PirVP genes causing AHPND identified in a new Vibrio species (Vibrio punensis) within the commensal Orientalis clade

Leda Restrepo1,2,3, Bonny Bayot1,4, Sebastián Arciniegas5, Leandro Bajaña3, Irma Betancourt1, Fanny Panchana1 & Alejandro Reyes Muñoz2,3,6

Acute hepatopancreatic necrosis disease (AHPND) has extended rapidly, causing alarming shrimp mortalities. Initially, the only known causative agent was Vibrio parahaemolyticus carrying a plasmid coding for the mortal toxins PirVP. Recently, it has been found that the plasmid and hence the disease, could be transferred among members of the Harveyi clade. The current study performs a genomic characterization of an isolate capable of developing AHPND in shrimp. Mortality studies and molecular and histopathological analyses showed the infection capacity of the strain. Multilocus sequence analysis placed the bacteria as a member of the Orientalis clade, well known for containing commensal and even probiotic bacteria used in the shrimp industry. Further whole genome comparative analyses, including Vibrio species from the Orientalis clade, and phylogenomic metrics (TETRA, ANI and DDH) showed that the isolate belongs to a previously unidentified species, now named Vibrio punensis sp. nov. strain BA55. Our findings show that the gene transfer capacity of Vibrio species goes beyond the clade classification, demonstrating a new pathogenic capacity to a previously known commensal clade. The presence of these genes in a different Vibrio clade may contribute to the knowledge of the Vibrio pathogenesis and has major implications for the spread of emerging diseases.
isolates because of the presence of two homologous genes related to the insecticidal toxin genes, PirA and PirB (Pir<sup>10</sup>), described for the first time in *Photurisbus* sp.<sup>1</sup>. The toxin coding genes are located on a plasmid (pVAHPND<sup>10</sup>) and the plasmid has been identified as being present in all AHPND-causing bacteria.<sup>2</sup>

Until now, *V. parahaemolyticus* strains containing plasmids with the genes for putative virulence have been described in Thailand<sup>3</sup>, Mexico<sup>4</sup>, China<sup>5</sup>, Vietnam<sup>6</sup> and South America<sup>7</sup>. Although there is genetic variability in the plasmid sequences, which has led to a description of a Mexican type, characterized by a 4,243 bp Tn3-like transposon insertion and a 9 bp small sequence repeat (SSR), both features being absent from the Asian type<sup>8</sup>. However, all plasmids contain the toxin genes<sup>12</sup> and a group of transposase-coding sequence related with horizontal gene transferance (HGT).<sup>7</sup>

Sawabe <i>et al</i>.<sup>13</sup> argued that the HGT within the *Vibrio* genus might occur between sister species, such as *V. cholerae* and *V. mimicus* (*Cholerae clade*) or *V. harveyi* and *V. campbellii* (*Harveyi clade*). To date, no evidence of HGT has been observed between species belonging to distantly related clades. Recently, the presence of the pVAHPND has been discovered not only among *V. parahaemolyticus* strains, but also in other related species, in particular: *V. harveyi*, *V. owensii* and *V. campbellii*<sup>8,14–16</sup>, all species belonging to the *Harveyi clade*. For this reason, this clade is known to be the pathogenic clade for AHPND.

Most *Vibrio* species in the *Orientalis* clade are not causative agents of diseases, except *V. tubiashi* and *V. sin-aloensis*, which have been associated with mortalities of bivalves and fishes<sup>17–21</sup>. In particular, there is no record of pathogens for crustaceans within this clade. The current study describes a new *Vibrio* species, *Vibrio punensis* sp. nov. strain BA55, belonging to the *Orientalis* clade and closely related to *V. orientalis* and *V. hepatarium*. Moreover, *V. punensis* sp. nov. strain BA55 carries the plasmid containing the toxin genes shown to be the cause of AHPND in shrimp. Our findings show that the gene acquisition capacity of *Vibrio* species goes beyond the clade boundaries that have been previously shown, demonstrating new infective capacity to a previously known commensal clade. The presence of these genes in a *Vibrio* clade not previously reported as pathogenic to crustaceans may contribute to the understanding of the *Vibrio* pathogenesis and has major implications for the spread of emerging diseases.

**Results**

**Pir<sup>10</sup> gene detection, histopathology analysis and typing using RAPDs.** Shrimp collected during a mortality event in 2015 in a South American farm showed the symptomatology of AHPND, with external signs of empty stomach, lethargy, discoloration of the hepatopancreas, with a white membrane of smooth consistency expanding through it (Fig. 1A,B). In the longitudinal sections of the shrimp, a whitish coloration was present in the hepatopancreas, indicating a degenerative disease in this organ (Fig. 1A).

Histopathologic evaluation showed severe necrosis of the hepatopancreas tubules, with detachment of the epithelial cells from the membrane to the lumen (Fig. 1C), making it impossible to discriminate between the distinct types of cells that are affecting the integrity of the tubules (Fig. 1E). A marked difference was observed when compared to hepatopancreas tubules of healthy shrimp, where it was possible to discriminate between embryonic and secretory cells, both appearing to be in good condition, without any detachment and maintaining the tubule structure without deformities (Fig. 1D). Severe hepatopancreas necrosis limited the observation of lipid cells due to the cellular detachment caused by a bacterial infection, evidencing the formation of melanized haemocytic nodules in the middle part of the hepatopancreas tubules (Fig. 1F).

Bacterial recovery from infected animals was dominated by a single morphotype, named strain BA55, which was confirmed to contain the Pir<sup>10</sup> genes associated with AHPND (Fig. 2A). RAPD (randomly amplified polymorphic DNA), a simple and cost-efficient assay to evaluate genetic variability, was used to discriminate between related *Vibrio* species. A unique pattern for the identified BA55 strain was observed when compared to different isolates of *Vibrio* spp. previously characterized at CENAIM (Fig. 2B,C), suggesting that the disease was caused by a strain with unique molecular characteristics different to previously isolated bacteria from the *Harveyi* clade. Furthermore, full 16S Sanger sequencing confirmed that the isolated strain did not belonged to the *Harveyi* clade (Fig. S1).

**Bacterial bioassay shows BA55 strain causes AHPND in shrimp.** The BA55 strain was used <i>in vivo</i> to challenge susceptible shrimp in triplicates starting at 2 × 10<sup>6</sup> CFU ml<sup>–1</sup>. Shrimp mortality was observed after 10 h post infection, whereas the *V. parahaemolyticus* BA94C2 strain, at the same concentration, caused mortality after 8 h (Fig. 3). No mortalities were reported for the negative controls. Shrimp cumulative mortalities at 70 h post infection in both infected treatments were high (BA55: 95.2 ± 0.8%; BA94C2: 99.8 ± 0.7%), with a significant difference between treatments (P = 0.005, Kruskal-Wallis test). Moribund shrimp from the BA55 infected treatment exhibited the same symptoms as observed on diseased *P. vannamei* collected from the shrimp farm. Moribund shrimp from different replicates infected with the BA55 strain were used for isolating the predominant *Vibrio* strains and recovering them in pure culture. The isolated strains were then further tested with the RAPD assay. The DNA banding pattern in these colonies was the same as those obtained from the BA55 strain isolated from the diseased cultured shrimp (Fig. 4) and used as an inoculum, thus confirming Koch's postulates and confirming that the BA55 strain is a causative agent of AHPND. Additionally, the *PirA* and *PirB* toxin genes were detected by PCR in the isolated colonies recovered from moribund shrimp during the challenge, whereas the macerates of the negative controls remained negative (Fig. S2).

**De novo assembly of the BA55 strain genome sequence.** Total DNA extracted from the isolated strain was used for <i>de novo</i> genome sequencing and assembled using 19,434,590 IlliVuna PE reads. A total of 75 contigs were obtained. Annotation and comparison to reference *Vibrio* spp. genomes showed evidence of two chromosomes (Chr 1: 3.2 Mbp long and Chr 2: 1.5 Mbp long) and a plasmid (pVAHPND: ~69 Kbp long) containing the toxin *Pir*, with two subunits: *PirA* and *PirB*. The average coverage was 46.3 ×, G + C content was 44.5%, 4,494 genes were predicted, and the total genome size was 4.7 Mbp (Table 1). An initial evaluation using BLAST among
the predicted genes and a set of 76 other reference *Vibrio* genomes available in public databases (Table S1) showed no significant difference (less than 1% in the percent identity) among the scores obtained from the different *Vibrio* species, suggesting that this strain is equally distant from the previously known *Vibrio* species, potentially constituting an undescribed species.

**Phylogenetic analysis of the BA55 strain.** The Maximum Parsimony (MP), Maximum Likelihood (ML) and Neighbor Joining (NJ) analysis of 16S rDNA showed a high identity between all *Vibrio* sequences analyzed and low support for the branches in all the inference methods used, suggesting a low resolution to resolve intra and inter-specific phylogenetic relationships within the *Vibrio* genus. We found that the closest neighbor of the strain BA55 was *V. brasiliensis* strain LMG 20546 (75% bootstrap values; Fig. S3) from the *Orientalis* clade. Importantly, although the 16S rDNA gene is commonly used for phylogenetic analyses, the sequence identities

---

**Figure 1.** Photographs from healthy *P. vannamei* shrimp and shrimp infected with BA55 strain carrying *PirV* genes associated with AHPND. (A) Left shrimp view. Gross signs of AHPND-infected shrimp (left): pale, atrophied hepatopancreas surrounded with a white membrane with smooth consistency (black arrow). Normal shrimp (right): normal size hepatopancreas with brownish color. (B) Right shrimp view. Gross signs of AHPND-infected shrimp (left): complete hepatopancreas destruction (black arrow). Normal shrimp (right): normal size hepatopancreas with brownish color. (C) Hematoxylin and eosin-stained histological sections of the hepatopancreas of *P. vannamei* from sick shrimps. AHPND pathology characterized by sloughing of hepatopancreatic tubule epithelial cells (black arrow). (D) Hematoxylin and eosin-stained histological sections of the normal shrimp hepatopancreas. (E) Histological sections show severe necrosis of the hepatopancreas tubules (black arrow), making it impossible to discriminate the different types of cells. (F) Histological sections show cellular detachment caused by a bacterial infection, evidencing the formation of melanized haemocytic nodules (black arrow).
among *Vibrio* species tend to be very high making this gene an unsuitable marker for phylogenetic analysis within the genus, further confirmed by the low branching support and the incongruence on the clustering with the traditional *Vibrio* clades.

**Figure 2.** Identification of an isolate BA55 strain carrying *Pir*<sup>V</sup> genes associated with AHPND in *P. vannamei*. (A) Detection of *Pir*<sup>V</sup> genes. Lane 1: *V. parahaemolyticus* not containing the *Pir*<sup>V</sup> genes; lane 2: *V. harveyi* not containing the *Pir*<sup>V</sup> genes and lane 3: non-template control; lane 4: *V. parahaemolyticus* containing the *Pir*<sup>V</sup> genes; lane 5 and 6: PCR results from two independent DNA extractions from strain BA55; lane M: DL2000 DNA marker. (B) UBC101 amplicon profiles from the different isolates, lane 1: *V. parahaemolyticus*; lane 2: *V. harveyi*; lane 3: *V. campbellii*; lane 4: *V. orientalis*; lane 5: *V. hepatarius*; lane 6: BA55 strain. UBC 457 amplicon profiles from the different isolates, lane 1: *V. parahaemolyticus*; lane 2: *V. harveyi*; lane 3: *V. campbellii*; lane 4: *V. orientalis*; lane 5: *V. hepatarius*; lane 6: BA55 strain; lane M1: DL10000 DNA marker; lane M and M2: DL1000 DNA marker.

**Figure 3.** Shrimp cumulative mortality after being challenged with BA55 and *Vibrio parahaemolyticus* BA94C2 strains. Negative control was TSB 2% NaCl. Bars indicate standard deviations.

**Figure 4.** DNA banding pattern of bacterial isolates obtained from challenged shrimps infected with the pathogenic BA55 and *V. parahaemolyticus* BA94C2 strains. From challenged shrimp infected with the BA55 strain; Shrimp 1, lane 1: Large size and convex isolated (LCI); lane 2: Small size and convex isolated (SCI); lane 3: Large and plain isolated (LPI). Shrimp 2, lane 4: LCI; lane 5: SCI; lane 6: LPI. From challenged shrimp infected with the *V. parahaemolyticus* BA94C2 strain; Shrimp 3, lane 7: Large size isolated (LI); lane 8: Medium size isolated (MI); lane 9: Small size isolated (SI). Inoculum controls from BA55 strain, lane 10 and lane 11. Positive control, lane +. Lane M1: DL 10000 DNA marker; lane M2: DL1000 DNA marker.
In order to gain a higher phylogenetic resolution, the phylogenetic trees derived from the concatenated nucleotide sequences of six commonly used MLST genes (ftsZ, gapA, gyrB, mreB, topA, and 16S rRNA genes) showed that the strain BA55 branched deep within the Orientalis clade, indicating a sister group relationship with *V. orientalis* and *V.* hepatarius. In fact, these three species form a monophyletic group within the Orientalis clade, distant from other known AHPND-causing bacteria and all the *Vibrio* within the Harveyi clade (Fig. 5). In addition, MP, ML, NJ and Bayes analyses revealed consistent phylogenetic tree topologies. The BA55 strain was phylogenetically distant from the Anguillarum clade (89.01% ± 3%), *Vulnificus* clade (87.69% ± 1%) and Cholerae clade (81.46% ± 3%), and more closely related to members of the Orientalis clade: *V.* hepatarius (96.32%), *V.* orientalis (95.57%) and *V.* tubiashii (89.85%). The closest clades to the BA55 strain were the Coralliilyticus and the Harveyi clades (90.12% ± 2% and 89.23% ± 4%), the latter being the pathogenic clade for AHPND, containing *V. parahaemolyticus*, *V.* *harveyi*, *V.* owensii and *V.* campbellii, the species where the presence of the pVAHPND plasmid has been previously reported\[^{14-16}\]. Percentages shown are the average sequence similarity, calculated with the proportion of nucleotide sites that differ between sequences and divided by the total number of sites for the comparison; in our case, based on the six protein-coding housekeeping genes of the BA55 strain.

Also, we show the phylogenetic reconstruction supporting the taxonomic position of the strain BA55, well-differentiated within the Orientalis clade (Fig. 5). Phylogenetic trees based on the concatenated partial sequences of the five housekeeping genes reconstructed by the NJ, ML and Bayes methods supported the

| Attributes          | Values          |
|---------------------|-----------------|
| Assembly size (bp)  | 4,727,353       |
| Total number of contigs | 75              |
| Contig N50 (bp)     | 206,237         |
| L50                 | 10              |
| GC content %        | 44.5            |
| Number of CDS       | 4,494           |
| Number of tRNAs     | 142             |
| Number of rRNAs     | 20              |

Table 1. Genome characteristics of the strain BA55 draft genome.
Table 2. Clades and subclades used for MLST analysis. aGC content calculated based on six genes. bMLST percentage of concatenate similarity between sequences. cAAI Raw average amino acid identity (10). dPhi test. The phi test was conducted for clades that included at least four species. eHabitat described for Sawabe et al.13.

| Clade     | Species included                                                                 | No. of species | GC (mol%)a | MLST (%)b | AAI (%)c | Phi test (P value)d | Habitat*          |
|-----------|----------------------------------------------------------------------------------|---------------|------------|-----------|----------|---------------------|--------------------|
| Anguillarum | V. anguillarum, V. aestuarianus and V. ordalii                                   | 3             | 43–46      | 95.5      | 99       | 87.0–98.3 95.8–99.8 | Brackish water, seawater and fish |
| Cholerae   | V. cholerae, V. mimicus, V. furnissii, V. fluvialis, V. metchnikovii and V. cincinnatiensis | 6             | 44–50      | 87        | 96.3     | 83.4–94.4 93.5–99.8 | Brackish water, seawater |
| Coralliilyticus | V. coralliilyticus and V. neptunianni                                              | 2             | 45–47      | 97        | 99.7     | 94.2–99.5         | Brackish water, bivalves and rotifers |
| Harveyi    | V. harveyi, V. campbellii, V. parahaemolyticus, V. alginolyticus, V. mytili, V. natriegens, and V. rotiferianus | 7             | 42–48      | 91.3      | 97.8     | 86.7–96.0 96.9–99.9 | Seawater, salt marsh mud and marine animals |
| Orientalis | V. orientalis, V. hepatarius, V. brasilienensis, V. sinaloensis, V. tubiashi and BA55 strain | 6             | 43–46      | 95.9      | 99.2     | 100                | Brackish water and seawater |
| Splendida   | V. splendidus, V. croosoutrea, V. cyclotrophicus, V. chagasii, V. fortis, V. kanaloua, V. lentus, V. gigantis, V. pelagius, V. taeniensis and V. pomeroyi | 11            | 39–47      | 98.2      | 99.8     | 88.8–93.7 97.1–98.9 | Seawater and marine animals |
| Vulnificus  | V. vulnificus and V. navarrensis                                                 | 2             | 45–48      | 93.6      | 98.8     |                    | Sewage, seawater and oyster |
| Damselae   | P. damselae                                                                       | 1             | 42         |           |          |                    | Seawater and fish    |

independent and robust position of the strain BA55 within the Orientalis clade, especially the closest position relatives to V. orientalis and V. hepatarius and maintaining a low percentage of similarity with V. tubiashi, V. bivalvica and V. europeaus. V. crossai maintains its position with V. brasilienensis.

For calibration of the evolutionary rates and estimation of the divergence times, we used a set of representative genomes from the Vibrio genus and analyzed the rate of amino acid substitutions in the six housekeeping genes. The lowest radiation time value for our strain BA55 was obtained with V. hepatarius (Table 2), revealing that these species are closely related. The different phylogenetic trees reconstructed from species of the Harveyi and Orientalis clades showed a consistent branch pattern, in particular for the relative position of the BA55 strain within Orientalis clade (Fig. 5).

Sequence analysis of pV_AHPND in the strain BA55. The plasmid pV_AHPND found in the strain BA55 was assembled in a single 69,163 bp contig, with a mean GC content of 44.9% and 53 putative ORFs. A homologue of the insecticidal Photorhabdus assemblage in a single 69,163 bp contig, with a mean GC content of 44.9% and 53 putative ORFs. A homologue of the insecticidal Photorhabdus assemblage in a single 69,163 bp contig, with a mean GC content of 44.9% and 53 putative ORFs. A homologue of

Comparative genome analysis. The comparative genomic analyses supported the hypothesis of the strain BA55 as a member of the Orientalis clade. The evidence came from multiple genome alignments and the evaluation of genomic architectures using Blast. The pairwise genome alignment between the genomes of the strain BA55 with V. tubiashi (Orientalis clade) and V. parahaemolyticus (Harveyi clade) suggested a closer relationship to V. tubiashi than to V. parahaemolyticus (Fig. 7A) as there was a higher number of rearrangements with the V. parahaemolyticus strain (Fig. 7B). The closer similarity was observed both when comparing the genomes at the nucleotide and amino acid level. Blast analysis showed a higher average percent identity with V. tubiashi (91%) compared to V. parahaemolyticus (88%) (Fig. 8A). At the amino acid level, the shared genes with a RAST annotated function between V. parahaemolyticus and the strain BA55 contained mainly essential genes that are needed for growth or viability (that were also shared with V. tubiashi and are likely part of a Vibrio core genome) and only 42 genes uniquely shared with V. parahaemolyticus, whereas V. tubiashi and the strain BA55 shared genes that are necessary for adaptation, including the genes for growth in different environments, which are associated with biol film formation and bacterial communication like the Acetyltransferase SyPM (Fig. 8B inset). This suggests the existence of gene clusters unique to the Orientalis strains distinct from the other Vibrio's core genes. Thirty-two genomic islands (GIs) were detected for the strain BA55 (Table S2) including sequences coding for mobile genetic elements, as prophages and transposons, and several recombination-related proteins as integrases, recombinases and transposases. The gene comparison represented by the Venn diagram showed different number of shared genes between pair of genomes, which is due to homologs within the same genome (paralogs) (Fig. 8B inset).
PirVP gene causing acute hepatopancreatic necrosis disease identified in a novel species from the Orientalis clade. In order to calculate the distances among the genomes on seven members of the Orientalis clade (V. hepatarius, V. sinaloensis, V. tubiashii, V. orientalis, V. brasiliensis and BA55 strain) and one member of the Harveyi clade (V. parahaemolyticus) we used the Genome-to-Genome Distance Calculator (GGDC 2.1)\(^\text{20}\). The first evaluated method was the \textit{in silico} DDH (DNA-DNA hybridization) analysis. Our results show that the BA55 strain displayed DDH values below 70% with every other strain analyzed. This cut-off has been traditionally used for the delineation of species in prokaryotes, for both \textit{in silico}\(^\text{21}\) and experimental analysis\(^\text{22}\) (Fig. 9A). For all pairwise comparisons made, the highest value obtained was 36% with \textit{V. orientalis} (Table S3). In addition, the percentage of differences between G+C content was >1%, corresponding to values obtained traditionally between distinct species\(^\text{20}\).

ANI calculations were performed using MUMmer (ANIm) and BLAST (ANIb) algorithms\(^\text{23}\). Previous studies using ANIm and ANIb for species classification in the Orientalis clade had suggested a species boundary of 95–96%\(^\text{20}\). Our results are consistent with those observations given, that all known strains within a given species showed pairwise ANIm values above the threshold, except for \textit{V. sinaloensis} strain DSM21326, concordant to the DDH results (Fig. 9B, Table S3) furthermore, the ANIb results confirm that BA55 is a species distinct from others available, with an ANIb value of <79% and an ANIm of <86% (Fig. 9B, Table S3). The discrepancy between the ANIb and ANIm values obtained is consistent with previous reports from Li \textit{et al}\(^\text{24}\) and Dubert \textit{et al}\(^\text{25}\) where they demonstrated the lack of agreement between ANIb and ANIm when the compared genomes show low correlation values (ANIb <75% and <77%, respectively), in our case the ANIb value is <79%.

Furthermore, it has been argued that the oligonucleotide frequencies carry a species-specific signal\(^\text{26}\). For this reason, statistical calculation of oligonucleotide frequencies (TETRA) from pairwise comparisons was performed. TETRA values indicated that the strain BA55 was more related to \textit{V. orientalis} strain CIP102891 (Fig. 9C). When comparing with both DDH and ANI cut-offs for species demarcation, TETRA values were above 99.5%, supporting their affiliation to the same species and between the Orientalis clade, this value can be proposed as a plausible cut-off. In contrast, the highest values for the BA55 strain were 93% for the comparison with \textit{V. orientalis} and 92.4% with \textit{V. sinaloensis} (Table S3). Together with the results based on \textit{in silico} DDH and ANI we also suggest that \textit{V. sinaloensis} strain DSM21326 should be reclassified, as it does not comply with the species described cut-offs as the other strains of \textit{V. sinaloensis} does.

Based on all previously reported evidence and the nucleotide composition values, we proposed the strain BA55 as a new species named \textit{Vibrio punensis sp. nov.} strain BA55, belonging to the Orientalis clade. This novel species carries the plasmid pV\textit{AHPND} giving it the pathogenic characteristics that had been only previously reported on the Harveyi clade.

Discussion

The visible symptoms observed in dying shrimp collected during the challenge test were similar to those observed in diseased shrimp collected from farms. The results of our challenge test indicated that the isolated BA55 strain triggered a high mortality, although lower than the strain BA94C2, and similar to other AHPND strains reported by Tran \textit{et al}\(^\text{6}\) and Nunan \textit{et al}\(^\text{4}\), where shrimp reached 100% mortality at 48 hours post infection trough immersion assays in...
Figure 7. Whole genome alignments between *V. punensis* strain BA55, *V. tubiashii* strain ATCC 19109 and *V. parahaemolyticus* RIMD 2210633. (A) Dot-plots of nucleotide identities of the BA55 strain against *V. tubiashii* strain ATCC 19109 (left) and BA55 strain against *V. parahaemolyticus* RIMD 2210633 (right). (B) Nucleotide-based multiple genome alignment of the same genomes. Homologous blocks are shown as identically colored regions and linked across the genomes. Rearrangements are shown as homologous regions in different genomic locations.

Figure 8. Comparative analysis of BA55 strain with *Vibrio tubiashii* ATCC 19109 and *Vibrio parahaemolyticus* RIMD 2210633. (A) Circles 1 and 2 show the BLAST comparison with *V. tubiashii* (green) and *V. parahaemolyticus* (purple). (B) Venn diagram showing unique and shared genes based on the RAST subsystems annotation among the three *Vibrio* species used before (BA55 strain, *V. tubiashii* and *V. parahaemolyticus*). Intersects show different number of genes for each genome due to potential paralogs within the genomes.
experimental tanks, at an approximate bacterial density of $2 \times 10^6 \text{ CFU ml}^{-1}$. The results obtained from the challenge test using the BA55 strain compared with the control, and the fact that it contains the PirA and PirB genes suggest that the toxin, as is the case in other AHPND-causing Vibrios, is responsible for the mortality of the infected shrimp.

Current microbial taxonomy is being revolutionized by the generation of full-length microbial genomes from uncultured microorganisms and full genome assembly from metagenomic projects requiring a full set of new taxonomical tools and standards. To date, DNA-DNA hybridization (DDH) has been the “gold standard” for bacterial species demarcation, as it provides a numerical threshold for a species boundary. Nevertheless, some authors suggest the necessity of a new approach due to the labor-intensive and error-prone nature of DDH experiments. According to Richter and Rosselló-Móra et al., the scientific community needs methods that offer a similar resolution of DDH and simultaneously allow the construction of databases to retrieval of any information for comparative purposes. Therefore, the average nucleotide identity (ANI) method was developed and it seems to be the best alternative for a gold standard method. Richter and Rosselló-Móra; Ramasamy et al. and Meier-Kolthoff et al. among others, have made efforts to validate ANI as a mirror for DDH and that in conjunction with TETRA values, has been proposed as a plausible substitute for DDH, although further testing should be performed in particular clades. Given the mounting evidence, the recent use of such metrics for proposing other Vibrio clades and species, and the results shown in our study, we propose the BA55 strain as a novel species, named Vibrio punensis sp. BA55.

Researchers have proposed that HGT drives evolution by incorporating genetic material from one organism to another. In this specific case, it is plausible that through HGT, the Vibrio punensis sp. nov. strain BA55 had become pathogenic. This shows that the gene transfer and the capacity of this new species to use these foreign genes is offering a new infective capacity to a species and a clade that has not previously been reported as a pathogen in crustaceans. Previously, it was only reported that species of the Harveyi clade present the ability to use PirP genes, carried in plasmids, to cause AHPND. The current phylogenetic study revealed that these genes could be found in new species and thus enhance their infective capacity, thereby potentially increasing the complexity of causative agents of AHPND and aggravating the threat to the cultured shrimp industry.

Current developments in sequencing technologies and microbial ecology are changing the perception that we had about commensals and pathogens and blurring the lines separating them. Common aquatic environments are composed of thousands of different bacterial species displaying complex interactions and where selective pressures award those capable of gaining new properties that enhance the fitness of the surviving species. The Vibrio genus is a large and complex taxon that includes free-living bacteria found in aquatic environments but with the potential of residing in different animal hosts. The specific interactions with the host ranges from beneficial, as those described in V. hepatarius used as probiotic in shrimp to pathogenic, such as V. cholerae, V. parahaemolyticus, V. vulnificus and V. alginolyticus. Once related species with different adaptation strategies to an environment collocate within a confined space under strong selection (such as different Vibrio species within the gut of a crustacean animal), it constitutes the perfect setup for the occurrence of HGT. This scenario could be the cause for a previously commensal bacterium to become pathogenic. The same principle applies to any other environment where commensal or symbiotic microorganisms potentially interact with pathogens, thus, increasing the chances of horizontal gene transfer of such virulence markers, which not necessarily limited to toxins, but also to potential antibiotic resistance in environments such as the human gut.

Materials and Methods
Isolation of bacterial strains. On July 2015, stomachs and hepatopancreas from AHPND positive shrimp collected during a mortality event in 2015 in a South American farm were removed and separately macerated to obtain mixed cultures. These cultures were subdused to subculture on TCBS (thiosulfate citrate bile salts sucrose) agar plates to obtain individual colonies and were further purified on TSA (tryptic soy agar) plates and pure...
isolates were then cryopreserved at −80 °C. A total of 39 bacterial strains were isolated from *P. vannamei* juvenile shrimp. Bacterial strains were characterized using morphological and biochemical criteria following the schemes of Alsina & Blanch and bacterial typing were conducted by 16S rRNA sequencing. Amongst those, a predominant bacterial strain coded as BA55 was selected for further analysis.

**PirVP gene detection.** Bacterial samples were centrifuged at 4,000 g for 10 minutes and decanted. The resulting pellet was resuspended in 500 μl extraction STE buffer (10 mM Tris-HCl, 1 mM EDTA and 100 mM NaCl, and 0.2 mg/ml proteinase K; Invitrogen, Carlsbad, CA) and incubated at 55 °C for 2 hours. After cell lysis, purification was carried out with phenol-chloroform and precipitation with ethanol, following procedures by Maniatis et al. with minor modifications. Each supernatant was removed and extraction with an equal volume of phenol-chloroform-isooamyl alcohol (25:24:1) was performed, followed by extraction with chloroform-isooamyl alcohol (24:1). DNA was recovered by the addition of 1 volume of isopropanol followed by centrifugation at 10,000 g for 10 min. The DNA pellet was washed with 70% ethanol, dried, and resuspended in 50 μl of ultrapure water pH 7.0. DNA extracts were stored at −20 °C for further analysis. DNA concentrations were determined using a NanoDrop 8000 Spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific Inc., Wilmington, USA).

The presence of the main genes associated to AHPND (PirVT) were tested on all 39 isolated bacterial strains using a nested PCR method, with primers previously described, to obtain amplified products of 1,269 and 230 bp (Table S4). Likewise, PirA and PirB genes were amplified separately with specific primers aimed at generating PCR products of 284 and 392 bp for PirA and PirB, respectively (Table S4). RAPD analysis. Two primers (UBC101, 5'-GGCCCTGGAG-3' and UBC 457, 5'-GCAGCCCTGTG-3') were selected from an initial screening with 10 decanucleotide primers, for the evaluation of RAPD pattern bands of the bacterial strains: BA55, AHPND *V. parahaemolyticus* BA94C2 and pathogenic non-AHPND strains (*V. parahaemolyticus*, *V. harveyi*, *V. campbellii*, acquired from the Microbiology Laboratory, Universidad Santiago de Compostela and *V. hepatarius* acquired from the Microbiology Laboratory, CENAIM-ESPOL). All strains were grown overnight at 28 °C on TSA. One colony of each strain was suspended in 200 μl STE buffer and the DNA extraction was performed as described above. PCR was carried out in a 10 ml reaction volume containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 3 mM MgCl2, 100 mM of each dNTP, 0.4 mM of the arbitrary primer, 2 μl of genomic DNA and 1 Taq DNA polymerase (Invitrogen). Amplifications were performed using a Thermo Fisher thermoblock under the following conditions: 4 min initial denaturation at 94 °C, 45 sec annealing at 46 °C, and 1 min elongation at 72 °C, with a final elongation step at 72 °C for 5 min. The PirA and PirB genes were amplified separately with specific primers aimed at generating PCR products of 284 and 392 bp for PirA and PirB, respectively (Table S4).

**Immersion challenge test.** The pathogenicity of the BA55 strain was evaluated by an immersion challenge test as described by Tran et al., with minor modifications. Briefly, a single colony of the BA55 strain was picked and re-suspended in 30 ml of sterile TSB (tryptic soy broth culture medium with 2% NaCl). The culture was incubated for 18 h at 28 °C on a rotary shaker (200 rpm) and the bacterial density was determined by a microplate luminometer (Varioskan ™, Thermo Fisher Scientific). One ml of this solution was transferred to 50 ml of TSB medium and cultured as described above for 10h. The suspension was plated onto TSA after serial dilutions to determine the colony-forming units of the isolate (CFU ml⁻¹). The *V. parahaemolyticus* BA94C2 strain was used as positive control under the same conditions.

For the infection, each strain was cultured in TSB for 12 h at 28 °C, to reach 2 × 10⁹ CFU ml⁻¹. Then, a 15 min immersion was performed on flasks containing 40 healthy shrimps (2.5 ± 0.5 g) per flask and a solution of bacterial suspension with saline water, to achieve a bacterial density of approximately 2 × 10⁸ CFU ml⁻¹. Immersed animals were transferred into experimental aquariums filled with 40 L of filtered and UV sterilized seawater and continuously aerated. After transfer, a solution of bacterial suspension was immediately added directly to the experimental tanks containing clean seawater (filtered and UV sterilized), to reach an approximate bacterial density of 2 × 10⁷ CFU ml⁻¹. A negative control (40 shrimp immersed in sterile TSB, without bacterial strains) was also included. All treatments, including the control, had 10 replicates. During the assay, shrimp were monitored for mortality every 2 h. Difference of cumulative mortality at 70 h post infection among infected treatments was analyzed with the non-parametric Kruskal–Wallis test by using the R software.

**Re-isolation of bacteria.** Moribund shrimp from infected treatments were collected 12 h post infection. Samples from the hepatopancreas and stomachs were removed and macerated in sterile saline solution. One hundred microliter of serial dilutions (10⁻¹, 10⁻² and 10⁻³) containing macerated hepatopancreas and stomachs were inoculated on TCBS agar and incubated at 28 °C for 18 h, and the three most predominant colonies were purified on TSA plates (Table S5). Following this, the DNA of the colonies was extracted and stored for subsequent comparisons with the tested isolates using RAPD assay, and PirVT genes detection, as described above. Macerates of shrimp hepatopancreas and stomachs of negative controls were also analyzed for PirVT genes detection. Shrimps were analyzed individually.

**Histopathologic analysis.** Shrimp samples were preserved with Davidson AFA fixative for histopathology analysis. Tissues were processed in accordance with the histological routine procedure. Sections were cut at 5 μm and stained with Mayer-Bennet hematoxylin and eosin. Tissues were examined for histopathological changes.

**Genome sequencing and de novo assemble.** The genome of the predominant bacteria strain, BA55, was sequenced using Illumina MiSeq PE150 platform. The library was constructed with Illumina Paired-End DNA Sample Kit (Illumina Cambridge Ltd, UK). Raw Illumina FASTQ files were demultiplexed and quality-filtered
using FastQC\textsuperscript{38}. Any reads containing ambiguous base calls or barcode errors were discarded. \textit{De novo} assembly was performed using Spades\textsuperscript{39}. The generated contigs were used as seeds into Contiguator\textsuperscript{40} to polish it, through an alignment of raw reads on contigs by BLAST\textsuperscript{41}. The complete genome sequence was deposited at DDBJ/EMBL/GenBank under the accession PRJNA412371.

**Genome annotation.** The genome of the BA55 strain was annotated using GLIMMER\textsuperscript{42} and NCBI Prokaryotic Genome Annotation Pipeline\textsuperscript{43}. In addition, other 76 \textit{Vibrio} genomes from the GenBank database (Table S1) were used for multilocus sequence typing (MLST) phylogeny. These 76 genomes were re-annotated by RAST\textsuperscript{44} and the RNA regions were characterized using RNAmes\textsuperscript{45}. Then, all the predicted ORFs were translated and compared by a homology search (BLAST) against non-redundant protein databases from NCBI (E-value threshold = 10\textsuperscript{−6} and minimal alignment length ≥80%), Swiss-Prot\textsuperscript{46}, Clusters of Orthologous Groups (COG)\textsuperscript{47}, Kyoto Encyclopedia of Genes and Genomes (KEGG)\textsuperscript{48} and Gene Ontology (GO)\textsuperscript{49}. To evaluate the functional and evolutionary relationship between the 76 \textit{Vibrio} genomes and the assembled genome of the BA55 strain, a comparison among those genomes was performed to evaluate sequence similarities.

**16S rDNA and multilocus sequence typing phylogeny.** An initial phylogenetic analysis was performed with a dataset of 862 16S rDNA sequences obtained from the NCBI Reference Sequence Database (RefSeq), together with the 16S rDNA sequences extracted from the 77 complete \textit{Vibrio} genomes (including the genome of the BA55 strain). Before performing all the phylogenetic analyses, a dereplication of the sequences by species was performed, retaining only one representative sequence per specie, obtaining a final set of 216 sequences (Fig. S5). All non-redundant sequences were aligned with MEGA 6.0\textsuperscript{46} using the MUSCLE algorithm. Phylogenetic trees were constructed using Maximum Parsimony (MP), Maximum Likelihood (ML) and Neighbor Joining (NJ) methods. We use JModeltest 2.0\textsuperscript{50} to test the evolution models based on the hierarchical likelihood ratio test, determining that the TM\textsubscript{+G} model best fit the data\textsuperscript{51}. The nucleotide substitution model chosen was consistent with previous reported models\textsuperscript{13}. The evolutionary distances between the species of \textit{Vibrio} were estimated with Mr. Bayes, considering the branch-length and divergence time for model selection across a wide range of phylogenetic and evolutionary models\textsuperscript{52}.

To establish a more robust inference of the evolutionary history of the \textit{Vibrio} genus, the same phylogenetic analyses described before were repeated, using a MLST analysis, by evaluating partial sequences of six commonly used, phylogenetic marker genes: ftsZ, gapA, gyrB, mreB, topA, and 16S rRNA from \textit{Vibrio} species (70 sequences) reported by Sawabe et al.,\textsuperscript{13} complemented by the 77 genomes used for re-annotation (including the genome of the BA55 strain). GenBank accession numbers are listed in Tables S1 and S6. We used the TCS program\textsuperscript{53} to estimate a phylogenetic network estimation among all \textit{Vibrio} species used for MLST analysis, to choose the 21 more related to the BA55 strain.

Recombination was evaluated with the Phi test using the concatenated sequences of \textit{Vibrios}, reported by Sawabe et al.\textsuperscript{13}. Recombination was detected in gyrB (P = 6.4 × 10\textsuperscript{−3}), gapA (P = 6.8 × 10\textsuperscript{−7}) and topA (P = 4.5 × 10\textsuperscript{−2}) in agreement with the conflicting phylogenetic splits (Table 2). The analysis was performed with SplitsTree\textsuperscript{46}, a framework for building phylogenetic trees and networks. Detecting recombination is basically a statistical endeavor and ideally \textit{in vitro} experimental work should be carried out in order to confirm the ability of \textit{Vibrios} to carry out recombination in the loci analyzed\textsuperscript{13}.

**Comparative genome analysis.** MAUVE software\textsuperscript{55} was used to perform whole genome alignments and comparisons. The genome of the BA55 strain was aligned to the two most cured, well-annotated and complete genomes of the \textit{Harveyi} and \textit{Orientalis} clades: \textit{V. parahaemolyticus} RIMD 2210633 and \textit{V. tubihshii} ATCC 19109, respectively, to evaluate gene conservation among the \textit{Harveyi} and \textit{Orientalis} clades. Genes previously predicted, were mapped in this comparison, for the identification of genomic islands (GIs) and several common characteristics of the genome, such as abnormal sequence composition or the presence of mobile genetic elements using Pfam. The gene comparison was represented by a Venn diagram (Fig. 6 inset) only on the genes that belong to a subsystem annotation in RAST.

Dot plots were generated by Gepard\textsuperscript{56} using default parameters with the ordered contigs produced by MAUVE for each genome.

**Genomic islands identification.** Genomic islands were predicted in the draft genome of BA55 strain using IslandPick, IslandPath, DIMOB and SIGI-HMM algorithms, performed in an integrated interface from IslandViewer\textsuperscript{57}.

**Average nucleotide identity, correlation indexes and DDH estimates.** Current bioinformatics methods allow replacement of the wet-lab DNA:DNA hybridization (DDH) estimates by in-silico genome-to-genome comparison and transforms the distance from high-scoring segments pairs to values analogous to DDH using a generalized linear model\textsuperscript{15}, this logistic regression model is inferred from an empirical reference dataset comprising real DDH values and pairwise genome sequences comparison. DDH \textit{in silico} was used to obtain a better resolution of discrimination and simultaneously allow the construction of databases that permit the retrieval of any information for comparative purposes\textsuperscript{59}.

In addition to DDH \textit{in silico}, the metrics ANI\textsubscript{b}, ANIm and TETRA have been recently developed and are widely used as metrics to measure the distance between closely related species, generating boundaries and differentiating isolates of the same species to those of different species. The average nucleotide identity (ANI) was measured between pairs of genomes, based on the BLAST algorithm (ANIB)\textsuperscript{59}, as well as on the MUMmer aligning tool (ANIm)\textsuperscript{60}. In addition, we calculated the tetranucleotide frequencies (TETRA), a statistical method which is an alignment-free process. The values for ANIB, ANIm and TETRA for the \textit{Vibrio} genome comparisons were calculated for all possible bacterial pairs, including all species in \textit{Orientalis} clade (five members), one species of \textit{Harveyi} clade (\textit{V. parahaemolyticus}) and our BA55 strain. All comparisons were represented as heatmaps using R statistical software\textsuperscript{59}.
Equipment and adjustments

Histological Images:
Biological Microscope OLYMPUS CX31, UIS (Universal Infinity System) optical system. Infinity capture imaging software Lumenera Corporation, Trademark INFINITY 2.
Size: 1616 × 1216 pixels
Resolution: 96 DPI

Gel Images:
Equipment ENDUROtm GDS TOUCH Labnet
The Endurotm GDS Touch Acquisition Software.
Size: 768 × 526 pixels
Resolution: 96 DPI

Organism Images:
Ilumina Nokia 630
Size: 2592 × 1956 pixels
Resolution: 72 DPI.

The Fig. 1 is a merge of captures using photoshop CC 2015. The images were left in the same size 750 × 578 pixels. The text, arrows and contrast were modified in photoshop and the final merge was left in 300 DPI.

The Fig. 2 is a merge of captures using photoshop CC 2015. The images were left in the same size 2400 × 1644 pixels. The text, arrows and contrast were modified in photoshop. The final figure was left in 300 DPI.

Data Availability Statement
The genome sequences used in the current study are available on the NCBI Genome Database under the accession number PRJNA412371.

References
1. Hong, X., Lu, L. & Xu, D. Progress in research on acute hepatopancreatic necrosis disease (AHPND). Aquaculture International. 24, 577–593 (2016).
2. De La Peña, L. D. et al. Acute hepatopancreatic necrosis disease (AHPND) outbreaks in Peneaus vannamei and P. monodon cultured in the Philippines. Dis. Aquat. Organ. 116, 251–254 (2015).
3. Soto-Rodriguez, S. A., Gomez-Gil, B., Lozano-Olvera, R., Betancourt-Lozano, M. & Morales-Covarrubias, M. S. Field and experimental evidence of Vibrio parahaemolyticus as the causative agent of acute hepatopancreatic necrosis disease of cultured shrimp (Litopenaeus vannamei) in northwestern Mexico. Appl. Environ. Microbiol. 81, 1689–1699 (2015).
4. Nunan, L., Lightner, D., Pantoja, C. & Gomez-Jimenez, S. Detection of acute hepatopancreatic necrosis disease (AHPND) in Mexico. Dis. Aquat. Organ. 111, 81–86 (2014).
5. Sawabe, T. et al. Updating the Vibrio clades defined by multilocus sequence phylogeny: Proposal of eight new clades, and the description of Vibrio tritonius sp. nov. Front. Microbiol. 4 (2013).
6. Tran, L. et al. Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. Dis. Aquat. Organ. 105, 45–55 (2013).
7. Han, J.-E., Tang, K.-F. J., Tran, L. H. & Lightner, D. V. Photorhabdus insect-related (Pir) toxin-like genes in a plasmid of Vibrio parahaemolyticus, the causative agent of acute hepatopancreatic necrosis disease (AHPND) of shrimp. Dis. Aquat. Organ. 113, 33–40 (2015).
8. Han, J.-E., Tang, K.-F. J. & Lightner, D. V. Genotyping of virulence plasmid from Vibrio parahaemolyticus isolates causing acute hepatopancreatic necrosis disease in shrimp. Dis. Aquat. Organ. 115, 245–251 (2015).
9. Gomez-Gil, B., Soto-Rodriguez, S., Lozano, R. & Betancourt-Lozano, M. Draft Genome Sequence of Vibrio parahaemolyticus Strain M0605, Which Causes Severe Mortalities of Shrimps in Mexico. Genome Announc. 2, e0055–14 (2014).
10. Yang, Y. et al. Draft Genome Sequences of Four Strains of Vibrio parahaemolyticus, Three of Which Cause Early Mortality Syndrome/ Acute. Genome 2, 2–3 (2014).
11. Restrepo, L., Bayot, B., Betancourt, I. & Pinzon, A. Draft genome sequence of pathogenic bacterium Vibrio parahaemolyticus strain Ba94C2, associated with acute hepatopancreatic necrosis disease isolate from South America. Genomics Data 9, 143–144 (2016).
12. Waterfield, N., Kamita, S. G., Hammock, B. D. & Firestone-Constant, R. The Photorhabdus Pir toxins are similar to a developmentally regulated insect protein but show no juvenile hormone esterase activity. FEMS Microbiol. Lett. 245, 47–52 (2005).
13. Sawabe, T., Kita-Tsukamoto, K. & Thompson, E. L. Inferring the evolutionary history of Vibrios by means of multilocus sequence analysis. J. Bacteriol. 189, 7932–7936 (2007).
14. Kondo, H., Van, P. T. & Dang, L. T. Draft Genome Sequence of Non-Vibrio parahaemolyticus Acute Diseased Shrimp in Vietnam. Genome Announc. 3, 104–2015 (2015).
15. Liu, L. et al. Draft Genome Sequence of Vibrio owensii Strain SH-14, which causes shrimp acute hepatopancreatic necrosis disease. Genome Announc. 3, 3354 (2015).
16. Dong, X. et al. Complete genome sequence of Vibrio campbellii strain 20130629003501 isolated from shrimp with acute hepatopancreatic necrosis disease. Gut Pathog. 9 (2017).
17. Hada, H. S., West, P. A., Lee, J. V., Stemmler, J. & Colwell, R. R. Vibrio tubiashii sp. nov., a pathogen of bivalve mollusks. Int J of Syst Bacteriol. 34, 1–4 (1984).
18. Gomez-Gil, B. et al. Vibrio sinaloensis sp. nov., isolated from the spotted rose snapper, Lutjanus guttatus Steinbacher, 1869. International journal of systematic and evolutionary microbiology. 58, 1621–1624 (2008).
19. Xiao, J. et al. Shrimp AHPND-causing plasmids encoding the PirAB toxins as mediated by pirAB-Tn903 are prevalent in various Vibrio species. Sci. Rep. 7, 42177 (2017).
20. Meier-Kolthoff, J. P., Auch, A. F., Klenk, H. P. & Göker, M. Genome sequence-based species delimitation with confidence intervals and improved distance functions. BMC bioinformatics. 14, 60 (2013).
21. Meier-Kolthoff, J. P., Klenk, H. P. & Göker, M. Taxonomic use of DNA G+C content and DNA-DNA hybridization in the genomic age. Int J Syst Evol Microbiol 64, 352–356 (2014).
22. Prado, S., Dubert, J. & Barja, J. L. Characterization of pathogenic vibrios isolated from bivalve hatcheries in Galicia, NW Atlantic coast of Spain. Description of Vibrio tubiashii subsp. europaensis subsp. nov. Syst Appl Microbiol 38, 26–29 (2015).
23. Richter, M. & Rosselló-Móra, R. Shifting the genomic gold standard for the prokaryotic species definition. Proceedings of the National Academy of Sciences. 45, 19126–19131 (2009).
24. Li X., Huang Y., Whitman W. B. The relationship of the whole genome sequence identity to DNA hybridization varies between genera of prokaryotes. Antonie van Leeuwenhoek 107:241–249 (2015).
25. Dubert, J. et al. Reclassification of the larval pathogen for marine bivalves Vibrio tubiashii subsp. europaeus as Vibrio europaeus sp. Nov. *International Journal of Systematic and Evolutionary Microbiology* 66, 4791–4796 (2016).
26. Richter, M. & Rossello-Mora, R. Shifting the genomic gold standard for the prokaryotic species definition. *Proc. Natl. Acad. Sci.* 106, 19126–19131 (2009).
27. Ramasamy, D. et al. A polyphasic strategy incorporating genomic data for the taxonomic description of novel bacterial species. *International journal of systematic and evolutionary microbiology* 64, 384–391 (2014).
28. Meier-Kolthoff, J. P., Göker, M., Sproer, C. & Klenk, H. P. When should a DDH experiment be mandatory in microbial taxonomy? *Archives of Microbiology* 195, 413–418 (2013).
29. Kim, M., Oh, H. S., Park, S. C. & Chun, J. Toward a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes. *International journal of systematic and evolutionary microbiology* 64, 346–351 (2014).
30. Thompson, F. L. et al. *Vibrio fori* sp. nov. and *Vibrio hepatarius* sp. nov., isolated from aquatic animals and the marine environment. *Int. J. Syst. Evol. Microbiol.* 53, 1495–1501 (2003).
31. Heidelberg, J. F. et al. DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. *Nature* 406, 477–483 (2000).
32. Makino, K. et al. Genome sequence of *Vibrio parahaemolyticus*: A pathogenic mechanism distinct from that of *V cholerae*. *Lancet* 361, 743–749 (2003).
33. Oliver, J. D. Wound infections caused by *Vibrio vulnificus* and other marine bacteria. *Epidemiol. Infect.* 133, 383–391 (2005).
34. Alina, M. & Blanch, A. R. A set of keys for biochemical identification of environmental *Vibrio* species. *J. Appl. Bacteriol.* 76, 79–85 (1994).
35. Kembel, S. W. et al. *Picante*: R tools for integrating phylogenies and ecology. *Bioinformatics* 26, 1463–1464 (2010).
36. Tang, S. et al. Genetic heterogeneity of the tropical abalone (*Haliotis asinina*) revealed by RAPD and microsatellite analyses. *BMB Reports* 38, 182–190 (2005).
37. Bell, T. A. A handbook of normal penaeid shrimp histology. *World Aquaculture Society.Baton Rouge, LA* 114 (1988).
38. Andrews, S. *FQC*: A quality control tool for high throughput sequence data. *http://www.Bioinformatics.Babraham.Ac.Uk/Projects/*
39. Galardini, M., Biondi, E. G., Bazzicalupo, M. & Mengoni, A. *CONTIGuator*: a bacterial genomes finishing tool for structural insights *et al.*
40. Aziz, R. K. *et al.* *RNAmmer*: Consistent and rapid annotation of ribosomal RNA genes. *Nucleic Acids Res.* 35, 3100–3108 (2007).
41. Altschul, S. F. *et al.* *Gapped BLAST* and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Research* 25, 3389–3402 (1997).
42. Delcher, A. L., Bratke, K. A., Powers, E. C. & Salzberg, S. L. Identifying bacterial genes and endosymbiont DNA with Glimmer. *Bioinformatics* 23, 673–679 (2007).
43. Tatusova, T. *et al.* NCBi prokaryotic genome annotation pipeline. *Nucleic Acids Res.* 44, 6614–6624 (2016).
44. Aziz, R. K. *et al.* The RAST Server: Rapid Annotations using Subsystems Technology. *BMC Genomics* 9, 75 (2008).
45. Lagesen, K. *et al.* *RNAmmer*: Consistent and rapid annotation of ribosomal RNA genes. *Nucleic Acids Res.* 35, 3100–3108 (2007).
46. Tatusov, R. L. The COG database: a tool for genome-scale analysis of protein functions and evolution. *Nucleic Acids Res.* 28, 33–36 (2000).
47. Kanehisa, M. The KEGG resource for deciphering the genome. *Nucleic Acids Res.* 32, 277D–280 (2004).
48. Ashburner, M. *et al.* Gene Ontology: tool for the unification of biology. *Nat. Genet.* 25, 25–29 (2000).
49. Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. *MEGA6*: Molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 30, 2725–2729 (2013).
50. Darrida, D., Taboada, G. L., Doallo, R. & Posada, D. *jModelTest* 2: more models, new heuristics and parallel computing. *Nat. Methods* 9, 772–772 (2012).
51. Posada, D. Using *MODELLTEST* and PAUP* to Select a Model of Nucleotide Substitution. In *Current Protocols in Bioinformatics* 10.1002/0471258953.b0605s00 (2003).
52. Hueslenbeck, J. P. & Ronquist, F. *MRBAYES*: Bayesian inference of phylogenetic trees. *Bioinformatics* 17, 754–755 (2001).
53. Clement, M., Posada, D. & Crandall, K. A. TCS: A computer program to estimate gene genealogies. *Mol. Ecol.* 9, 1657–1659 (2000).
54. Huyn, D. H., & Bryant, D. Estimating phylogenetic trees and networks using SplitsTree 4. *Manuscript in preparation, software available from www.splitsTree.org* (2005).
55. Darling, A. C., Mau, B., Blattner, F. R. & Perna, N. T. Mauve: Multiple alignment of conserved genomic sequence with rearrangements. *Genome Res.* 14, 1394–1403 (2004).
56. Krumsiek, J., Arnold, R. & Rattie, T. Gepard: A rapid and sensitive tool for creating dotplots on genome scale. *Bioinformatics* 23, 1026–1028 (2007).
57. Langille, M. G. & Brinkman, F. S. *IslandViewer*: An integrated interface for computational identification and visualization of genomic islands. *Bioinformatics* 25, 664–665 (2009).
58. Auch, A. F., von Jan, M., Klenk, H. -P. & Göker, M. Digital DNA-DNA hybridization for microbial species delineation by means of genome-to-genome sequence comparison. *Stand. Genomic Sci.* 2, 117–134 (2010).
59. Stackebrandt, E. *et al.* Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *Int. J. Syst. Evol. Microbiol.* 52, 1043–1047 (2002).
60. Kurtt, S. *et al.* *Versatile and open software for comparing large genomes*. *Genome Biol.* 5, R12 (2004).

**Acknowledgements**

We thank Betsy Andrade and Karina Reyes for their technical assistance in this study. We would like to thank Dr. Andres Pinzón for his help in the preliminary analysis and Dr. Stanislaus Sonnenholzner and Nicolas Malo for their kind reviewing of the manuscript and suggestions. Additionally, we acknowledge the High-Performance Computing Service at Universidad de los Andes in Bogotá, Colombia, (http://hpc.uniandes.edu.co) for providing HPC resources that have contributed to the research results reported in this work. This work was funded by the Secretaria de Educación Superior, Ciencia, Tecnología e Innovación (SENESCYT) in the framework of the PIC-14-CENAIM-003 Project “Desarrollo e implementación de métodos de control y prevención de enfermedades en especies acuáticas de uso comercial y uso potencial en maricultura y repoblación”.

**Author Contributions**

A.R. designed this study. L.R. assembled the sequences and performed related experiments. L.R. analyzed the data and S.A. performed the phylogenetic analysis. L.R.B., L.B. and E.P. performed laboratory experiments. A.R. modified the manuscript. L.R. and B.B. wrote the manuscript. Contributed reagents/materials/analysis tools and acquired funding: B.B. and A.R. All authors reviewed the manuscript.
Additional Information
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-30903-x.

Competing Interests: The authors declare no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2018