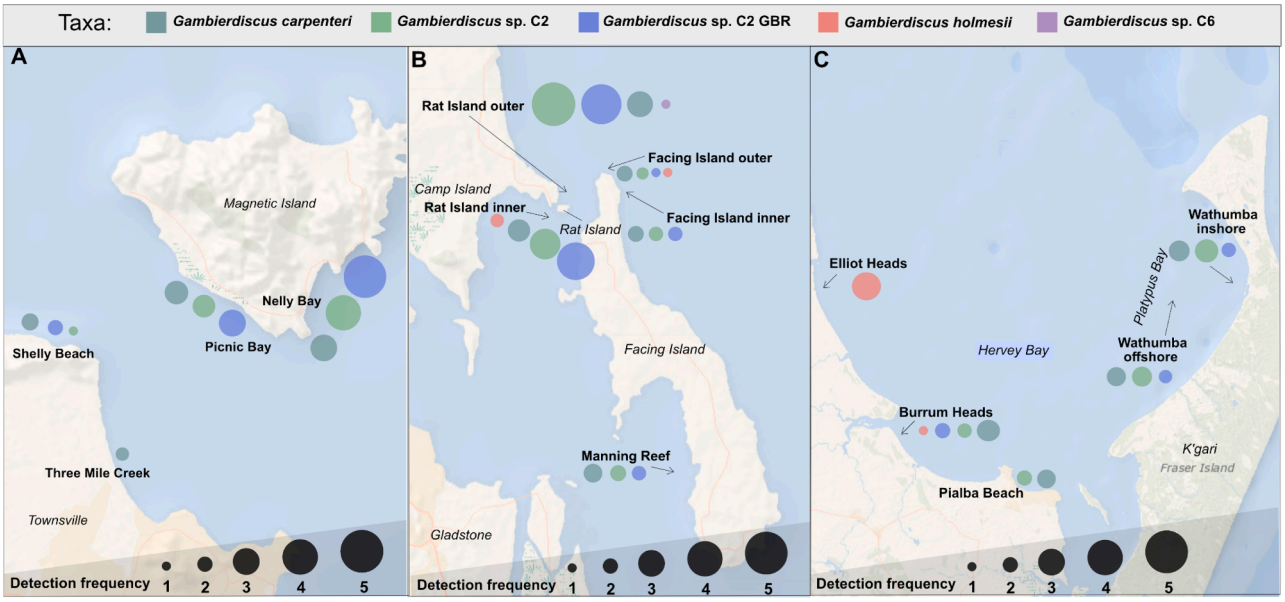

Fig. 5. Spatial distribution and replicate detection frequency (number of positive detections per site) of *Gambierdiscus* taxa across three coastal locations in Queensland: (A) Townsville, (B) Gladstone, and (C) Hervey Bay. Bubble size corresponds to detection frequency (see legend, bottom), and colours represent different *Gambierdiscus* taxa (see legend, top).

on both axes, indicating a weaker response to the measured variables. *Gambierdiscus* sp. C6 projected opposite the macroalgal host and aligned more closely with site, indicating a site-specific distribution with limited influence from macroalgae. *G. holmesii* also projected strongly along axis 1, consistent with a site-specific association, but was positioned in the upper right quadrant of the CCA where habitat contributed to its placement. This suggests that although site was the strongest driver, habitat type exerted a secondary influence. Overall, these results

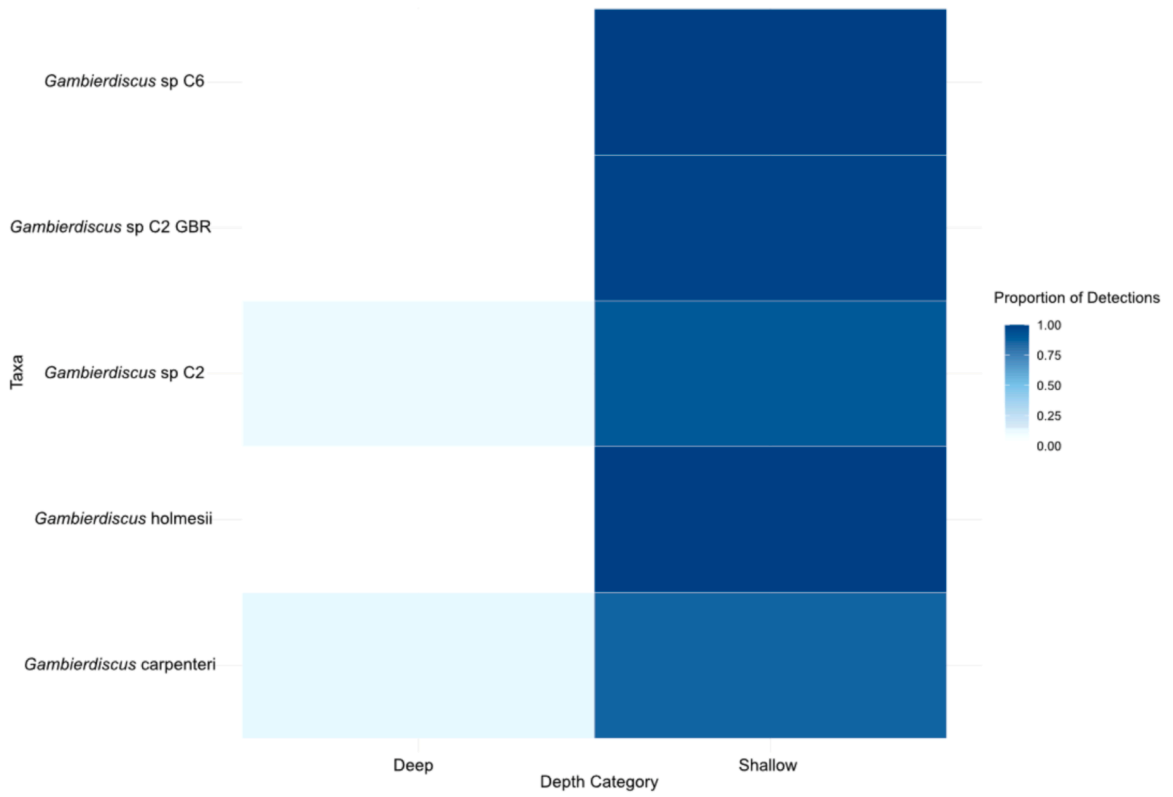

Fig. 6. Heatmap showing the proportion of *Gambierdiscus* taxon detections standardised within each taxon across depth categories ("Shallow": 0.2–4.2 m; "Deep": 12.6–18 m). Colours represent the relative proportion of positive detections at each depth per taxon, with darker shades indicating higher proportions.

indicate that *Gambierdiscus* distribution is primarily structured by site-specific environmental conditions, with additional contributions from macroalgal hosts and habitat type.

To explore macroalgal influences further, a second CCA was performed using macroalgal species as explanatory variables (Fig. 9). Macroalgal associations were found to structure *Gambierdiscus* communities, with axis 1 explaining 60.42% of the variation ($p = 0.022$) and axis 2 explaining 29.87% ($p = 0.001$). *Asparagopsis taxiformis* appeared to be a preferred host for *Gambierdiscus* sp. C2 (axis 2 score = 1.939), suggesting a potential ecological association. In contrast, *Cladophora* was more closely associated with *G. holmesii* (axis 1 score = 4.945). *Gambierdiscus* sp. C2 GBR showed a strong negative association with both axes, suggesting avoidance or exclusion from these macroalgal hosts. While *Gambierdiscus* sp. C6 showed strong responses, it did not align with any specific macroalga. Other taxa such as *Cystoseira, Halimeda*, and *Lobophora* had negligible influence, suggesting a limited role in structuring assemblages. These findings highlight that while some *Gambierdiscus* taxa show strong macroalgal preferences, others appear more generalist or influenced by factors beyond host availability.

4. Discussion

This study provides the first broad-scale molecular assessment of *Gambierdiscus* diversity along Queensland's inshore coastline, revealing pronounced regional variation in species composition and several novel biogeographic patterns. A previously unreported lineage within Clade II, designated Clade II GBR, was identified and may represent a distinct group restricted to the Great Barrier Reef. Gladstone emerged as the most taxonomically diverse region and marks the first confirmed record of *Gambierdiscus* in the area. Notably, *G. holmesii*, a recently discovered producer of P-CTXs (Murray et al., 2025), was detected for the first time in inshore coastal waters, specifically at Hervey Bay and Gladstone. Across all sites, *G. carpenteri* and *Gambierdiscus* sp. C2 were dominant, while other taxa such as *G. holmesii* and *Gambierdiscus* sp. C6 appeared more locally restricted. Taxa composition was strongly influenced by macroalgal host, with shallow coral reef habitats supporting the highest species diversity and detection frequency, suggesting their role as ecological hotspots.

4.1. Phylogenetic structure and sequence variant patterns in *Gambierdiscus*

This study revealed strong phylogenetic and sequence-level structure within *Gambierdiscus* Clade II, including the detection of a genetically distinct group, here referred to as Clade II GBR. Phylogenetic analysis consistently resolved this cluster as a separate lineage from other Clade II taxa and also other known clades within the genus *Gambierdiscus*. The haplotype network further supported this structure by revealing a dense cluster of variants separated by at least five mutational steps from other Clade II sequences. Unlike *G. carpenteri* (Clade II), which had several dominant haplotypes surrounded by many low-abundance singletons, *Gambierdiscus* sp. Clade II GBR exhibited a more diffuse haplotype pattern with no central dominant type, and a lower but more even distribution of read counts. These results indicate that Clade II GBR is genetically distinct and likely represents a potentially undescribed lineage within *Gambierdiscus*.

Accurate species delimitation in microorganisms such as *Gambierdiscus* is particularly challenging when relying on short, conserved markers like the 18S rRNA gene (Ott et al., 2022; Pappalardo et al., 2021). However, 18S remains a widely used marker for dinoflagellates such as *Gambierdiscus*, with a well-curated reference sequence database and primer sets that have been tested in previous studies, including Funaki et al. (2022), which successfully revealed high species diversity in Japanese waters. This study assessed whether ASVs within and across Clade II and Clade II GBR could be resolved into distinct lineages or species. The Poisson Tree Processes (PTP) model seemingly over-split

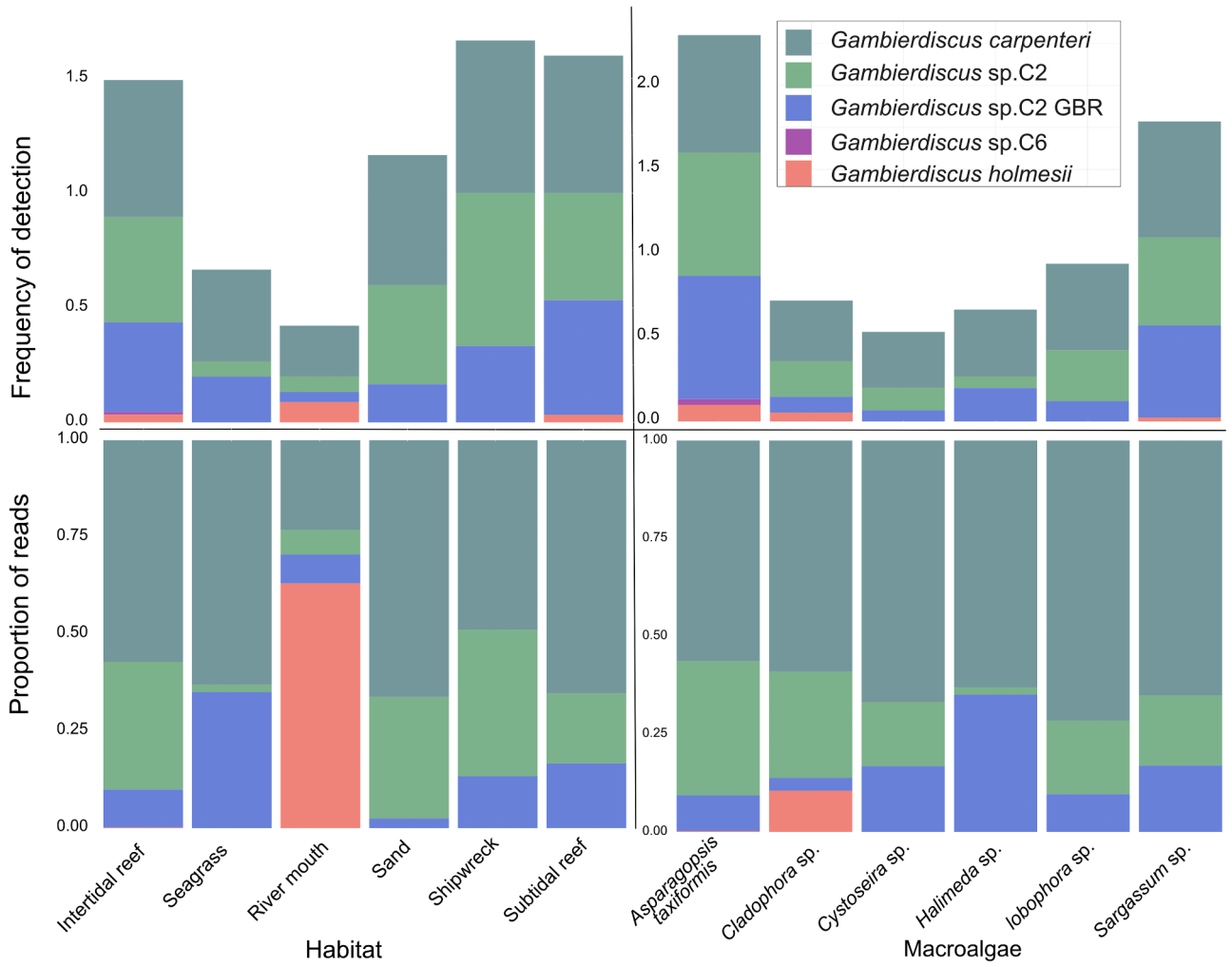

Fig. 7. Frequency of detection of *Gambierdiscus* taxa across habitat types (top left) and macroalgal hosts (top right), shown as the proportion of replicate samples in which each taxon was detected. The bottom panels display proportional read abundances for habitat (bottom left) and macroalgae (bottom right). Colours represent *Gambierdiscus* taxa as indicated in the legend.

the dataset, assigning nearly every ASV to its own species. This result likely stems from the model's assumption of a single substitution rate across the tree. This can lead to inflated species counts in datasets with rate heterogeneity or fine-scale intraspecific variation, a limitation that has been highlighted in previous studies (Rabosky et al., 2017; Zheng et al., 2024). In contrast, the MPTP model, accounts for heterogeneity in substitution rates, and aligned more closely with known phylogenetic clades, supporting *Gambierdiscus* Clade II GBR as a distinct lineage separate from known taxa within Clade II. This is similar to findings by Pappalardo et al. (2021) suggesting that short-read markers may be more effective for delineating ecotypes or evolutionary clades rather than true species. As such, we recommend that species delimitation in *Gambierdiscus* using short sequences be approached cautiously, and that models incorporating rate heterogeneity, such as MPTP, be favoured for identifying biologically meaningful groups.

Expanding on this, our study observed high Amplicon Sequence Variance (ASV) richness particularly at Rat Island for taxa in Clade II (175 unique ASVs), which may reflect a combination of ecological and genomic factors. The presence of intra-genomic variability, particularly in multi copy genes such as 18S ribosomal DNA (rDNA), is common in dinoflagellates (Lin, 2011; Wang et al., 2020). In species such as *Gambierdiscus*, high rDNA copy numbers coupled with relaxed concerted evolution (a process where rDNA copies are not fully homogenised within the genome) can lead to multiple slightly divergent sequences within a single individual, thus inflating ASV counts (Gaiani et al., 2021; Kretzschmar et al., 2019; Vandersea et al., 2012). This process may partly explain the high ASV richness observed in *G. carpenteri* and other Clade II haplotypes, where dominant haplotypes were surrounded by singletons, suggesting minor variants branching from a central ribotype. However, ecological factors likely also contribute, including local adaptation and fine-scale microhabitat variation, and should not be discounted.

In addition to the widespread presence of *G. carpenteri*, a substantial number of ASVs could not be confidently assigned to described species but consistently clustered within or near Clade II based on phylogenetic analysis. Many of these unidentified ASVs formed a distinct cluster referred to in this study as *Gambierdiscus* Clade II GBR. While they often co-occurred with *G. carpenteri* and other Clade II ASVs, these ASVs typically exhibited lower detection frequency and were restricted to fewer sites. The group was most common in Townsville and, to a lesser extent, Gladstone, with only two haplotypes detected from Hervey Bay. In contrast, the dominant Clade II haplotypes, including those of *G. carpenteri*, were more prevalent in Gladstone, with some also present in Townsville and Hervey Bay. The lower detection and more restricted distribution of Clade II GBR may suggest ecological differentiation and raises the possibility that these ASVs represent distinct strains, cryptic species, or regionally adapted phylotypes within the broader Clade II lineage.

Comparable findings have been reported in other studies where high-throughput sequencing revealed unidentified *Gambierdiscus* genotypes

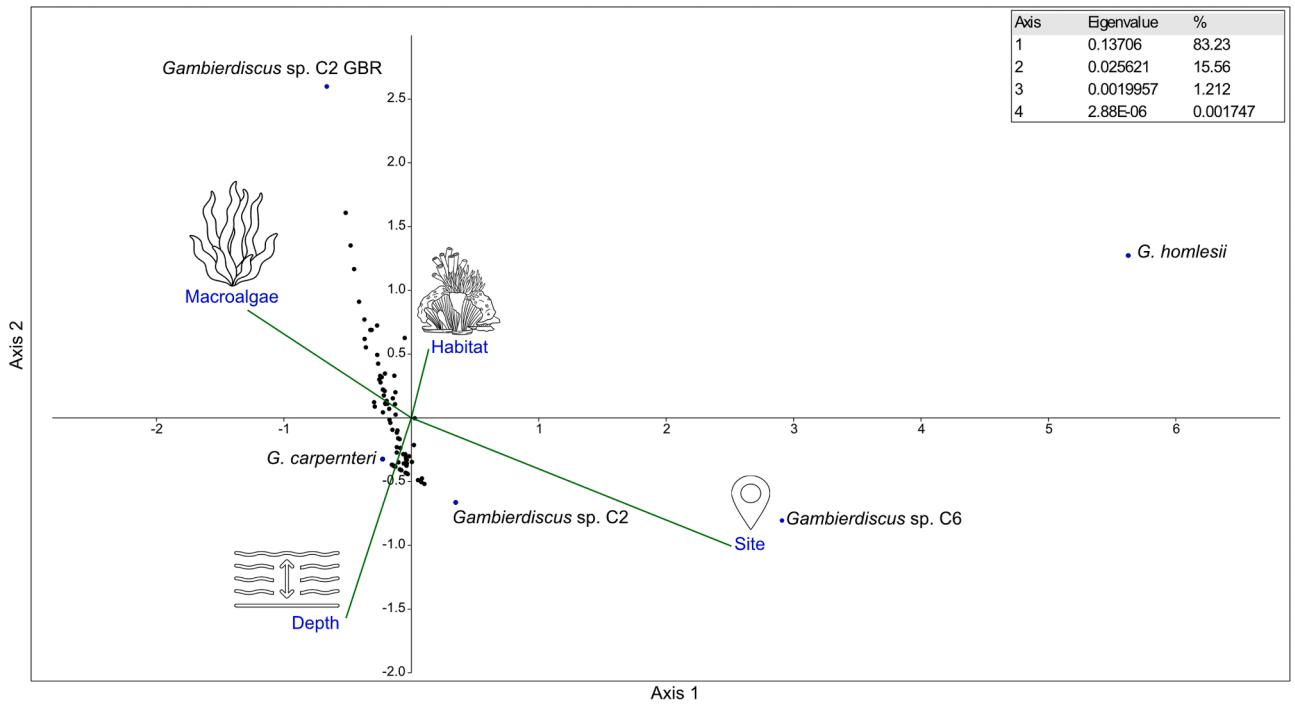

Fig. 8. Canonical Correspondence Analysis (CCA) showing the relationship between *Gambierdiscus* taxa and environmental variables (site, habitat, depth, and macroalgal host). Each black point represents an individual sample. Blue text labels denote environmental variables, while green arrows indicate the direction and strength of their associations with *Gambierdiscus* distribution. Taxa (*G. carpenteri, G. holmesii, Gambierdiscus* sp. C2, *Gambierdiscus* sp. C2 GBR, and *Gambierdiscus* sp. C6) are plotted with blue dots.

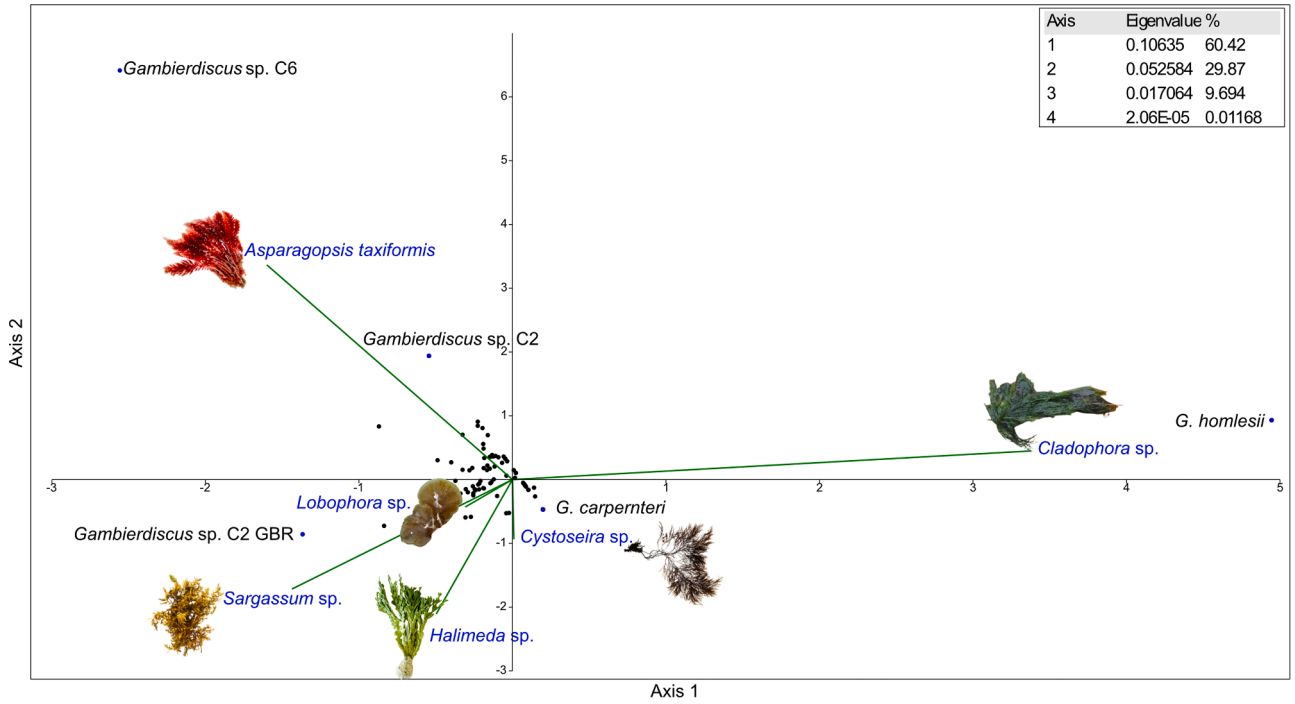

Fig. 9. Canonical Correspondence Analysis (CCA) showing the relationship between *Gambierdiscus* taxa and macroalgal host. Each black point represents a sample, and blue text labels denote macroalgal genera, with corresponding photographic images for visual reference. Green lines represent the direction and strength of the association between algal hosts and the distribution of *Gambierdiscus* taxa. Individual taxa (*G. carpenteri, G. holmesii, Gambierdiscus* sp. C2, sp. C2 GBR, and sp. C6) are plotted with blue dots.

with distinct biogeographic patterns (Funaki et al., 2022; Nishimura et al., 2013; Richlen et al., 2008). For example, cryptic *Gambierdiscus* lineages in Japan were found to cluster into five distinct phylotypes based on SSU, LSU, and ITS regions, including three novel clades with restricted regional distributions (Nishimura et al., 2013). These were hypothesised to represent new species or ecotypes, likely shaped by local environmental conditions. Funaki et al. (2022) extended these observations by identifying several phylotypes with distinct distributional

patterns across subtropical and temperate Japanese waters, including '*Gambierdiscus* sp. Clade VI_1' found in both temperate and subtropical waters, which formed a new clade (VI). In our study we detected a sequence that grouped into this clade (VI) and was observed only at a single site (Rat Island outer reef in Gladstone; subtropics).

Despite the insights offered by this approach, short-read amplicon data have limitations when used to delineate species in *Gambierdiscus*. The complex, variable genome of this genus and the presence of intra-genomic sequence variants means that short regions may not capture the full extent of genomic or functional divergence. As a result, diversity may be either under or overestimated. The combination of phylogenetic, haplotype, and ASV-based approaches in this study provides valuable insight into lineage structure but also highlights the need to integrate multiple markers, longer-read sequencing data and ecological metadata in future research. This will improve our ability to accurately define species boundaries and understand their potential role in ciguatera risk across the region.

4.2. *Gambierdiscus* distribution along the Queensland coast

Gambierdiscus were detected across all surveyed regions along the Queensland coast, demonstrating a broad distribution from tropical to subtropical latitudes. However, pronounced regional variation was evident in species composition and frequency of detection. Subtropical Gladstone and Hervey Bay supported a greater diversity of *Gambierdiscus* taxa compared to the more northerly tropical sites near Townsville. This pattern contrasts with the commonly held view that *Gambierdiscus* species are most prolific in warm, tropical reef environments (Chinain et al., 2023; Funaki et al., 2022; Litaker et al., 2010), and suggests that subtropical habitats along the southern Queensland coast may also provide under-recognised ecological niches for these dinoflagellates.

Gladstone emerged as both the most taxonomically diverse region, with all five *Gambierdiscus* taxa detected. This study represents the first confirmed record of the genus from the Gladstone area. High detection frequencies were particularly concentrated around Rat Island (northern Facing Island), indicating this site may act as a local biodiversity hotspot. Gladstone is subject to substantial industrial pressures, including dredging, sedimentation, and reduced coral cover (Ghosh et al., 2022; McIntosh et al., 2019). Such disturbances may increase macroalgal growth by reducing coral competition and altering substrate availability. Degraded reefs often support higher macroalgal biomass, providing extensive substrate for epiphytic dinoflagellates such as *Gambierdiscus* (Clements et al., 2018; Gingold et al., 2014; Rongo and van Woesik, 2013; Sotka and Hay, 2009). The combination of habitat heterogeneity, abundant macroalgae, and subtropical environmental conditions likely contributes to the elevated diversity observed in this region.

Gambierdiscus holmesii was detected for the first time in inshore coastal waters of Queensland, specifically in Gladstone (Rat Island inner reef and Facing Island outer reef) and Hervey Bay (Burrum Heads and Elliot Heads). This expands its known distribution beyond offshore reefs of the GBR such as Heron Island, Orpheus Island, and Beaver Reef, where it was previously reported (Kretzschmar et al., 2019; Murray et al., 2025). Although *G. holmesii* was not detected in Platypus Bay, which is considered a ciguatera hotspot, it was present at river-influenced sites on the opposite side of Hervey Bay, suggesting potential adaptation to estuarine or nutrient-rich coastal conditions. Platypus Bay has long been implicated in ciguatera outbreaks prompting refusal of fish exportation from the area (Lewis and Endean, 1983, 1984; Sydney Fish Market, 2015). Prior studies confirmed high densities of *Gambierdiscus* and CTX-like activity in wild and cultured samples, although the responsible species were not identified at the time (Holmes et al., 1991, 2021). The recent confirmation that *G. holmesii* produces M-seco-CTX4A/B, underscores the toxic potential of this species and highlights the significance of its detection in human-accessible, coastal environments. Its presence in a region historically linked to ciguatera, combined with its confirmed toxicity, warrants further monitoring to determine whether *G. holmesii* contribute to toxin production within Hervey Bay and Gladstone.

One species that has been shown to have large ranges is *G. carpenteri*. In our study it was detected in high detection frequency and at every site, except one, Elliot Heads (Hervey Bay) along the Queensland coast. Its widespread global distribution highlights its adaptability to diverse environmental conditions. This distribution pattern aligns with previous studies suggesting that *G. carpenteri* exhibits a broad ecological tolerance and is commonly detected across tropical and subtropical reef systems, along with detections in temperate waters (16.5–17°C) (Kohli et al., 2014; Sparrow et al., 2017; Vacarizas et al., 2018). Although we did not explicitly factor temperature into this study, the detection of *G. carpenteri* and other Clade II taxa at nearly all sites demonstrates their adaptability to a range of temperature profiles (Townsville, 24–30°C; Gladstone, 20–28°C; Hervey Bay, 18–26°C). This adaptability may be further supported by warming ocean conditions; for example, the strengthening of the East Australian Current has extended warmer waters further south, and Australia's temperate seas have warmed by ~0.75 °C per century since the early 1900s. Such trends could be facilitating the southward expansion and detection of *Gambierdiscus* beyond their historical range (Farrell et al., 2017; Kohli et al., 2014; Suthers et al., 2011).

The toxicity of *G. carpenteri* strains has been shown to vary across regions, with distinct toxin profiles reported from different geographic locations. Strains from the Gulf of California, Hawaii and the Philippines have demonstrated CTX-like and MTX-like activities, with high toxicity values in mouse bioassays (Malto et al., 2022; Pisapia et al., 2017; Ramos-Santiago et al., 2024; Vacarizas et al., 2018). In contrast, strains from Australia and the Caribbean primarily produce maitotoxin-3 (MTX-3) and related compounds but lack detectable levels of ciguatoxins (CTXs) (Kohli et al., 2014; Larsson et al., 2018; Litaker et al., 2017).

These observations support the idea that *G. carpenteri* is not only broadly distributed, but also genetically and functionally diverse. The variability in toxin production across regions suggests that strain-level genetic differences (as discussed previously) is shaped by local evolutionary processes and/or environmental pressures, as previously proposed by Vacarizas et al. (2018). However, this pattern of regional variation is not unique to *G. carpenteri*. Other species such as *G. holmesii, G. lapillus*, and cryptic taxa within Clade II also exhibit biogeographic structuring and variable toxicity profiles. These findings highlight the broader need for strain-level characterisation across all *Gambierdiscus* species, particularly within Australia, where relatively few isolates have undergone comprehensive toxicological analysis. Understanding the ecological and genetic diversity of these dinoflagellates is essential for assessing the spatial risk of ciguatera poisoning and informing targeted monitoring and management strategies along the Queensland coast and beyond.

4.3. *Gambierdiscus* macroalgal and habitat associations

Macroalgal hosts play a critical role in shaping the distribution, abundance, and epiphytic behaviour of *Gambierdiscus* species, with varying degrees of host specificity observed across taxa (Parsons et al., 2011; Rains and Parsons, 2015). For example, *G. belizeanus* demonstrated preferential attachment and growth on *Polysiphonia* and *Dictyota* species, and this was also linked with increased toxicity potential, suggesting certain host macroalgal species could elevate CP risk (Rains and Parsons, 2015). Similarly, Parsons et al. (2011) found that *Gambierdiscus toxicus* proliferated on some hosts (e.g., *Galaxaura* and *Jania* spp.) while avoiding or exhibiting high mortality on others (e.g., *Portieria hornemannii*) suggesting that physical and chemical traits of macroalgae influence host suitability. In our study, *Gambierdiscus carpenteri* was dominant across most macroalgal types, suggesting a generalist strategy consistent with previous reports of its tolerance to varied environmental conditions (Kohli et al., 2014; Sparrow et al., 2017). In contrast, ASVs that could only be assigned to Clade II (*Gambierdiscus* sp. C2) and

Gambierdiscus sp. C6, showed a strong preference for *Asparagopsis taxiformis*, a red alga. Mustapa et al. (2019) also observed enhanced growth of certain *Gambierdiscus* species on red and brown macroalgae. While our study was limited to sampling during the wet season, seasonal shifts in macroalgal communities could influence *Gambierdiscus* dynamics. Many macroalgal taxa display strong seasonal patterns in abundance and composition, which may alter the availability of preferred hosts and thus impact *Gambierdiscus* distribution and ciguatera risk (Chen et al., 2021; Rains and Parsons, 2015). Disturbance events such as coral bleaching can also lead to macroalgal proliferation, potentially expanding habitat for epiphytic dinoflagellates (Bruno et al., 2009; Perkins et al., 2024). Seasonal variation in canopy-forming species such as *Sargassum* also influences associated epifaunal productivity and trophic dynamics with peaks in biomass in summer months (Chen et al., 2021). Together, these findings highlight that both disturbance and seasonal processes can reshape macroalgal host availability, with potential consequences for *Gambierdiscus* community structure and ciguatera risk. Future studies spanning seasonal cycles and post-disturbance conditions will be critical to capture these dynamics.

Our results demonstrated that all five *Gambierdiscus* taxa detected in this study exhibited a clear preference for shallow marine habitats (0–5 m), consistent with previous research highlighting the shallow-water affinity of *Gambierdiscus* species (Funaki et al., 2022; Litaker et al., 2010). Detections were substantially higher in shallow samples compared to deeper sites (12–18 m). Notably, *G. holmesii* and *Gambierdiscus* sp. C6 were completely absent from deeper waters, suggesting a stricter depth limitation or lower tolerance to deeper conditions such as reduced light or temperature.

Despite this overall shallow-water pattern, ASVs assigned to *Gambierdiscus* from within Clade II (*G. carpenteri, Gambierdiscus* sp. C2, and *Gambierdiscus* sp. C2 GBR) were still detected at deeper sites (13–17 m), albeit at less frequency. This suggests some taxa possess broader depth tolerances, potentially enabling their persistence under lower light or alternative environmental conditions. Such findings are consistent with *Gambierdiscus* detections in mesophotic zones elsewhere. For instance, Tester et al. (2013) documented *Gambierdiscus* spp. at 40–60 m in the Flower Garden Banks National Marine Sanctuary (Texas, USA) following CP events linked to fish caught in deeper waters. Similarly, Funaki et al. (2022) detected *Gambierdiscus* spp. at approximately 30 m depth in Japanese coastal waters, including *G. jejuensis* and an unknown *Gambierdiscus* phylotype, both again from Clade II. Yoshimatsu et al. (2016) demonstrated that some species could maintain growth even at 87 m under controlled conditions. These findings collectively challenge the historical view that *Gambierdiscus* is limited to shallow reef environments and highlight the need to consider vertical distribution when assessing potential CP risk.

However, it remains unclear whether deeper detections represent viable, actively growing populations or low-level passive dispersal. Factors such as light availability, substrate type, and hydrodynamic conditions at depth may influence the ability of these taxa to establish and persist. In our dataset, the sharp drop in detections in deeper sampling sites and the absence of several taxa from deeper samples suggest that deeper environments may serve as marginal or suboptimal habitats rather than core habitats for most *Gambierdiscus* species.

The distribution of *Gambierdiscus* taxa across different habitats revealed distinct patterns in detection frequency and proportional read abundances, suggesting ecological preferences among species. Consistent with previous studies, *Gambierdiscus* spp. were most frequently detected in shallow reef-associated environments (Chinain et al., 2021; Funaki et al., 2022; Litaker et al., 2010). These habitats provide favourable substrate complexity and microenvironments that support *Gambierdiscus* colonisation and persistence. Among taxa, *G. carpenteri* and *Gambierdiscus* sp. C2 were the most broadly distributed, dominating detections across nearly all habitat types. This aligns with the known generalist nature of species within Clade II has been reported in diverse habitats (Funaki et al., 2022; Kohli et al., 2014; Perkins et al., 2025; Ramos-Santiago et al., 2024; Sparrow et al., 2017; Vacarizas et al., 2018). In contrast, *G. holmesii* displayed a highly restricted distribution, with its highest detection frequency observed at river mouth sites, an unexpected finding given that it has only previously been detected in offshore coral reef habitats with little freshwater influence (Kretzschmar et al., 2019). The presence of *G. holmesii* in a riverine-influenced environment suggests a broader environmental tolerance than previously recognised, or possible adaptation to estuarine-associated substrates.

These patterns highlight how habitat type may play a significant role in structuring *Gambierdiscus* communities, with certain taxa displaying potential ecological specialisation. Artificial or disturbed habitats, such as shipwrecks, supported only minimal *Gambierdiscus* detections in this study. However, elsewhere, artificial substrates such as petroleum platforms have been shown to support high *Gambierdiscus* biomass in otherwise unsuitable soft-bottom environments (Sparrow and Heimann, 2016; Villareal et al., 2007), underscoring the potential of anthropogenic structures to influence habitat availability.

These findings, together with previous studies, underscore that both habitat type and macroalgal host are key factors influencing *Gambierdiscus* community composition and distribution. The dominance of generalist taxa like *G. carpenteri* across diverse habitats and macroalgae reflects broad ecological plasticity, whereas the habitat-restricted patterns of *G. holmesii* and macroalgal-specific preferences of *Gambierdiscus* sp. C2 and sp. C6 suggest niche partitioning or functional specialisation. The strong association of certain taxa with specific macroalgae, such as *Asparagopsis taxiformis*, and habitats, such as river mouths, may have implications for localised CP risk. Together, these patterns highlight the need to consider both substrate availability and macroalgal composition in monitoring frameworks. Future studies should continue to explore how macroalgal-microalgal interactions influence *Gambierdiscus* growth, toxicity, and transfer into food webs, ultimately shaping spatial and temporal variation in ciguatera poisoning risk.

4.4. Conclusions and future directions

This study revealed previously undocumented diversity within *Gambierdiscus* along the inshore Queensland coastline, including the discovery of a potential genetically distinct group (Clade II GBR) and the first confirmed detection of *Gambierdiscus* in the Gladstone region. These findings expand the known biogeographic range of the genus in Australia, including *Gambierdiscus holmesii* a known CTX producer detected further south than its known range. The high ASV richness observed, particularly in within Clade II, likely reflects a combination of true ecological diversity and intragenomic variation, complicating species delimitation based on short-read amplicon data. The results also highlight the limitations of commonly used models, such as PTP, and demonstrate the value of approaches that account for substitution rate heterogeneity, like MPTP, in resolving fine-scale lineage structure.

Looking ahead, there is a clear need to integrate long-read sequencing technologies, multi-gene phylogenies such as LSU (28S) or ITS, and strain-level toxin profiling to better define species boundaries and assess ciguatera risk. Particular attention should be given to cryptic or regionally restricted lineages, such as Clade II GBR, to determine their distribution, ecological role, and toxic potential. Future studies should also investigate in situ cell densities and intragenomic variability to evaluate whether read counts from metabarcoding data can reliably serve as proxies for organism abundance, an area in which this study did not investigate. In addition, macroalgal host associations should be further explored to understand how substrate specificity influences community composition, abundance, and toxin production. Although temperature was not assessed in this study, future research should examine the occurrence and abundance of *Gambierdiscus* species and macroalgae they are associated with across seasonal and temperature gradients to better understand thermal influences on their distribution. Ultimately, future research should aim to develop a multi-scale, integrative monitoring framework that combines molecular detection, toxin

analysis, ecological data, and predictive modelling. This will be essential for accurately assessing the public health risk from ciguatera poisoning in Australian coastal waters.

CRediT authorship contribution statement

Joseph C. Perkins: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Kyall R. Zenger:** Writing – review & editing, Validation, Supervision, Funding acquisition, Conceptualization. **Angela Capper:** Writing – review & editing, Methodology, Data curation. **Yang Liu:** Writing – review & editing, Supervision, Conceptualization. **Jan M. Strugnell:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.hal.2025.103006.

Data availability

Data will be made available on request.

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