Vibrio caribbeanicus sp. nov., isolated from the marine sponge *Scleritoderma cyanea*

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A Gram-negative, oxidase-positive, catalase-negative, facultatively anaerobic, motile, curved rod-shaped bacterium, strain N384^T, was isolated from a marine sponge (*Scleritoderma cyanea*; phylum Porifera) collected from a depth of 795 feet (242 m) off the west coast of Curaçao. On the basis of 16S rRNA gene sequencing, strain N384^T was shown to belong to the genus *Vibrio*, most closely related to *Vibrio brasiliensis* LMG 20546^T (98.8 % similarity), *Vibrio nigripulchritudo* ATCC 27043^T (98.5 %), *Vibrio tubiashii* ATCC 19109^T (98.6 %) and *V. sinaloensis* DSM 21326^T (98.2 %). The DNA G+C content of strain N384^T was 41.6 mol%. An analysis of concatenated sequences of five genes (*gyrB*, *rpoA*, *pyrH*, *mreB* and *ftsZ*; 4068 bp) demonstrated a clear separation between strain N384^T and its closest neighbours and clustered strain N384^T into the 'Orientalis' clade of vibrios. Phenotypically, the novel species belonged to the arginine dihydrolase-positive, lysine decarboxylase- and ornithine decarboxylase-negative (A+/L−/O−) cluster. The novel species was also differentiated on the basis of fatty acid composition, specifically that the proportions of iso-$C_{13:0}$, iso-$C_{15:0}$, $C_{15:0}$, iso-$C_{16:0}$, $C_{16:0}$, iso-$C_{17:0}$, $C_{17:1}\omega 8c$ and $C_{17:0}$ were significantly different from those found in *V. brasiliensis* and *V. sinaloensis*. The results of DNA–DNA hybridization, average nucleotide identity and physiological and biochemical tests further allowed differentiation of this strain from other described species of the genus *Vibrio*. Collectively, these findings confirm that strain N384^T represents a novel *Vibrio* species, for which the name *Vibrio caribbeanicus* sp. nov. is proposed, with the type strain N384^T (=ATCC BAA-2122^T=DSM 23640^T).

Abbreviations: ANI, average nucleotide identity; HGT, horizontal gene transfer; ILD, incongruence length difference; ML, maximum-likelihood; MLSA, multilocus sequence analysis; MP, maximum parsimony.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene, *topA*, *ftsZ*, *gapA*, *gyrB*, *mreB*, *pyrH*, *recA* and *rpoA* gene sequences derived in this study are HM771333–HM771388 and HQ890462–HQ890466. The accession numbers for the sequences of strain N384^T are GU223601 (16S rRNA), HM771337 (*topA*), HM771358 (*ftsZ*), HM771363 (*gapA*), HM771368 (*gyrB*), HM771373 (*mreB*), HM771378 (*pyrH*), HM771383 (*recA*), HM771388 (*rpoA*) and AEIU00000000 (whole-genome shotgun sequence). The accession numbers for the whole-genome shotgun sequences of *V. brasiliensis* LMG 20546^T, *V. sinaloensis* DSM 21326^T, *V. splendidus* ATCC 33789, *V. ichthyoenteri* ATCC 700023^T, *V. scophthalmi* LMG 19158^T, *V. orientalis* ATCC 33934^T, *V. tubiashii* ATCC 19109^T and *V. nigripulchritudo* ATCC 27043^T are AEVS00000000, AEVT00000000, AFWG00000000, AFWF00000000, AFWE00000000, AFWH00000000, AFWI00000000 and AFWJ00000000.

Five supplementary figures and three supplementary tables are available with the online version of this paper.

The family *Vibrionaceae* is a member of the class *Gammaproteobacteria* and consists of the genera *Vibrio*, *Photobacterium*, *Salinivibrio*, *Grimontia*, *Enterovibrio* and *Aliivibrio* (Chimetto *et al.*, 2011). At the time of writing, the genus *Vibrio* consisted of 89 species (http://www.vibriobiology.net). Some vibrios, such as *Vibrio cholerae*, *V. parahaemolyticus* and *V. vulnificus*, are well-known human pathogens (FDA, 2004). Generally, *Vibrio* species are halophilic, mesophilic and chemo-organotrophic in nature and have a facultatively fermentative metabolism (Thompson *et al.*, 2004). Typically, vibrios are inhabitants of aquatic environments that show a remarkable degree of biodiversity, persisting in a variety of geographical locales and eukaryotic hosts, including corals, molluscs, sponges and zooplankton (Thompson *et al.*, 2004).

Sponges can maintain diverse symbiotic microbe populations that differ in composition from the microbial communities

of surrounding seawaters (Taylor *et al.*, 2007). Novel bacteria isolated from sponges have been reported previously (Ahn *et al.*, 2011; Olson *et al.*, 2007; Yoon *et al.*, 2011). Strain N384^T was isolated from a marine sponge (*Scleritoderma cyanea*) collected in May 2000 from a depth of 795 feet (242 m) off the west coast of Curaçao using the *Johnson-Sea-Link* manned submersible (Sfanos *et al.*, 2005). A previous study suggested, based on 16S rRNA gene sequencing, that strain N384^T might represent a novel species of the genus *Vibrio* (Hoffmann *et al.*, 2010a). In the present study, detailed genotypic and phenotypic analyses were performed that confirm that strain N384^T does indeed represent a novel, previously unidentified *Vibrio* species.

Isolation of strain N384^T from *Scleritoderma cyanea* was carried out as described previously (Sfanos *et al.*, 2005). *Vibrio brasiliensis* LMG 20546^T, *V. splendidus* ATCC 33789, *V. scophthalmi* LMG 19158^T, *V. hepatarius* LMG 20362^T, *V. ichthyoenteri* ATCC 700023^T, *V. orientalis* ATCC 33934^T, *V. tubiashii* ATCC 19109^T, *V. nigripulchritudo* ATCC 27043^T, *V. coralliilyticus* ATCC BAA-450^T and *V. sinaloensis* DSM 21326^T were obtained from the Belgian Co-ordinated Collection of Microorganisms (BCCM, Ghent, Belgium), the American Type Culture Collection (ATCC, Manassas, VA, USA) or the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). Bacteria 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 or on trypticase soy agar (TSA; Oxoid) supplemented with 1 % NaCl (w/v) at 28 °C for 24 h.

Phenotypically, strain N384^T can be assigned clearly to the genus *Vibrio* (Baumann *et al.*, 1984; Noguerola & Blanch, 2008). Gram status was determined as Gram-negative using the KOH method, as described by Buck (1982). Catalase activity was determined to be negative for gas production in 3 % (v/v) H_2O_2. Oxidase activity was confirmed as positive using the Pathotec cytochrome oxidase test (Remel). Bacterial morphology and the presence of flagella were confirmed by electron microscopy using a JEOL 1011 transmission electron microscope operating at an accelerating voltage of 80 kV. Motility was tested on motility agar (1 % tryptone, 1.5 % NaCl, 0.3 % agar) after incubation overnight at 28 °C. Strain N384^T was confirmed to be a motile, slightly curved rod possessing a polar flagellum (Fig. S1, available in IJSEM Online). Growth at 0–10.0 % (w/v) NaCl, 5–40 °C and pH 4.0–12.0 (adjusted using HCl and KOH) was recorded for 5 days with shaking. Media used for growth studies in different NaCl concentrations were prepared in-house with individual components (pancreatic digest of casein, papaic digest of soybean meal, dipotassium phosphate and glucose) to match the concentrations noted in commercially available TSB without NaCl. Growth of strain N384^T occurred in TSB supplemented with 1 % NaCl (w/v) after 24 h between 10–35 °C and at 0.5–8.0 % NaCl and pH 6.0–10.0. Additionally, growth was tested on thiosulfate-citrate-bile salts-sucrose agar (TCBS agar; Difco), which is highly selective for the isolation of vibrios. Consistent with the properties of the genus *Vibrio*, the novel strain grew on TCBS agar, forming green colonies, indicating the absence of sucrose fermentation.

Antibiotic sensitivity was established using the disc susceptibility assay, as described by the CLSI (2006). Sensitivity to the vibriostatic agent O/129 (2,4-diamino-6,7-diisopropylpteridine) was determined using Oxoid discs containing 10 and 150 μg. Strain N384^T was susceptible to piperacillin (100 μg), cefoxitin (30 μg), ceftazidime (30 μg), cephalothin (30 μg), imipenem (10 μg), amikacin (30 μg), gentamicin (10 μg), tetracycline (30 μg), ciprofloxacin (5 μg), chloramphenicol (30 μg) and trimethoprim-sulfamethoxazole (1.25/23.75 μg) but showed an intermediate response to ampicillin (10 μg). Additionally, strain N384^T was susceptible to the vibriostatic agent O/129 at both 10 and 150 μg.

Biochemical characterization was performed using the standardized API 20E, API 20NE, API ZYM and API 50CH identification systems (bioMérieux) and Biolog GN1, GN2 and GN3 microtitre plates with incubation at 28 °C, according to the manufacturers' instructions, except that sterile 1.5 % (w/v) NaCl was used to prepare the inocula. Type strains of closely related *Vibrio* species (*V. brasiliensis* LMG 20546^T, *V. hepatarius* LMG 20362^T, *V. orientalis* ATCC 33934^T, *V. tubiashii* ATCC 19109^T, *V. nigripulchritudo* ATCC 27043^T, *V. coralliilyticus* ATCC BAA-450^T and *V. sinaloensis* DSM 21326^T) were included in the phenotypic analyses. Biochemical characteristics that are important in differentiating strain N384^T from closely related *Vibrio* species are listed in Table 1. Surprisingly, strain N384^T did not reduce nitrate to nitrite and did not produce indole, whereas most vibrios are positive for these attributes. Strain N384^T belongs, phenotypically, to the arginine dihydrolase-positive, lysine decarboxylase- and ornithine decarboxylase-negative (A+/L−/O−) cluster, like its nearest neighbours, *V. brasiliensis* and *V. sinaloensis*. Although phenotypically similar to its nearest neighbours, membership of the A+/L−/O− cluster can be used to differentiate strain N384^T from vibrios of the Harveyi clade, which mostly clustered in the A−/L+/O+ group (Chimetto *et al.*, 2011).

Several other biochemical attributes of strain N384^T were evaluated, all at 30 °C. Among those, DNase activity was tested on TSA containing 1.5 % NaCl, DNA and methyl green and confirmed to be positive. Assays of amylase activity (starch) on nutrient agar supplemented with potato starch, according to the FDA Bacteriological Analytical Manual, yielded negative results. Furthermore, Tween 80 hydrolysis (lipase) by N384^T was shown to be negative by two separate methods (Kilburn *et al.*, 1973; Oliver *et al.*, 1986). Casein hydrolysis by N384^T was negative on heart infusion agar containing 1 % NaCl and 1 % skimmed milk, as described by Hülsmann *et al.* (2003). Strain N384^T was unable to ferment glucose or produce gas using the method of Baumann & Baumann (1981).

The cellular fatty acid composition was determined for N384^T, *V. brasiliensis* LMG 20546^T and *V. sinaloensis* DSM

Table 1. Phenotypic characteristics that differentiate *V. caribbeanicus* sp. nov. from type strains of phylogenetically related *Vibrio* species

Strains: 1, *V. caribbeanicus* sp. nov. N384^T; 2, *V. brasiliensis* LMG 20546^T; 3, *V. sinaloensis* DSM 21326^T; 4, *V. tubiashii* ATCC 19109^T; 5, *V. orientalis* ATCC 33934^T; 6, *V. hepatarius* LMG 20362^T; 7, *V. coralliilyticus* ATCC BAA-450^T; 8, *V. nigripulchritudo* ATCC 27043^T. All data were obtained in this study. All strains were negative for ornithine decarboxylase.

Characteristic	1	2	3	4	5	6	7	8
Growth in/at:								
8 % (w/v) NaCl	+	+	+	−	−	+	+	−
0.5 % (w/v) NaCl	+	+	+	+	−	−	+	+
10 °C	+	−	−	−	+	−	−	−
35 °C	+	+	+	+	−	−	+	−
37 °C	−	+	+	−	−	−	−	−
pH 6	+	+	+	+	+	+	+	−
Indole production	−	+	+	+	+	+	+	+
Nitrate reduction	−	+	+	+	+	+	+	+
Arginine dihydrolase	+	+	+	+	−	+	+	−
Lysine decarboxylase	−	−	−	−	+	−	−	−
Hydrolysis of:								
Gelatin	−	+	+	+	+	+	+	+
Aesculin	−	+	+	−	−	−	−	+
Enzyme activities of:								
Esterase (C4)	+	−	+	−	−	+	+	+
Lipase (C14)	−	−	−	−	−	+	+	−
β-Galactosidase	−	+	+	+	−	+	+	+
Trypsin	+	−	−	−	−	−	−	−
Tryptophan deaminase	−	−	+	+	−	−	+	−
Fermentation of:								
Mannitol	−	+	+	+	+	+	+	−
Amygdalin	−	+	+	+	+	+	−	+
Sucrose	−	+	+	+	+	+	+	+
Melibiose	−	−	+	+	−	−	−	+
Arabinose	−	−	−	−	−	−	−	+
Assimilation of:								
Glucose	+	+	−	−	−	−	+	−
Mannose	−	+	+	−	−	−	−	−
Malate	−	+	−	+	−	−	−	−

21326^T using bacteria grown for 24 h at 28 °C on TSA containing 1 % NaCl (w/v). Fatty acid methyl ester (FAME) analysis was performed as described previously (Hoffmann *et al.*, 2010b). FAMEs were analysed by gas chromatography with flame-ionization detection (GC-FID), using the rapid Microbial Identification System software (RBTR20; MIDI Inc.) to identify the relative amounts of fatty acids. Differences in fatty acids among bacterial strains were assessed by ANOVA (Snedecor & Cochran, 1980). Values are expressed as means with standard deviations. The Duncan multiple comparison method was used to differentiate among means for variables that were significantly different (Snedecor & Cochran, 1980). The major fatty acids were $C_{12:0}$, iso-$C_{13:0}$, $C_{12:0}$ 3-OH, $C_{14:0}$, $C_{15:0}$, $C_{16:0}$, iso-$C_{17:0}$, $C_{18:1}\omega7c$, summed $C_{14:0}$ 3-OH/iso-$C_{16:1}$ and summed $C_{16:1}\omega6c/C_{16:1}\omega7c$, accounting for 87.33 % of the total fatty acids (Table S1). FAME analysis could be used to differentiate strain N384^T from other closely related *Vibrio* species. The fatty acid profile from strain N384^T showed significant differences from those of *V. brasiliensis* LMG 20546^T and *V. sinaloensis* DSM 21326^T. The major fatty acids used to distinguish strain N384^T from closely related vibrios were iso-$C_{13:0}$, $C_{15:0}$, $C_{16:0}$ and iso-$C_{17:0}$. Representative amounts of these fatty acids were significantly higher in strain N384^T compared with *V. brasiliensis* LMG 20546^T and *V. sinaloensis* DSM 21326^T, while amounts of iso-$C_{14:0}$, iso-$C_{14:0}$ 3-OH and iso-$C_{16:0}$ were significantly lower in N384^T (Table S1).

DNA–DNA hybridization between N384^T and *V. brasiliensis* LMG 20546^T, *V. splendidus* DSM 19640^T, *V. orientalis* DSM 19142^T, *V. tubiashii* DSM 19142^T, *V. nigripulchritudo* DSM 21607^T, and *V. sinaloensis* DSM 21326^T was performed in duplicate by the Identification Service at the DSMZ. DNA used for each analysis was purified at the DSMZ, as described by Cashion *et al.* (1977). Hybridization was carried out as described by De Ley *et al.* (1970) incorporating the modifications described by Huß *et al.* (1983). DNA from strain N384^T showed relatively low DNA–DNA relatedness with *V. brasiliensis* LMG 20546^T, *V. splendidus* ATCC 33789, *V. orientalis* ATCC 33934^T, *V. tubiashii* ATCC 19109^T, *V. nigripulchritudo* ATCC 27043^T and *V. sinaloensis* DSM 21326^T (6.2 ± 1.05, 19.0 ± 0.85, 11.3 ± 2.75, 16.2 ± 3.40, 20.6 ± 4.35 and 25.2 ± 4.00 %, respectively), significantly below the recommended cut-off threshold value of 70 % DNA–DNA hybridization for the identification of bacterial species (Wayne *et al.*, 1987). These results further support the notion that N384^T represents a species distinct from either of its nearest neighbours, as well as from *V. splendidus*.

16S rRNA gene sequence analysis was performed as described previously (Hoffmann *et al.*, 2010c). Multilocus sequence analysis (MLSA) of eight housekeeping genes encoding DNA gyrase B subunit (*gyrB*), topoisomerase I (*topA*), recombination repair protein (*recA*), a cell division protein (*ftsZ*), actin-like cytoskeleton protein (*mreB*), glyceraldehyde-3-phosphate dehydrogenase (*gapA*), RNA polymerase alpha-subunit (*rpoA*) and uridylate kinase (*pyrH*), was carried out as described previously (Sawabe *et al.*, 2007; Thompson *et al.*, 2005). PCR amplicons were sequenced by Molecular Cloning Laboratories (MCLAB, San Francisco, CA, USA). DNA sequences were edited and assembled using the DNASTAR Lasergene SeqMan II 5.07 sequence analysis software (accession numbers listed in Table S2).

16S rRNA gene sequences and DNA sequences for the MLSA study were aligned using MAFFT (Katoh *et al.*, 2002). Phylogenetic analyses were performed using PAUP* version 4.10b (Swofford, 2002), the GARLI software (Zwickl, 2006) and MrBayes version 3.1.2 (Huelsenbeck & Ronquist, 2001). Maximum-parsimony (MP) trees were sought using heuristic search methods combined with tree bisection-reconnection

branch swapping and 1000 random, stepwise addition replicates. When necessary, a posteriori reweighting based on the RCI (base weight=10) was performed in order to reduce the number of equally parsimonious trees. Bootstrap analyses were performed with 1000 replicates being considered. Distance matrices were generated according to the Jukes–Cantor correction in MEGA version 4 (Tamura *et al.*, 2007). Congruence between genes was assessed using the incongruence length difference (ILD) test (Farris *et al.*, 1994). ILD tests were performed in PAUP* version 4.10b with 1000 data partitions using branch and bound searches (Allard *et al.*, 1999).

Maximum-likelihood (ML) trees were generated in GARLI and visualized in PAUP* version 4.10b. The shortest MP tree was used for a starting topology for the evaluation of 16 nested models of sequence evolution (Frati *et al.*, 1997; Sullivan *et al.*, 1997; Swofford *et al.*, 1996). Parameter space was searched for the best tree with simultaneous estimation for model parameters using a ML search. Twenty runs were performed. Branch support was determined by 100 ML bootstrap iterations with Bayesian posterior probability. The parameters of sequence evolution estimated from the final ML tree were used for Bayesian character support methods with one million generations, discarding 25 % of the tree samples.

After analysing and assembling the 1470 bp 16S rRNA gene sequence of strain N384^T, the consensus sequence was used to query the GenBank database at NCBI using BLAST to identify those strains with the highest sequence identity. Sequence searches at the NCBI database demonstrated that strain N384^T belongs to the genus *Vibrio*. Moreover, the analysis (query coverage 100 %) revealed that *V. brasiliensis* LMG 20546^T, *V. sinaloensis* CAIM 695, *V. tubiashii* ATCC 19109^T and *V. nigripulchritudo* ATCC 27043^T were the *Vibrio* type strains most closely related to strain N384^T, sharing 98.7, 98.6, 98.6 and 98.3 % 16S rRNA gene sequence identity, respectively.

A previous study showed an MP tree derived from 16S rRNA gene sequences from 72 different *Vibrio* species and 23 *Vibrio* isolates, including N384^T. The report suggested that strain N384^T might represent a novel *Vibrio* species (Hoffmann *et al.*, 2010a). In this study, an analysis of 16S rRNA gene sequences from N384^T and strains of 24 *Vibrio* species allowed construction of an MP tree (Fig. 1) and an ML tree (Fig. S2). The two phylogenetic trees had similar topologies, showing divergence of strain N384^T, and clustered strain N384^T into the 'Orientalis' clade of vibrios (Sawabe *et al.*, 2007).

MP trees were individually constructed for each of the eight housekeeping genes evaluated in this study (*topA*, *gyrB*, *recA*, *rpoA*, *ftsZ*, *mreB*, *pyrH* and *gapA*) (Fig. S3). Phylogenetic analysis of *pyrH*, *gyrB*, *ftsZ* and *mreB* sequences revealed substantial divergence of strain N384^T from other vibrios, consistently yielding more than 15 % sequence divergence from adjacent strains in the trees and further supporting its species-level variation among vibrios. Interestingly, not all of the eight genes placed strain N384^T adjacent to members of the 'Orientalis' clade, despite clear evolutionary kinship between this strain and other 'Orientalis' group species based on 16S rRNA gene sequences and the majority of the housekeeping genes reported here. For example, trees from *gapA* and *recA* grouped N384^T with

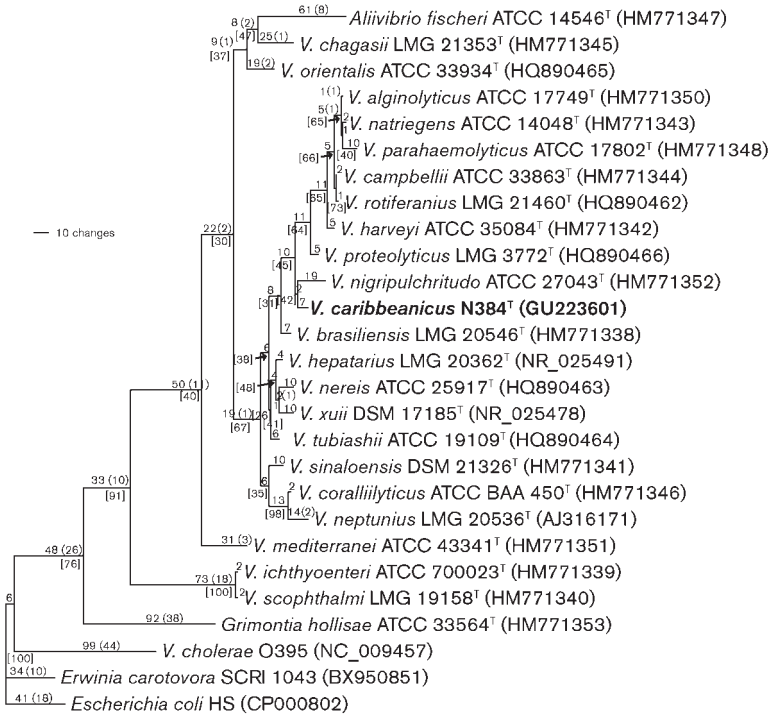

Fig. 1. MP tree derived from 16S rRNA gene sequences, showing the relationship between *V. caribbeanicus* sp. nov. and 24 other *Vibrio* species. Individual branch lengths are presented above each branch; the numbers of unambiguous substitutions that mapped to the tree only once and are greater than zero are given in parentheses. Measures of clade confidence are reported in brackets below each node in the form of bootstrap values (1000 iterations). The tree was rooted with *Escherichia coli* HS and *Erwinia carotovora* SCRI1043. Bar, 10 changes.

Vibrio alginolyticus ATCC 17749^T, while *topA* clustered our strain more closely with *Aliivibrio fischeri* ATCC 7744^T. Sequence comparisons further underscored this observation, revealing unusually high similarity between N384^T and *V. alginolyticus* ATCC 17749^T (99.9 %) and between N384^T and *A. fischeri* ATCC 7744^T (99.9 %).

Given the divergence of strain N384^T from other known species, it was intriguing to find extensive genetic homogeneity among alleles from such disparate vibrios. Depauperate genetic diversity among genes may signal a role for horizontal gene transfer (HGT), which could account for sequence similarity between diverged species. That is, the horizontal reintroduction of one or a few preferred alleles among these species would prevent allelic diversification and account for such unusual homogeneity (Patel & Loeb, 2000). It has long been noted that HGT can have a homogenizing effect on specific regions of the bacterial chromosome (Dykhuizen & Green, 1991), a thesis supported by empirical studies of *polA* in *Escherichia coli* (Brown *et al.*, 2001).

In order to examine the likelihood of HGT among these sequences, we applied the ILD test, which evaluates the null hypothesis of phylogenetic congruence between sequences from different genes (Farris *et al.*, 1994). Rejection of the null often supports a role for HGT between bacterial species and strains (Brown *et al.*, 2002; Lecointre *et al.*, 1998). ILD testing was conducted on strain N384^T, six members of the 'Orientalis' clade and *V. nigripulchritudo*, as well as *V. alginolyticus* and *A. fischeri* (Table 2). Simultaneous ILD testing of the eight genes partitioned separately revealed substantial incongruence (P=0.001). To isolate the genes responsible for the incongruence, each of the eight genes was partitioned against a combined matrix of the remaining seven. As expected from earlier observations in the gene trees, *topA*, *recA* and *gapA* all revealed the highest levels of incongruence with the remaining housekeeping genes, signalling a substantial role for HGT in the structuring of alleles from these three genes. Subsequent removal of these genes revealed a marked improvement in congruence among the remaining five. In order to improve phylogenetic accuracy using our MLSA dataset, *topA*, *recA* and *gapA* were removed from the combined gene analysis. It has long been noted that 'combining data (genes) with different histories to produce a single reconstruction ... not only obscures an important feature of history but runs the risk of producing a reconstruction that fails to represent either history' (Bull *et al.*, 1993).

Combined MLSA from concatenated sequences of the remaining five housekeeping genes (total alignment length 4068 bp) revealed similar results to the 16S rRNA gene sequence analysis. The construction of an MP tree (Fig. 2) and an ML tree (Fig. S4) showed a clear separation of N384^T from its nearest neighbours and also clustered strain N384^T into the 'Orientalis' clade of vibrios. The concatenated MLSA approach has been reported as a valuable technique for the identification of vibrios, as well as for identifying novel *Vibrio* species (Chimetto *et al.*, 2011). Thompson *et al.* (2009) suggested that *Vibrio* species are defined as a group of strains that share more than 95 % similarity among MLSA datasets. Consequently, strains below this threshold value would qualify as members of separate species. As can be observed in Fig. 2, the results obtained for strain N384^T show that the strain is <95 % similar to its nearest neighbour based on reported branch lengths. Moreover, distance matrices for 16S rRNA gene sequences and the MLSA combined sequence dataset (data not shown) provide additional evidence that supports substantial genetic divergence of N384^T from other closely related *Vibrio* species.

Shotgun genome sequencing for N384^T, *V. brasiliensis* LMG 20546^T, *V. splendidus* ATCC 33789, *V. orientalis* ATCC 33934^T, *V. tubiashii* ATCC 19109^T, *V. nigripulchritudo* ATCC 27043^T and *V. sinaloensis* DSM 21326^T was carried out with a Genome Sequencer FLX 454 Life Sciences (Roche) using the GS FLX Titanium Sequencing kit XLR70

Table 2. ILD values among eight housekeeping loci compared with five housekeeping loci in ten vibrio type strains

Values represent *P*-values for 1000 ILD partitions using the partition homogeneity test and the branch and bound search option available in PAUP* v.4.03b. *V. caribbeanicus* sp. nov. N384^T, *V. alginolyticus* ATCC 17749^T, *Aliivibrio fischeri* ATCC 7744^T, *V. nigripulchritudo* ATCC 27043^T, *V. brasiliensis* LMG 20546^T, *V. hepatarius* LMG 20362^T, *V. xuii* LMG 21346^T, *V. tubiashii* ATCC 19109^T, *V. sinaloensis* DSM 21326^T and *V. orientalis* ATCC 33934^T were included in each comparison.

Eight loci	*P*-value	Five loci	*P*-value
gyrB, *rpoA*, *ftsZ*, *mreB*, *pyrH*, *topA*, *recA*, *gapA*	0.001	*gyrB*, *rpoA*, *ftsZ*, *mreB*, *pyrH*	0.005
gyrB	0.058	*gyrB*	0.044
rpoA	0.005	*rpoA*	0.539
ftsZ	0.003	*ftsZ*	0.223
mreB	0.056	*mreB*	0.274
pyrH	0.085	*pyrH*	0.011
topA	0.001		
recA	0.001		
gapA	0.001		

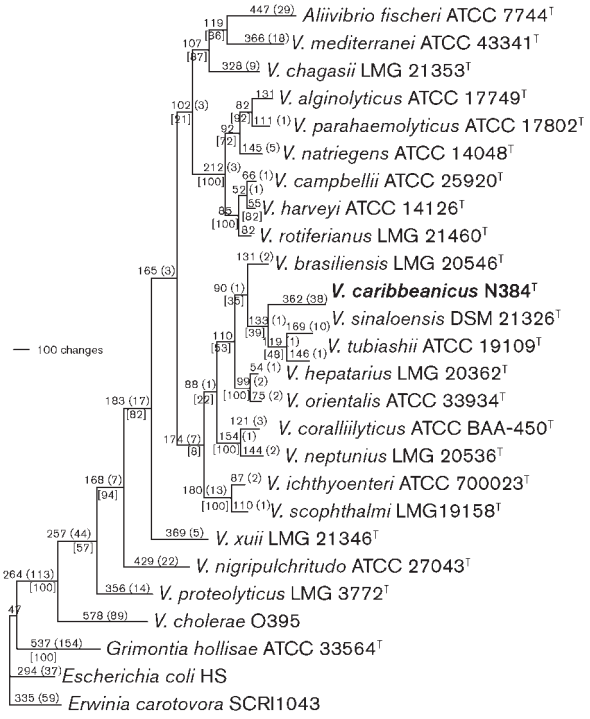

Fig. 2. MP tree derived from concatenated sequences of *gyrB*, *rpoA*, *ftsZ*, *mreB* and *pyrH* genes (total length 4068 bp) showing the relationship between *V. caribbeanicus* sp. nov. and 23 other *Vibrio* species. See Fig. 1 for other details. Bar, 100 changes.

according to the manufacturer's recommended protocol. Contigs were assembled using Roche Newbler software (version 2.3). Sequences were annotated using the NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP; http://www.ncbi.nlm.nih.gov/genomes/static/Pipeline.html) (locus tag VIB2010; NCBI project number 49601) and have been deposited at DDBJ/EMBL/GenBank. The new genomic sequence for N384^T had 102 contigs with a total length of approximately 4 403 195 bp. The mean contig size was 43 168 bp, with the N_{50} (50 % of the genome is contained in contigs of size N or greater) contig size being 122 512 bp. The mean DNA G + C content of strain N384^T was calculated to be 41.6 mol% using MEGA version 4.0, which falls within the range of 38–51 mol% typically observed for vibrios (Farmer *et al.*, 2005).

The availability of complete bacterial genomes has provided new possibilities for bacterial species classification. One way has been to measure the average nucleotide identity (ANI), a highly accurate technique that measures genetic and evolutionary distance between two genomes (Konstantinidis & Tiedje, 2005) using recently improved methods (Goris *et al.*, 2007). Goris *et al.* (2007) demonstrated that 70 % DNA–DNA hybridization corresponds to 95 % ANI. The ANI determined in this study using the complete genome sequences of *Vibrio* strains available at NCBI and whole-genome sequences from N384^T, *V. brasiliensis* LMG 20546^T, *V. splendidus* ATCC 33789, *V. orientalis* ATCC 33934^T, *V. tubiashii* ATCC 19109^T, *V. nigripulchritudo* ATCC 27043^T and *V. sinaloensis* DSM 21326^T are listed in Table S3. These ANI values clearly indicate that N384^T has an ANI less than 95 % to other closely related vibrios, again confirming species-level distinctiveness.

A protein BLASTatlas was constructed using *V. parahaemolyticus* RMD 2210633 as the reference genome. The BLASTatlas plots were constructed as described previously (Hallin *et al.*, 2008) using software available at http://www.cbs.dtu.dk/services/gwBrowser/index.php. The genomes of N384^T, *V. brasiliensis* LMG 20546^T and *V. sinaloensis* DSM 21326^T were annotated and compared to the reference chromosomes one and two from *V. parahaemolyticus* RIMD 2210633 (GenBank accession numbers BA000031 and BA000032). *V. parahaemolyticus* RIMD 2210633 genes were blasted against the other genomes using TBLASTX with the percentage identity for each gene being plotted in the outer circle. The BLASTatlas was generated to provide a global visualization of the differences in gene content between our novel strain and the genomes from the two closest relatives, *V. brasiliensis* LMG 20546^T and *V. sinaloensis* DSM 21326^T (Fig. S5). Since BLASTatlas only compares protein-encoding genes, intergenic regions, tRNAs and rRNAs are visualized as 'gaps' in the query genomes. Notable is the fact that chromosome one (containing genes for growth and viability) is more conserved than chromosome two (containing genes for adaptation to environmental change); however, the BLASTatlases for both chromosomes show numerous and substantial compositional differences between coding regions of the N384^T genome and the genomes of *V. parahaemolyticus* RIMD 2210633, *V. brasiliensis* LMG 20546^T and *V. sinaloensis* DSM 21326^T.

This study has analysed strain N384^T exhaustively, both phenotypically and genotypically, to determine whether there is sufficient evidence to support this strain as a member of a novel and previously uncharacterized *Vibrio* species. Phenotypically, there are several traits that are observed in strain N384^T that are unique to the strain, indicating non-conformity with previously established *Vibrio* species. Furthermore, the genotypic analyses demonstrate that strain N384^T satisfies all of the previously described requirements for documenting that an isolate does, in fact, represent a novel species. Taken together, these data confirm that strain N384^T represents a novel species that belongs to the genus *Vibrio* and for which the name *Vibrio caribbeanicus* sp. nov. is proposed.

Description of *Vibrio caribbeanicus* sp. nov.

Vibrio caribbeanicus (ca.rib.be.a′ni.cus. N.L. masc. adj. *caribbeanicus* of the Caribbean Sea).

Cells are slightly curved, Gram-negative, motile rods with one polar flagellum (approx. 1.0 μm wide and 2.5–3.1 μm long). Good growth occurs on marine agar and on TSA supplemented with 1 % NaCl (w/v) at 28 °C for 24 h. Colonies are

cream coloured, translucent and smooth-rounded with a diameter of 1–2 mm, motile but not swarming. Growth occurs at 10–35 °C, at NaCl concentrations of 0.5–8.0 % and at pH 6.0–10.0. Optimal growth is observed at 28 °C with 1.5 % NaCl (w/v) at pH 7. Colonies are green, convex, round and about 1 mm in size on TCBS agar after 48 h incubation at 28 °C. Bioluminescence is not observed. Susceptible to the vibriostatic agent O/129 (at both 10 and 150 μg) and shows an intermediate response to ampicillin (10 μg). Catalase-negative, oxidase-positive and belongs to the arginine dihydrolase-positive, lysine decarboxylase- and ornithine decarboxylase-negative (A+/L−/O−) cluster. Positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, trypsin and DNase activities and D-glucose oxidation. Negative for glucose fermentation and gas formation (anaerobic conditions). Negative for lipase (C14), amylase activity (starch), valine arylamidase, cystine arylamidase, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, urease, tryptophan deaminase and α-fucosidase activities. Negative for citrate utilization, nitrate reduction to nitrite, production of indole, H_2S and acetoin (Voges–Proskauer), hydrolysis of gelatin, casein and aesculin, fermentation of D-mannitol, inositol, D-sorbitol, L-rhamnose, sucrose, melibiose, amygdalin and L-arabinose and assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetylglucosamine, maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid. In tests with Biolog GN1, GN2 and GN3 microtitre plates, utilizes *N*-acetyl-D-glucosamine, L-proline, DL-alanine, trehalose, DL-serine, L-threonine, L-glutamic acid, L-asparagine, adenosine, 2-deoxyadenosine, inosine, L-alanyl glycine, DL-malic acid, D-ribose, D-fructose, acetoacetic acid, acetic acid, α-D-glucose, glycyl L-proline, L-proline, DL-serine, L-tryptophan, L-threonine, L-tyrosine, L-citrulline, D-glucosamine, D-galactosamine, D-mannosamine, L-arginine, glycine, ammonia (as nitrogen source), L-cysteine and cytidine, but cannot utilize Tweens 20, 40 or 80, D-galactose, DL-arabinose, D-mannose, DL-fucose, glycerol, D-sorbitol, D-xylose, cellobiose, DL-aspartic acid, D-gluconic acid, DL-α-glycerol phosphate, gelatin, dextrin, methyl β-D-galactoside, β- or γ-hydroxybutyric acids, hydroxy-L-proline, guanosine, L-histidine, L-isoleucine, L-methionine, L-phenylalanine, L-homoserine, D-saccharic acid, thymidine, 1,2-propanediol, lactose, uridine, D-glucose 1-phosphate, D-threonine, glycogen, allantoin, methylamine or hydroxylamine. The fatty acid profile consists of $C_{12:0}$, iso-$C_{13:0}$, $C_{12:0}$ 3-OH, $C_{14:0}$, iso-$C_{15:0}$, $C_{15:0}$, $C_{16:0}$, iso-$C_{17:0}$, $C_{17:1}\omega8c$, $C_{17:0}$, $C_{18:1}\omega7c$, summed $C_{14:0}$ 3-OH/iso-$C_{16:1}$ and summed $C_{16:1}\omega6c/C_{16:1}\omega7c$. The DNA G+C content of the type strain is 41.6 mol%.

The type strain, N384^T (=ATCC BAA-2122^T=DSM 23640^T), was isolated from a marine sponge (*Scleritoderma cyanea*; phylum Porifera) collected from a depth of 795 feet (242 m) off the west coast of Curaçao (Van Soest *et al.*, 2008).

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