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

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The GenBank/EMBL/DDBJ accession number for genome is WEKT00000000 and for the 16S rRNA is ON406572.

Abstract

A new species of *Vibrio* (CAIM 722^T, = SW9, = DSM 24596) 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.

Keywords

AAI, ANI, GGDC, MLSA, *Vibrio*, 139 single-copy genes, Porteresiae clade

Author Notes

The GenBank/EMBL/DDBJ accession number for genome is WEKT00000000 and for the 16S rRNA is ON406572.

Abbreviation

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 genes.

Introduction

The genus *Vibrio* was originally proposed in 1854 [1]; at the time of writing, the genus *Vibrio* comprises 128 bacterial species (http://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 *V. fischeri*, [4], to the pathogenic interactions between a variety of *Vibrio* species and marine species. For example, *V. alginolyticus* is a pathogen associated with bivalves [5], *V. ordalii* is a fish pathogen [6], *V. harveyi, V. campbellii* and *V. 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, a polyphasic taxonomy has been proposed as an effective way for phylogenetic classification and identification of bacteria, and has allowed for the 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.

Material and methods

Strain CAIM 722^T (= SW9, = DSM 24596) was isolated from water from a shrimp pond (*Penaeus vannamei*) located in Los Mochis, Sinaloa, Mexico in April 2003. The strain was obtained from the Collection of Aquatic Important Microorganisms (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 (TSA, 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. Brie y, 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). TSB was used to determine salt tolerance (0–10 % NaCl), growth at different temperatures (4, 20, 30, 37, 40 °C) and 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). Draft genome of CAIM 722^{T} was sequenced with the Illumina Miniseq platform (300 cycles, 2 × 150 bp). 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 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), after ltering, the pair-end reads were assembled using SPAdes 3.13.0 [12]. Genomic annotation was carried out using Prokka [13] and the basic statistics about the genome were extracted using quast software version 5.0.2 [14] and 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 from Campbell et al., [17] was used. The MLSA analysis, was performed by using eight housekeeping gene sequences (*ftsZ, gapA, gyrB, mreB, pyrH, recA, rpoA*, and *topA*) and 16S rRNA gene [18] all the gene sequences were extracted from the genome. The phylogenetic

trees were reconstructed using the Maximum Likelihood algorithm with MEGA ver.6. [19] of concatenated genes. Genome sequences used in this study are listed in the supplementary Table S1. Genome relatedness was evaluated with closely related species with Average Nucleotide Identity (ANI), OrthoANI as described by Lee et al. [20], and GGDC genome-relatedness; whereas Average Amino Acid Identity (AAI) was calculated with GET_HOMOLOGUES v. x86_64-20171023 with the following settings: -M –t 0 –n -A [21]. In silico phenotyping was performed using Traitar (v1.20) [22].

Results and Discussion

Physiology and Chemotaxonomy

Strain CAIM 772^T was isolated from water of a shrimp pond (*Penaeus vannamei*) 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 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 D-galactose, D-glucose, D-mannose, esculin, D-cellobiose, lactose, maltose, D-trehalose, D-sucrose and D-fucose. Produce acid phosphatase, alkaline phosphatase, esterase lipase (C8), leucine arylamidase, naphthol-AS-BI-phosphohydrolase, b-galactosidase and esterase (C4). Several phenotypic traits were found that differentiate *V. eleionomae* sp. nov. from closely related species (Table 1and Fig. S1). The most outstanding feature was a weak grow as green colonies on TCBS agar.

In silico phenotyping

The genome of *V. eleionomae* sp. nov. was positive for phenotypic features related to growth; growth at 42 °C, on MacConkey agar, on ordinary blood agar, and bile-susceptible (Fig. S1). Positive results were predicted for the following enzymes, alkaline phosphatase, lipase, nitrate to nitrite, and negative for coagulase production, pyrrolidonyl-beta-naphthylamide related to

carboxylic acid. Positive for malonate, and tartrate use. Genome was positive for Voges–Proskauer, esculin hydrolysis, L-arabinose, glucose fermentation, methyl red, D-mannitol, D-mannose, maltose, sucrose, melibiose, trehalose and salicin, and negative for glycerol, casein hydrolysis. The genome was positive for indole, proteolysis, and catalase; and negative for spore formation, and hydrogen sulfide.

16S RNA 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 SCG 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 *Vibrio* genus. It has been shown that these types of analyses are useful for taxonomic and phylogenetic studies of the *Vibrionaceae* family, since they can detect small changes between phylogenetically close strains [11, 14, 27, 28, 29, 30].

Phylogenetic analyses

In recent taxonomic studies, single copy genes (SCG) have been used to infer robust phylogenies at the genome level [11, 14, 23, 27, 29, 30]. The phylogenetic reconstruction with 139 SCG showed that CAIM 722^T, is a new species that belonging to *Vibrio* genus, forming an independent branch and these analyses clearly place it as a member of the Porteresiae clade [27,28], 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 5283001bp and was formed of 132 contigs with a coverage of 37×. The N50 value was 65885 and the G+C content 42.47mol%. The N's per 100 kbp was 3.71 and the largest contig 267289. The completeness Check M was 100 % and contamination check M is 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 values between 84% and 72% (Fig. S3). OrthoANI values were always below 85% (Fig. S3). The estimated DDH values between CAIM 722^T and the other members of the Porteresiae clade, were between a value of 24% and 15% (Fig. S4). The Average Amino Acid Identity (AAI) varied between 90% and 72% (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 [23, 31]. This confirms that CAIM 722^T strain does not belong to any the species described above, representing a novel species of the genus *Vibrio*.

The phenotypic results, the phylogenetic analyzes (16S rRNA, MLSA, and SGC) and the analysis of genomic comparisons (ANI, OrthoANI, AAI, and GGDC) support the classification of the isolate analyzed as a new species of the genus *Vibrio*, whose name is called *Vibrio eleionomae* sp. nov. it is proposed.

Protologue

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, = DSM 24596) grow as a weak green colony on TCBS agar. Growth occurs at 1 - 10% de NaCl. No growth was observed at NaCl concentrations of 0 % or higher. Optimal pH was established at a range of 4.5 to 8.5. Grow at 20, 30 and 35 °C, but not at 4 and 40 °C, can utilize mannitol, sucrose, glucose, but the results for Voges–Proskauer, urease, citrate, gelatinase are negative, Ferments amygdalin and arabinose. Does not produce

arginine dehydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophane deaminase, indole production and H2S production; fermentation of inositol, sorbitol, rhamnose, melibiose and reduction of nitrate to nitrite are positive.

Strain CAIM 772T ferments D-galactose, D-glucose, D-mannose, esculin, D-cellobiose, lactose, maltose, D-trehalose, D-sucrose and D-fucose; no fermentation was observed for glycerol, D,L-arabinose, D,L-ribose, L-xylose, D-fructose, L-rhamnose, inositol, mannitol, sorbitol, amygdalin, salicin, D-melibiose, methyl-αD-mannopyranoside, methyl-αD-glucopyranoside, inulin, D,L-arabitol mannitol, sorbitol, inositol, inulin, D- turanose, adonitol and N-acetyl-D-glucosamine are negative.

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

The type strain is CAIM 722^T (= SW9, = DSM 24596) was isolated from water of a shrimp pond (*Penaeus vannamei*) in Los Mochis, Sinaloa, Mexico in April 2003. The genome accession number of the type strain CAIM 722^T is WEKT00000000.

Compliance with Ethical Standards

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.

Conflict of interest

The authors of this paper deny any financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

Funding information

This study was funded by Centro de Investigación en Alimentación y Desarrollo.

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Table 1 Phenotypic traits that distinguish *V. eleionomae* sp. nov. CAIM 722^T from type strains of closely related *Vibrio* species.

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

Test	**1**	**2**	**3**	**4**	**5**
Arginine dihydrolase	+	-[a]	-[a]	-[a]	-[a]
Lysine decarboxylase	-	-[a]	-	-[a]	-
Oxidase	-	-	+[a]	+[a]	+[a]
Growth on TCBS	w	-	-	-	-
Voges-Proskauer	v[a]	+[a]	+[a]	+[a]	+[a]
Catalase	+[a]	+[a]	+[a]	+[a]	-
Citrate	-	-	+[a]	+[a]	+[a]
Nitrate to nitrite	+	+[a]	-	ND	+[a]
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. [22], Rameshkumar et al. [23], Guo et al. [24] and Sawabe et al. [25], respectively.

+ positive, - negative, ND no data available, v variable results, w weakly.

[a] These results coincide with the results for the *in silico* phenotyping (Fig. S1).

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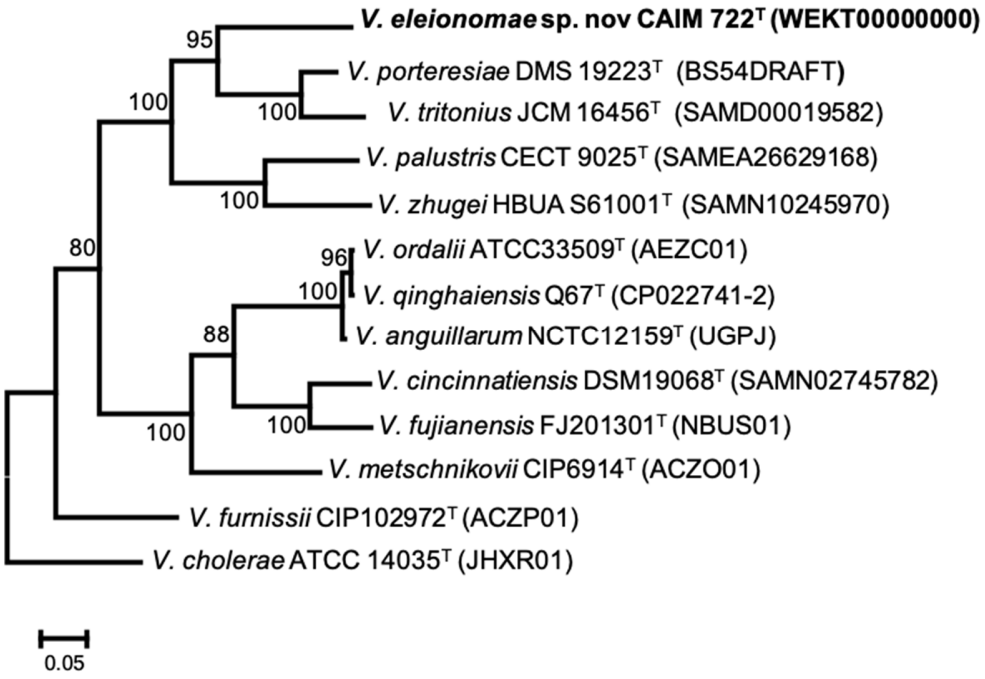

Figure 1. Phylogenetic tree based on partial 16S rRNA gene sequences obtained by the Maximum likelihood method based on the Jukes-Cantor model. Genome sequence accession numbers are given in parentheses. Numbers at nodes denote the level of bootstrap based on 1000 replicates; only values greater than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

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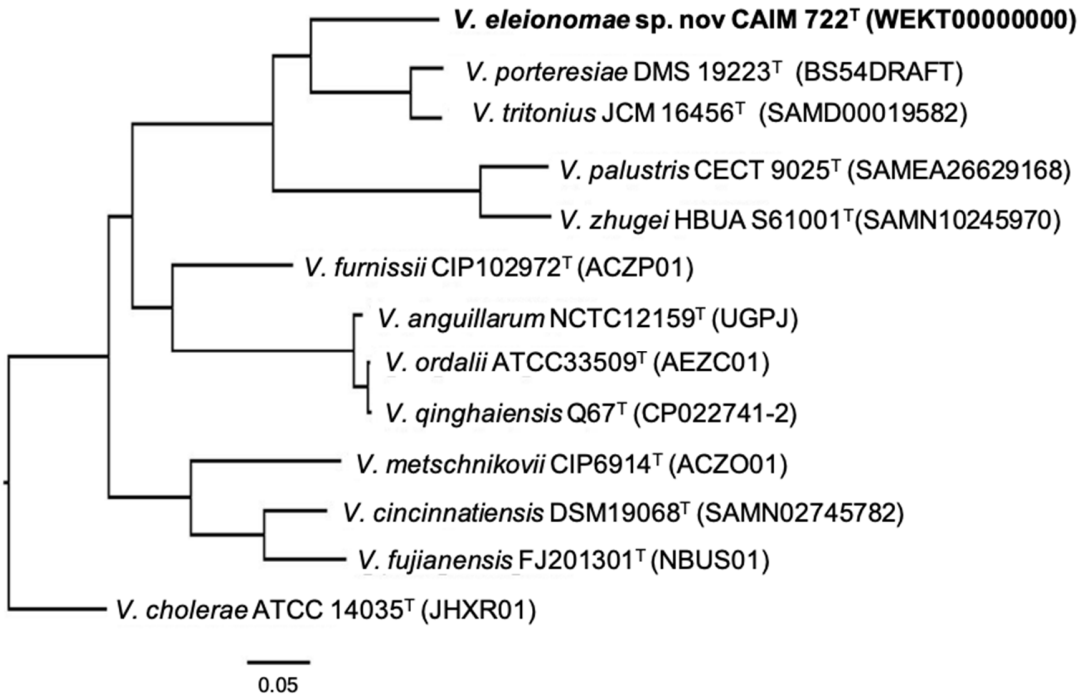

Figure 2. Phylogenetic tree based on concatenated sequences of 139 single-copy genes (SCG) of type strains more closely related of the genus *Vibrio* to *V. eleionomae* sp. nov. CAIM 722^T by Maximum likelihood method based on the Jones-Taylor-Thorton model. Strain and genome sequence accession number are presented next to the species and after the parenthesis the clade. *Vibrio cholerae* ATCC14035^T was used as outgroup.

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