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Detection and diversity of pathogenic *Vibrio* from Fiji

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Summary

Here we investigate the diversity of pathogenic *Vibrio* species in marine environments close to Suva, Fiji. We use four distinct yet complementary analyses – biochemical testing, phylogenetic analyses, metagenomic analyses and molecular typing – to provide some preliminary insights into the diversity of vibrios in this region. Taken together our analyses confirmed the presence of nine *Vibrio* species, including three of the most important disease-causing vibrios (i.e. *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*), in Fijian marine environments. Furthermore, since toxigenic *V. parahaemolyticus* are present on fish for consumption we suggest these bacteria represent a potential public health risk. Our results from Illumina short read sequencing are encouraging in the context of microbial profiling and biomonitoring. They suggest this approach may offer an efficient and cost-effective method for studying the dynamics of microbial diversity in marine environments over time.

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Introduction

Members of the genus *Vibrio* are Gram-negative, motile rods ubiquitous in marine environments (Farmer, 1992). Farmer and colleagues (2003) recognize 12 *Vibrio* species as clinically important human pathogens. Disease-causing vibrios are most commonly associated with gastrointestinal infections. Two well-known examples are *V. cholerae* and *V. parahaemolyticus*. *Vibrio cholerae* is the causative agent of cholera. Seven documented cholera pandemics have claimed millions of lives and the disease continues to affect 3–5 million people annually (Blake, 1994; Wachsmuth *et al*., 1994; Faruque *et al*., 1998; Reeves and Lan, 1998; Gaffga *et al*., 2007). Both toxigenic and non-toxigenic strains of this species occur naturally in aquatic ecosystems and infection is often via contaminated water or food. *Vibrio parahaemolyticus* is a leading cause of bacterial gastroenteritis in the USA (e.g. Daniels *et al*., 2000; McLaughlin *et al*., 2005) and Asia (e.g. Wong *et al*., 1999; Chiou *et al*., 2000). In this case infections are commonly the result of consuming contaminated seafood (Chan *et al*., 1989; Kagiko *et al*., 2001). In addition to gastrointestinal infections vibrios are also associated with infections of wounds, blood, ears, eyes and skin (Penland *et al*., 2000; Oliver, 2005; Oliver and Kaper, 2005; Pruzzo *et al*., 2005).

Several methods are available for the identification of vibrios from clinical and environmental samples. These include phenotypic characterization (e.g. Myhr *et al*., 1991; Alsina and Blanch, 1994; Noguerola and Blanch, 2008), DNA–DNA hybridization (e.g. Wang *et al*., 2011), 16S ribosomal sequences (e.g. Dorsch *et al*., 1992), amplified fragment length polymorphism (e.g. Jiang *et al*., 2000) and multi-locus sequence analysis (e.g. Thompson *et al*., 2005; 2007a,b; Sawabe *et al*., 2007). These approaches have done much to improve our understanding of vibrio diversity. Yet all these methods rely on culturing of the bacteria prior to characterization and this is now widely recognized as biasing the outcomes of subsequent diversity analyses (e.g. Thompson *et al*., 2004). In contrast, environmental metagenomics provides a tool with which to survey the diversity of microbial communities without the need to culture the bacteria first (Green Tringe *et al*., 2005; Hugenholtz and Tyson, 2008; Huson *et al*., 2009). This methodology has already been used to investigate the structure of microbial communities in marine water column and soil environments (e.g. Allen

and Banfield, 2005; Daniel, 2005; Delong, 2005). An extension of describing microbial communities from individual samples is to monitor the structure of such assemblages using samples drawn over time; metagenomics has considerable potential in this context (Lazarevic *et al*., 2009; Caporaso *et al*., 2010; Qin *et al*., 2010; Rodriguez-Brito *et al*., 2010). To date this approach has not been applied to long-term monitoring (e.g. for water quality management) mainly due to the cost of the high-throughput sequencing technologies on which these analyses are now based. However, rapid methodological developments, in particular the development of cheaper short read sequencing protocols, suggests that it may become cost-effective to use metagenomics for the routine monitoring of microbial communities.

Little is known about the distribution and prevalence of disease-causing *Vibrio* species in the South Pacific. In the present study we use four distinct yet complementary analyses – biochemical testing, phylogenetic analyses, metagenomic analyses and molecular typing – to investigate the diversity of vibrios in this region. In the first part of our study, we combine analyses of fish-associated isolates with those of water column DNA diversity of pathogenic *Vibrio* species present in Fijian marine environments and assess the potential health risks posed by these bacteria. In the second part we use the results of our metagenomic analyses to examine the potential and limitations of short read sequencing for routine monitoring of aquatic bacterial communities.

Results

Phenotypic identification of Vibrio *species*

Colony growth on selective TCBS agar suggests that *Vibrio* species are common on fish available from retail outlets in Suva, Fiji. In the present study we detected vibrios on 88.9% (160/180) of the sampled fish; these bacteria were cultured from all retail locations (roadside fish stalls, local fish markets and fish shops) and all three regions of the fish tested (skin, gills and the gut cavity).

Biochemical tests suggest a number of *Vibrio* species were present on sampled fish. Tests provided unequivocal identifications for just a small portion of isolates – *V. cholerae* (2 isolates), *V. metschnikovii* (9 isolates), *V. mimicus* (12 isolates) and *V. navarrensis* (46 isolates). For the remaining 256 isolates our tests are consistent with several candidate species (see *Supporting information* for a complete list of the biochemical test results). Often the unequivocally identified species were not among the candidate species thus suggesting additional diversity is associated with these fish. While we were unable to describe the fish-associated *Vibrio* community in full, our results indicate the presence of potentially pathogenic species. All four of the unequivocally identified species are known to infect humans (Farmer *et al*., 2003) while several pathogens are among the candidate species for the unidentified isolates (e.g. *V. parahaemolyticus* and *V. vulnificus*).

Phylogenetic analyses of V. parahaemolyticus *isolates*

We used phylogenetic analyses of 16S, *recA* and *pyrH* sequences to further examine the diversity of presumptive *V. parahaemolyticus* isolated from fish (see *Supporting information*). Partial 16S rDNA sequences from 22 accessions share 100% identity with previously published *V. alginolyticus*, *V. diabolicus*, *V. natriegens* and *V. parahaemolyticus* sequences. These accessions belong to a clade containing additional representatives of *V. alginolyticus* and *V. parahaemolyticus* with the remaining core group species (i.e. *V. campbellii*, *V. harveyii*, *V. mytili* and *V. rotiferianus*) more distantly related (Fig. 1; Tables S3 and S4). Whereas our 16S analysis did not distinguish between *V. parahaemolyticus* and its close relatives, analyses of *recA* and *pyrH* sequences recovered well-supported clades corresponding to each of the included species (Figs 2 and 3; Table S3 and S4). Fijian *recA* and *pyrH* sequences are a combination of novel forms (e.g. Fiji^{8} and Fiji^{17} for *recA*) and those encountered in previous analyses (e.g. Fiji^{2} is equivalent to *V. parahaemolyticus* SG259).

Although most Fijian accessions were associated with the *V. parahaemolyticus* clade, Fiji^{12} and Fiji^{21} are more closely related to sequences from *V. alginolyticus* in *recA* and *pyrH* analyses (Figs 2 and 3). The *recA* sequence for one non-Fijian *V. parahaemolyticus* accession (strain 28; Gonzalez-Escalona *et al*., 2008) also grouped with the *V. alginolyticus* clade (Fig. 2).

Metagenomic analysis of seawater samples

Metagenomic analyses of total community DNA were used to investigate *Vibrio* diversity in the water column close to Suva (see *Supporting information*). For single end analyses a random sample of 2×10^5 75 bp reads was analysed using MEGAN (Huson *et al*., 2011). MEGAN assigned 23 561 (11.8%) of reads to the NCBI taxonomic hierarchy. *Vibrio* and the related genus *Photobacterium* were both identified in this sample. Reads were assigned to five *Vibrio* species – *V. cholerae*, *V. harveyi*, *V. parahaemolyticus*, *V. shilonii* and *V. vulnificus* – and to strains within three of these (Fig. 4). The number of reads assigned to nodes and the number of phylogenetic lineages identified increased substantially when reads were treated as paired ends (i.e. sequences from each end of a DNA fragment are considered jointly). When the same sample of 2×10^5 reads was analysed in this way, the number of assigned fragments increased to 42 354

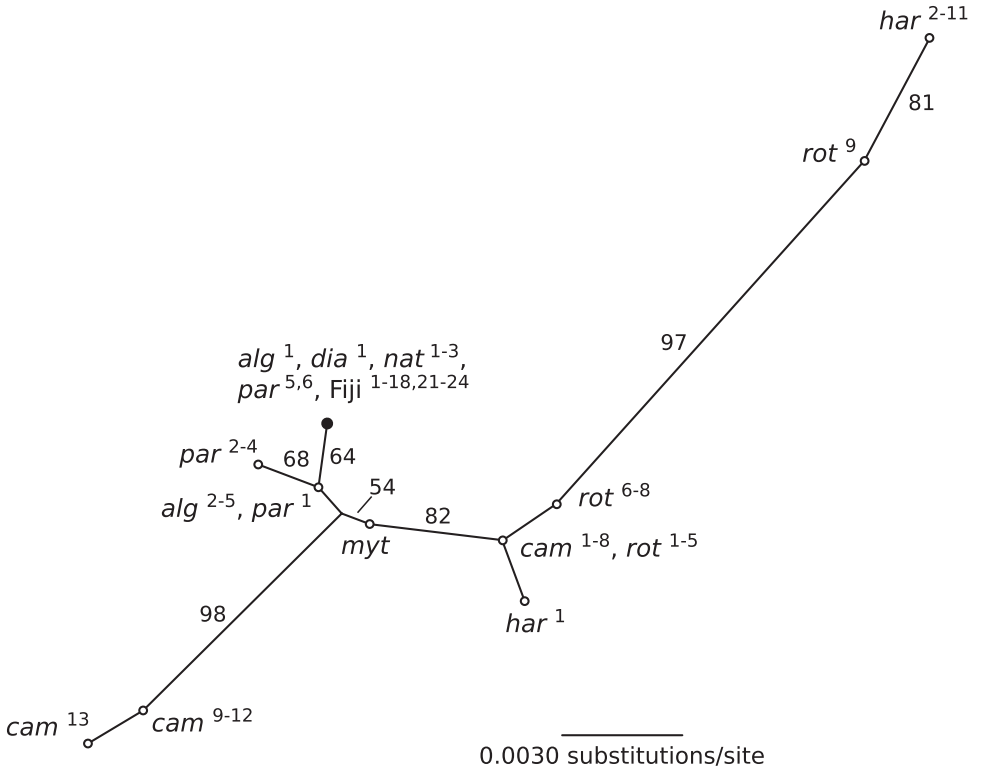

Fig. 1. Maximum likelihood tree based on partial 16S rDNA sequences for presumed *V. parahaemolyticus* isolates and representatives of several closely related *Vibrio* species. Black circles denote nodes with at least one Fijian isolate and white circles those with only non-Fijian species/strains. Species/strains/isolate labels are: *alg*, *V. alginolyticus*; *cam*, *V. campbellii*; *dia*, *V. diabolicus*; Fiji, presumed *V. parahaemolyticus* isolate from Fiji; *har*, *V. harveyi*; *myt*, *V. mytili*; *nat*, *V. natriegens*; *par*, *V. parahaemolyticus*; *rot*, *V. rotiferianus*. Superscript numerals denote strains as described in the Tables S1 and S2. Bootstrap support values are noted when greater than 50%.

(21.2%) with reads assigned to three genera of *Vibrionaceae* (i.e. *Aliivibrio*, *Photobacterium* and *Vibrio*), six *Vibrio* species (i.e. those identified in the single read analysis plus *V. splendidus*) and four *Vibrio* strains (Fig. 4).

The vibrio community identified by metagenomic analyses contained three important human pathogens, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*. In addition, *V. harveyii* has been found to sporadically infect humans. The two remaining species, *V. shilonii* and *V. splendidus*, are non-pathogenic (Farmer *et al*., 2003).

Molecular typing of pathogenicity loci

Both biochemical testing and metagenomic analyses identified potentially pathogenic *Vibrio* species but neither approach specifically evaluates whether these bacteria carry the genetic determinants of pathogenicity. For one species, *V. parahaemolyticus*, we examined the pathogenicity of Fijian strains using molecular typing of loci with known or putative roles in the pathogenicity (see *Supporting information*).

We first used a pair of *V. parahaemolyticus*-specific PCR markers to confirm the identity of presumptive *V. parahaemolyticus* isolates. Results for the *tlh* and *toxR* markers suggested all but one isolate (i.e. Fiji[21]) was *V. parahaemolyticus* (Table S5). Pathogenicity typing used markers for two key pathogenicity loci (i.e. *tdh* and *trh*) plus six loci (i.e. ure, VPA1321, VPA1339, VPA1346, VPA1376 and MTase) with known or putative roles in pathogenicity. The thermostable direct haemolysin (*tdh*) locus was detected for all the confirmed *V. parahaemolyticus* isolates; all the isolates lacked the *tdh*-related haemolysin (*trh*) locus (Table S5). Clinical strains of *V. parahaemolyticus* commonly express one or both of these proteins with *tdh*-producing strains identified in > 90% of cases (Caburlotto *et al*., 2009). For the remaining pathogenicity loci results varied; up to 22 isolates testing positive for any given locus (Table S5).

In contrast, molecular typing of DNA isolated from our seawater samples did not produce positive amplifications for the two *V. parahaemolyticus*-specific nor for any of the eight pathogenicity loci (see *Supporting information*). Since metagenomic analyses indicate *V. parahaemolyticus* is present in the water column we suspect the issue here is sensitivity of the staining protocols used to visualize PCR products. It appears that amplification of loci did not increase copy number sufficiently for taxa-specific and

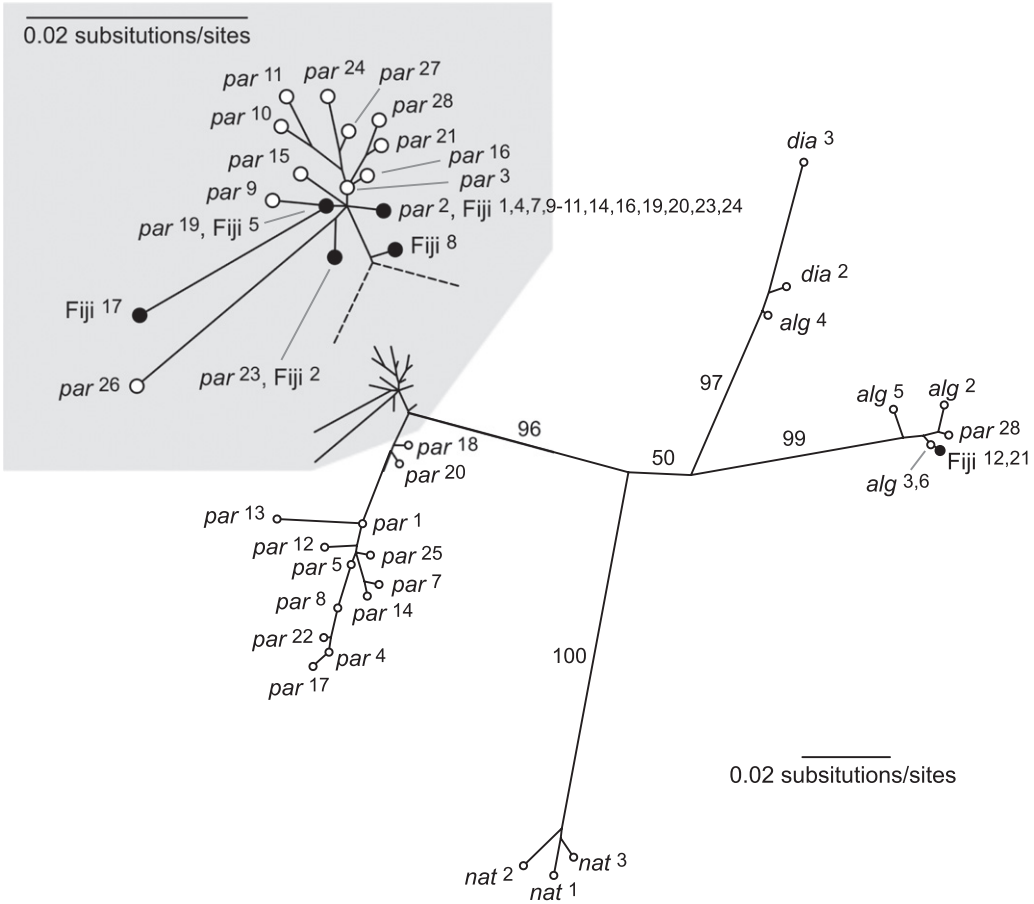

Fig. 2. Maximum likelihood tree based on partial *recA* sequences for presumed *Vibrio parahaemolyticus* isolates and representatives of several closely related *Vibrio* species. Species, strains and isolates are denoted as in Fig. 1. Bootstrap support values are noted when greater than 50%.

pathogenicity markers to be visualized using ethidium bromide and SBYR green staining protocols.

Discussion

Diversity of pathogenic vibrios in Fijian marine environments

Our analyses of isolates from commercially available fish and DNA extracted from the water column confirmed the presence of nine *Vibrio* species in marine environments close to Suva. However, further studies of vibrio diversity in the Pacific are warranted, as we cannot rule out the presence of other taxa. Among those identified from Fiji are three of the most important disease-causing vibrio species. Specifically, we detected *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* in the water column and two of these, *V. cholerae* and *V. parahaemolyticus*, on commercially available fish. Species that more sporadically infect humans were also found (e.g. *V. metschnikovii* and *V. mimicus*).

Phylogenetic analyses and molecular typing of *V. parahaemolyticus* isolates further suggest that this species is represented by several variants in the region. For *recA* and *pyrH* we identified both widespread and novel sequence types. Given the importance of mobile genetic elements and horizontal gene transfer in the evolution of *Vibrio* (e.g. Hazen *et al*., 2010; Pascual *et al*., 2010) these may be best interpreted as suggesting a diverse genetic background for this species in Fijian marine environments. Indeed our results for two fish-associated isolates (e.g. Fiji[21] and Fiji[21]) suggest horizontal genetic exchange. Based on biochemical testing both these isolates are presumptively *V. parahaemolyticus* yet phylogenetic analyses and molecular typing are contradictory. These tests suggest genetic elements have been acquired from *V. alginolyticus*; Pascual and colleagues (2010) have recently reported a similar result for the *rctB* gene. Interestingly, we did not unequivocally identify *V. alginolyticus* in our samples suggesting these transfers may not be specific to Fijian strains.

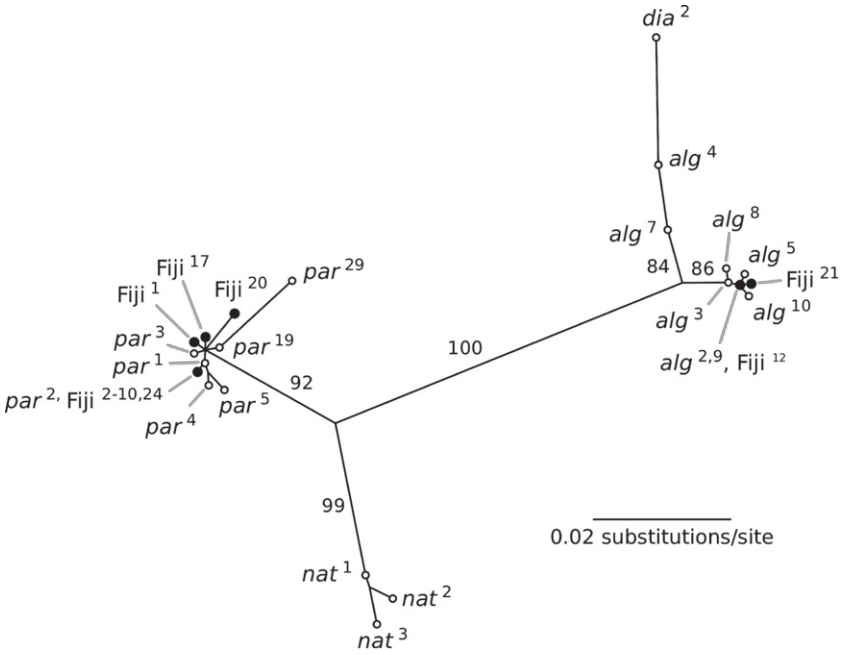

Fig. 3. Maximum likelihood tree based on partial *pyrH* sequences for presumed *Vibrio parahaemolyticus* isolates and representatives of several closely related *Vibrio* species. Species, strains and isolates are denoted as in Fig. 1. Bootstrap support values are noted when greater than 50%.

In general our results suggest a number of pathogenic vibrios occur naturally in Fijian marine environments. In the case of *V. parahaemolyticus* we also show that toxigenic forms of this species are also present. That we find toxigenic vibrios associated with commercially available fish in Fiji is consistent with findings from various other studies (e.g. Chan *et al*., 1989; El hadi *et al*., 2004; Gopal *et al*., 2005). While there are currently no data directly linking gastrointestinal illness to *Vibrio* infection in Fiji such illnesses are common (Fiji Centre for Communicable Disease Control, 2009a,b; 2010). Given that raw fish is frequently eaten in Fiji our results strongly suggest *Vibrio* is a potential health risk.

The potential of high-throughput sequencing approaches for biomonitoring

Our MEGAN analyses indicate that a relatively small sample of short DNA sequences (2×10^5 75 bp reads) is sufficient to distinguish closely related *Vibrio* species and identify strain level lineages. Further, as predicted by Mitra and colleagues (2010), when we treat reads as paired ends the number of DNA fragments that can be unambiguously assigned to the NCBI taxonomic hierarchy is substantially increased. Our results are encouraging since it had been thought that only analyses of long read sequences would provide the resolution necessary to describe the composition of naturally occurring microbial communities.

If, as our analyses suggest, it is possible use short read sequencing to determine the structure of naturally occurring microbial communities this would increase the cost-effectiveness of monitoring such assemblages over time using metagenomics (c.f. long read-based protocols). Further, if it can be shown that a small sample of short reads is sufficient to consistently describe community structure this would result in further cost reductions. For example, the current capacity of Illumina's MiSeq sequencer is 5×10^6 150 bp paired-end reads per run. Assuming 2×10^5 reads are sufficient to describe a community up to 25 indexed samples could be analysed on a single flow cell lane. These observations suggest that it may become cost-effective to use metagenomics for the routine monitoring of microbial communities in the near future.

Limitations to overcome with high-throughput sequencing approaches

One limitation illustrated by the present study involves the detection of toxigenic forms. While we identified potentially pathogenic *Vibrio* species using MEGAN our analyses did not detect genetic loci specifically associated with pathogenicity. Clearly the ability to identify such loci is important in the context of monitoring programmes. The result is, however, not unexpected given the relatively small sample of reads analysed; identifying specific loci is likely to require a larger number of reads than does identifying taxa. While one could increase the number of reads analysed (e.g. from 2×10^5 to 3×10^7 reads, approximately the number of reads generated our Illumina GAII runs) there is no guarantee that this approach would result in detection of individual genes from an environmental sample. We think it is more effective to combine

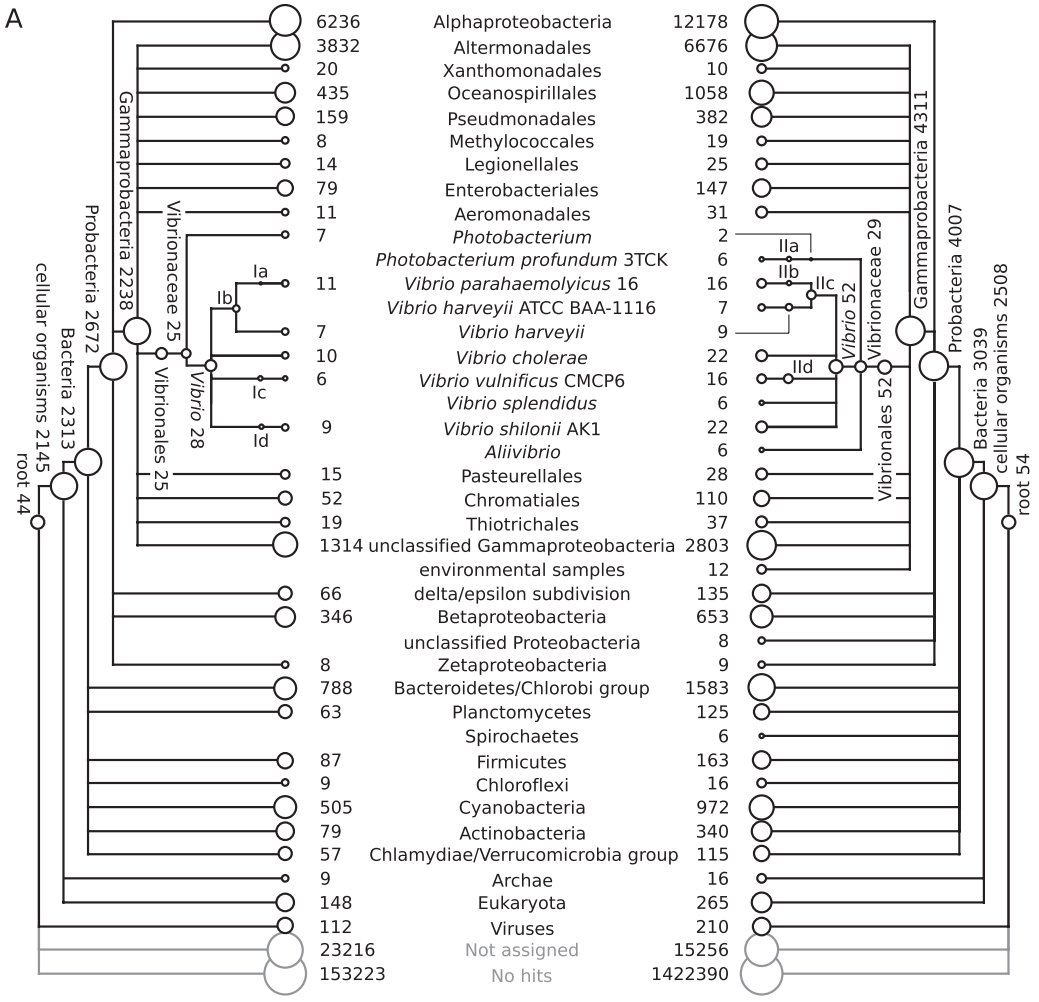
A
6236 Alphaproteobacteria 12178
3832 Altermonadales 6676
20 Xanthomonadales 10
435 Oceanospirillales 1058
159 Pseudmonadales 382
8 Methylococcales 19
14 Legionellales 25
79 Enterobacteriales 147
11 Aeromonadales 31
7 Photobacterium 2
Photobacterium profundum 3TCK 6
11 Vibrio parahaemolyicus 16 16
Vibrio harveyii ATCC BAA-1116 7
7 Vibrio harveyii 9
10 Vibrio cholerae 22
6 Vibrio vulnificus CMCP6 16
Vibrio splendidus 6
9 Vibrio shilonii AK1 22
Aliivibrio 6
15 Pasteurellales 28
52 Chromatiales 110
19 Thiotrichales 37
1314 unclassified Gammaproteobacteria 2803
environmental samples 12
66 delta/epsilon subdivision 135
346 Betaproteobacteria 653
unclassified Proteobacteria 8
8 Zetaproteobacteria 9
788 Bacteroidetes/Chlorobi group 1583
63 Planctomycetes 125
Spirochaetes 6
87 Firmicutes 163
9 Chloroflexi 16
505 Cyanobacteria 972
79 Actinobacteria 340
57 Chlamydiae/Verrucomicrobia group 115
9 Archae 16
148 Eukaryota 265
112 Viruses 210
23216 Not assigned 15256
153223 No hits 1422390
root 44
cellular organisms 2145
Bacteria 2313
Probacteria 2672
Gammaprobacteria 2238
Vibrionales 25
Vibrionaceae 25
Vibrio 28
Ia
Ib
Ic
Id
Gammaprobacteria 4311
Probacteria 4007
Bacteria 3039
cellular organisms 2508
root 54
Vibrionaceae 29
Vibrio 52
Vibrionales 52
IIa
IIb
IIc
IId
B
Number of reads
Paired-end reads
Single reads
284 138
138 122
179 90
54 28
22 14
16 11
16 7
7 0
22 10
29 11
16 6
6 0
22 13
22 9
Vibrionales
Vibrionaceae
Vibrio
Vibrio harveyi group
Vibrio parahaemolyticus
Vibrio parahaemolyticus 16
Vibrio harveyi
Vibrio harveyi ATCC BAA-1116
Vibrio cholerae
Vibrio vulnificus
Vibrio vulnificus CMCP6
Vibrio splendidus
Vibrio shilonii
Vibrio shilonii AK1

Fig. 4. MEGAN profile of taxonomic diversity based on GAII sequencing of microorganisms from seawater samples.
A. MEGAN trees describing the taxonomic affiliation of GAII reads identified by BLASTX. The left hand tree is based on analysis of single reads, right hand tree on paired end reads. Size of circles at nodes is proportional to the number of sequence reads assigned to the corresponding node (the actual number of reads assigned to each node is also given). Some taxon names and associated read numbers have been omitted for clarity. These nodes are labelled as follows: Ia, *Vibrio parahaemolyticus* (3 reads); Ib, *V. harveyi* group (7 reads); Ic, *V. vulnificus* (5 reads); Id, *V. shilonii* (4 reads); IIa, *Photobacterium profundum* (4 reads); IIb, *V. parahaemolyticus* (6 reads); IIc, *V. harveyi* group (16 reads); IId, *V. vulnificus* (13 reads). 'Not assigned' and 'No hits' categories include reads for which the bit scores of matches were below the threshold or were unmatched to sequences in the NCBI-nr database respectively.
B. Numbers of reads assigned to selected nodes of the *Vibrionales* in single and paired end MEGAN analyses. In this graph, read numbers are cumulative; for example, the value *Vibrionaceae* is the sum of reads specifically assigned to this node plus all taxonomic levels contained within this group (i.e. genera, species and strains).

metagenomic analyses that describe the overall structure of the microbial community with molecular typing of specific loci to determine whether toxigenic forms are present. However, the detection of PCR products with standard staining protocols is a problem. A much more sensitive approach is to use the high-throughput sequencing apparatus to detect PCR products. This could easily be done by spiking the environmental DNA sample prior to sequencing with aliquots of PCR amplifications for pathogenicity loci; in each case the template for PCR amplifications would be the same environmental DNA sample.

A second potential issue is completeness of the reference database used by MEGAN. If target organisms are poorly represented then both the number and phylogenetic resolution of assignments are likely to be limited (Huson *et al*., 2007; Morgan *et al*., 2010). Here we used a database (NCBI non-redundant protein, February 2010 version) that contained 57 complete *Vibrio* genomes. All the strain-level lineages identified by MEGAN are represented by complete genome sequences (e.g. *V. harveyi* ATCC BAA-1116 and *V. parahaemolyticus* 16) suggesting that the database needs to contain complete, or nearly so, genomes in order to differentiate very closely related taxa. At face value this is a potential weakness of the methodology since not all lineages will be represented in this way. However, we suspect that the clinical importance of pathogens, which are often the targets of monitoring programmes, will result in them being over-represented in databases and thus readily identified. More generally, the continued growth of reference databases should reduce the problem of under-representation over time.

Conclusion

Our study provides a first insight into the diversity of *Vibrio* species in Fijian marine environment. Understanding both the distribution and diversity of vibrios in the Pacific Islands is an important step towards managing the health risks posed by these bacteria. Metagenomic analyses based upon high-throughput sequencing protocols will likely have an important role to play in helping assess the risks posed by vibrios and other disease-causing microbes in naturally occurring communities.

Acknowledgements

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Experimental procedures.

Table S1. Biochemical test results for colonies that were green or green/blue on selective TCBS agar.

Table S2. Biochemical test results for colonies that were yellow on selective TCBS agar.

Table S3. GenBank accession details for DNA sequences from non-Fijian *Vibrio* species/strains included in phylogenetic analyses.

Table S4. GenBank accession details for DNA sequences from presumed *Vibrio parahaemolyticus* isolates included in phylogenetic analyses.

Table S5. Results of molecular typing for isolates presumed to be *Vibrio parahaemolyticus*.

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