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Kiloniella majae sp. nov., isolated from spider crab (*Maja brachydactyla*) and pullet carpet shell clam (*Venerupis pullastra*)☆

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ABSTRACT

Ten Gram-negative, rod-shaped and motile bacterial strains were isolated from spider crab (M27.10, M27.11a, F36.1, F36.4, M56.1, F76.17b, M146.1, M166.3 and M166.6) and pullet carpet shell clam (SBRF 1.10) collected in the coast of Galicia. Analyses of the 16S rRNA genes showed that the strains belong to the genus *Kiloniella* and have high similarity with the species *Kiloniella spongiae* (99.44–99.86%) and *Kiloniella litopenaei* (99.0–99.5%). Strains M56.1^T (=CECT 9195, =LMG 29925), M146.1 (=CECT 9193, =LMG 29926) and SBRF 1.10 (=CECT 9194, =LMG 29927) were selected on the basis of genotyping by enterobacterial repetitive intergenic consensus PCR (ERIC-PCR). Phylogenetic analysis based on concatenated sequences of the genes *gyrB*, *ftsZ*, *rpoD* and *mreB* showed that the isolates form a differentiated branch within the genus *Kiloniella*. Moreover, the average nucleotide identity (ANIm, ANIb and OrthoANI) and *in silico* estimated DNA–DNA reassociation values between selected Galician isolates and *Kiloniella* species were below the established cut-off for species deliniation. The results obtained in the genetic and phenotypical analyses indicate that the isolates represent a new species of the genus *Kiloniella*, for which the name *Kiloniella majae* sp. nov. is proposed with strain M56.1^T (=CECT 9195^T, =LMG 29925^T) as the type strain.

The genus *Kiloniella* and its type species *Kiloniella laminariae* were created by Wiese et al. [19] to allocate an α-proteobacterium strain obtained from the marine macroalga *Laminaria saccharina* in the Baltic Sea. The taxon was described as a mesophilic, chemoheterotrophic bacterium with typical marine, moderately halotolerant growth response. It presents aerobic and facultatively anaerobic metabolism with nitrate as electron acceptor. Cell morphology vary from spiral to vibrioid shape, even occasionally rod-like or filamentous, being motile by means of flagella. These authors suggested that the genus constitutes the type genus of a new family *Kiloniellacae* in a new order *Kiloniellales*.

A second species, namely *Kiloniella spongiae*, was described within the genus in January 2015 [20]. This description was based on the polyphasic characterization of a strain isolated from a marine sponge at Uljin country in the coastal area of the East Sea, Korea. *K. spongiae* was described as a Gram-negative, facultatively anaerobic, motile and rod-shaped bacterium positive for oxidase and catalase activities. The description of the later species leads to emend the description of the genus *Kiloniella*, adding the characterization of the polar lipids and major respiratory quinones, as well as the predominant fatty acids [20].

A third species isolated from the gut microbiota of Pacific white shrimp, *Litopenaeus vannamei*, was reported in December 2015 [18] and was named *Kiloniella litopenaei*. This species was described as a Gram-negative, rod-shaped, aerobic and facultatively anaerobic bacteria, with nitrate or nitrite as electron acceptor and positive for oxidase and catalase activities. Moreover, average nucleotide identity (ANI) and estimated DNA–DNA hybridization (eDDH) values between *K. litopenaei* and *K. laminariae* were respectively 81,7% and 71.3%, and between *K. litopenaei* and *K. spongiae* were 24.6% and 18.3%.

In the present work, a polyphasic approach was employed to characterize ten isolates resembling the characteristics of the genus *Kiloniella* that were obtained during previous surveys of the microbiota associated to spider crab (*Maja brachydactyla*) and cultured pullet carpet shell clam (*Venerupis pullastra*) in the coast of Galicia (NW Spain). Isolates M27.10, M27.11a, F36.1, F36.4, M56.1^T (=CECT 9195^T =LMG 29925^T), F76.17b, M146.1 (=CECT 9193 =LMG 29926), M166.3 and M166.6 were obtained from crab in O Freixo (A Coruña), and isolate SBRF 1.10 (=CECT 9194 =LMG 29927) from clams in

☆ The nucleotide sequences of the 16S rRNA gene obtained in this study for the *Kiloniella majae* isolates are available under GenBank accession numbers LT717337–LT717346. Accession numbers of whole genome sequences of M56.1^T, M146.1 and SBRF 1.10 are MTSZ00000000, MTSX00000000 and MTSY00000000, respectively. The versions described in this paper are versions MTSZ01000000, MTSX01000000 and MTSY01000000, respectively.

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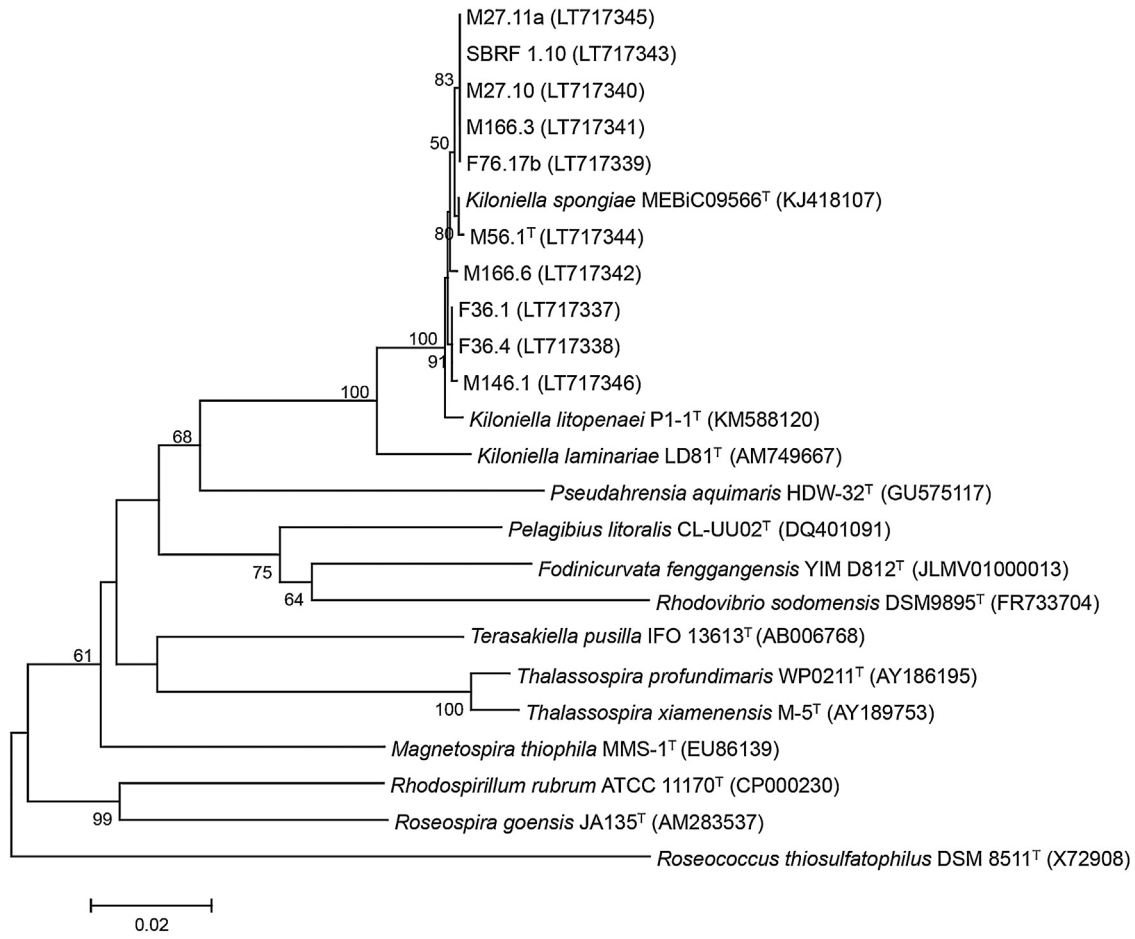

Fig. 1. Phylogenetic reconstruction based on the concatenated sequences of the 16S rRNA gene by the NJ algorithm, showing the position of the Galician *Kiloniella* isolates from spider crab and clam. Bootstrap values (expressed as percentages of 1000 replications) are shown at the nodes. Bar, 0.02 substitutions per nucleotide position.

Camariñas (A Coruña). All strains were cultured on marine agar (MA, Difco) at 24 ± 1 °C for 48 h. Cultures were stored at −80 °C in marine broth (MB, Difco) supplemented with 15% of glycerol.

The 16S rRNA gene of the ten strains was amplified and sequenced using the universal primer pair pA (5′-AGAGTTTGATCCTGGCTCAG-3′) and pH (5′-AAGGAGGTGATCCAGCCGCA-3′) as described by Hutson et al. [6]. Sequence data analyses were performed using DNASTAR Lasergene SEQMAN program. Sequence similarities were determined using the EzTaxon-e server (www.eztaxon-e.ezbioclouf.net) and the BLASTN program. Sequences were aligned using CLUSTAL W tool [7] and phylogenetic trees were constructed using the neighbour-joining (NJ) and maximum-likelihood (ML) algorithms (MEGA version 6.06). Distance matrices were calculated by using Kimuraís two-parameter correction and stability of the groups was estimated by bootstrap analysis (1000 replicates) using the MEGA version 6.06 [16].

16S rRNA gene sequences obtained showed similarities from 99.4 to 100% among the Galician isolates, indicating a close relationship. In addition, similarities among these strains and the type strain of *K. laminariae* DSM 19542^T ranged from 96.5 to 96.7% revealing that the ten strains belonged to the *Kiloniella* genus. Similarities of Galician isolates and *K. spongiae* and *K. litopenaei* ranged from 99.4 to 99.86% and from 99.0 to 99.5% respectively. Phylogenetic analyses based on the 16S rRNA gene sequences, using both NJ and ML algorithms, showed that the type strains of *K. spongiae* MEBiC09566^T and *K. litopenaei* P1-1^T clustered together with our isolates (Fig. 1). Therefore, the use of other methods is required to accurately identify the Galician isolates due to the low discriminative power of 16S rRNA gene.

The isolates were examined using phenotypic traits basically as reported by MacFaddin [10] and Romalde et al. [14]. Biochemical and physiological test included colony morphology and motility, Gram-staining, oxidation/fermentation reaction, Vogues Proskauer, indol reaction, nitrate reduction, production of oxidase, catalase, arginine dihydrolase, lysine and ornithine descarboxylases. In addition, susceptibility to the vibriostatic agent O/129 (2,4-diamino-6,7-diisopropylpteridine; 10 and 150 μg/disc) was evaluated. Flagella were visualized using the Leifson method [9]. Gelatinase, lipase, and hydrolysis of aesculin and starch activities were analyzed on MA plates supplemented with 0.4%, 1%, 0.1% and 0.4% (w/v) of substrates, respectively. Temperature (4–44 °C) and pH (3–10) ranges were determined on MA. Salinity range was tested on basal medium agar (1 g/l yeast extract [Oxoid]; 4 g/l neopeptone [Difco]; 15 g/l agar [Pronadisa]) plates supplemented with 0.5 to 10% (w/v) sea salts (Sigma). All tests were performed at 24 ± 1 °C unless indicated. Further biochemical characterization was carried out using API 20E, API 20NE and API ZYM strips (bioMérieux) according to the manufacturerís instructions, with the exceptions of the use of 1% (w/v) sea salts as inoculation solution and the incubation temperature that was fixed at 24 ± 1 °C.

All strains were Gram-negative, motile, oxidase and catalase positive, and resistant to the vibriostatic agent O/129. Results were variable for indol reaction. Moreover, all strains were no reactive for the aminoacid decarboxilation and were negative for the oxidation/fermentation reaction. Hydrolysis of gelatin, Tween 80, starch