



Sources of variation in community composition of the hindgut microbiota in two tropical *Kyphosus* species

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Abstract Gut microbiota play a fundamental role in the nutrition of many vertebrate herbivores through foregut and hindgut fermentation of plant carbohydrates. Some species of marine herbivorous fishes contain moderate to high levels of short-chain fatty acids in the hindgut, indicating the importance of hindgut fermentation. Herbivorous fish hindgut microbiota are diverse and can vary with geographic location, but data on the scale of geographic variation involving a few km of separation are limited. Here, we used the 16S rRNA gene to describe community composition of the gut microbiota of the herbivorous species *Kyphosus vaiigiensis* and *K. cinerascens* collected in the vicinity of Lizard Island, northern Great Barrier Reef, Australia, in 2011 and 2017. Microbiota community structure differed between posterior hindgut sections, host species, sampling years and two mid-shelf and outer reef locations approximately 20 km apart. Hindgut bacterial community composition varied remarkably between mid-shelf and outer reef locations, and among individual fish on the mid-shelf reef. In both

fish species, the most abundant phyla were Pseudomonadota, Bacillota and Bacteroidota, followed by Spirochaetota, Thermodesulfobacteriota and Verrucomicrobiota. There were no clear differences between the host species in terms of the relative abundance and composition of bacterial genera in outer reef samples. In contrast, the dominant genera differed between mid-shelf samples of *K. cinerascens* and *K. vaiigiensis*, being *Endozoicomonas*-like (Pseudomonadota) and *Brevinema* (Spirochaetota), respectively. *Endozoicomonas* are emerging as important symbionts in many marine hosts worldwide and are thought to be important in the coral sulphur cycle. Differences in microbiota composition were not associated with variation in fish condition, suggesting that the different microbial taxa perform equivalent functional roles.

Keywords Kyphosidae · Herbivory · Microbiome · ASVs · Coral reef · *Endozoicomonas*

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Introduction

Gut-associated bacteria play fundamental roles in metazoan physiology (Lee and Hase 2014) and nutrition (Stevens and Hume 1998), such as contributing to the digestion of plants in many herbivores (Choat and Clements 1998; Greene et al. 2020). Algal carbohydrates represent a major source of potential energy for marine herbivorous fishes, which different fish taxa exploit to varying degrees (Krogdahl et al. 2005; Clements et al. 2014, 2017). For example, some taxa including mugilids, scarine parrotfish and some grazing acanthurids employ mechanical trituration in a pharyngeal mill or gizzard-like stomach and depend mainly on rapid gut throughput and digestion of protein, lipid and soluble carbohydrate (Clements et al. 2014). Other marine herbivorous

fish taxa, e.g. kyphosids, odacine labrids, aplodactylids and nasine acanthurids, have lengthier gut retention times and rely on gastrointestinal fermentation to salvage energy from refractory carbohydrate (Choat and Clements 1998; Mountfort et al. 2002). Given the algae-rich diet of the latter group of fish taxa, carbohydrate fermentation represents a key process in the marine environment (Choat and Clements 1998). Short-chain fatty acid (SCFA) profiles from the gut of species of the families Acanthuridae, Siganidae, Pomacanthidae and Kyphosidae suggest that the anaerobic microbial populations inhabiting the intestine of these species play a significant role in the nutritional ecology of the host (Clements and Choat 1995; Clements et al. 2009). These fish species contain the highest level of SCFAs in posterior gut sections, also defined as sections IV and V of the intestine (Clements and Choat 1995, 1997), indicating that hindgut sections have the highest levels of microbial fermentation.

Fish gut microbial communities can be highly diverse (Wang et al. 2018) and differ among fish species in taxonomic composition (Sullam et al. 2012). Three bacterial phyla, Pseudomonadota, Fusobacteriota and Bacillota (formerly Proteobacteria, Fusobacteria and Firmicutes, respectively), generally dominate the gut microbiota of fish (Tarnecki et al. 2017), with Vibrionales tending to be the most abundant order in many marine fish taxa (Sullam et al. 2012). Trophic level is also known to play a role in shaping the gut microbiota (Sullam et al. 2012). Although there are remarkable differences among trophic levels in fish gut community composition, common microbial taxa are present in carnivores, omnivores and zooplanktivores (Egerton et al. 2018). For example, the family Vibrionaceae, and the genera *Aeromonas* and *Pseudomonas*, are frequently reported in fish gut microbiome studies (Egerton et al. 2018), regardless of fish trophic level.

Dietary composition and geographic location influence microbial community composition in these fish gut communities (Tarnecki et al. 2017; Parata et al. 2020). In general, four processes, namely environmental selection, historical contingency, ecological drift and dispersal, interact with each other in different ways to influence microbial community assembly in the host gut (Costello et al. 2012). These processes can all lead to geographic variation in gut microbial community composition (Sullam et al. 2012; Taranaki et al. 2017; Jones et al. 2018; Senghor et al. 2018; Arroyo et al. 2019). Despite our increasing knowledge of fish gut microbiota, we still lack information on the influence of geographic location on gut community composition, and whether such variation influences community function, such as digestion of foods including seaweed and its constituent carbohydrates.

The family Kyphosidae has a worldwide tropical marine distribution and also occurs in temperate Australasia (Knudsen et al. 2019). Herbivorous *Kyphosus* spp. display high

concentrations of SCFA in the hindgut (Clements and Choat 1995, 1997; Choat and Clements 1998; Clements et al. 2017), indicating that gut microbial communities play an important role in nutrient assimilation from seaweeds. The tropical *Kyphosus cinerascens* and *K. vaigiensis* display some of the highest levels of carbohydrate fermentation products among other coral reef associated herbivorous fishes and related omnivorous and planktivorous taxa (Clements et al. 2017). However, levels of SCFA do not differ between the posterior gut segments IV and V in *K. vaigiensis*, whereas in *K. cinerascens* section V has the highest levels of SCFA in the gut (Clements and Choat 1995, 1997). Most *Kyphosus* species have specialized gut anatomy associated with microbial fermentation (Clements and Choat 1997; Johnson and Clements 2021); however, the high level of SCFAs is not restricted to distinct hindgut structures (i.e. hindgut chamber) (Clements and Choat, 1997). In *K. vaigiensis*, the terminal part of the intestine (section V) forms a hindgut chamber or caecal pouch (Rimmer and Wibe 1987); this feature is absent in *K. cinerascens* (Clements 1997). The two species also exhibit differences in diet. Previous studies conducted at Lizard Island, Australia, show that *Kyphosus vaigiensis* has a diet dominated by phaeophytes, while *K. cinerascens* consumes a high proportion of rhodophytes (Clements and Choat 1997; Choat et al. 2002).

The present study used 16S rRNA gene sequences to characterize hindgut microbial community composition in *K. cinerascens* and *K. vaigiensis* collected in 2011 and 2017 from two locations in the vicinity of Lizard Island, Great Barrier Reef, Australia. We sought to determine the differences in community composition between: (1) posterior hindgut sections IV and V, (2) the two *Kyphosus* species, and (3) mid-shelf reef and outer reef locations separated by approximately 20 kms.

Material and methods

Sample collection

Adult specimens of *K. cinerascens* ($n=10$) and *K. vaigiensis* ($n=8$) were collected by spear gun while snorkelling from the northern Great Barrier Reef, Australia (14° 40' S, 145° 28' E). Fish were collected from two locations, the Lizard Island complex (mid-shelf reef) and the adjacent outer reefs (Fig. 1 and Table 1) during March 2011 and December 2017 with some crossover between years and locations (Supplementary Table 1). Fish were transported on ice to the laboratory at the Lizard Island Research Station (LIRS). Fish length and weight were recorded for each specimen, and intestinal content was collected from posterior gut sections IV and V following Choat et al. (2002). Gut content (digested and partially digested material in the lumen) samples were fixed

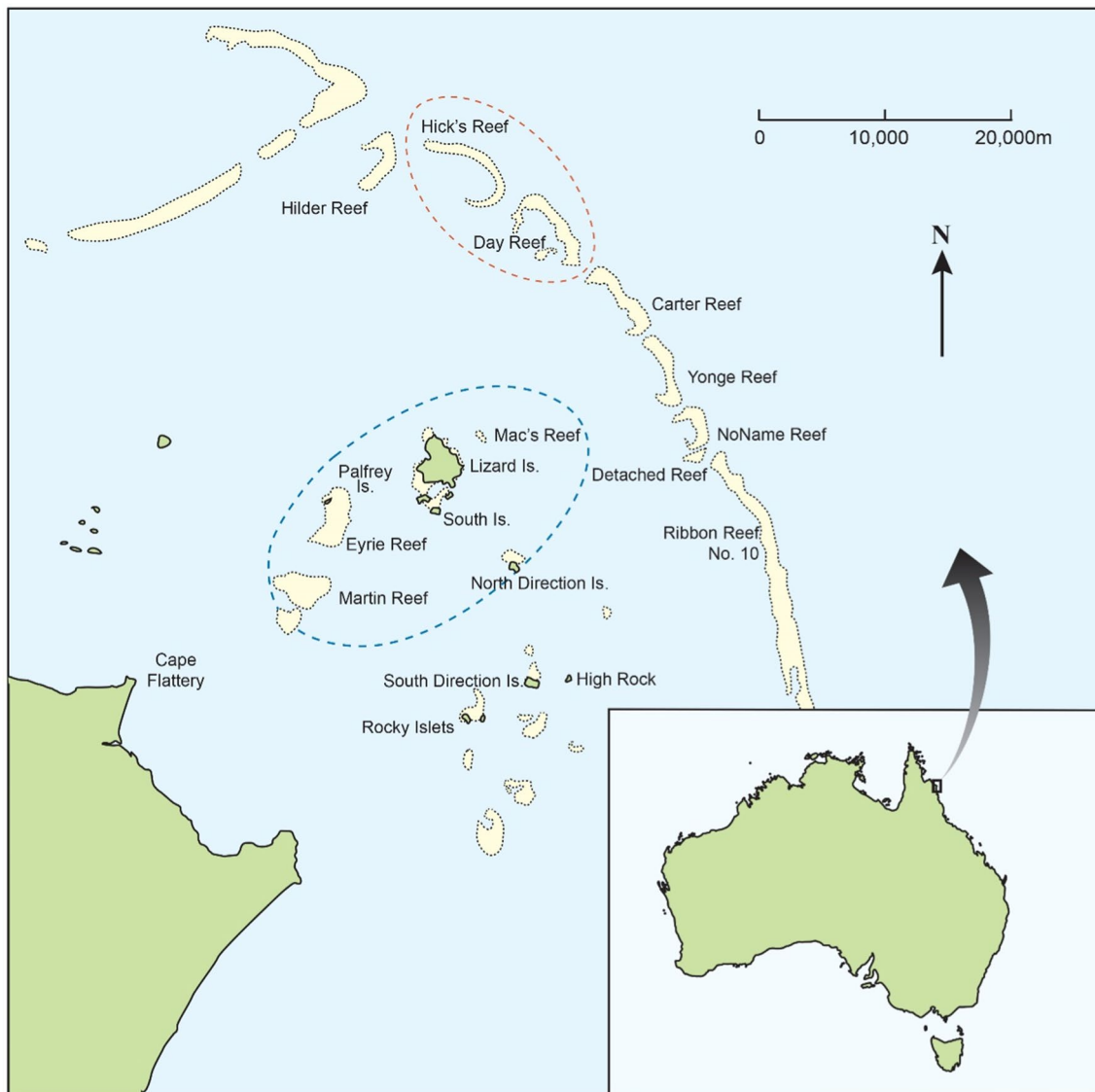


Fig. 1 Study location for sample collection on the northern Great Barrier Reef, Australia. Blue-dashed circle indicates mid-shelf reef collection area; red-dashed circle indicates outer reef collection area

Table 1 Details of fish species, geographic location, number of samples and IDs. Fish collected in 2011 have labels starting with M, while fish collected in 2017 have a G

Hindgut section	<i>Kyphosus cinerascens</i> (n = 10)		<i>Kyphosus vaigiensis</i> (n = 8)	
	IV	V	IV	V
Mid-shelf reef IDs	(n = 4); G160IV, G161IV, G179IV, G184IV	(n = 3); G160V, G161V, G184V	(n = 5); G157IV, G158IV, G159IV, G162IV, M241IV	(n = 5); G157V, G158V, G159V, G162V, M241V
Outer Reef IDs	(n = 5); G135IV, G138IV, M96IV, M103IV, M235IV	(n = 5); G138V, M96V, M97V, M103V, M235V	(n = 2); M90IV, M104IV	(n = 3); M90V, M104V, M106V
Total per section	9 section IV	8 section V	7 section IV	8 section V

in 80% ethanol and shipped to the University of Auckland, New Zealand, for further analysis in PC1 laboratories following standard, clean laboratory procedures.

DNA extraction

Sufficient DNA for sequencing was obtained from 16 samples each of gut sections IV and V (see Table 1). Prior to DNA extraction, the 80% ethanol-fixed samples were pelleted for three minutes at $20,800\times g$ (Eppendorf 5804 centrifuge, Hamburg, Germany) and supernatant was removed. DNA extractions were carried out according to the DNeasy PowerSoil DNA Isolation Kit (Qiagen, Hilden, Germany) manufacturer's instructions.

PCR amplification and sequencing

The hypervariable V3-V4 region of the 16S rRNA gene was amplified from the hindgut samples. For the 2011 samples, PCR amplification was performed using the following Illumina tagged and barcoded primers: 341F (5'-CCTACG GGNGGCWGCAG-3') forward (Herlemann et al. 2011) and 806R (5'-GGACTACHVGGGTWTCTAAT-3') reverse (Caporaso et al. 2011). For the 2017 samples, the forward primer was the same, while the reverse primer was changed to 785R (5'-GACTACHVGGGTATCTAATCC-3') (Herlemann et al. 2011). The primer set in 2017 was changed as the 341F/785R primer pair was suggested to yield improved coverage of bacterial taxa by Klindworth et al. 2013. PCR reactions (25 μ L) for the 2011 samples were carried out using 1 \times HotStarTaq Plus Master Mix in triplicate (Qiagen), and for the 2017 samples using 2 \times KAPA HiFi HotStart (KAPA Biosystems, Woburn, USA) (single reaction per sample). For the 2011 samples, thermocycling conditions consisted of an initial denaturation step of 5 min at 94 $^{\circ}$ C, followed by 30 cycles of 94 $^{\circ}$ C for 30 s, 50 $^{\circ}$ C for 45 s, 68 $^{\circ}$ C for 90 s, and a final extension of 10 min at 68 $^{\circ}$ C. For the 2017 samples, the denaturation step was 3 min at 95 $^{\circ}$ C, followed by 25 cycles of 95 $^{\circ}$ C for 30 s, 55 $^{\circ}$ C for 30 s, 72 $^{\circ}$ C for 30 s, and a final extension of 5 min at 72 $^{\circ}$ C. For the 2011 samples, the resulting amplicons were cleaned using ZR-96 DNA Clean and Concentrator-5 (Deep Well) kits (Zymo Research), and amplicons from the 2017 samples were cleaned using Agencourt AMPure XP magnetic beads (Beckman Coulter, CA, USA).

In silico PCR testing of both primer sets (2011 and 2017) against the SILVA database 138 (as used for taxonomic assignment) resulted in only a 0.55% difference in the number of organisms (reference sequences) targeted relative to the size of the entire SILVA database, indicating that the 2011 and 2017 primer sets yield highly similar results. Only 5 taxa differed between the two primer sets, all of which were members of phylum Chloroflexota class Anaerolineae

(genera *OLB13*, *Thermomarinilinea*, *Bellilinea*, *ADurb. Bin120*, and *RBG-16-58-14*). For both sets of samples, library preparation and 2 \times 300 bp paired end sequencing was performed using the Illumina MiSeq platform with V3 chemistry at Auckland Genomics (University of Auckland, NZ).

16S rRNA gene amplicon analysis

For both set of samples, raw sequences were filtered using Cutadapt v3.3 (Marcel 2011; Callahan et al. 2016) to remove primers and adapters. The following analysis was carried out in R v4.0.1 using DADA2 pipeline v1.16.0 (Callahan et al. 2016). Sequencing reads were quality filtered and trimmed using filterAndTrim, dereplicated using derepFastq and merged using mergePairs. The amplicon sequence variant (ASV) table was generated using makeSequenceTable and chimeras removed using RemoveBimeraDenovo command. Taxonomy of ASVs was assigned using DADA2 (Callahan et al. 2016) with SILVA SSU Ref NR99 v138 database (Quast et al. 2013). Finally, sequences were rarefied to 1234 with QIIME v2020.6 (Bokulich et al. 2018), which was the overall sample minimum value, leaving 2875 ASVs across samples. Phyla taxonomy was reevaluated and updated using the updated NCBI phylum taxonomy (Oren and Garrity 2021).

Fish and microbial community analysis

The length–weight relationship (LWR) for each fish species was calculated following Ricker (1973). QGIS 3.2 Bonn (QGIS Development Team, 2018) was used to differentiate the fish collection locations. Hindgut microbial community and alpha diversity indices, observed ASVs, Chao1, Shannon (Shannon and Weaver 1949) and Pielou's evenness (Pielou 1969), were plotted using R v4.0.1 with Rstudio software version 1.2.1335 and R packages ggplot2 v3.3.5 and vegan v2.5–7. Bacterial community beta diversity was plotted with nonmetric multidimensional scaling (NMDS) of weighted UniFrac distances (Lozupone and Knight 2005). Statistical analysis of alpha diversity indices and NMDS was performed in R using analysis of variance (ANOVA) and permutational multivariate analysis of variance (PERMANOVA, through adonis2), respectively.

Endozoicomonas phylogenetic tree

A phylogenetic tree was constructed using the 18 ASV *Endozoicomonas*-like sequences and three *Actinobacter* ASV sequences (used as an outgroup to root the tree) recovered from this study and 14 reference sequences of full length 16S rRNA recovered from GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>). Taxonomy of ASVs

was annotated based on the SILVA SSU Ref NR99 v138 database (Quast et al. 2013). Sequences were aligned with MUSCLE using Geneious (v2020.05, <https://www.geneious.com>). 16S full-length amplicons were trimmed to increase the quality of the alignment with the regions V3-V4 of the ASVs. A maximum-likelihood phylogenetic tree was built using FastTree v2.1.10-gimkl-2018b (Price et al. 2009, 2010). Finally, the tree was visualized using iTOL (Letunic and Bork 2021).

Results

Hindgut microbiota composition

Based on 16S rRNA gene sequence data, the relatively dominant phyla, on average, across most fish were Pseudomonadota, Bacillota and Bacteroidota, followed by less abundant phyla that included Spirochaetes, Thermodesulfobacteriota and Verrucomicrobiota (Fig. 2). The dominant bacterial taxa in *Kyphosus* species differed between the two geographic areas and gut sections (Fig. 2). In the mid-shelf reef, Pseudomonadota were the most abundant phylum in fish hindgut sections IV ($72.8 \pm 7.7\%$, mean \pm SE) and V

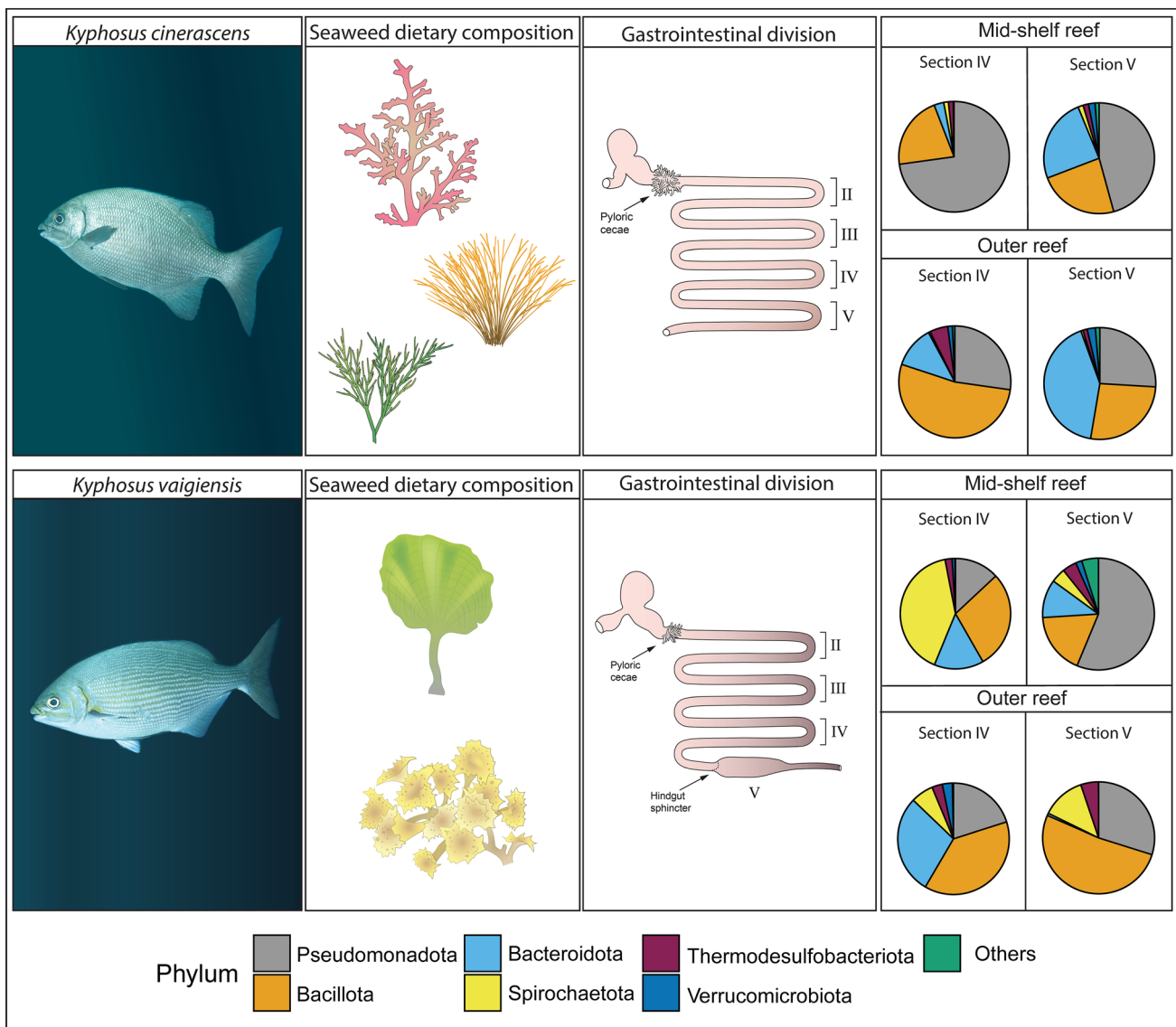


Fig. 2 From left to right: photographs of *Kyphosus* study species; illustrations of their main dietary components: *Laurencia majuscula*, *Sphacelaria tribuloides* and *Cladophora rugulosa* for *Kyphosus cinerascens* and *Udotea argentea* and *Turbinaria ornata* for *K. vaigien-*

sis (following Choat et al. 2002); gastrointestinal tract morphology and sampling regions for the two study species; and mean ASVs relative abundances for sections IV and V from mid-shelf and outer reef samples of each fish species

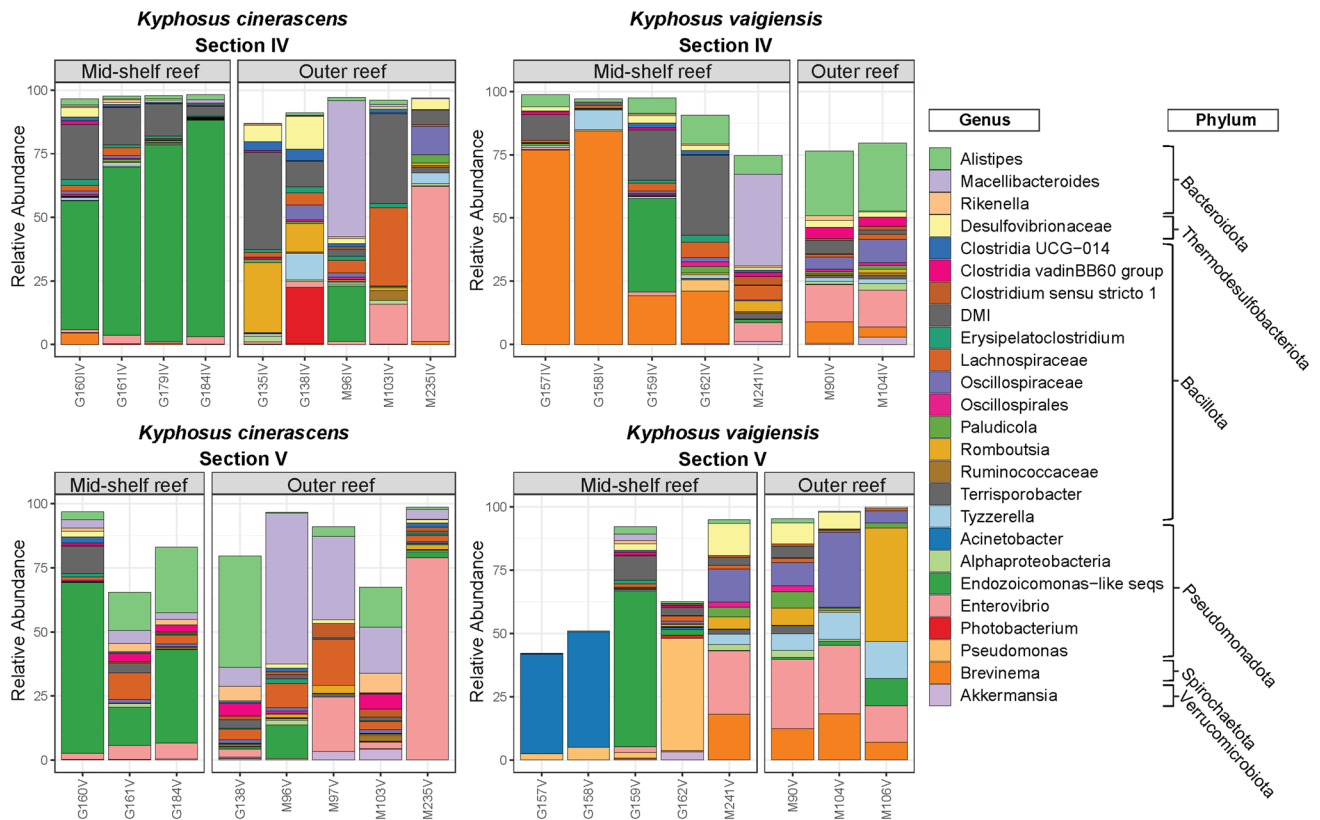


Fig. 3 Relative abundance of the 25 most abundant bacterial genera in gut sections IV and V of *K. cinerascens* and *K. vaigiensis* from mid-shelf and outer reef samples. Data is based on 16S rRNA gene

ASVs, and relative abundances are summed to genus level or the nearest higher rank. Labels with M= samples collected in 2011; those with G=2017

($45.7 \pm 13.4\%$, mean \pm SE) of *K. cinerascens*, and in section V ($56.3 \pm 7.1\%$, mean \pm SE) of *K. vaigiensis*. In contrast, in the outer reef, Bacillota were highly abundant in the communities of both sections IV ($38.3 \pm 5.1\%$, mean \pm SE) and V ($51.7 \pm 7.7\%$, mean \pm SE) of *K. vaigiensis* and in section IV ($52.8 \pm 12.4\%$, mean \pm SE) of *K. cinerascens*. While Thermodesulfobacteriota were not the most abundant members of the hindgut communities, they comprised up to 5% of some communities. They tended to be more abundant in section IV than V of both fish species, regardless of location fish were collected, whereas Spirochaetes exhibited the greatest difference between fish species, as they tended to be more abundant in *K. vaigiensis* from the mid-shelf reef, particularly in section IV ($40.7 \pm 16.7\%$, mean \pm SE).

In *K. cinerascens* from mid-shelf reef *Endozoicomonas*-like sequences (Pseudomonadota) were numerically dominant in section IV of all fish studied, where they comprised over 50% of each community (all 2017 samples), and up to 85.2% in sample G184IV (Fig. 3). They were also very abundant in the corresponding section V communities (also 2017 samples), but strikingly less abundant overall in the outer reef samples of the same fish species, regardless of year sampled (2011 or 2017). Gut communities of *K. cinerascens*

from the outer reef exhibited no clear trends in microbial community composition and varied considerably between individual fish. For example, the most abundant genera were *Macellibacteroides* in section IV sample M96IV (53.6%), and *Enterovibrio* in section V sample M235V (78.9%), yet their relative abundance was very low in the other samples from the same gut sections (Fig. 3).

Remarkably, the *Endozoicomonas*-like genus was also very abundant (61.5%) in one individual of *K. vaigiensis* (section V, mid-shelf reef). The gut microbial community was highly distinct between *K. vaigiensis* collected at mid-shelf reef (section V) and outer reef (sections IV and V samples) locations, and the prevalent gut taxa were *Oscillospiraceae* and *Enterovibrio* (Fig. 3). Other prevalent taxa associated with *K. vaigiensis* were *Acinetobacter* and *Pseudomonas*. While not possible to compare differences in sampling location in *K. vaigiensis* without considering the potential for temporal effects (all but one fish from the mid-shelf reef were from 2017 and both fish from the outer reef were from 2011), we found the genus *Brevinema* (Spirochaetes) dominated or were prominent in certain groups of samples and not others. In particular, *Brevinema* dominated section IV gut communities of *K. vaigiensis* from the

mid-shelf reef, but were largely absent from section V samples from the same fish species and year (2017). *Brevinema* were also notable community members in six out of seven 2011 samples in sections IV and V (all out reef and one of two mid-shelf reef samples), indicating considerable variation across gut sections and time or space.

One possible reason for the difference in microbial composition between fish collected from mid-shelf reef and outer reef sites across two different sampling years could be variation in fish condition, dysbiosis or an aberrant gut microbiota in some of the fish in one of the two locations. However, our data show that mid-shelf reef and outer reef fish all fell on the same length–weight line ($R^2=0.9479$ for *K. cinerascens* and $R^2=0.9423$ for *K. vaigiensis*, Fig. 4), indicating that all fish were in similar condition.

Alpha and beta diversity analysis

Weighted UniFrac beta diversity was used to analyse the differences in microbial community structure between hindgut sections, fish species, sampling year and geographic location. Findings indicate that no one factor alone explained all variation, and factors contributing to variation differed between posterior gut sections in the study fish. Again,

geographic location (PERMANOVA, p -value < 0.05), sampling years (PERMANOVA, p -value < 0.01) and fish (PERMANOVA, p -value < 0.001) appeared to influence the gut microbiota of section IV in the two fish species, wherein the outer reef samples (which are a mix of 2011 and 2017 and fish species) clustered together, while mid-shelf samples from both fish species formed a discrete cluster (all 2017, Fig. 5). In contrast, community structure in hindgut section V (Fig. 5) was significantly influenced by fish species (PERMANOVA, p -value < 0.01).

To evaluate alpha diversity, we assessed a range of metrics based on 16S rRNA gene ASVs. Diversity indices (Shannon and Pielou) and richness estimates (Chao1, ASVs richness) of combined sections IV and V showed no significant difference between location, time or fish species (Fig. 6a), as also confirmed using ANOVA (p -value > 0.1). Despite sampling different locations and years, microbial community alpha diversity of combined gut sections appeared to be broadly similar (based on ASVs, Fig. 6a). However, when considering genus-level data, where sections IV and V were treated separately (Fig. 3), there is a discernible difference in alpha diversity among samples, particularly in section IV. We therefore also calculated alpha diversity metrics (richness and Shannon index) for each gut section separately based

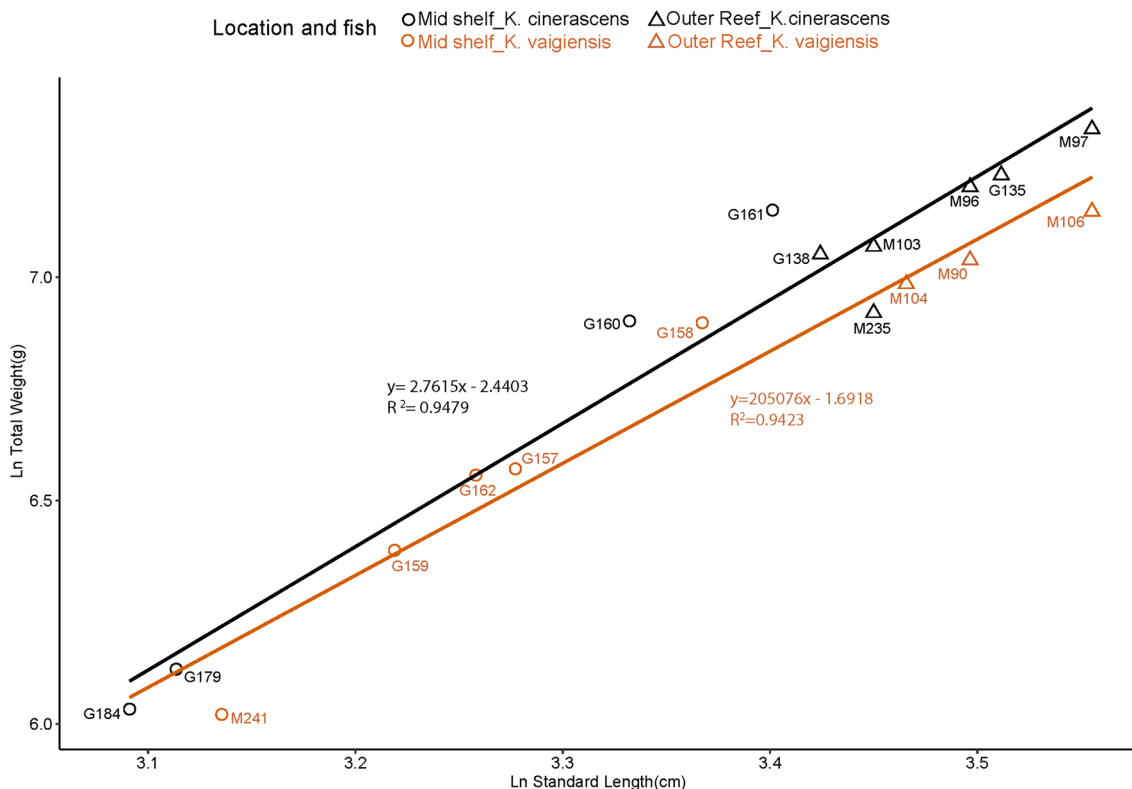


Fig. 4 Relationship between length and weight of *Kyphosus* specimens. *K. vaigiensis* shown in orange and *K. cinerascens* in black. Circles represent mid-shelf specimens, and triangles represent outer reef

specimens. The length–weight relationship is based on the equation $\log(W) = \log a + b \log (L)$ (a is the intercept and b is the slope of the equation)

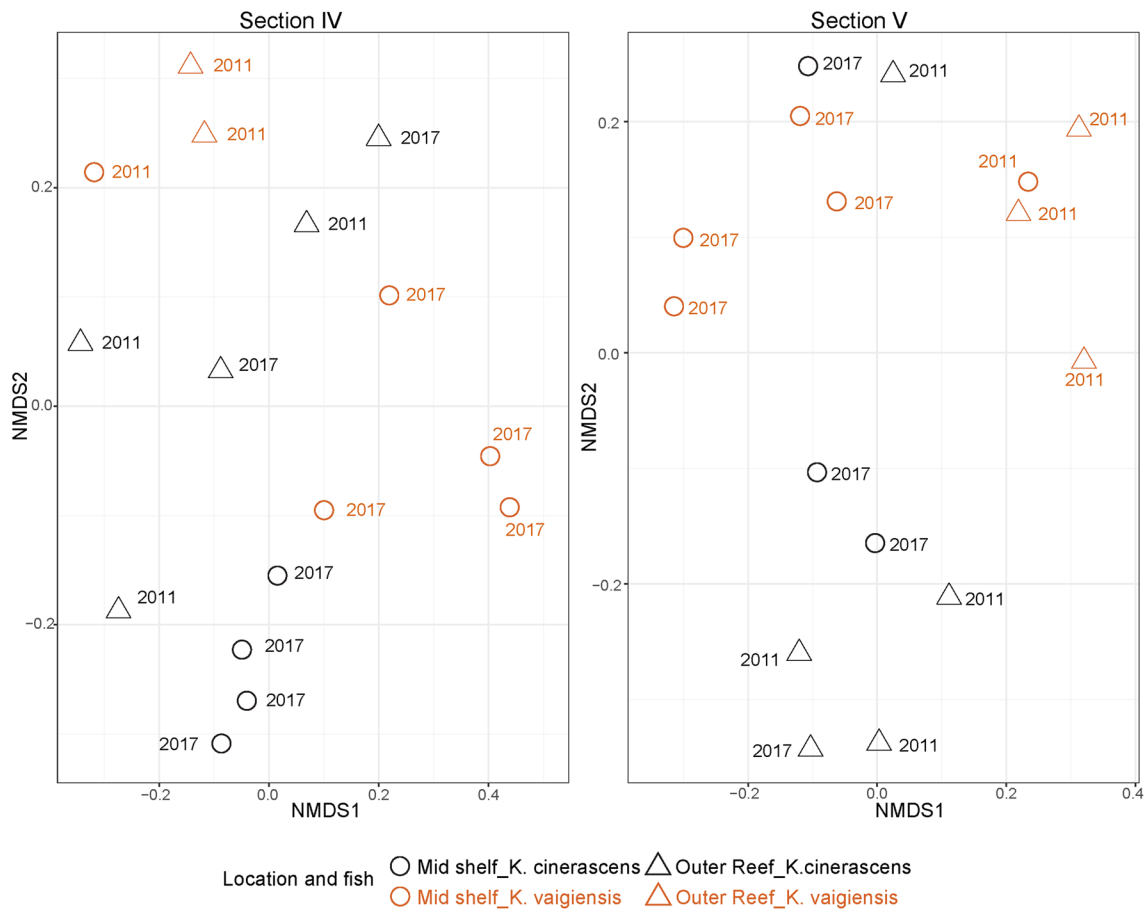


Fig. 5 Weighted UniFrac NMDS for sections IV (left, stress value=0.213) and section V (right, stress value=0.181). Colours represent fish species: *K. cinerascens* black; *K. vaigiensis* orange. Symbols show location: mid-shelf reefs circle, outer reef triangle

on ASVs (Fig. 6b). These confirmed statistically significant differences in alpha diversity between fish species at each location in section IV across sampling years, but not section V (Fig. 6b, Shannon index). Overall, alpha diversity results revealed similar richness between communities when sections were combined (Fig. 6a), although this was largely due to equivalent community diversity in section V among fish from the two locations. Microbial communities in hindgut section IV were highly distinct due to low alpha diversity in fish from the mid-shelf reef (Fig. 3).

Endozoicomonas phylogenetic tree

To examine the relationship of fish-associated *Endozoicomonas*-like sequences to previously described *Endozoicomonas*, we built a phylogenetic tree with reference sequences of *Endozoicomonas* from GenBank. The *Endozoicomonas*-like ASV sequences displayed high similarity and formed a distinct clade to that containing reference sequences of *Endozoicomonas* (Fig. 7). Accordingly, we designated

these *Kyphosus*-associated ASVs as *Endozoicomonas*-like throughout our study.

Discussion

Microbiota community composition in this study differed considerably between posterior hindgut sections, fish species, two mid-shelf and outer reef locations approximately 20 km apart, and sampling years. The dominant phyla in both fish species were Pseudomonadota, Bacillota and Bacteroidota, followed by Spirochaetota, Thermodesulfobacteriota and Verrucomicrobiota. No single microbial genus was dominant across individual fish of the same species in the microbial composition of outer reef samples. Conversely, the dominant genera differed between mid-shelf samples of *K. cinerascens* and *K. vaigiensis*, being *Endozoicomonas* (Pseudomonadota) and *Brevinema* (Spirochaetota), respectively.

Bacillota and/or Pseudomonadota (formerly Firmicutes and Proteobacteria) taxa are often the dominant groups in herbivorous fishes such as surgeonfish (Miyake et al. 2015;

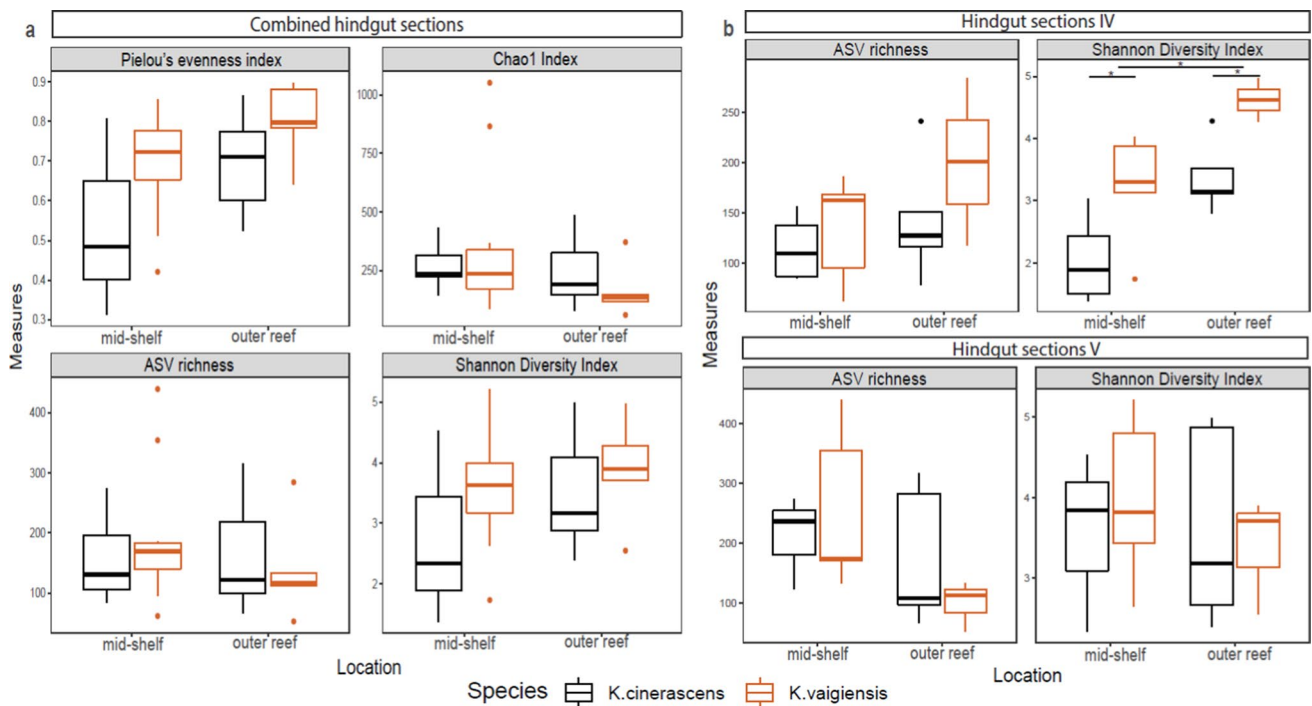


Fig. 6 **a** Boxplots of alpha diversity indices (Pielou's evenness, Chao1, Richness (ASVs) and Shannon) of combined hindgut sections IV and V; **b** alpha diversity (ASVs richness and Shannon diversity)

of sections IV and V separately. Colours represent *K. cinerascens* (black) and *K. vaigiensis* (orange) in mid-shelf and outer shelf reef locations

Ngugi et al 2017; Nielsen et al. 2017; Parata et al. 2020) and butterflyfish (Clever et al. 2020). Bacillota are associated with high-fibre diets and SCFA production in humans (Nobel et al. 2018), and were associated with carbohydrase production in surgeonfish (Ngugi et al. 2017). Bacillota were the most abundant taxon in the temperate fish species *Kyphosus sydneyanus*, *Odax pullus* and *Aplodactylus arcidens* (Clements et al. 2007), all of which have diets dominated by macroalgae and moderate to high levels of SCFA in the hindgut (Mountfort et al. 2002). Although *K. vaigiensis* and *K. cinerascens* both contain high levels of SCFA in the hindgut (Clements et al. 2017), in our present study Bacillota were only dominant in fish collected from outer reef sites.

Spirochaetes were also a notable phylum distinguishing the gut communities in *K. vaigiensis* from those in *K. cinerascens*, particularly in section IV from mid-shelf reef. Sparagon et al. (2022) examined the gut microbiota of five *Kyphosus* individuals collected in Hawaii, and based on their supplementary photo of their *Kyphosus* specimens we were able to identify three individuals (fish 4, 5 and 8) as *K. vaigiensis* and one individual (fish 6) as *K. cinerascens*. The gut microbiota of some of the samples from these fish species reported by Sparagon and colleagues were in some cases similar to the gut microbiome of *K. vaigiensis* and *K. cinerascens* in our study (i.e. the presence of the Spirochaete genus *Brevinema* and the Bacteroidota genus

Alistipes). Spirochaetes are often reported as pathogenic in humans (Lee and Hampson. 1994); however, they can play a beneficial role in termites and in other terrestrial herbivores through acetogenesis and dinitrogen fixation (Ohkuma et al. 2015; Tokuda et al. 2018; Blankenchip et al. 2018). Spirochaetes have also been suggested to play an important ecological role in herbivorous fish (Clements et al. 2009; Parata et al. 2020).

A number of studies collectively show that the gut microbial community structure is distinct between species of tropical herbivorous fish (Smriga et al. 2010; Miyake et al. 2015; Jones et al. 2018; Scott et al. 2020). For example, according to Scott et al. 2020, the most abundant phyla in the gut microbiota of the herbivorous fish *Acanthurus coeruleus*, *Acanthurus tractus*, *Scarus taeniopterus*, *Sparisoma aurofrenatum* and *Sparisoma viride* were Proteobacteria, Firmicutes and Planctomycetes (now known as Pseudomonadota, Bacillota and Planctomycetota, respectively; Oren and Garrity 2021). While species composition was relatively consistent between these fish species, the relative abundance of each microbial taxonomic group differed strongly (e.g. *Sp. aurofrenatum* harboured a less diverse intestinal microbiome than *Sc. Taeniopterus* or *A. tractus*) (Scott et al. 2020). This indicates considerable functional diversity within coral reef fish, as also suggested by our results (i.e. we found different bacterial taxa within individual fish of the same

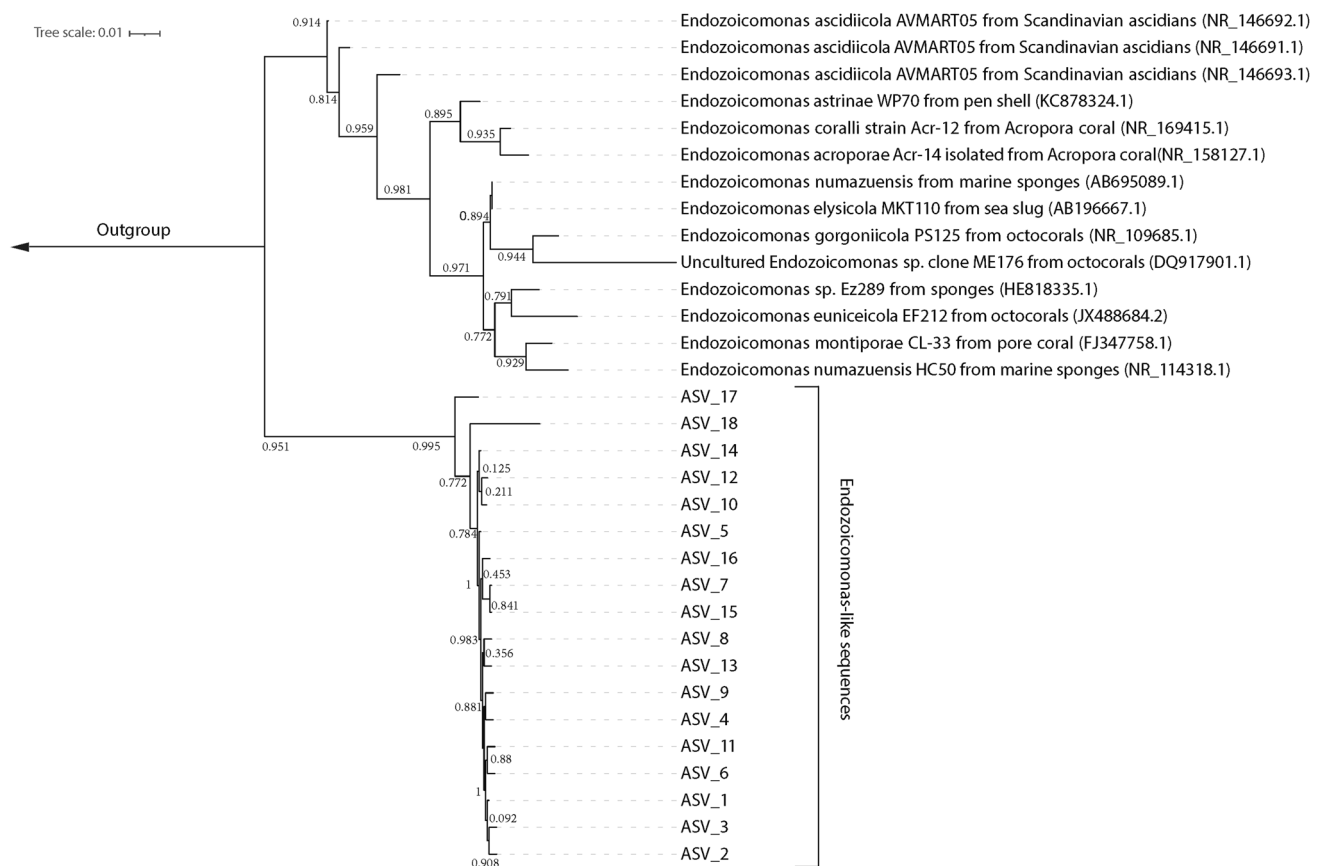


Fig. 7 Phylogenetic tree of 18 ASVs of *Endozoicomonas*-like 16S rRNA sequences recovered in the present study and 14 reference sequences from GenBank of 16S rRNA sequences of *Endozoico-*

monas associated with different marine organisms. The scale bar corresponds to 0.1 substitutions per nucleotide position. 1000 bootstraps (values represented on the tree)

species, suggesting that different microbial taxa can perform equivalent functional roles). Microbial communities in faecal samples of *Acanthurus nigricans* were dominated by Pseudomonadota (non-Vibrionaceae), Bacteroidota (formerly Bacteroidetes), and Bacillota, while those in *Chlorurus sordidus* (now *C. spilurus*) and *Lutjanus bohar* were dominated by Pseudomonadota-Vibrionaceae (Smriga et al. 2010). These observations are consistent with findings here, with an overall high abundance of Pseudomonadota, Bacillota and Bacteroidetes in *K. cinerascens* and *K. vaigiensis* gut microbial communities. Additionally, we found that the relative abundances of microbial taxa in *K. cinerascens* and *K. vaigiensis* were influenced by different factors, including gut sections, host fish species, geographic locations and sampling years. Similarly, Jones et al. (2018) reported that latitudinal gradient influences the gut microbiota of the rabbitfish *Siganus fuscescens* collected from over 2000 km on the coast of Western Australia.

Variation in community composition of gut microbiota could drive host niche differentiation (Greene et al. 2020). For example, the gut microbiomes in some Madagascar lemurs can vary depending on host diet, gut morphology

and geographic location (Greene et al. 2020). Similarly, in our study the dominant genera, *Endozoicomonas*-like and *Brevinema*, varied depending on host fish species, hindgut section and location or time (Fig. 3). Both *Endozoicomonas* and *Brevinema* were the most abundant groups in butterflyfish on Caribbean coral reefs (Clever et al. 2020), and *Brevinema* was one of the most prevalent genera in surgeonfish from the Great Barrier Reef (Parata et al. 2020).

Endozoicomonas are emerging as diverse and important symbionts in many marine hosts worldwide (Neave et al. 2016) and were one of the most abundant genera in planktivorous damselfish and cardinalfish collected from Lizard Island (Parris et al. 2016), where the present study was conducted. *Endozoicomonas* species have been suggested to play a role in the coral sulphur cycle via dimethyl sulfoniopropionate (DMSP) metabolism to produce dimethyl sulphide (DMS) (Tandon et al. 2020). DMSP is also produced in significant quantities by red algae (Dacey et al. 1994), a major dietary component of *K. cinerascens* (Choat et al. 2002). We examined the phylogenetic relationship of the *Endozoicomonas*-like sequences in our data set to reference *Endozoicomonas* sequences from other marine taxa to

explore patterns of host-symbiont association. The *Endozoicomonas*-like ASVs formed a distinct clade to that containing the reference *Endozoicomonas* sequences, indicating that our ASV sequences comprise a novel *K. cinerascens*-associated clade that is closely related to *Endozoicomonas* found in other marine animals.

Differences in populations of gut symbionts of marine herbivorous fish have been detected previously between mid-shelf and outer shelf reef locations on the northern Great Barrier Reef (Arroyo et al. 2019), showing that it is possible that geographic comparisons could be confounded by temporal factors. The Lizard Island Complex and nearby outer reefs were affected by two major cyclones in 2014 and 2015, and a mass bleaching event in 2016 (Madin et al. 2018; Zawada et al. 2019). Our 2011 samples were collected prior to these disturbances, while the 2017 sampling was collected one year after the bleaching event. It is possible that these disturbances influenced community assembly in our gut communities, either through historical contingency, ecological drift or dispersal (Costello et al. 2012), and so we explored this in more detail.

We observed a strong difference in community composition at genus level between sections IV and V in *K. vaigiensis* from mid-shelf reef (Fig. 3). This could be due to the presence in this species of a hindgut chamber (Clements and Choat 1997). Specimens of *Kyphosus cinerascens* lack a hindgut chamber, and share broadly similar bacterial taxa between sections IV and V, particularly on the mid-shelf reefs, such as the genus *Endozoicomonas*-like. This may indicate that the presence of a hindgut chamber that is separated from the proximal intestine by a sphincter increases total microbiota diversity. Community composition also differed markedly between the two host species. This could be due to a diet dominated by green (*Udotea argentea*) and brown (*Turbinaria ornata*) algae in *K. vaigiensis* (Choat et al. 2002) (Fig. 2), and by a mixture of red (*Laurencia majuscola*), brown (*Sphacelaria tribuloides*) and green (*Cladophora rugulosa*) algae in *K. cinerascens* (Choat et al. 2002) (Fig. 2). Gut morphology and diet have a strong influence on microbial community composition in animals (Ley et al. 2008), especially herbivores (Greene et al. 2020; Escalas et al. 2021). In addition, we observed variation among individual fish in their gut microbiota for both fish species and gut sections, especially in *K. cinerascens* from the outer reefs and in *K. vaigiensis* from mid-shelf reefs (Fig. 3). Although individual conspecific fish displayed variation in gut microbial communities, these gut communities should be consistent in overall metabolic function, as both fish species have consistently high levels of SCFA in the hindgut (Clements and Choat 1995, 1997).

The importance of gut microbiota to nutrition in kyphosid fishes (Mountfort et al. 2002; Clements et al. 2014) raised the possibility that highly variant gut communities may

influence fish condition and health. We tested this by examining the relationship between length and weight for all of our sampled fish (Fig. 4). Fish that are nutritionally compromised would be expected to weigh less for a given length than fish with a healthy gut microbiota. However, we detected no difference in length/weight between either sampling locations or sampling years. This suggests that the considerable variation in gut microbiome community composition did not influence community function, and that community assembly is labile and driven by factors other than microbiota phylogeny.

This study suggests that spatial variation, even over scales of only tens of kilometres, can influence gut community composition in herbivorous fishes. There is a significant difference in the hindgut microbial community composition in *K. cinerascens* between mid-shelf and outer-shelf reefs, as seen in *Naso tonganus* over the same spatial scales (Arroyo et al. 2019), while marked variation in *K. vaigiensis* may be attributed to either spatial or temporal factors, similarly to the variation in microbial composition observed in *S. fuscescens* with latitudinal changes in Jones et al. (2018). We also observed considerable interspecific variation in the hindgut microbiota of fish living in the same geographic area at the same time, i.e. outer-shelf reef for *K. cinerascens* and mid-shelf reef for *K. vaigiensis*. Our data also suggest that the *Endozoicomonas*-like genus, which dominated communities in mid-shelf samples of *K. cinerascens*, most likely makes an important contribution to metabolic function and immune defence, as *Endozoicomonas* does in corals and other invertebrates (Neave et al. 2016; Tandon et al. 2020; Jensen et al. 2021).

Future work should examine the level of spatial and temporal variation in gut community composition in other herbivorous fishes over larger spatial scales, while taking into account the influence of potential spatial and/or temporal variation in diet. Our study suggests that while there must be strong environmental selection for functionality in these gut communities, this is not strictly tied to particular bacterial taxa, and the effects of variation in many factors including diet and location, and the interaction of different processes including historical contingency, random sampling and dispersal may also influence community assembly (Costello et al. 2012).

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Data availability All raw reads associated with this work have been deposited at the National Center for Biotechnology Information (<https://www.ncbi.nlm.nih.gov/bioproject/>) under BioProject ID: PRJNA783643.

Declarations

Conflicts of interest The authors declare that they have no conflicts of interest.

Ethics approval Fish collection was covered by James Cook University Animal Ethics Committee approvals A1641 and A2237.

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