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Vibrio eleionomae sp. nov., isolated from shrimp (*Penaeus vannamei*) pond water

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Abstract

A novel *Vibrio* strain (CAIM 722^T=SW9^T=DSM 24596^T) was isolated in 2003 from water of a shrimp (*Penaeus vannamei*) culture pond located in Los Mochis, Sinaloa, Mexico, and taxonomically characterized using a polyphasic approach. The 16S rRNA gene sequence clustered within those of the genus *Vibrio*, showing high similarity to the type strains of the Porteresiae clade. Multilocus sequence analysis using eight housekeeping genes (*ftsZ*, *gapA*, *gyrB*, *mreB*, *pyrH*, *recA*, *rpoA*, *topA* and 16S rRNA) and phylogenetic analysis with 139 single-copy genes showed that the strain forms an independent branch. Whole genome sequencing and genomic analyses (average nucleotide identity, OrthoANI, average amino acid identity and *in silico* DNA–DNA hybridization) produced values well below the thresholds for species delineation with all methods tested. In addition, a phenotypic characterization was performed to support the description and differentiation of the novel strain from related taxa. The results obtained demonstrate that the strain represent a novel species, for which the name *Vibrio eleionomae* sp. nov. is proposed.

INTRODUCTION

The genus *Vibrio* was originally proposed in 1854 [1] and, at the time of writing, the genus comprises 144 valid bacterial species (www.bacterio.net/vibrio.html). They are Gram-negative, fermentative, motile, halophilic and found in estuarine and marine habitats [2].

Some *Vibrio* species remain in planktonic form or closely associated with marine plants and animals [3]. These associations range from the bioluminescence symbiosis of *Vibrio fischeri* [4], to the pathogenic interactions between a variety of *Vibrio* species and marine species. For example, *Vibrio alginolyticus* is a pathogen associated with bivalves [5], *Vibrio ordalii* is a fish pathogen [6], and *Vibrio harveyi*, *Vibrio campbellii* and *Vibrio parahaemolyticus* are pathogens for shrimp [7–9].

However, within this genus there are closely related species that are difficult to identify. Due to ambiguity in correct taxonomic classification, polyphasic taxonomy has been proposed as an effective way for phylogenetic classification and identification of bacteria, and has allowed for reliable taxonomic identification [10]. To further ascertain the taxonomic position of newly described species, whole-genome sequencing is now required.

In this study, with a genomic approach based on genome-wide parameters and the analysis of phenotypic, genotypic and phylogenetic characteristics, the identification and classification of strain CAIM 722^T was done. The results of these analyses support the description of a novel species, for which the name *Vibrio eleionomae* sp. nov. is proposed.

METHODS

Strain CAIM 722^T (= SW9^T=DSM 24596^T) was isolated from water from a shrimp (*Penaeus vannamei*) pond located in Los Mochis, Sinaloa, Mexico, in April 2003. The strain was obtained from the Collection of Aquatic Important Microorganisms

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Keywords: AAI; ANI; GGDC; MLSA; 139 single-copy genes; porteresiae clade; *Vibrio*.
Abbreviations: AAI, average amino acid identity; ANI, average nucleotide identity; DDH, DNA–DNA hybridization; GGDC, Genome-to-Genome Distance Calculator; MLSA, multilocus sequence analysis; SCG, single-copy gene.
The GenBank/EMBL/DDBJ accession number for genome is WEKT00000000 and for the 16S rRNA is ON406572.
Four supplementary figures and two supplementary tables are available with the online version of this article.

(CAIM; registered at the WFFC with number 813; Unit for Aquaculture and Environmental Management, CIAD, Mazatlán, Sinaloa, Mexico). It was grown on tryptone soy agar (Oxoid) supplemented with 2% NaCl (w/v) and incubated at 30 °C for 24 h.

Phenotypic characterization was performed as described previously [11] with minor modifications as follows. Briefly, the following tests were performed: Gram staining, catalase and oxidase activities, cell morphology, motility, oxidation/fermentation test, Voges–Proskauer test, utilization of citrate, arginine dehydrolase, lysine and ornithine decarboxylation, and nitrate reduction. Further characterization was done using API 20E, API 50E and API ZYM tests strips (bioMérieux). Tryptone soy broth was used to determine salt tolerance (0–10% NaCl) and growth at different temperatures (4, 20, 30, 37, 40 °C) and pH (pH 4–10).

The Promega kit (Wizard Genomic DNA Purification Kit) was used to extract the Genomic DNA of the isolate. The integrity, purity and quantity of extracted DNA were checked by agarose gel electrophoresis visualization and measuring the absorbance with a spectrophotometer DS-11 Fx (DeNovix, A260/A280 ratio). The draft genome of CAIM 722^T was sequenced with the Illumina Miniseq platform (300 cycles, 2×150 bp). A genomic DNA library was prepared using the Nextera XT Library Preparation Kit, using a single enzymatic 'tagmentation' reaction. The sequence data was checked using FASTQC (www.bioinformatics.babraham.ac.uk/projects/fastqc/), after filtering, the pair-end reads were assembled using SPAdes 3.13.0 [12]. Genomic annotation was carried out using Prokka [13] and basic information about the genome was extracted using QUAST software version 5.0.2 [14]. To predict the integrity and contamination of the genome CheckM2 version 1.2 was used [15].

Phylogenomic analyses were done with Anvio version 5.5 [16]; the phylogenomics workflow (http://merenlab.org/2017/06/07/phylogenomics/) was followed and the HMM profile for 139 single-copy genes (SCGs) from Campbell *et al.* [17], was used. Multilocus sequence analysis (MLSA) was performed by using eight housekeeping gene sequences (*ftsZ*, *gapA*, *gyrB*, *mreB*, *pyrH*, *recA*, *rpoA* and *topA*) and the 16S rRNA gene [18]. All of the gene sequences were extracted from the genome. Phylogenetic trees were reconstructed by using the maximum-likelihood algorithm with MEGA version 6 [19] and concatenated genes. The genome sequences used in this study are listed in supplementary Table S1.

Genome relatedness with closely related species was evaluated in terms of average nucleotide identity (ANI), OrthoANI as described by Lee *et al.* [20], and Genome-to-Genome Distance Calculator (GGDC) relatedness; whereas average amino acid identity (AAI) was calculated with GET_HOMOLOGUES version x86_64–20171023 with the following settings: -M –t 0 –n -A [21]. *In silico* phenotyping was performed using Traitar (version 1.20) [22].

RESULTS AND DISCUSSION

Physiology and chemotaxonomy

Strain CAIM 772^T was isolated from water of a shrimp (*Penaeus vannamei*) pond sampled in Los Mochis, Sinaloa, Mexico. The strain studied here had phenotypic characteristics that placed it as a member of the genus *Vibrio*, as it is a Gram-negative small

Table 1. Phenotypic traits that distinguish strain CAIM 722^T from the type strains of closely related *Vibrio* species

Strains:1, CAIM 722^T (=SW9^T=DSM 24596^T); 2, *V. palustri* CECT 9025^T; 3, *V. porteresiae* DMS 19223^T; 4, *V. zhugei* HBUA S61001^T; 5, *V. tritonius* JCM 16456^T.

Characteristic	1	2	3	4	5
Arginine dihydrolase	+	–*	–*	–*	–*
Lysine decarboxylase	–	–*	–	–*	–
Oxidase	–	–	+*	+*	+*
Growth on TCBS	W	–	–	–	–
Voges–Proskauer	V*	+*	+*	+*	+*
Catalase	+*	+*	+*	+*	–
Citrate	–	–	+*	+*	+*
Nitrate to nitrite	+	+*	–	ND	+*
Salinity growth range (%)	0.5–10	1–10	5.0–7.5	4.0–9.0	0.5–6.0
pH growth range	4.5–8.5	6.0–8.0	5.5–9.0	4.0–10	4.5–9.0
Temperature growth range (°C)	15–37	15–26	20–37	8–37	25–30

Data in column 1 is from this study; data in columns 2–5 are from Lucena *et al.* [29], Rameshkumar *et al.* [27], Guo *et al* [30] and Sawabe *et al.* [31], respectively. +, Positive; –, negative; ND, no data available; V, variable results; W, weak.
*These results coincide with the results for the *in silico* phenotyping (Fig. S1).

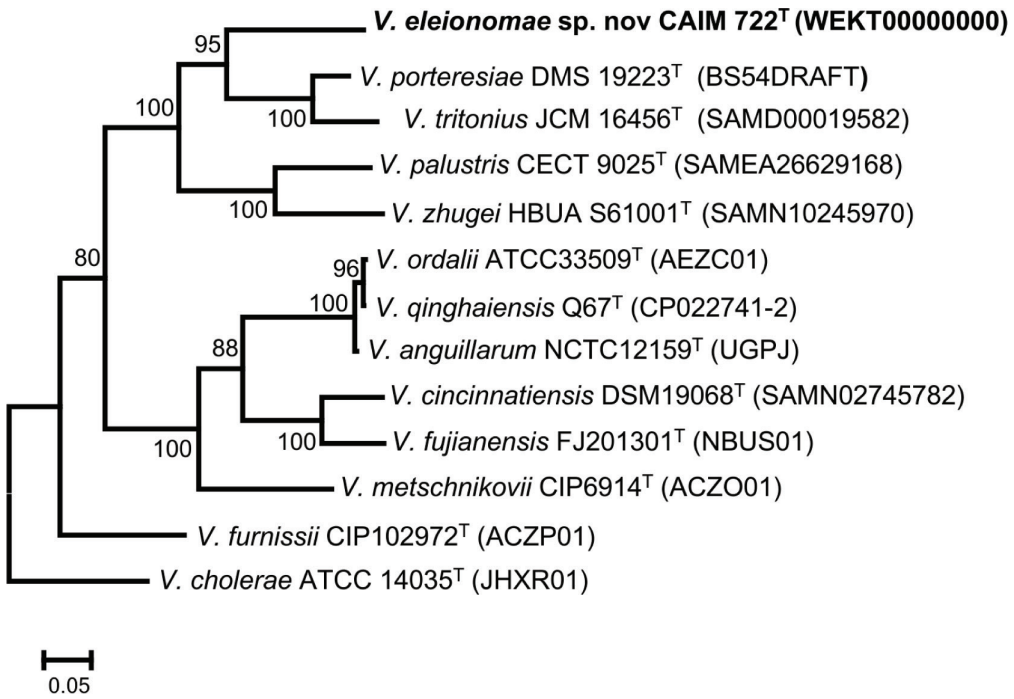

Fig. 1. Phylogenetic tree based on partial 16S rRNA gene sequences obtained by the maximum-likelihood method based on the Jukes–Cantor model. Genome sequence accession numbers are given in parentheses. Numbers at nodes denote the level of bootstrap based on 1000 replicates; only values greater than 50% are shown. *Vibrio cholerae* ATCC14035^T was used as an outgroup. Bar 0.5% estimated sequenced divergence. Scale bar, base substitutions per site.

bacillus, motile, negative for the oxidation/ fermentation test, sensitive to the vibriostatic agent O/129 (at 10 and 150 µg), oxidase negative, and catalase positive. Strain CAIM 772^T can utilize galactose, glucose, mannose, aesculin, cellobiose, lactose, maltose, trehalose, sucrose and fucose. It produces acid phosphatase, alkaline phosphatase, esterase lipase (C8), leucine arylamidase, naphthol-AS-BI-phosphohydrolase, β-galactosidase and esterase (C4). Several phenotypic traits were found that differentiate strain CAIM 772^T from its closely related species (Table 1 and Fig. S1, available in the online version of this article). The most outstanding feature was weak growth as green colonies on Thiosulphate-Citrate-Bile Salts-Sucrose (TCBS) agar.

In silico phenotyping

The genome of strain CAIM 722^T was positive for the following phenotypic features related to growth: growth at 42 °C, on MacConkey agar, on ordinary blood agar, and in bile-susceptible medium (Fig. S1). Positive results were predicted for the following enzyme activity: alkaline phosphatase, lipase, and nitrate to nitrite; and negative results were predicted for coagulase production and pyrrolidonyl-β-naphthylamide related to carboxylic acid. Positive for malonate and tartrate use. The genome was positive for Voges–Proskauer, aesculin hydrolysis, L-arabinose, glucose fermentation, methyl red, mannitol, mannose, maltose, sucrose, melibiose, trehalose and salicin, and negative for glycerol and casein hydrolysis. The genome was positive for indole, proteolysis and catalase; and negative for spore formation and hydrogen sulphide.

16s rRNA gene phylogeny

The phylogenetic analyses based on 16S rRNA gene sequences, MLSA (sequence similarity of less than 77.6% for 16S rRNA and 83% for MLSA, Table S2) and SCGs showed the same clustering (Fig. 1, Fig. S2 and Fig. 2, respectively), supporting that strain CAIM 722^T does not belong to any of the species previously described of the genus *Vibrio*. It has been shown that these types of analyses are useful for taxonomic and phylogenetic studies of the family *Vibrionaceae*, since they can detect small changes between phylogenetically close strains [11, 14, 23–26].

Phylogenetic analyses

In recent taxonomic studies, SCGs have been used to infer robust phylogenies at the genome level [11, 14, 23, 25–27]. The phylogenetic reconstruction with 139 SCGs showed that CAIM 722^T represents a new species belonging to the genus *Vibrio*, forming an independent

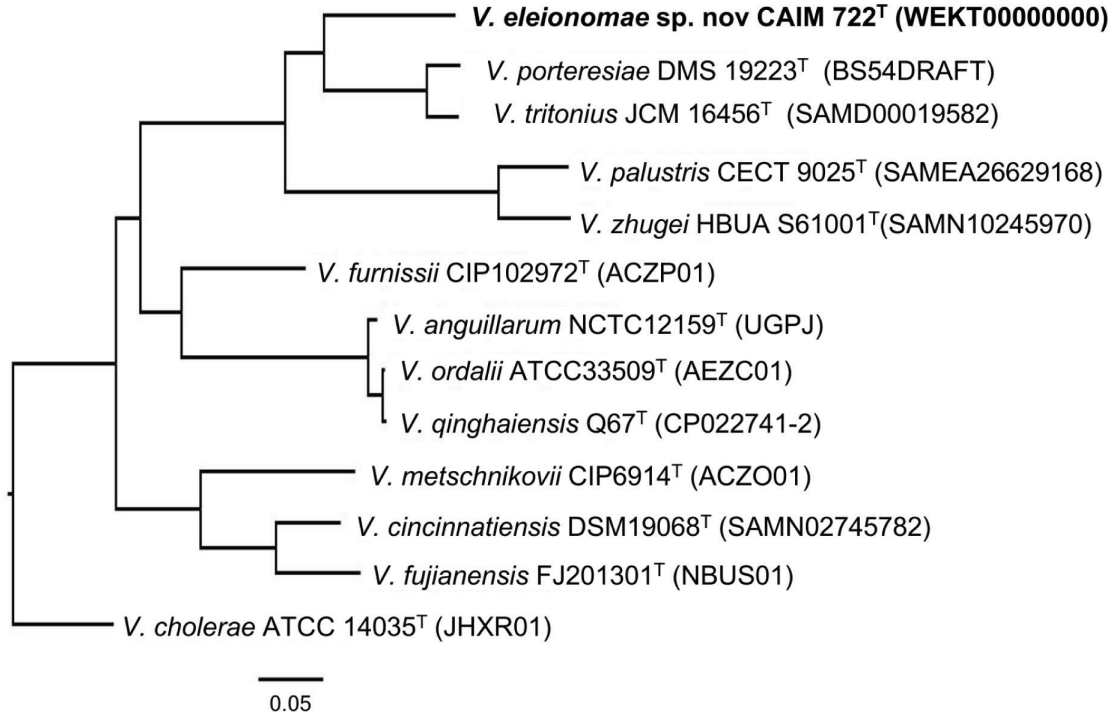

Fig. 2. Phylogenetic tree based on the concatenated sequences of 139 single-copy genes of type strains of the genus *Vibrio* closely related to strain CAIM 722^T by the maximum-likelihood method based on the Jones–Taylor–Thornton model. Strain and genome sequence accession number are presented next to the species and after the parenthesis the clade. *Vibrio cholerae* ATCC14035^T was used as outgroup.

branch, and these analyses clearly place it as a member of the Porteresiae clade [23, 24], with *V. porteresiae* DMS 19223^T, *V. tritonius* JCM 16456^T, *V. palustris* CECT 9025^T and *V. zhugei* HBUA S61001^T .

Genome features

According to the genome sequence analyses, the draft genome sequence of strain CAIM 722^T had a total length of 5283001 bp and was formed of 132 contigs with a coverage of 37×. The N50 value was 65885 and the G+C content 42.47 mol%. The N per 100 kbp was 3.71 and the largest contig was 267289. Completeness evaluated with Check M was 100% and contamination was 0.65%.

Phylogenomic analyses

Genomic comparisons between CAIM 722^T and the type strains of closely related species, namely *V. porteresiae* DMS 19223^T, *V. tritonius* JCM 16456^T, *V. palustris* CECT 9025^T and *V. zhugei* HBUA S61001^T, produced ANIb a minimum value of 72% and a maximum of 84% (Fig. S3). OrthoANI values were always below 85% (Fig. S3). The maximum estimated DDH value between CAIM 722^T and the other members of the Porteresiae clade, was only 24% (Fig. S4). The AAI varied from 72% to a maximum of 90% (Fig. S4). All indexes have values well under the threshold for same species delimitation, which is 70% for DDH and 95–96.0% for ANI, OrthoANI and AAI [27, 28]. This confirms that strain CAIM 722^T does not belong to any the species described above, representing a novel species of the genus *Vibrio*.

The phenotypic results, the phylogenetic analyses (16S rRNA, MLSA and SCG) and the genomic comparisons (ANI, OrthoANI, AAI and GGDC) support the classification of the isolate as representing a new species of the genus *Vibrio*, for which the name *Vibrio eleionomae* sp. nov. is proposed.

DESCRIPTION OF *VIBRIO ELEIONOMAE* SP. NOV.

Vibrio eleionomae (e.lei.o.no'mae. Gr. fem. n. *Eleionome*, nymph of marshes, ponds and wetlands; N.L. gen. n. *eleionomae*, of *Eleionome*, nymph of marshes, ponds and wetlands, referring to the habitat of the bacteria).

Strain CAIM 722^T (=SW9^T=DSM 24596^T) grows weakly as a green colony on TCBS agar. Growth occurs at 1–10% NaCl. No growth is observed at NaCl concentrations of 0% or higher. Optimal pH for growth is pH 4.5–8.5. Grows at 20, 30 and 35 °C, but not at 4 and 40 °C. Utilizes mannitol, sucrose and glucose, but the results for Voges–Proskauer, urease, citrate and gelatinase are negative. Ferments

amygdalin and arabinose. Does not produce arginine dehydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophane deaminase, indole production and H2S; fermentation of inositol, sorbitol, rhamnose, melibiose and reduction of nitrate to nitrite are positive.

Strain CAIM 772^T ferments galactose, glucose, mannose, aesculin, cellobiose, lactose, maltose, trehalose, sucrose and fucose; no fermentation of glycerol, D,L-arabinose, D,L-ribose, L-xylose, fructose, L-rhamnose, inositol, mannitol, sorbitol, amygdalin, salicin, melibiose, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, inulin, D,L-arabitol mannitol, sorbitol, inositol, inulin, turanose, adonitol and *N*-acetyl-D-glucosamine is observed.

Produces acid phosphatase, alkaline phosphatase, esterase lipase (C8), leucine arylamidase, naphthol-AS-BI-phosphohydrolase, β-galactosidase and esterase (C4), but not lipase (C14), α-chymotrypsin, α-fucosidase, α-galactosidase, valine arylamidase, β-glucuronidase, β-glucosidase, α-glucosidase, α-mannosidase, *N*-acetyl-β-glucosaminidase, trypsin or cystine arylamidase.

The type strain, CAIM 722^T (=SW9^T=DSM 24596^T), was isolated from water of a shrimp pond (*Penaeus vannamei*) in Los Mochis, Sinaloa, Mexico, in April 2003.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
We followed all procedures for care and use of animals according to the related Mexican guidelines and policies stated in NOM-033-ZOO-1995 and NOM-062-ZOO-1999.

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