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Genetic and phylogenetic evidence for horizontal gene transfer among ecologically disparate groups of marine *Vibrio*

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Abstract

Vibrio represents a diverse bacterial genus found in different niches of the marine environment, including numerous genera of marine sponges (phylum Porifera), inhabiting different depths and regions of benthic seas, that are potentially important in driving adaptive change among *Vibrio* spp. Using 16S rRNA gene sequencing, a previous study showed that sponge-derived (SD) vibrios clustered with their mainstream counterparts present in shallow, coastal ecosystems, suggesting a genetic relatedness between these populations. Sequences from the *topA*, *ftsZ*, *mreB*, *rpoD*, *rctB* and *toxR* genes were used to investigate the degree of relatedness existing between these two separate populations by examining their phylogenetic and genetic disparity. Phylogenies were constructed from the concatenated sequences of the six housekeeping genes using maximum-parsimony, maximum-likelihood and neighbour-joining algorithms. Genetic recombination was evaluated using the incongruence length difference test, Split decomposition and measuring overall compatibility of sites. This combined technical approach provided evidence that SD *Vibrio* strains are largely genetically homologous to their shallow-water counterparts. Moreover, the analyses conducted support the existence of extensive horizontal gene transfer between these two groups, supporting the idea of a single panmictic population structure among vibrios from two seemingly distinct, marine environments.

The genus *Vibrio* shows remarkable biodiversity in a variety of geographical locales and eukaryotic hosts (Thompson et al., 2004a). Several *Vibrio* species are known pathogens of fishes, corals, molluscs and humans (Cotanche et al., 1994; Dziejman et al., 2002; Thompson et al., 2004a). Environmental characteristics, such as temperature, salinity and pH, are all important factors in the ecology of vibrios (Thompson et al., 2004b; Beaz-Hidalgo et al., 2010).

Associations among many of these vibrios with both phyto- and zooplanktonic organisms have been documented (Lipp et al., 2002; Huq et al., 2005; Turner et al., 2009). Interestingly, vibrios have been shown to express a specific chitinase that enables the bacteria to metabolize zooplankton (e.g. copepods) exoskeletons, an important feature that mediates *Vibrio* distribution and spread (Heidelberg et al., 2002; Hunt et al., 2008). Indeed, as some vibrios are pathogenic to humans or other aquatic eukaryotes, dispersion of these pathogens within planktonic blooms carries significant health and socio-economic impact (Colwell and Huq, 1994; Huq et al., 2005).

In addition to their association with planktonic blooms, vibrios are consistently found in benthic zones, particularly ocean sediments (Kaneko and Colwell, 1978). Furthermore, it has been reported that *Vibrio* isolates can be obtained from the phylum Porifera both from shallow marine niches and from greater depths (i.e.

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ranging from 13 to 908 m) (Santavy et al., 1990; Hentschel et al., 2001; Webster et al., 2001; Brück et al., 2010; Hoffmann et al., 2010b; Kumaran et al., 2010). Sponges are sessile, filter-feeding organisms that comprise a very diverse component of marine benthic communities which form close associations with a wide variety of micro-organisms (Taylor et al., 2007). Previous reports have noted the specific roles that certain *Vibrio* species play within sponge microcosms, being implicated in both secondary metabolism and the synthesis of antibacterial compounds (Elyakov et al., 1991; Oclarit et al., 1994; Webster et al., 2001). The extensive biological and genetic diversity among vibrios associated with this unique marine environment is only now being realized (Hoffmann et al., 2010b), and the genetic and ecological relationship between sponge-derived (SD) vibrios and their nearshore counterparts (i.e. vibrios associated with planktonic blooms) remain largely unclear. Many SD vibrios appear to belong to the Harveyi clade (Hoffmann et al., 2010b), currently comprising 11 *Vibrio* species (Cano-Gomez et al., 2010; Chimetto et al., 2010; Lin et al., 2010; Yoshizawa et al., 2010). Consequently, elucidation of *Vibrio* population dynamics within these natural marine environments has become an important area of study.

Here we provide extensive phylogenetic and genetic evidence for horizontal gene transfer (HGT) among SD vibrios and their nearshore counterparts, supporting the existence of a panmictic population structure between these two groups of *Vibrio* strains.

Materials and methods

Bacterial strains, characterization and DNA isolation

The 23 *Vibrio* isolates previously characterized by Hoffmann et al. (2010b) as well as an additional 29 vibrios collectively used in this study were isolated from marine sponges (52) and a sea cucumber (one) from depths between 0.3 and 750 m at several geographical locales (Table 1) using previously described methods (Sfanos et al., 2005). Briefly, samples (1 cm^3) were removed aseptically from freshly collected sponges and ground in sterile artificial seawater using a sterilized Waring blender (see Supporting information). The resulting suspension was serially diluted and plated onto various nutrient agars. Plates were incubated at 20–22 °C for 1–6 months after which time each colony type was isolated and entered into the Harbor Branch Marine Microbial Culture Collection (Gunasekera et al., 2005).

Reference strains from the Harveyi clade (Table 2), originally isolated from a variety of different globally distinct locations and used to represent nearshore strains, were obtained from the American Type Culture Collection (ATCC) and Belgian Co-ordinated Collection of Microorganisms (BCCM). Freeze-dried (lyophilized) cultures were revived according to protocols provided by the ATCC and BCCM curators. 16S rRNA gene sequencing was used to further confirm the identity of the reference strains. Of particular note, in the course of evaluating the reference strain collection, it was determined that the MLSA typing scheme data flagged ten separate *V. harveyi* strains as being misidentified, with seven associating strongly with *V. campbellii*, and the remaining three clustering tightly with *V. communis* (Hoffmann et al., 2012).

Vibrio strains were cultured in trypticase soy broth (TSB; Oxoid) supplemented with 1% NaCl (w/v) at 28 °C with shaking (112 r.p.m.) for 24 h and on trypticase soy agar (TSA; BD, Franklin Lakes, NJ, USA) supplemented with 1% NaCl (w/v) at 28 °C for 24 h. Additionally, growth on thiosulfate-citrate-bile salts-sucrose (TCBS; BD, Franklin Lakes, NJ, USA) agar, which is highly selective for the isolation of vibrios, was tested. Genomic DNA was isolated from pure *Vibrio* cultures using the MasterPure™ DNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA).

Multiplex PCR

Testing for the presence of the thermostable-direct hemolysin (*tdh*), *tdh*-related hemolysin (*trh*), the *V. vulnificus* hemolysin gene (*vllY*) and cytolysin-hemolysin (*vvhA*), *V. mimicus* hemolysin gene (*vmhA*) and the *V. cholerae* toxin (*ctx*) was accomplished using a multiplex PCR system containing target-specific primers at 200, 300, 300, 100, 100 and 500 nM, respectively. PCR was performed in a 50-μL volume with HotStarTaq Master Mix (Qiagen, Valencia, CA, USA) containing 400 μM dNTP (each of dATP, dCTP, dGTP and dTTP), 5 U of HotStart *Taq* Polymerase, 1 × *Taq* polymerase buffer, 3.0 mM $MgCl_2$ and ~100 ng of DNA template. The optimized amplification programme was initiated with an enzyme activation cycle at 95 °C for 15 min, followed with ten cycles at 95 °C for 30 s, 68–59 °C (decreasing at 1 °C per cycle) for 30 s and 72 °C for 1 min. Once completed, full target amplification was achieved with 27 cycles of 95 °C for 30 s, 59 °C for 30 s and 72 °C for 1 min. The process was finished with a single cycle at 72 °C for 7 min and stored at 4 °C until analysed.

Thermolabile hemolysin (*tlh*) was tested for using a second PCR system. Primer sequences were specifically designed to target *tlh* alleles specific to those species comprising the Harveyi clade. PCR was performed in a 50-μL volume containing 300 μM dNTP, 5 U of HotStart *Taq* Polymerase, 1 × *Taq* polymerase buffer, 2.0 mM $MgCl_2$ and 500 nM concentration of each *tlh*-specific primer with ~100 ng of DNA template. The amplification programme was 95 °C for 15 min,

Table 1
Benthic *Vibrio* isolates used in this study

Microbe ID*	Taxa of source	Geographical source	Depth (m)	GenBank accession no. of isolate	Closest GenBank match	Similarity (%)	GenBank accession no. of relative
L647	*Corallistes* sp.	USA, Florida, Gulf of Mexico	204	GU223581	*V. campbellii* ATCC 33863	99	HM771344
K875	*Erylus* sp.	Bahamas	143	GU223582	*V. campbellii* ATCC BAA-1116	100	CP000789
N376	*Scleritoderma cyanea*	Curaçao	242	GU223583	*V. harveyi* EHP7	99	FJ227113
N377	*Scleritoderma cyanea*	Curaçao	242	GU223584	*V. harveyi* EHP7	99	FJ227113
J036	*Axinella* sp.	USA, Florida, Atlantic seaboard	46	GU223585	*V. communis* R-40900	99	GU078670
J231	Halichondrida	Puerto Rico	144	GU223586	*V. shilonii* SW-2*	100	AY911395
K882	*Erylus* sp.	Bahamas	143	GU223587	*V. communis* LMG 20370	99	AJ345066
K878	*Erylus* sp.	Bahamas	143	GU223588	*V. campbellii* ATCC BAA-1116	100	CP000789
K921	*Erylus* sp.	Bahamas	143	GU223589	*V. harveyi* SW-3	99	AY911369
K873	*Erylus* sp.	Bahamas	143	GU223590	*V. communis* R-40501	99	GU078674
K872	*Erylus* sp.	Bahamas	143	GU223591	*V. campbellii* ATCC BAA-1116	100	CP000789
H455	*Bubaris* sp.	USA, Florida, Atlantic seaboard	79	GU223592	*V. gigantis* CAIM 25	99	EF094888
A975	Pachastrellidae	Barbados	125	GU223593	*V. parahaemolyticus* CM12	100	EU660326
N382	*Scleritoderma cyanea*	Curaçao	242	GU223594	*V. harveyi* EHP7	99	FJ227113
N380	*Scleritoderma cyanea*	Curaçao	242	GU223595	*V. harveyi* EHP7	99	FJ227113
K350	*Scleritoderma cyanea*	Curaçao	242	GU223596	*V. harveyi* EHP7	99	FJ227113
J207	*Higginsia strigilata*	USA, Florida, Atlantic seaboard	61	GU223597	*V. harveyi* LA08005	100	GQ180186
J555	Halichondrida	Puerto Rico	224	GU223598	*V. mediterranei* ATCC 43341	100	HM771351
K324	*Scleritoderma cyanea*	Curaçao	242	GU223599	*V. harveyi* EHP7	99	FJ227113
K323	*Scleritoderma cyanea*	Curaçao	242	GU223600	*V. harveyi* EHP7	99	FJ227113
N384	*Scleritoderma cyanea*	Curaçao	242	GU223601	*V. brasiliensis* LMG 20546	98	HM771338
N418	*Scleritoderma cyanea*	Curaçao	242	GU223602	*V. scophthalmi* LMG 19158	99	HM771340
J462	Pachastrellidae	Puerto Rico	379	GU223603	*Aliivibrio fischeri* ATCC 14546	100	HM771347
C259	*Chondrosia collectrix*	Bahamas	0.3	JF836186	*V. harveyi* LA08005	100	GQ180186
F048	*Topsentia bahamensis*	Bahamas	21.3	JF836179	*V. communis* LMG 20370	99	AJ345066
F052	Porifera	Bahamas	21.3	JF836182	*V. communis* R-40900	100	GU078670
F064	Porifera	Bahamas	15.2	JF836180	*V. communis* LMG 20370	99	AJ345066
F077	Aplysiniidae	Bahamas	83.8	JF836183	*V. communis* R-40900	100	GU078670
F078	Aplysiniidae	Bahamas	83.8	JF836184	*V. communis* R-40501	99	GU078674
B840	*Discodermia* sp.	Bahamas	182.9	JF836166	*V. alginolyticus* HN08335	99	FJ906749
M028	Choristida	Bahamas	410.0	JF836187	*V. harveyi* LA08005	100	GQ180186
P274	*Discodermia* sp	Bahamas	158.5	JF836181	*V. communis* LMG 20370	99	AJ345066
V013	*Leiodermatium* sp.	Bahamas	630.9	JF836168	*V. harveyi* EHP7	99	FJ227113
V014	*Leiodermatium* sp.	Bahamas	630.9	JF836169	*V. harveyi* EHP7	99	FJ227113
V056	*Leiodermatium* sp.	Bahamas	630.9	JF836170	*V. harveyi* EHP7	99	FJ227113
V058	*Leiodermatium* sp.	Bahamas	630.9	JF836171	*V. harveyi* EHP7	99	FJ227113
W016	Porifera	USA, Florida, Gulf of Mexico	70.4	JF836172	*V. parahaemolyticus* CM12	100	EU660326
W017	Porifera	USA, Florida, Gulf of Mexico	70.4	JF836173	*V. parahaemolyticus* CM12	100	EU660326
W018	Porifera	USA, Florida, Gulf of Mexico	70.4	JF836174	*V. parahaemolyticus* CM12	100	EU660326
W019	Geodiidae	Florida, Dry Tortugas	115.5	JF836175	*V. parahaemolyticus* CM12	100	EU660326
W020	Geodiidae	Florida, Dry Tortugas	115.5	JF836176	*V. parahaemolyticus* CM12	100	EU660326

Table 1
(*Continued*)

Microbe ID*	Taxa of source	Geographical source	Depth (m)	GenBank accession no. of isolate	Closest GenBank match	Similarity (%)	GenBank accession no. of relative
W046	Geodiidae	USA, Florida Miami Terrace Reef	304.5	JF836177	*V. parahaemolyticus* CM12	100	EU660326
W047	Geodiidae	USA, Florida Miami Terrace Reef	304.5	JF836178	*V. harveyi* EHP7	99	FJ227113
J608	Scleritodermidae	Puerto Rico	364.2	JF836167	*V. alginolyticus* HN08801	99	FJ906750
J821	Holothuroidea	Puerto Rico	299.6	JF836185	*V. communis* R-40504	99	GU78675
D725	*Poecilastra* sp.	Madiera	364	JF836195	*V. scophthalmi* LMG 19158	99	HM771340
V007	*Leiodermatium* sp.	Bahamas	630.9	JF836190	*V. gigantis* CAIM 25	99	EF094888
V097	*Lyssacinosida*	Florida, St. Augustine	751	JF836188	*V. penaeicida* DSM 14398	99	AJ421444
V098	*Lyssacinosida*	USA, Florida, St. Augustine	751	JF836189	*V. penaeicida* DSM 14398	99	AJ421444
W048	Geodiidae	USA, Florida Miami Terrace Reef	305	JF836191	*V. splendidus* PB1-10rrnG	99	EU091331
W208	*Smenospongia* sp.	Bahamas	723	JF836192	*V. splendidus* PB1-10rrnG	99	EU091331
W221	*Smenospongia* sp.	Bahamas	723	JF836193	*V. splendidus* PB1-10rrnG	99	EU091331
W676	Raspailliidae	USA, Florida Pourtales Terrace	300	JF836194	*V. splendidus* PB1-10rrnG	99	EU091331

*The microbe ID was given by HBOI and entered into the Harbor Branch Marine Microbial Culture Collection (Gunasekera et al., 2005).

followed with ten cycles at 95 °C for 30 s, 65–56 °C (decreasing at 1 °C per cycle) for 20 s and 72 °C for 45 s. Afterwards, complete amplification was achieved with 35 cycles of 95 °C for 30 s, 56 °C for 20 s and 72 °C for 45 s. The process was finished with a single cycle at 72 °C for 1 min and stored at 4 °C. All PCR reactions contained primers for amplification of the 16S rRNA gene as a reaction-positive control. The primer sequence, concentration of each primer and the amplification size for each target are listed in Table 3.

16S rRNA gene sequencing, ISR-typing

Sequence analysis of the 16S rRNA gene and 16S–23S rRNA intergenic spacer region (ISR) were performed as previously described (Hoffmann et al., 2010a). The primer sequences and concentration for each primer are listed in Table 3. 16S rRNA gene amplicons were sequenced by Amplicon Express (Pullman, WA). Sequence similarities were determined using the Basic Local Alignment Search Tool (BLAST) operated by the National Center for Biotechnology Information (NCBI) website (Altschul et al., 1990). ISR PCR amplicons were resolved by capillary gel electrophoresis using an Agilent BioAnalyzer 2100 and the Agilent DNA 7500 Assay Protocol (Agilent Technologies, Santa Clara, CA, USA).

Multi-locus sequence analysis

Multi-locus sequence analysis (MLSA) of six housekeeping genes, encoding topoisomerase I (*topA*), a cell division protein (*ftsZ*), actin-like cytoskeleton protein (*mreB*), RNA polymerase σ^{70} factor (*rpoD*), replication origin-binding protein (*rctB*) and transmembrane regulatory protein (*toxR*), was carried out, as previously described for *topA*, *ftsZ* and *mreB* (Thompson et al., 2007) and for *rctB*, *rpoD* and *toxR* (Pascual et al., 2010). Primer sequences, concentrations and amplification size for each target are listed in Table 3. To minimize PCR products derived from mispriming events, the actual amplification was initiated with a ''touchdown'' PCR step consisting of ten cycles starting at an annealing temperature of 9 °C above the targeted annealing temperature and decreasing at 1 °C per cycle until the desired annealing temperature had been achieved. PCR amplicons were sequenced by Molecular Cloning Laboratories (MCLAB, San Francisco, CA, USA). DNA sequences were edited and assembled using the Geneious Pro v.5.3.5 (Drummond et al., 2011) software. All DNA sequences for the MLSA analyses were aligned using MAFFT (Katoh et al., 2002).

Phylogenetic analyses

Phylogenetic analyses were performed using PAUP* (Phylogenetic Analysis Using Parsimony) ver. 4.10b (Swofford, 2003), MEGA (Molecular Evolutionary Genetics Analysis) ver. 4 (Tamura et al., 2007), GARLI (Genetic Algorithm for Rapid Likelihood Inference) ver. 0.951-GUI (Zwickl, 2006) and MrBayes ver. 3.1.2 (Huelsenbeck and Ronquist, 2001). Most-parsimonious (MP) trees were sought using heuristic search methods combined with tree bisection-reconnection branch (TBR) swapping and 100 random, stepwise addition replicates. Strict consensus methods were applied to

Table 2
Bacterial reference strains used in this study

Strain	Species	Origin
ATCC 33840	*V. alginolyticus*	Blister, Florida, USA
ATCC 17749	*V. alginolyticus*	Spoiled horse mackerel causing food poisoning, Japan
ATCC 43515	*V. alginolyticus*	Shark mouth, Bahamas
ATCC 17750	*V. alginolyticus*	Gill of horse mackerel
ATCC 51160	*V. alginolyticus*	Stainless steel plate immersed in Biscayne Bay, Florida, USA
ATCC 33839	*V. alginolyticus*	Seawater, Oahu, Hawaii, USA
ATCC 33787	*V. alginolyticus*	Seawater, Oahu, Hawaii, USA
LMG 11650	*V. alginolyticus*	Seawater, Oahu, Hawaii, USA
LMG 2174	*V. alginolyticus*	Port Hueneme, CA, USA
LMG 3418	*V. alginolyticus*	Cured hides, South Africa
LMG 10943	*V. alginolyticus*	Surface water, Red Sea
LMG 11213	*V. alginolyticus*	Sri Lanka
LMG 23675	*V. alginolyticus*	Seawater, Japan
LMG 23676	*V. alginolyticus*	Seawater, Japan
LMG 23870	*V. alginolyticus*	Clam, Spain
AK 1296-A2-1	*V. alginolyticus*	Oyster, Alaska, USA/2004
ATCC 33863	*V. campbellii*	Seawater, Oahu, Hawaii, USA
ATCC 33865	*V. campbellii*	Seawater, Oahu, Hawaii, USA
LMG 11216	*V. campbellii*	Seawater, Hawaii, USA
LMG 11256	*V. campbellii*	Seawater, Hawaii, USA
LMG 11257	*V. campbellii*	Seawater, Hawaii, USA
LMG 21361	*V. campbellii*	Seawater, Mexico
LMG 21362	*V. campbellii*	Seawater, Mexico
LMG 21363	*V. campbellii*	Diseased *Penaeus monodon* juvenile, Negros Island, Philippines
ATCC BAA-1116*	*V. campbellii*	Marine ocean isolate, 1993
LMG 11658*	*V. campbellii*	Seawater, Oahu, Hawaii, USA
LMG 22891*	*V. campbellii*	Shrimp, Mexico
LMG 22893*	*V. campbellii*	Diseased shrimp (*Penaeus* sp.) isolate, Mexico/1995
LMG 16862*	*V. campbellii*	Oyster, Spain
LMG 16863*	*V. campbellii*	Oyster, Spain
LMG 16829*	*V. campbellii*	Black tiger prawn, Thailand
LMG 11660*	*V. campbellii*	Salton Sea, California, USA
LMG 11659*	*V. communis*	Seawater, Hawaii, USA
LMG 10948*	*V. communis*	Seawater, Hawaii, USA
LMG 4043*	*V. communis*	Seawater, Israel
LMG 25430	*V. communis*	Mucus of the endemic coral *Mussismilia hispida*, São Paulo, Brazil
LMG 20370	*V. communis*	Digestive gland of white shrimp (*Litopenaeus vannamei*), Ecuador
ATCC 35084	*V. harveyi*	Brown shark (*Carcharhinus plumbeus*) kidney isolate, Baltimore, USA/1982
ATCC 43516	*V. harveyi*	Shark mouth, Bahamas
ATCC 33868	*V. harveyi*	Seawater, Puerto Rico
LMG 4044	*V. harveyi*	Dead amphipod (*Talorchestia* sp.) isolate, Woods Hole, USA/1935
ATCC 17802	*V. parahaemolyticus*	Shirashu food poisoning, Japan/1951
ATCC 43996	*V. parahaemolyticus*	Cockles causing fatal food poisoning, England
ATCC 27519	*V. parahaemolyticus*	Shrimp involved in food poisoning in Louisiana, USA
ATCC BAA-239	*V. parahaemolyticus*	Faeces, human, Calcutta, India
ATCC 14048	*V. natriegens*	Mud from a salt marsh, Sapelo Island, USA
ATCC 33898	*V. natriegens*	Seawater, Oahu, Hawaii, USA
LMG 21460	*V. rotiferianus*	Rotifer (*Brachionus plicatilis*) water isolate, Ghent, Belgium/1999

*Originally deposited as *V. harveyi* (Hoffmann et al., 2011; Lin et al., 2010).

reduce the number of equally parsimonious cladograms, such that they could be assembled into a single tree. Neighbour-joining (NJ) tree analyses were performed using the Jukes–Cantor correction available in PAUP*. Bootstrap analyses for character support for tree nodes were determined using 1000 replications for both the MP and NJ analyses. Further genetic distances were generated according to the Jukes–Cantor correction in MEGA.

Maximum-likelihood (ML) trees were generated in GARLI and visualized in PAUP*. The optimal models of nucleotide substitution were estimated with jModeltest ver. 0.1.1 (Posada, 2008) using the Akaike Information Criterion (AIC). Parameter space was searched for the best tree with simultaneous estimation for model parameters using an ML search. Twenty runs were performed. Branch support was determined by 100 ML bootstrap iterations with Bayesian posterior probability.

Table 3
List of primers used in this study

Gene	Sequence (5′–3′)	Concentration (nM)	Amplification size (bp)
5′ 16S*	GTTTGATCATGGCTCAGATTG	300	1408
3′ 16S*	CTACCTTGTTACGACTTCACC-	300	
5′ 16S (16S.6)*	ACTGGGGTGAAGTCGTAACA	300	16S–23S ISR
3′ 23S (23S.1)*	CTTCATCGCCTCTGACTGC	300	16S–23S ISR
5′ *rpoD* (70F)†	ACGACTGACCCGGTACGCATGTAYATGMGNGARATGGGNACNGT	1000	780
3′ *rpoD* (70R)†	ATAGAAATAACCAGACGTAAGTTNGCYTCNACCATYTCYTTYT	1000	
5′ *rctB* (rctBs)†	ATHGARTTYACNGAYTTYCARYTNCAY	1000	591
3′ *rctB* (rctBas)†	YTTNCTYTGHATNGGYTCRAAYTCNCCRTC	1000	
5′ *toxR* (toxRs)†	GANCARGGNTTYGARGTNGAYGAYTC	1000	477
3′ *toxR* (toxRas)†	TTDKKTTGNCCNCYNGTVGCDATNAC	1000	
5′ *topA* (400F)‡	GAGATCATCGGTGGTGATG	300	800
3′ *topA* (1200 R)‡	GAAGGACGAATCGCTTCGTG	300	
5′ *mreB* (12F)‡	ACTTCGTGGCATGTTTTC	300	1000
3′ *mreB* (999R)‡	CCGTGCATATCGATCATTTC	300	
5′ *ftsZ* (75F)‡	GCTGTTGAACACATGGTACG	300	750
3′ *ftsZ* (700R)‡	GCACCAGCAAGATCGATATC	300	
5′ *trh*	AARTGGTTAAGCGCCTATATGAC	300	274
3′ *trh*	ATATGTCCATTTCCGCTCTCATA	300	
5′ *tdh*	GGTTCTGATGAGATATTGTTTGTTG	200	332
3′ *tdh*	GGAATAGAATCTTCATCTTCACCAA	200	
5′16S	AAGAAGCACCGGCTAACTCC	150	205
3′16S	CGCATTTCACCGCTACACC	150	
5′*vllY*	GGAAGTCGCCAAGCATCG	300	875
3′*vllY*	CCTTCATTGCCTTTACGCTG	300	
5′*vvhA*	CGCCTTACCCTACTCTGCTG	100	373
3′*vvhA*	TGACATCGCTGACAATCGC	100	
5′*vmhA*	TAGCCAAGTCGCGGATAAC	100	445
3′*vmhA*	AGTACACTCTTCATCGCTGATTG	100	
5′*ctx*	AGCAGTCAGGTGGTCTTATGC	500	544
3′*ctx*	GTATTACTGATCGATGATCTTGGAG	500	
5′*tl*	ATCTTTAAYGCDTCMCAATGG	500	276
3′*tl*	ATGAARTCATTYARHCCAAACTC	500	
5′16S	AAGAAGCACCGGCTAACTCC	100	205
3′16S	CGCATTTCACCGCTACACC	100	

*Hoffmann et al. (2010a).

†Pascual et al. (2010).

‡Thompson et al. (2007).

The parameters of sequence evolution estimated from the final ML tree were used for Bayesian character support methods with one million generations and discarding 25% of the tree samples.

To assess the extent of recombination between SD and nearshore vibrios, the powerful principles of congruence and compatibility were used. Congruence between the six housekeeping genes, *ftsZ*, *mreB*, *topA*, *rctB*, *rpoD* and *toxR*, was assessed using the incongruence length differences (ILD) test, also known as the Partition Homogeneity Test, available in PAUP* (Farris et al., 1994). ILD tests were individually performed on strains in group 1 (*V. campbellii*), group 2 (*V. communis*), group 3 (*V. harveyi*) and group 4 (*V. alginolyticus*), initially with SD and reference strains together and then separately, with 1000 data partitions using heuristic search methods and ten random, stepwise addition replicates (Allard et al., 1999). Single, six-way partitions were conducted with all six housekeeping genes run simultaneously against one another to look for all possible incongruences in the data matrix. Split decomposition was performed using the SplitsTree ver. 4.11.3 (Huson and Bryant, 2006) computer software. All analyses were done using Hamming distances with informative sites only. Additionally, the overall compatibility of sites was measured using COMPATDNA, a Windows-based program (Bell et al., 2010) based on the compatibility algorithm of Jakobsen and Easteal (1996). Only binary sites (informative sites containing exactly two distinct nucleotides) were included in the analysis.

The nucleotide sequences determined in this study are available at GenBank under accession numbers: *rctB*, JF836196–JF836281; *ftsZ*, JF836282–JF836367; *rpoD*, JF930347–JF930406, JQ015323–JQ015348; *mreB*, JF93 0407–930491, JQ015322; *topA*, JF930492–JF930552, JQ015349–JQ015373; *toxR*, JF930553–JF930638.

Results

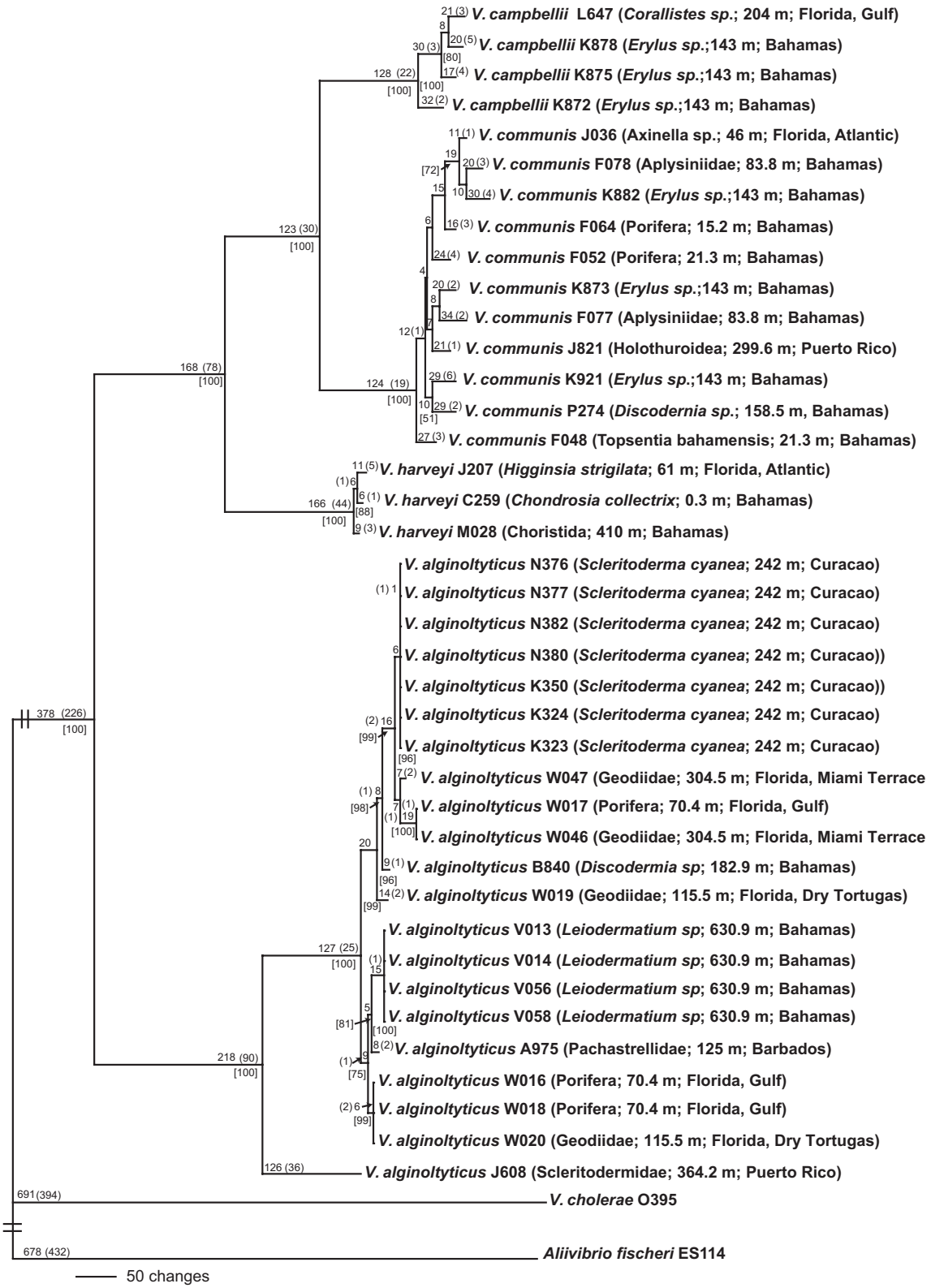
Fifty-three *Vibrio* isolates were recovered from marine sponges (phylum Porifera) at the Harbor Branch Oceanographic Institution. 16S rRNA gene sequence analysis of 23 of these *Vibrio* isolates was reported previously (Hoffmann et al., 2010b) with 16S rRNA gene sequences from the remaining 30 isolates being added in the course of this study. Sequence comparisons using BLAST at the NCBI database demonstrated that 39 of these vibrios clustered within the *Vibrio* Harveyi clade (Table 1). Among the remaining SD strains, one isolate was found related to *Aliivibrio fischeri*, one to *V. brasiliensis*, two to *V. scophthalmi*, two to *V. gigantis*, two to *V. mediterranei*, two to *V. penaeicida* and four to *V. splendidus*.

The 39 Harveyi clade-associated SD *Vibrio* isolates were analysed in more detail. DNA sequence analysis of six housekeeping genes, *ftsZ*, *mreB*, *topA*, *rctB*, *rpoD* and *toxR*, resulted in 4299 characters, of which 1076 were parsimony-informative. MP analysis of the six housekeeping genes yielded ten equally most parsimonious trees 3589 steps in length, with a consistency (CI) and retention (RI) index of 0.711 and 0.908, respectively. All most parsimonious trees partitioned the 39 Harveyi clade-associated SD *Vibrio* isolates into four distinct clades strongly supported with 100% bootstrap support. An example of one such most parsimonious tree, which includes taxa source, geographical location and depth for each SD *Vibrio* isolate, is shown in Fig. 1.

The 39 Harveyi clade-associated SD *Vibrio* isolates were further characterized by comparison with 48 nearshore Harveyi clade reference strains using MLSA and ISR typing. Phylogenetic analysis of the matrix from six housekeeping genes, consisted of 4299 characters, of which 1363 were parsimony-informative, yielded ten trees with a tree length of 5907, CI of 0.504 and RI of 0.899. The strict consensus tree, which had the same topology as the semi-strict consensus tree, had resolved 43 nodes with 36 showing bootstrap support $> 50\%$ (Fig. 2a). The MP tree topology was further supported by both ML (Fig. 2b) and NJ analyses (Fig. S1). Note that all three analyses provided equally well-resolved phylogenies that were congruent entirely among the major *Vibrio* Harveyi lineages.

Phylogenetic analysis separated the 87 *Vibrio* strains into seven distinct groups (Fig. 2). Sixteen reference strains (nine *V. campbellii* strains, seven *V. harveyi*) and four SD *Vibrio* isolates comprised group 1, while group 2 contained three *V. harveyi* and two *V. communis* reference strains and 11 SD *Vibrio* isolates. Four *V. harveyi* reference strains and three SD *Vibrio* isolates fell into group 3 and all 16 *V. alginolyticus* reference strains along with 21 SD *Vibrio* isolates comprised group 4. Group 5 included the four *V. parahaemolyticus* reference strains, while groups 6 and 7 retained two *V. natriegens* and a single *V. rotiferianus*, respectively. Notably, none of the last three groups (5–7) consisted of any SD vibrios. It was also notable that MP trees generated from the *topA*, *ftsZ*, *toxR*, *rctB* and *rpoD* genes, when analysed separately, largely supported with high bootstrap support the combined six-gene MLSA tree (Hoffmann et al., 2011). Moreover, the topology of the SD vibrios was determined to be the same as when the analysis was performed using only the 39 SD vibrios (Fig. 1). As MLSA data subsequently demonstrated that the *V. harveyi* strains segregating into groups 1 and 2 actually represented *V. campbellii* and *V. communis*, respectively (Hoffmann et al., 2012); taken together, these results suggest that group 1 comprised *V. campbellii*, group 2 *V. communis*, a novel *Vibrio* species recently described by Chimetto et al. (2010), group 3 *V. harveyi*, group 4 *V. alginolyticus*, group 5 *V. parahaemolyticus*, group 6 *V. natriegens* and group 7 *V. rotiferanius*.

Genetic distances between the seven distinct *Vibrio* clades are shown in Table 4 and support the phylogenetic partitions revealed above. Mean divergence among the seven clades ranged from 7.61 to 19.89%, while intraclade variation varied from as low as 0.42% in the *V. harveyi* group (group 3) to 3.04% in the *V. alginolyticus* group (group 4), the most extensive intraclade divergence among the seven groups reported here. Recalculated intraclade genetic distance for the four SD-containing clades minus the SD isolates yielded increased mean divergences for each group, excluding group 3 (*V. harveyi*). Accordingly, intraclade distances for SD vibrios only revealed again lower mean divergences within group 1 (*V. campbellii*), group 2 (*V. communis*) and group 4 (*V. alginolyticus*) than when reference strains and SD isolates were calculated together, albeit that the *V. harveyi* group was slightly increased. These results were supported by the minimum–maximum pairwise genetic distances found within each group. The maximum pairwise distance (Table 4) in each group was not represented with an SD strain. That is, the greatest divergence between strains involved reference strains only but no single SD strain. Rather, with the exception of *V. harveyi* group 3, greatest diversity was found between reference strains compared with themselves and not with SD strains, pointing to a remarkable lack of genetic diversity between SD strains and other known reference strains. Of note was the fact that each of the four groups was highly supported in both NJ and MP trees with bootstrap support values of 100% and ML trees with bootstrap support values of $> 70\%$ and posterior probabilities of 1 for the nodes at the base of the clades containing both SD vibrios and nearshore vibrios. As such, these observations supported the idea that both sets of strains are phylogenetically homogeneous and have not diverged along any significant evolutionary lines. Together, these data


21 (3) V. campbellii L647 (Corallistes sp.; 204 m; Florida, Gulf)
8
20 (5) V. campbellii K878 (Erylus sp.;143 m; Bahamas)
30 (3)
[80]
17 (4) V. campbellii K875 (Erylus sp.;143 m; Bahamas)
128 (22)
[100]
[100]
32 (2) V. campbellii K872 (Erylus sp.;143 m; Bahamas)
11 (1) V. communis J036 (Axinella sp.; 46 m; Florida, Atlantic)
19
[72]
20 (3) V. communis F078 (Aplysiniidae; 83.8 m; Bahamas)
15
10
30 (4) V. communis K882 (Erylus sp.;143 m; Bahamas)
6
16 (3) V. communis F064 (Porifera; 15.2 m; Bahamas)
123 (30)
[100]
24 (4) V. communis F052 (Porifera; 21.3 m; Bahamas)
4
20 (2) V. communis K873 (Erylus sp.;143 m; Bahamas)
8
34 (2) V. communis F077 (Aplysiniidae; 83.8 m; Bahamas)
12 (1)
21 (1) V. communis J821 (Holothuroidea; 299.6 m; Puerto Rico)
168 (78)
[100]
29 (6) V. communis K921 (Erylus sp.;143 m; Bahamas)
124 (19)
[100]
10
29 (2) V. communis P274 (Discodernia sp.; 158.5 m, Bahamas)
[51]
27 (3) V. communis F048 (Topsentia bahamensis; 21.3 m; Bahamas)
11 (5) V. harveyi J207 (Higginsia strigilata; 61 m; Florida, Atlantic)
(1) 6
6 (1) V. harveyi C259 (Chondrosia collectrix; 0.3 m; Bahamas)
166 (44)
[88]
[100]
9 (3) V. harveyi M028 (Choristida; 410 m; Bahamas)
V. alginoltyticus N376 (Scleritoderma cyanea; 242 m; Curacao)
(1) 1
V. alginoltyticus N377 (Scleritoderma cyanea; 242 m; Curacao)
V. alginoltyticus N382 (Scleritoderma cyanea; 242 m; Curacao)
6
V. alginoltyticus N380 (Scleritoderma cyanea; 242 m; Curacao))
V. alginoltyticus K350 (Scleritoderma cyanea; 242 m; Curacao))
V. alginoltyticus K324 (Scleritoderma cyanea; 242 m; Curacao)
378 (226)
[100]
(2) 16
[99]
V. alginoltyticus K323 (Scleritoderma cyanea; 242 m; Curacao)
[96]
7 (2) V. alginoltyticus W047 (Geodiidae; 304.5 m; Florida, Miami Terrace
(1) 8
[98]
7 (1) V. alginoltyticus W017 (Porifera; 70.4 m; Florida, Gulf)
(1) 19
[100]
V. alginoltyticus W046 (Geodiidae; 304.5 m; Florida, Miami Terrace
20
9 (1) V. alginoltyticus B840 (Discodermia sp; 182.9 m; Bahamas)
[96]
14 (2) V. alginoltyticus W019 (Geodiidae; 115.5 m; Florida, Dry Tortugas)
[99]
V. alginoltyticus V013 (Leiodermatium sp; 630.9 m; Bahamas)
127 (25)
[100]
(1) V. alginoltyticus V014 (Leiodermatium sp; 630.9 m; Bahamas)
15
V. alginoltyticus V056 (Leiodermatium sp; 630.9 m; Bahamas)
5
[81]
[100]
V. alginoltyticus V058 (Leiodermatium sp; 630.9 m; Bahamas)
8 (2) V. alginoltyticus A975 (Pachastrellidae; 125 m; Barbados)
218 (90)
[100]
(1) 9
[75]
V. alginoltyticus W016 (Porifera; 70.4 m; Florida, Gulf)
(2) 6
[99]
V. alginoltyticus W018 (Porifera; 70.4 m; Florida, Gulf)
V. alginoltyticus W020 (Geodiidae; 115.5 m; Florida, Dry Tortugas)
126 (36)
V. alginoltyticus J608 (Scleritodermidae; 364.2 m; Puerto Rico)
691 (394)
V. cholerae O395
678 (432)
Aliivibrio fischeri ES114
50 changes

indicate that SD strains and their nearshore counterparts are not subject to the forces of allopatric divergence.

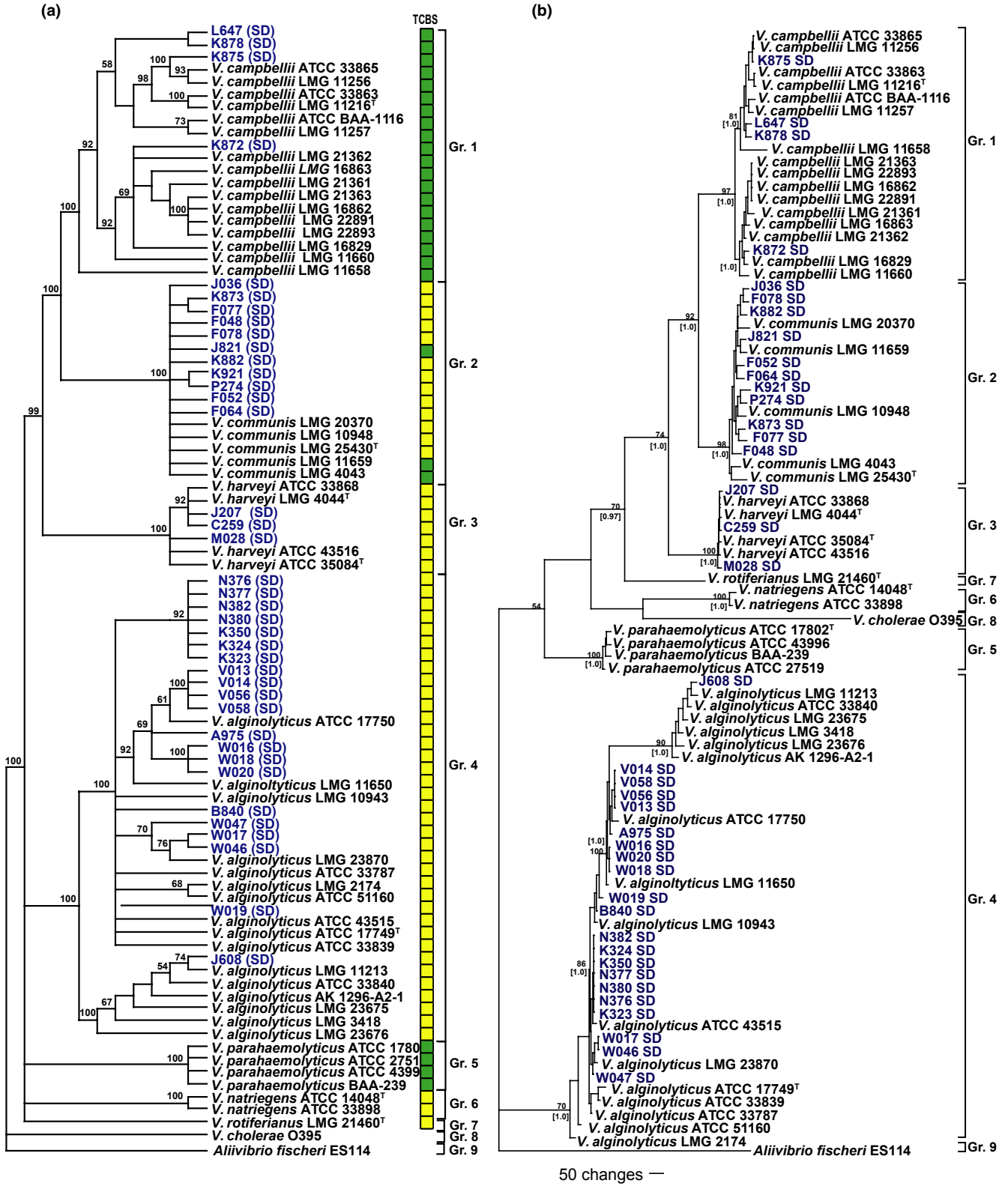
Both phylogenetic and genetic measures suggested that nearly all SD vibrios were distributed across and among other reference vibrios in four separate groups of Harveyi clade strains (Fig. 2). To further examine whether or not SD strains were genetically distinct from our reference isolates, we conducted several tests for recombination between these groups. That is, evidence for HGT and subsequent recombination would further support the notion that SD vibrios are part of a larger panmictic group of *Vibrio* strains that spans disparate marine ecologies. Repeatedly, HGT has been cited as the source of incongruence (phylogenetic discordance) between nucleotide sequences (Dykhuizen and Green, 1991; Lecointre et al., 1998; Brown et al., 2002). Specifically, the ILD test is a parsimony-based phylogenetic method that evaluates statistically the null hypothesis of congruence between different genes or distinct domains within the same gene (Farris et al., 1994). Importantly, ILD results, which are not quantifiable, show only that HGT is present between these strains (Brown et al., 2002).

First, ILD testing of all six concatenated housekeeping genes analysed for these strains separately led to the conclusion of incongruence for the *V. campbellii*, *V. communis* and *V. alginolyticus* groups ($P = 0.001$), pointing to extensive recombination among alleles for some or all of these genes. In contrast, the *V. harveyi* group ($P = 0.394$) was deemed to be congruent. To isolate the gene(s) responsible for the incongruence/congruence, each of the six housekeeping genes was partitioned against a combined matrix consisting of the remaining five. For the *V. campbellii* group, all six genes, *ftsZ* ($P = 0.002$), *mreB* ($P = 0.001$), *topA* ($P = 0.001$), *rctB* ($P = 0.001$), *rpoD* ($P = 0.009$) and *toxR* ($P = 0.002$), were highly discordant. Similar results were obtained for the *V. alginolyticus* group where five [*mreB* ($P = 0.001$), *topA* ($P = 0.001$), *rctB* ($P = 0.001$), *rpoD* ($P = 0.001$) and *toxR* ($P = 0.001$)] of the six genes showed incongruence, while *ftsZ* ($P = 0.519$) was deemed to be congruent with the combined housekeeping matrix. *ftsZ* ($P = 0.998$) and *rpoD* ($P = 0.747$) were congruent for the *V. communis* group while the remaining four housekeeping genes [*mreB* ($P = 0.001$), *topA* ($P = 0.002$), *rctB* ($P = 0.001$) and *toxR* ($P = 0.001$)] revealed significant incongruence. Only *rctB* ($P = 0.001$) was singled out as being incongruent for the *V. harveyi* group, while *ftsZ* ($P = 1.000$), *mreB* ($P = 0.489$), *topA* ($P = 0.830$), *rpoD* ($P = 0.883$) and *toxR* ($P = 0.797$) were congruent. When all possible pairwise ILD comparisons were performed, similar results were found for each of the four groups (Table 5).

Evidence for recombination was further affirmed using Compatibility of Sites analysis (Jakobsen and Easteal, 1996). Two sites are deemed compatible if the substitutions at these sites can be accounted for only once in a phylogeny. Incompatible nucleotide sites can be the result of HGT at a single site (Brown et al., 2002). Compatibility analysis demonstrated incongruence within three SD-containing groups, the *V. campbellii*, *V. communis* and *V. harveyi* groups. Overall compatibility for the *V. campbellii*, *V. communis*, *V. harveyi* group and *V. alginolyticus* groups was 69.7, 48.7, 43.8 and 85.8%, respectively. These values were remarkably low and depressed such that roughly half of the variable sites in the data matrix revealed strain incompatibility when compared with each other, particularly for the *V. communis* and *V. harveyi* groups. Note also that, when compatibilities were recalculated with SD strains removed, overall compatibility for *V. alginolyticus* and *V. campbellii* was higher at 86.4 and 73.4%, respectively. Figure 3 illustrates these differences, showing the compatibility matrix with and without SD *Vibrio* isolates for the *V. campbellii* group. With the exception of *ftsZ*, where compatibility remained unchanged, every other gene revealed an increased compatibility when SD *Vibrio* isolates were excluded, a finding further supporting a role for recombination between SD vibrios and other reference vibrios included here.

Finally, split decomposition was performed on the gene data from the four *Vibrio* groups containing SD strains. Split decomposition detects conflicting phylogenetic signals among DNA sequences by allowing the characters to be expressed in a network of relatedness rather than forced into a tree-like model of evolution (Huson, 1998; Brown et al., 2002). Split Tree analysis for the *V. campbellii*, *V. communis*, *V. harveyi* group and *V. alginolyticus* groups revealed additional evidence for HGT between sponge-associated *Vibrio* isolates and

Fig. 1. Maximum parsimony (MP) tree for the six-gene MLSA analysis. Shown is the first MP tree out of ten equally most-parsimonious trees generated from the concatenated gene sequences of *topA*, *ftsZ*, *mreB*, *rctB*, *rpoD* and *toxR* (total length 4299 bp) showing the relationship between the 39 sponge-derived SD *Vibrio* isolates. MP analysis was performed using Paup* ver. 4.0b10. Results yielded a tree having a length of 3589, consistency index (CI) of 0.711, retention index (RI) of 0.908 and homoplasy index (HI) of 0.289. Measures of clade confidence are reported in brackets below each node as bootstrap values (1000 iterations); values $< 50\%$ are not shown. Individual branch lengths are presented above each branch; the numbers of unambiguous substitutions that mapped to the tree only once and are > 0 are given in parentheses. The tree was rooted using *Aliivibrio fischeri* ES114 (CP000020, CP000021) and *V. cholerae* O395 (CP000626, CP000627). The taxa of source for each SD *Vibrio* isolate, geographical location and depth were mapped onto the tree.


(a)
TCBS
L647 (SD)
K878 (SD)
K875 (SD)
V. campbellii ATCC 33865
V. campbellii LMG 11256
V. campbellii ATCC 33863
V. campbellii LMG 11216T
V. campbellii ATCC BAA-1116
V. campbellii LMG 11257
K872 (SD)
V. campbellii LMG 21362
V. campbellii LMG 16863
V. campbellii LMG 21361
V. campbellii LMG 21363
V. campbellii LMG 16862
V. campbellii LMG 22891
V. campbellii LMG 22893
V. campbellii LMG 16829
V. campbellii LMG 11660
V. campbellii LMG 11658
J036 (SD)
K873 (SD)
F077 (SD)
F048 (SD)
F078 (SD)
J821 (SD)
K882 (SD)
K921 (SD)
P274 (SD)
F052 (SD)
F064 (SD)
V. communis LMG 20370
V. communis LMG 10948
V. communis LMG 25430T
V. communis LMG 11659
V. communis LMG 4043
V. harveyi ATCC 33868
V. harveyi LMG 4044T
J207 (SD)
C259 (SD)
M028 (SD)
V. harveyi ATCC 43516
V. harveyi ATCC 35084T
N376 (SD)
N377 (SD)
N382 (SD)
N380 (SD)
K350 (SD)
K324 (SD)
K323 (SD)
V013 (SD)
V014 (SD)
V056 (SD)
V058 (SD)
V. alginolyticus ATCC 17750
A975 (SD)
W016 (SD)
W018 (SD)
W020 (SD)
V. alginoltyticus LMG 11650
V. alginolyticus LMG 10943
B840 (SD)
W047 (SD)
W017 (SD)
W046 (SD)
V. alginolyticus LMG 23870
V. alginolyticus ATCC 33787
V. alginolyticus LMG 2174
V. alginolyticus ATCC 51160
W019 (SD)
V. alginolyticus ATCC 43515
V. alginolyticus ATCC 17749T
V. alginolyticus ATCC 33839
J608 (SD)
V. alginolyticus LMG 11213
V. alginolyticus ATCC 33840
V. alginolyticus AK 1296-A2-1
V. alginolyticus LMG 23675
V. alginolyticus LMG 3418
V. alginolyticus LMG 23676
V. parahaemolyticus ATCC 1780
V. parahaemolyticus ATCC 2751
V. parahaemolyticus ATCC 4399
V. parahaemolyticus BAA-239
V. natriegens ATCC 14048T
V. natriegens ATCC 33898
V. rotiferianus LMG 21460T
V. cholerae O395
Aliivibrio fischeri ES114
Gr. 1
Gr. 2
Gr. 3
Gr. 4
Gr. 5
Gr. 6
Gr. 7
Gr. 8
Gr. 9
(b)
V. campbellii ATCC 33865
V. campbellii LMG 11256
K875 SD
V. campbellii ATCC 33863
V. campbellii LMG 11216T
V. campbellii ATCC BAA-1116
V. campbellii LMG 11257
L647 SD
K878 SD
V. campbellii LMG 11658
V. campbellii LMG 21363
V. campbellii LMG 22893
V. campbellii LMG 16862
V. campbellii LMG 22891
V. campbellii LMG 21361
V. campbellii LMG 16863
V. campbellii LMG 21362
K872 SD
V. campbellii LMG 16829
V. campbellii LMG 11660
J036 SD
F078 SD
K882 SD
V. communis LMG 20370
J821 SD
V. communis LMG 11659
F052 SD
F064 SD
K921 SD
P274 SD
V. communis LMG 10948
K873 SD
F077 SD
F048 SD
V. communis LMG 4043
V. communis LMG 25430T
J207 SD
V. harveyi ATCC 33868
V. harveyi LMG 4044T
C259 SD
V. harveyi ATCC 35084T
V. harveyi ATCC 43516
M028 SD
V. rotiferianus LMG 21460T
V. natriegens ATCC 14048T
V. natriegens ATCC 33898
V. cholerae O395
V. parahaemolyticus ATCC 17802T
V. parahaemolyticus ATCC 43996
V. parahaemolyticus BAA-239
V. parahaemolyticus ATCC 27519
J608 SD
V. alginolyticus LMG 11213
V. alginolyticus ATCC 33840
V. alginolyticus LMG 23675
V. alginolyticus LMG 3418
V. alginolyticus LMG 23676
V. alginolyticus AK 1296-A2-1
V014 SD
V058 SD
V056 SD
V013 SD
V. alginolyticus ATCC 17750
A975 SD
W016 SD
W020 SD
W018 SD
V. alginoltyticus LMG 11650
W019 SD
B840 SD
V. alginolyticus LMG 10943
N382 SD
K324 SD
K350 SD
N377 SD
N380 SD
N376 SD
K323 SD
V. alginolyticus ATCC 43515
W017 SD
W046 SD
V. alginolyticus LMG 23870
W047 SD
V. alginolyticus ATCC 17749T
V. alginolyticus ATCC 33839
V. alginolyticus ATCC 33787
V. alginolyticus ATCC 51160
V. alginolyticus LMG 2174
Aliivibrio fischeri ES114
Gr. 1
Gr. 2
Gr. 3
Gr. 7
Gr. 6
Gr. 8
Gr. 5
Gr. 4
Gr. 9
50 changes

the reference strains in each of the four groups (Fig. 4). The split trees are consistent with the topology of the MP strict consensus tree, supporting the contention of past recombination events among these strains. Diamonds in the split trees represent parallel evolutionary pathways in the data and signal a reticulated phylogenetic history (i.e. HGT and recombination) for strains associated with these portions of the split tree networks.

Given the close genetic proximity of SD vibrios to the reference strains above, it was intriguing to explore more rapidly evolving genetic markers among these strains. Previously, we reported on the development of a rapid 16S–23S rRNA ISR typing method that was both stable and discriminatory among closely related *Vibrio* species (Hoffmann et al., 2010a). This approach was applied to the four groups in which SD vibrios were known to reside (i.e. the *V. communis*, *V. campbellii*, *V. harveyi* and *V. alginolyticus* groups). Several observations made from the phylogenetic and genetic analyses are also reflected in ISR-pattern variation among SD and reference strains (Fig. 5). That is, strain groups 2, 3 and 4 retained SD vibrios with distinct ISR profiles and others with ISRs identical to their reference *Vibrio* counterparts. For instance, *Vibrio* strain *V. communis* LMG 25430 and SD strain K882, both from group 2, shared identical ISR patterns, while group 4 strains *V. alginolyticus* LMG 11650 and SD strain B840 were also identical. Surprisingly, in group 3, reference *Vibrio harveyi* ATCC 35084^{T} shared a common ISR profile with strain M028, a sponge-derived *Vibrio* isolated from over 400 m below the ocean surface. As mentioned above, some SD vibrios did reveal ISR profiles distinct from those seen among reference strains. This was particularly true for SD isolates in the *V. campbellii* group (i.e. group 1). It should be noted, however, that many reference strains in this group also retained divergent ISR profiles from each other. That is, 16 different *V. campbellii* reference strains sorted into seven disparate ISR lineages based on pattern similarity, supporting the notion that *V. campbellii* is a relatively diverse species in and of itself within the Harveyi clade.

Phenotypic observations further supported the distinct groupings observed in the Harveyi clade tree (Fig. 1). Group 1 (*V. campbellii*), comprising both reference strains and SD *Vibrio* isolates, showed green colonies on TCBS agar, indicating that they were unable to ferment sucrose. The *V. communis* group comprised 13 strains that could ferment sucrose, forming yellow colonies on TCBS and three (one SD *Vibrio* and two reference) strains that were sucrose-negative. All members of the *V. harveyi* and *V. alginolyticus* groups were sucrose-positive.

Multiplex-PCR for *Vibrio*-associated virulence determinants of all SD *Vibrio* isolates failed to demonstrate the presence of the *trh*, *tdh*, *vlly*, *vvhA*, *vmhA* and *ctx* genes, while showing the presence of the 205-bp 16S control band for every strain tested. The multiplex-PCR showed positive results for *V. cholerae* strains ATCC 11679 and O395 for *ctx*, *V. mimicus* strain ATCC 33655 for *vmhA*, *V. vulnificus* strain ATCC 29306 for *vvhA*, strain *V. parahaemolyticus* K1314 for *trh*, *V. parahaemolyticus* strain AO-24491 for *tdh* and *V. parahamolyticus* strain 48057 for *tdh* and *trh*, further validating the results obtained for the SD *Vibrio* isolates (data not shown). Consistent with these findings, none of the reference isolates of the various groups was found to carry any of these determinants. In contrast to those results for other virulence genes, a PCR for detection of the thermolabile hemolysin (*tlh*) gene showed that all SD isolates, as well as reference strains, have *tlh*, a gene playing a potentially key role in virulence associated with aquatic organisms and possibly humans as well (Wang et al., 2007; Jia et al., 2010).

Discussion

In the present study, the potential for HGT between SD vibrios and their nearshore counterparts was investigated via phylogenetic and genetic analyses using a collection of 53 different SD *Vibrio* isolates obtained from benthic sponge sources located in different geo-

Fig. 2. (a) Most parsimonious, strict consensus tree for the six-gene MLSA analysis. Shown is the strict consensus tree derived from ten equally most-parsimonious trees generated from the concatenated gene sequences of *topA*, *ftsZ*, *mreB*, *rctB*, *rpoD* and *toxR* (total length 4299 bp). The tree depicts the relationship between the 48 Harveyi clade reference strains and 39 sponge-derived (SD) *Vibrio* isolates. MP analysis was performed using Paup* v.4.0b10. Results yielded a tree having a length of 5907, consistency index (CI) of 0.504, retention index (RI) of 0.899 and homoplasy index (HI) of 0.496. Measures of clade confidence are reported above each node as bootstrap values (1000 iterations); values $< 50\%$ are not shown. The tree was rooted using *Aliivibrio fischeri* ES114 (CP000020, CP000021) and *V. cholerae* O395 (CP000626, CP000627). Phenotypic observations are further depicted on the tree using coloured bars, shown on the right. Yellow bars identify those strains that were determined to ferment sucrose (positive), as established on TCBS agar, while the green bars indicate those strains that were unable to ferment sucrose (negative). (b) Maximum likelihood (ML) tree for the six-gene MLSA analysis. The ML tree derived from the concatenated gene sequences of *topA*, *ftsZ*, *toxR*, *rctB*, *rpoD* and *mreB* (total length 4299 bp) showing the relationship between 48 Harveyi clade reference strains and 39 sponge-derived (SD) *Vibrio* isolates. ML trees were generated in GARLI (Zwickl, 2006) and visualized in PAUP* ver. 4.0b10 (Swofford, 2003). ML bootstrap values are those shown without brackets. Bayesian nodal support values were calculated using MrBayes ver. 3.1.2 and are presented in brackets. The tree was rooted using *Aliivibrio fischeri* ES114 and *V. cholerae* O395. ML model/invariant sites/gamma shape parameter: GTR + I + G/0.3178/0.3912 Frequency A/C/G/T: 0.2926/0.2090/0.2209/0.2775; $-\ln L$ ML tree: 33302.89416.

Table 4
Genetic distances between the nine distinct *Vibrio* groups and intragroup diversity among SD *Vibrio* isolates individually and with their shallow-water counterparts

	Mean nucleotide diversity ± SE (%)*							
	V. campbellii [Gr. 1]	*V. communis* [Gr. 2]	*V. harveyi* [Gr. 3]	*V. alginolyticus* [Gr. 4]	*V. parahaemolyticus* [Gr. 5]	*V. natriegens* [Gr. 6]	*V. rotiferanius* [Gr. 7]	*V. cholerae* [Gr. 8]
V. campbellii [Gr. 1]								
V. communis [Gr. 2]	7.61 ± 0.35							
V. harveyi [Gr. 3]	10.35 ± 0.48	10.19 ± 0.48						
V. alginolyticus [Gr. 4]	15.92 ± 0.62	16.09 ± 0.58	16.34 ± 0.62					
V. parahaemolyticus [Gr. 5]	15.47 ± 0.63	15.12 ± 0.68	15.47 ± 0.65	14.57 ± 0.53				
V. natriegens [Gr. 6]	18.29 ± 0.72	18.15 ± 0.74	18.00 ± 0.70	17.12 ± 0.60	17.10 ± 0.68			
V. rotiferanius [Gr. 7]	14.63 ± 0.62	14.82 ± 0.62	15.62 ± 0.68	18.49 ± 0.72	17.47 ± 0.68	19.89 ± 0.74		
V. cholerae [Gr. 8]	31.37 ± 0.87	30.94 ± 0.90	30.70 ± 0.88	32.06 ± 0.87	31.59 ± 0.96	30.90 ± 0.91	31.97 ± 0.84	
A. fischeri [Gr. 9]	33.37 ± 1.16	33.18 ± 1.03	32.42 ± 1.16	32.41 ± 1.06	34.40 ± 1.17	33.74 ± 1.08	33.23 ± 1.01	38.57 ± 1.24
SD *Vibrio* isolates and reference strains	1.76 ± 0.13†	1.65 ± 0.12†	0.42 ± 0. 06†	3.04 ± 0.16†	0.79 ± 0.11†	0.80 ± 0.15§		
Reference strains	1.87 ± 0.20†	3.04 ± 0.16†	0.37 ± 0.06†	4.31 ± 0.19†				
SD *Vibrio* isolates	1.49 ± 0.13†	1.48 ± 0.12†	0.50 ± 0.09†	1.55 ± 0.09†				
SD *Vibrio* isolates and reference strains	0.00–3.86‡	0.94–2.55‡	0.31–0.61‡	0.00–7.77†				
Reference strains	0.00–3.86‡	1.64–2.55‡	0.31–0.49‡	0.45–7.77‡				
SD *Vibrio* isolates	0.97–1.90‡	0.94–2.17‡	0.40–0.61‡	0.00–7.05‡				

*Mean pairwise sequence variation for six genes among the nine *Vibrio* groups described in Fig. 1 and Fig. S1.

†Intragroup distance.

‡Minimum nucleotide distance and maximum nucleotide distance (%).

Table 5
ILD values for groups 1–4 including SD vibrios and nearshore vibrios

Locus	*P* value (1000 partitions)				
	mreB	*topA*	*rctB*	*rpoD*	*toxR*
V. campbellii					
ftsZ	0.002	0.001	0.001	0.004	0.001
mreB		0.001	0.003	0.009	0.001
topA			0.001	0.001	0.001
rctB				0.001	0.001
rpoD					0.001
V. communis					
ftsZ	*0.996*	0.215	0.209	0.461	0.375
mreB		0.017	0.004	0.690	0.001
topA			0.002	0.002	0.001
rctB				0.008	0.001
rpoD					0.019
V. harveyi					
ftsZ	1.000	1.000	1.000	1.000	1.000
mreB		0.853	0.061	0.706	0.643
topA			0.618	1.000	0.723
rctB				0.264	0.565
rpoD					0.223
V. alginolyticus					
ftsZ	0.187	0.110	0.006	0.186	0.005
mreB		0.001	0.001	0.001	0.001
topA			0.001	0.001	0.001
rctB				0.001	0.001
rpoD					0.001

graphical locations and at different depths (0.3–750 m). Preliminary results from 16S rRNA gene sequence analysis revealed that 39 of 53 (74%) SD isolates were members of the Harveyi clade (Sawabe et al., 2007), taxonomically akin to many shallow-water or nearshore *Vibrio* isolates that also reside among this group of *Vibrio* species. As members of the Harveyi clade are often difficult to identify to species level based solely on 16S rRNA gene sequences (e.g. *V. harveyi*, *V. communis* and *V. campbellii* share 16S rRNA gene sequence similarities > 99%) (Chimetto et al., 2010), an MLSA approach was applied to these 39 isolates to further distinguish the phylogenetic relatedness of SD and reference *Vibrio* strains. Thompson et al. (2007) and Pascual et al. (2010) introduced two distinct MLSA methods, both of which successfully discriminated vibrios at the species level in the Harveyi clade. Subsequently, Lin et al. (2010) applied both MLSA methods using three of the most conserved genes from each method in two separate analyses. Building upon these findings, we concatenated six genes that Lin et al. analysed separately into a single analysis. As expected, this combined MLSA approach offered a stronger degree of resolution at the species level than was obtained from 16S rRNA gene sequence analysis alone. Moreover, the data flagged ten separate *V. harveyi* strains (seven and three originally segregated to group 1 and 2, respectively) as being potentially misidentified (Hoffmann et al., 2012). Thus, these findings affirm this MLSA typing scheme for effectively differentiating members of the Harveyi clade. Collectively, analysis of the 4299-bp sequence identified the SD *Vibrio* isolates as representing *V. campbellii*, *V. communis*, *V. harveyi* and *V. alginolyticus*.

Additionally, we conducted several tests (i.e. ILD, split-decomposition and compatibility analysis) for recombination between SD and nearshore vibrios to provide evidence for HGT and subsequent recombination to determine if SD vibrios are genetically distinct. The tests for recombination used here are powerful strategies that are considered reliable and effective means for confirming HGT among closely related bacterial strains (Kashiwada et al., 1994; Liu et al., 1994; Hengstler et al., 2006; Huson and Bryant, 2006; Meyer et al., 2007). In near unanimity, the methods pointed to a role for recombination in the population structure of these *Vibrio* strains. First, although the *V. harveyi* group ($P = 0.394$) was deemed to be congruent, ILD testing of all six concatenated housekeeping genes analysed for the four *Vibrio* groups separately led to the conclusion of incongruence for the *V. campbellii*, *V. communis* and *V. alginolyticus* groups ($P = 0.001$). Previous reports have demonstrated that the null hypothesis of congruence cannot be rejected if there is no signal in the data (Brown et al., 2001). Moreover, noisy data sets are particularly difficult to establish congruence as they all appear congruent, even when that is not necessarily the case (Lecointre et al., 1998). Although noisy data sets cannot demonstrate significant incongruence with other data sets, a single structured incongruence can be detected even in the presence of contradictory signals, as is the case here, where the structured data presented revealed incongruence for three out of four groups, pointing to extensive recombination among alleles for some or all of these genes.

Evidence for recombination was further affirmed using Compatibility of Sites analysis. With rare exception, compatibility analysis, measured for each group with and without SD *Vibrio* isolates, yielded incongruence within three of the four groups of strains containing SD vibrios. Only *V. alginolyticus* showed congruence, although this was not a consequence of SD vibrios but, rather, appears due to the divergence noted between the two different *V. alginolyticus* subclades (Hoffmann et al., 2012). Overall compatibility for each subclade, including reference strains and SD isolates, was 66.0 and 44.7%, respectively, providing evidence for recombination within each of these subclades. Finally, split decomposition was performed on the gene data from the four *Vibrio* groups separately containing SD strains. Split Tree analysis revealed additional evidence for HGT between SD *Vibrio* isolates and nearshore vibrios for all four groups, supporting the contention of past recombination events among these strains.

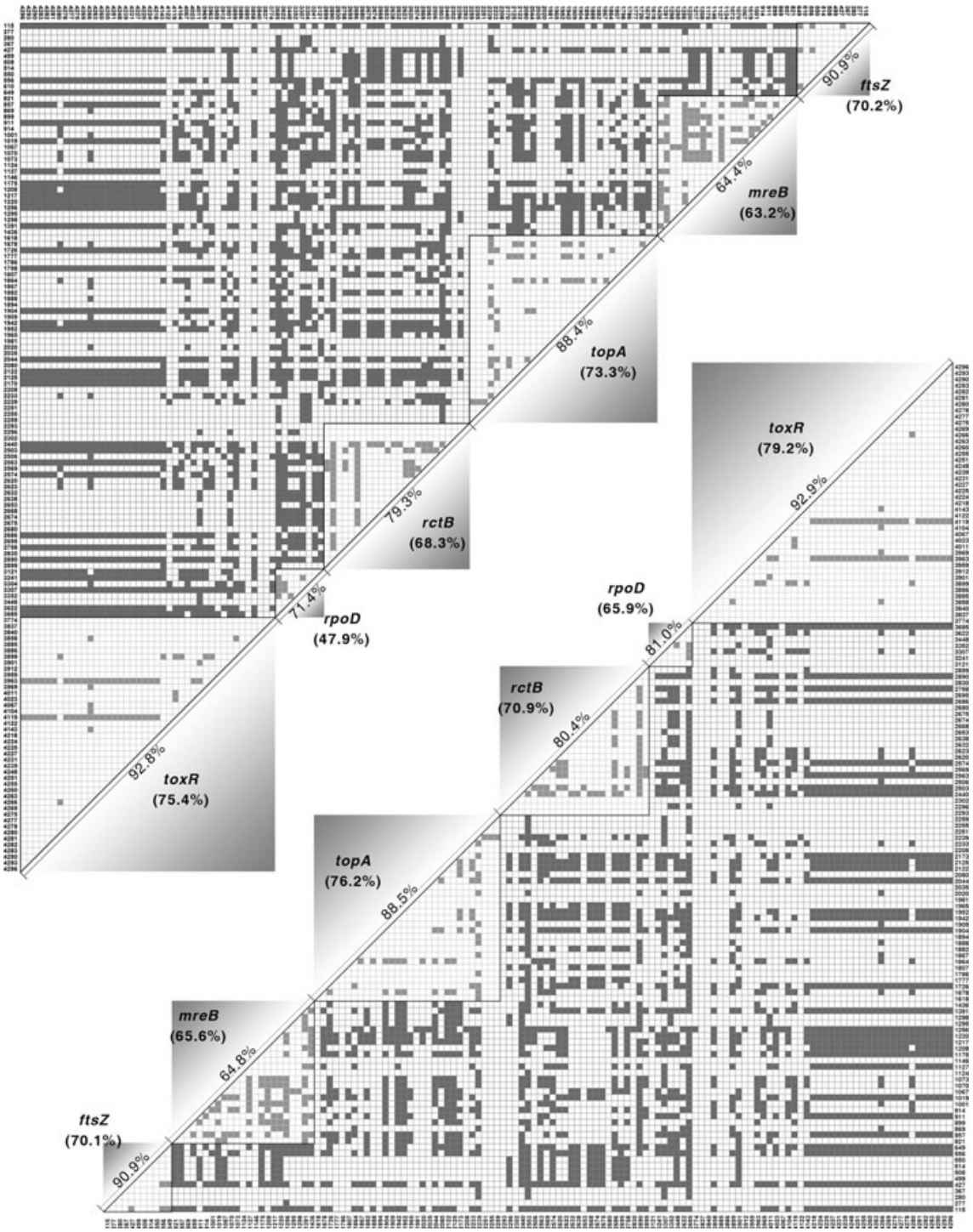


Fig. 3. Compatibility matrix analysis. Compatibility matrices of the *V. campbellii* group 1 strains using the concatenated six-gene sequence matrix showing pairwise comparisons of informative binary sites within (grey triangles) and between the genes indicated. The intergene compatibility scores are presented in parentheses below the gene indicated. Compatibilities for intragene comparison indicated for each gene are labelled on the diagonal line. The upper matrix represents collectively the reference strains from group 1 and the 39 sponge-associated *Vibrio* isolates, while the lower matrix represents only the reference strains. Both analyses were achieved using the program COMPATDNA (Bell et al., 2010).

Importantly, at least two out of three phylogenetic methods detected evidence for extensive recombination among SD vibrios and their nearshore counterpart strains, isolated from a variety of different globally distinct locations, further underlining the reticulate evolutionary relatedness of these two strain sets. It is

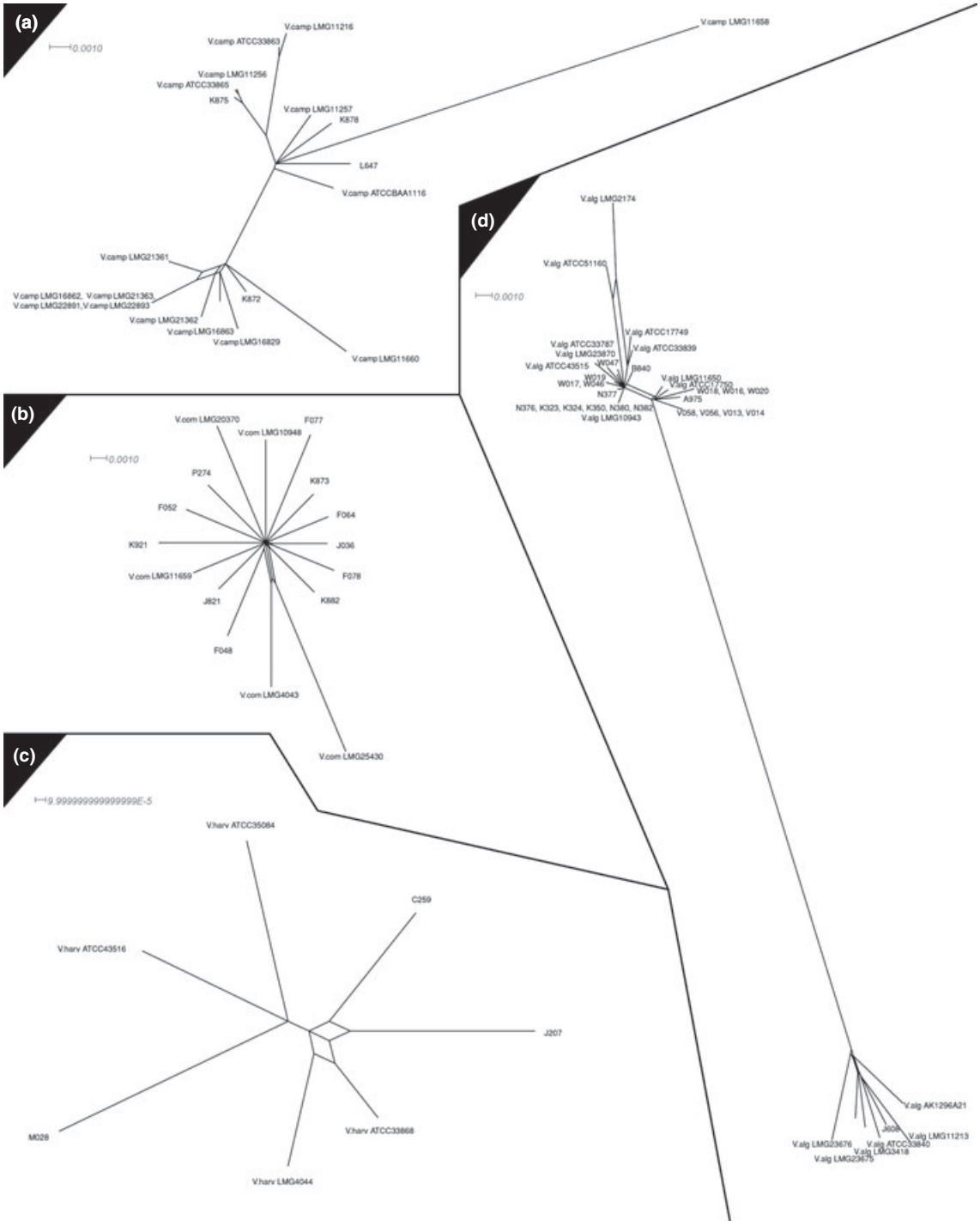


Fig. 4. Split decomposition analyses. Split decomposition analyses for the Harveyi clade reference strains and sponge-associated *Vibrio* isolates were generated using the concatenated six-gene sequence matrix and the SplitsTree software ver. 2.4 (Huson and Bryant, 2006). All graphs were drawn with equal edges to highlight parallel paths in the networks. Split graphs depicted are (a) *V. campbellii* group 1, (b) *V. communis* group 2, (c) *V. harveyi* group 3 and (d) *V. alginolyticus* group 4.

possible that some of the incongruence (i.e. HGT) observed may be attributed to deeper phylogenetic events in the radiation of the genus *Vibrio*, even perhaps to a time when SD vibrios and nearshore vibrios were indistinguishable both geographically or genetically. HGT among these strains could have been more

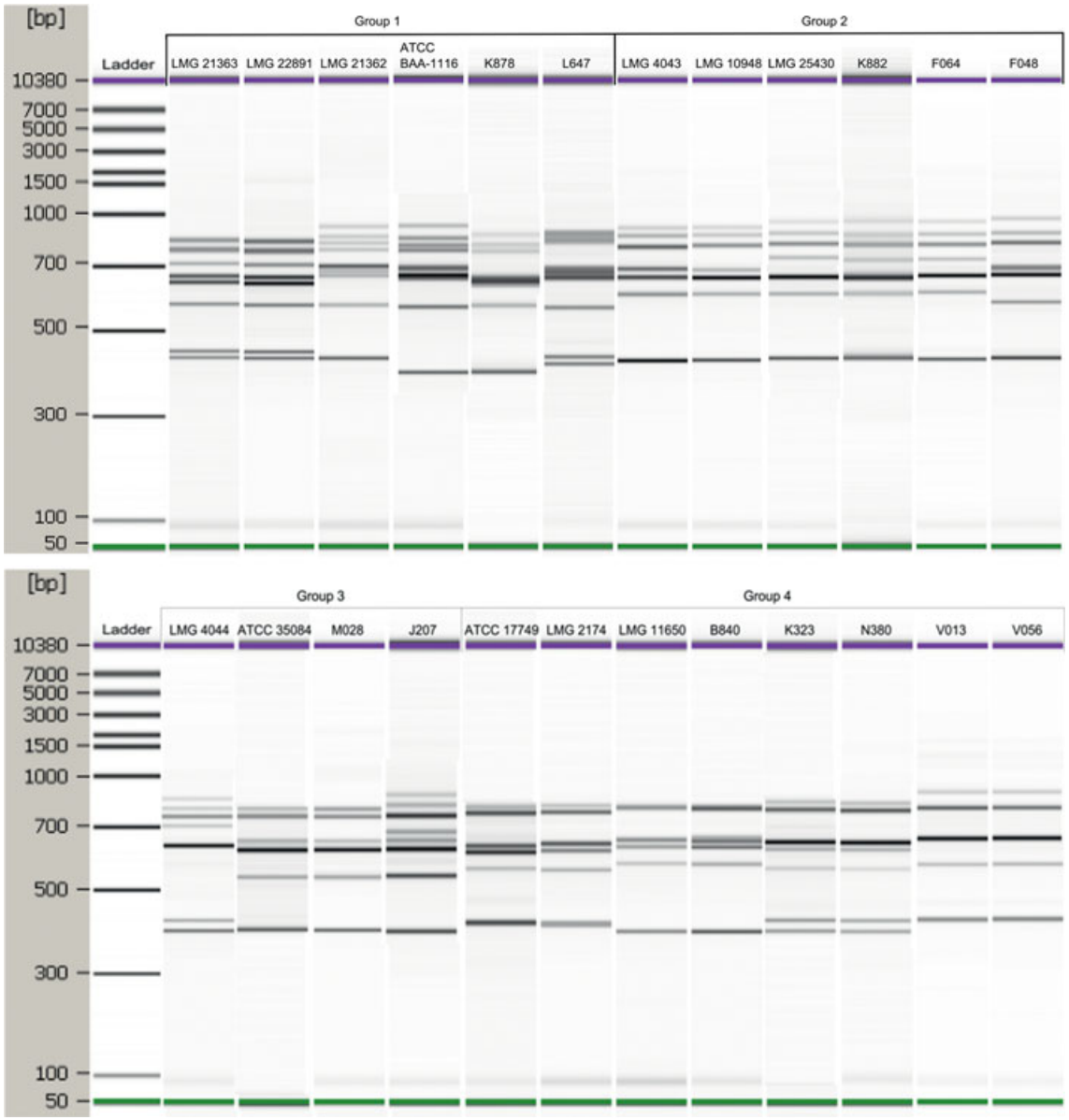


Fig. 5. Representative 16S–23S rRNA ISR-type patterns virtual gel image. The image shown was generated using selected sponge-derived (SD) *Vibrio* isolates and *Vibrio* reference species with an Agilent Bioanalyzer 2100. Molecular weight standards are shown on the left of each gel image. Gel image, upper. The *V. campbellii* group (group 1: lanes 1–6): lane 1, *V. campbellii* LMG 21363; lane 2, *V. campbellii* LMG 22891; lane 3, *V. campbellii* LMG 21362; lane 4, *V. campbellii* LMG BAA-1116; lane 5, SD *Vibrio* K878; lane 6, SD *Vibrio* L647. The *V. communis* group (group 2: lanes 7–12): lane 7, *V. communis* LMG 4043; lane 8, *V. communis* LMG 10948; lane 9, *V. communis* LMG 25430; lane 10, SD *Vibrio* K882; lane 11, SD *Vibrio* F064; lane 12, SD *Vibrio* F048. Gel image, lower. The *V. harveyi* group (group 3: lanes 1–4): lane 1, *V. harveyi* LMG 4044; lane 2, *V. harveyi* ATCC 35084; lane 3, SD *Vibrio* M028; lane 4, SD *Vibrio* J207. *V. alginolyticus* group (Group 4: lanes 5–12): lane 5, *V. alginolyticus* ATCC 17749; lane 6, *V. alginolyticus* LMG 2174; Ln 7. *V. alginolyticus* LMG 11650, Ln 8. SD *Vibrio* B840, Ln 9. SD *Vibrio* K323, lane 10, SD *Vibrio* N380; lane 11, SD *Vibrio* V013; lane 12, SD *Vibrio* V056.

ancestral during the radiation of these two populations but also could be the result of recent occurrences. Nonetheless, the integration of the ISR data, which evolves much more rapidly than most housekeeping genes, would argue that, in fact, some of the genetic exchange among these strains is a recent event such that ISR alleles are still shared by them and detectable as common alleles to both sets of strains. ISR analysis demonstrated that many SD vibrios are genetically indistinguishable from their shallow-water counterparts. Restated, these results support the hypothesis that SD and shallow-water vibrios are not two disparate

populations but, instead, are probably members of a larger single panmictic group.

Thus, regardless of whether the HGT events detected here are more ancestral or more recent, it does not exclude the fact that these two groups of vibrios have and may still be exchanging DNA across these expansive niches, a finding which has substantial implications for the movement and migration of vibrios globally. An example is provided by the recent emergence of the highly pathogenic *V. parahaemolyticus* O3:K6 clone Calcutta in India and its recent pandemic spread throughout South-East Asia, as well as the Atlantic and Gulf coast of the United States, and more recently, to Europe, Africa and South America (Gonzalez-Escalona et al., 2005; Martinez-Urtaza et al., 2005; Velazquez-Roman et al., 2012). Clearly, this raises the question about the dynamics, including environmental and genetic determinants, leading to the emergence, proliferation and transmission of *Vibrio* species pathotypes. Here we tested, using several highly recommended and commonly applied tests, the existence of recombination between the SD vibrios and their disparate nearshore vibrios, and found evidence suggesting the existence of a panmictic population structure, which may be a critical component of global distribution, such as was observed for the O3:K6 pandemic strain.

Members of the genus *Vibrio* have long been known to be associated with seasonal planktonic blooms (Authority, 2006), although the dynamics underpinning this cyclical association have not been fully described. Chitinous copepods appear well suited to harbour vibrios and thus serve as an important means of facilitating *Vibrio* growth (Romalde et al., 1990), distribution and spread (Maugeri et al., 2004; Lindsey et al., 2009; Turner et al., 2009). Moreover, the interface between copepod life cycles and marine deep-water environments has been noted (Rohatgi et al., 2009). This life cycle promotes close proximity between copepods to marine benthic environments, such as provided by sponges, that would be necessary to establish an association with "deep-water" vibrios. As the copepod life cycle also involves a shallow-water phase, this association provides a means by which vibrios may traverse disparate niches (i.e. nearshore versus deep-water niches). Previous work has shown that *Vibrio* species can be common sponge inhabitants, not typically represented in ambient sea water (Hentschel et al., 2001), and implicated in both secondary metabolism and synthesis of antibacterial compounds within the sponge (Elyakov et al., 1991; Oclarit et al., 1994). Additionally, in a previous study of SD *Vibrio* isolates, we noted the presence of several *Vibrio* species commonly observed in seasonal blooms (Hoffmann et al., 2010b), supporting the hypothesis that Porifera may serve as a reservoir for these vibrios (Brück et al., 2010; Kumaran et al., 2010). Interestingly, many vectors, such as copepods, have life cycles that could easily facilitate the dynamics necessary for deriving the bloom "isolates" from deep-water sources, such as the sponge.

Collectively, these data suggest that SD *Vibrio* strains are genetically homologous to their globally distributed, nearshore counterparts and that the two are part of a larger panmictic population whose dynamics are, quite possibly, facilitated as a consequence of the fortuitous copepod life cycle that overlaps these two disparate regions. Although it is entirely possible that several additional benthic zones could serve as reservoirs from which vibrios, as well as other bacterial species, might be derived, several *Vibrio* species commonly observed in seasonal blooms (Brück et al., 2010; Kumaran et al., 2010) have been isolated directly from sponge sources. While it remains to be determined whether copepods or other mechanisms (i.e. marine snow) effectively bridge these two marine ecologies, it becomes evermore apparent that transmission of vibrios, particularly of the Harveyi clade, observed in seasonal blooms probably includes a benthic ecological component, such as sponges.

Given the toxic nature of *Vibrio* blooms (Kangas et al., 2007; Grossart et al., 2010) and the pathogenesis associated with many of these bacteria, it is paramount that a better understanding of *Vibrio* persistence in these largely unexplored marine niches be garnered. This is particularly important considering that a recent *Vibrio* recovered from the sponge *Scleritoderma cyanea* was determined to represent a novel species (Hoffmann et al., 2011), raising the possibility that sponges support *Vibrio* speciation events, as well.

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Supporting information

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

Fig. S1. Neighbour joining (NJ) tree for the six-gene MLSA analysis.

Appendix S1. Protocol: *Vibrio* isolation from sponges.

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