



Phylogenomics-based reclassifications in the genus *Psychrobacter* including emended descriptions of *Psychrobacter pacificensis*, *Psychrobacter proteolyticus* and *Psychrobacter submarinus*

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Received: 22 February 2023 / Accepted: 9 August 2023 / Published online: 28 August 2023
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Abstract The taxonomic status of 43 *Psychrobacter* species was examined based upon the genome sequences of their type strains. Three groups of type strains were found to be conspecific, *Psychrobacter salsus* Shivaji et al. (Syst Appl Microbiol 27:628–635, 2004. 10.1078/0723202042369956) and *Psychrobacter submarinus* Romanenko et al. (Int J Syst Evol Microbiol 52:1291–1297, 2002. 10.1099/00207713-52-4-1291); *Psychrobacter oceani* Matsuyama et al. (Int J Syst Evol Microbiol 65:1450–1455, 2015. 10.1099/ijs.0.000118) and *Psychrobacter*

pacificensis Maruyama et al. (Int J Syst Evol Microbiol 50:835–846, 2000. 10.1099/00207713-50-2-835); and *Psychrobacter proteolyticus* Denner et al. (Syst Appl Microbiol 24:44–53, 2001. 10.1078/0723-2020-00006), *Psychrobacter marincola* Romanenko et al. (Int J Syst Evol Microbiol 52:1291–1297, 2002. 10.1099/00207713-52-4-1291) and *Psychrobacter adeliensis* Shivaji et al. (Syst Appl Microbiol 27:628–635, 2004. 10.1078/0723202042369956). For all three groups, the average nucleotide identity (ANI) and digital DNA–DNA hybridization (dDDH) values are > 97.69% and > 80.2%, respectively. This conclusion is supported by similarities in morphology, growth properties, and fatty acid compositions. Based on this evidence, we propose the reclassification of *Psychrobacter salsus* Shivaji

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Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10482-023-01871-8>.

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et al. (Syst Appl Microbiol 27:628–635, 2004. 10.1078/0723202042369956) as a later heterotypic synonym of *Psychrobacter submarinus* Romanenko et al. (Int J Syst Evol Microbiol 52:1291–1297, 2002. 10.1099/00207713-52-4-1291); *Psychrobacter oceani* Matsuyama et al. (Int J Syst Evol Microbiol 65:1450–1455, 2015. 10.1099/ijms.0.000118) as a later heterotypic synonym of *Psychrobacter pacificensis* Maruyama et al. (Int J Syst Evol Microbiol 50:835–846, 2000. 10.1099/00207713-50-2-835), and *Psychrobacter marincola* Romanenko et al. (Int J Syst Evol Microbiol 52:1291–1297, 2002. 10.1099/00207713-52-4-1291) and *Psychrobacter adeliensis* Shivaji et al. (Syst Appl Microbiol 27:628–635, 2004. 10.1078/0723202042369956) as later heterotypic synonyms of *Psychrobacter proteolyticus* Denner et al. (Syst Appl Microbiol 24:44–53, 2001. 10.1078/0723-2020-00006).

Keywords *Psychrobacter* · Phylogenomics · Reclassification · Average nucleotide identity · Digital DNA–DNA hybridization

Introduction

Psychrobacter, a genus within the family *Moraxellaceae* of the class *Gammaproteobacteria*, was proposed by Juni and Heym in 1986. This group includes various strains found in diverse environments like animals, sea ice, nonhost habitats, and food products (Juni and Heym 1986). Members of the group *Psychrobacter* are Gram-stain negative, osmotolerant, psychrophilic or psychrotolerant, aerobic and grow at temperatures between –10 and 42 °C (Kim et al. 2012). A few were isolated from humans and cause illnesses, including endocarditis and peritonitis (Bowman 2006). As of January 2022, 43 species of *Psychrobacter* have been taxonomically characterized and their names validly published (Parte et al. 2020). With the availability of whole-genome sequencing, comparison of the taxon-specific, conserved gene-contents is a valuable tool for examining taxonomic assignments. In this study, we performed phylogenomic analyses on the 42 available genomes of *Psychrobacter* spp., excluding *P. pocilloporae* S6-60^T, for which the genome sequence was not available.

Analyses based on phylogenomics and overall genome relatedness indices indicated that *P. salsus* is

a later heterotypic synonym of *P. submarinus*, and the union retains the name *P. submarinus*. *P. oceani* is a later heterotypic synonym of *P. pacificensis*. Lastly, *P. marincola* and *P. adeliensis* are later heterotypic synonyms of *P. proteolyticus*.

Materials and methods

The phylogenetic comparison of strains ($n=43$) was performed based on 16S rRNA gene sequences using *Geopsychrobacter electrodiphilus* A1^T as an outgroup to root the tree. The evolutionary distances were computed using the Jukes–Cantor method (Jukes and Cantor 1969) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated, leaving 914 positions in the final dataset. Evolutionary analyses were conducted in Mega11 (Tamura et al. 2021). The results were comparable when trees were drawn using neighbor-joining (Saitou and Nei 1987) and maximum parsimony approaches (Fitch 1977).

The genomic information for the type strains of the 42 species for which data was available was obtained from the NCBI genome database (Supplementary excel file 1). ANI was calculated using the BLAST (ANIb) in pyani with default parameters (Pritchard et al. 2019). The available genomes (Table 1) were subjected to the calculation of the overall genome-related index, which included average nucleotide identity (ANI), digital DNA–DNA hybridization (dDDH) calculated using the Genome-to-Genome Distance Calculator (GGDC) with blast+ and formula 2 (Goris et al. 2007). In addition, the AAI values were calculated using the Kostas web server (Konstantinidis and Tiedje 2005a).

For the core genome phylogeny, ORFs were predicted using Prodigal (Hyatt et al. 2010). All ortholog single-copy proteins were identified using OrthoMCL pipeline implemented in Get_homologues (Contreras-Moreira and Vinuesa 2013). The proteins were identified as a core only if present in all studied genomes and sharing a minimum 75% sequence identity. The proteins were aligned using MAFFT (Katoh et al. 2002), and multiple sequence alignments of all core amino acids were concatenated. Phylogeny of the concatenated core proteome was reconstructed using a maximum-likelihood approach using IQ tree v2.0.3 (Minh et al. 2020) with default parameters and based

Table 1 General genomic attributes of the whole-genome sequence assemblies of *Psychrobacter marincola*, *Psychrobacter adeliensis*, *Psychrobacter proteolyticus*; *Psychrobacter**salsus*, *Psychrobacter submarinus*; and *Psychrobacter oceani* and *Psychrobacter pacificensis*

Taxa	Type strain	Genome size (Mb)	Contigs	N50 (bp)	DNA G+C content (mol %)	GenBank accession number (genome)
<i>Psychrobacter oceani</i>	4K5 ^T	2.98	81	81,941	44.0	CAJHAM000000000
<i>Psychrobacter pacificensis</i>	NIBH-P2K6 ^T	3.17	44	442,556	44.0	CAJHAA000000000
<i>Psychrobacter adeliensis</i>	SJ-14 ^T	3.09	10	1,641,620	42.9	CAJGYR000000000
<i>Psychrobacter marincola</i>	KMM277 ^T	3.05	43	175,014	42.8	CAJHAI000000000
<i>Psychrobacter proteolyticus</i>	116 ^T	3.04	17	658,356	42.8	CAJHAS000000000
<i>Psychrobacter salsus</i>	DD48 ^T	2.89	68	114,010	44.8	CAJHAW000000000
<i>Psychrobacter submarinus</i>	KMM225 ^T	3.01	6	737,933	44.8	CAJHBQ000000000

on LG + F + I + G4 (Kalyanamoorthy et al. 2017) identified as the best-fit model. The robustness of tree topology was assessed using 1000 bootstrap replicates, and the resulting tree was visualized in iTOL (Letunic and Bork 2007). OrthoVenn2 at default parameters (Ling et al. 2019) was used for determining the orthologs and paralogs cluster in similar strains.

Results and discussion

In the 16S rRNA tree, several species were closely related, with gene sequence similarities > 98%. *P. salsus* DD48^T and *P. submarinus* KMM 225^T formed one clade, while *P. oceani* 4K5^T and *P. pacificensis* NIBH-P2K6^T formed another, having 16S rRNA gene sequence similarity values of 98.72 and 98.64%, respectively. It is important to highlight that *P. pocilloporae* S6-60^T falls within the established group of *P. pacificensis* species in the 16S rRNA gene tree. However, due to the unavailability of its genome sequence, a more in-depth investigation of this relationship could not be conducted. In addition, *P. adeliensis* SJ14^T and *P. marincola* KMM 277^T share 99.51 and 99.77% similarities with *P. proteolyticus* 116^T (Fig. 1).

Because 16S rRNA sequence similarity is not a reliable indicator at the species level, the ANIb was calculated for all pairs of the 42 species for which genomes were available. The ANIb values were > 95% between some pairs of species, indicating that they could be reclassified in the same species (Richter and Rosselló-Móra 2009, Fig. 2). For example, ANIb values of 99.89% and 99.93% were found

between *P. adeliensis* SJ14^T (CAJGYR000000000) and *P. marincola* KMM 277^T (CAJHAI000000000), respectively, when compared to *P. proteolyticus* 116^T (CAJHAS000000000). Additionally, the ANIb value between *P. adeliensis* SJ14^T and *P. marincola* KMM 277^T was 99.88% (Table 2). This high level of relatedness was further confirmed by dDDH values exceeding 99.5%. Similarly, the AAI values between these three strains were greater than 99.9%.

Furthermore, the genomes of *P. salsus* DD48^T (CAJHAW000000000) and *P. submarinus* KMM 225^T (CAJHBQ000000000) were very similar and shared values of 97.18% for ANIb, 76.8% for dDDH, and 96.93% for AAI (Table 2). Also found to be closely related were, *P. oceani* 4K5^T (CAJHAM000000000) and *P. pacificensis* NIBH-P2K6^T (CAJHAA000000000), with values of 97.69% for ANIb, 80.2% for dDDH, and 97.90% for AAI. The genome-related measures used for species identification, such as ANI, dDDH, and AAI values, exceeded the commonly accepted thresholds for species differentiation, which are > 95% for ANI, > 70% for dDDH, and > 95% for AAI values (Kim et al. 2014; Richter and Rosselló-Móra 2009; Konstantinidis and Tiedje 2005b). This indicates that the three groups of species belong to the same species.

The high similarities of the genome-related indices were also consistent with the similarity in size and mol % G+C for the genomes of each group (Table 1). These very similar genomes also shared most of their gene clusters as determined by OrthoVenn2 at default parameters (Ling et al. 2019), where each cluster consists of orthologs or paralogs (Supplementary Fig. 1). *P. salsus* DD48^T and *P. submarinus* KMM 225^T

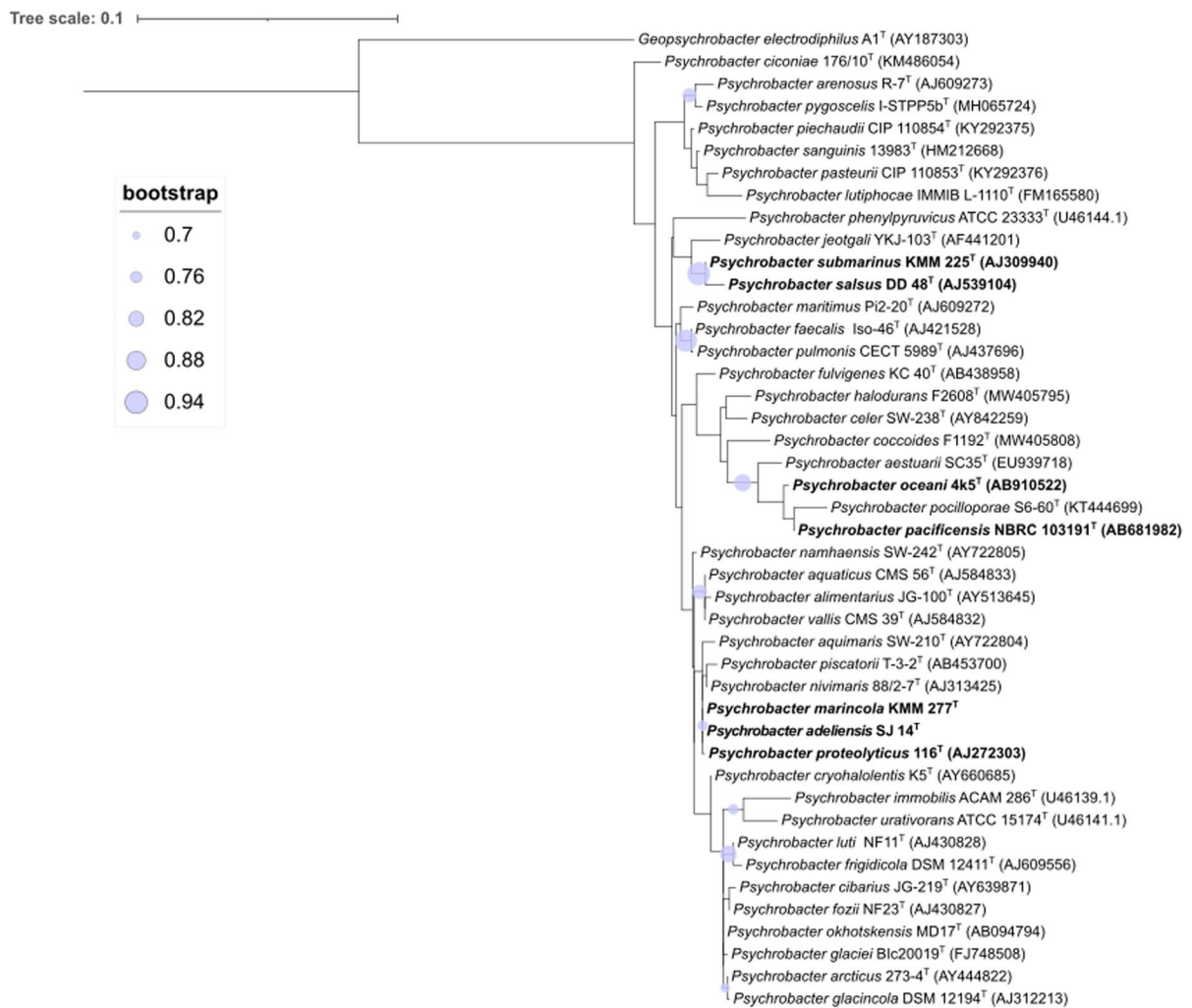


Fig. 1 Phylogenetic tree reconstructed using 16S rRNA gene sequences of 43 validly published species described in the genus *Psychrobacter* using the maximum likelihood algorithm (Welter et al. 2021) and Jukes-Cantor model of substitutions (Jukes and Cantor 1969). An additional sequence for *Geopsychrobacter electrodiphilus* A1^T was taken as an outgroup. The 16S rRNA genes for *Psychrobacter adeliensis* SJ14^T (CAJGYR010000004.1) and *Psychrobacter marincola* KMM

277^T (CAJHAI010000030.1) were obtained using RNAmmer (Lagesen et al. 2007). The percentages of replicate trees ($n=1000$) in which the associated taxa clustered together in the bootstrap test (>0.51) are shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree

share 2174 out of 2207 clusters; *P. oceani* 4K5^T and *P. pacificensis* NIBH-P2K6^T share 2332 out of 2373 clusters. *P. adeliensis* SJ14^T, *P. marincola* KMM 277^T and *P. proteolyticus* 116^T share 2497 out of 2501 clusters.

The phylogenomic analysis based on the core proteome of the strains under study confirmed that *P. salsus* DD48^T and *P. submarinus* KMM 225^T; *P. oceani* 4K5^T and *P. pacificensis* NIBH-P2K6^T; as well

as *P. adeliensis* SJ14^T, *P. marincola* KMM 277^T and *P. proteolyticus* 116^T formed monophyletic clades, indicative of a high level of genome sequence identity between these strains (Fig. 3).

Given the high level of genetic similarity, it was not surprising that the physiological and biochemical features of the strains possessed a high degree of phenotypic coherence (Supplementary tables 1 and 2). The few differences observed could be because

Table 2 ANiB (%) of closely related type strains based on whole-genome sequences of the genus *Psychrobacter*

Taxa	<i>Psychrobacter oceani</i> 4K5 ^T	<i>Psychrobacter adeliensis</i> SJ-14 ^T	<i>Psychrobacter marincola</i> KMM277 ^T	<i>Psychrobacter pacificensis</i> NIBH-P2K6 ^T	<i>Psychrobacter proteolyticus</i> 116 ^T	<i>Psychrobacter salsus</i> DD48 ^T	<i>Psychrobacter submarinus</i> KMM225 ^T
<i>Psychrobacter oceani</i> 4K5 ^T	100	82.37	82.42	97.69	82.40	78.05	78.33
<i>Psychrobacter adeliensis</i> SJ-14 ^T		100	99.88	82.55	99.89	76.62	76.48
<i>Psychrobacter marincola</i> KMM277 ^T			100	82.47	99.93	76.29	76.24
<i>Psychrobacter pacificensis</i> NIBH-P2K6 ^T				100	82.45	78.26	78.39
<i>Psychrobacter proteolyticus</i> 116 ^T					100	76.42	76.38
<i>Psychrobacter salsus</i> DD48 ^T						100	97.18
<i>Psychrobacter submarinus</i> KMM225 ^T							100

of intraspecies differences or differences in the laboratory conditions. Further, these observations are well-supported by Welter et al.'s recent study, which elucidates a link between ecological niches and *Psychrobacter*'s evolutionary history (Welter et al. 2021). According to this study, *P. salsus* DD48^T and *P. submarinus* KMM 225^T, as well as *P. adeliensis* SJ14^T, *P. marincola* KMM 277^T, and *P. proteolyticus* 116^T, all belong to the Restricted ecotype (RE), which can only grow between 4 and 25 °C. However, *P. oceani* 4K5^T and *P. pacificensis* NIBH-P2K6^T belong to the Flexible ecotype (FE), which can grow between 4 and 37 °C (Welter et al. 2021).

As per the priority of prokaryotic names governed by the International Code of Nomenclature of Prokaryotes (Parker et al. 2019), when species are united, the earliest validly published name is used for the union. *P. submarinus* was validly published in 2002 (Romanenko et al. 2002) and *P. salsus* was validly published in 2004 (Shivaji et al. 2004). Therefore, *P. salsus* is a later heterotypic synonym of *P. submarinus*, and the union retains the name *P. submarinus*. Similarly, *P. pacificensis* was validly published in 2000 (Maruyama et al. 2000) and *P. oceani* was

validly published in 2015 (Matsuyama et al. 2015). Thus, *P. oceani* is a later heterotypic synonym of *P. pacificensis*. Lastly, *P. proteolyticus* was validly published in 2001 (Denner et al. 2001), whereas *P. marincola* and *P. adeliensis* were validly published in 2002 and 2004, respectively (Romanenko et al. 2002; Shivaji et al. 2004). Thus, *P. marincola* and *P. adeliensis* are later heterotypic synonyms of *P. proteolyticus*. To reflect these species unions, emended descriptions are provided.

Emended description of *Psychrobacter submarinus*

The description is as before (Romanenko et al. 2002) with the following modifications:

Psychrobacter salsus (Shivaji et al. 2004) is later heterotypic synonym of *Psychrobacter submarinus* Romanenko et al. (2002). The type strain is DSM 14161^T (=KMM 225^T), and strain DD 48 (=DSM 15338=MTCC 4826) is an additional strain of *Psychrobacter submarinus*. The additional strain grows at the maximum temperature of 30 °C and optimum growth is observed at 22 °C, while growth of strain KMM 225^T was observed at 4–35 °C.

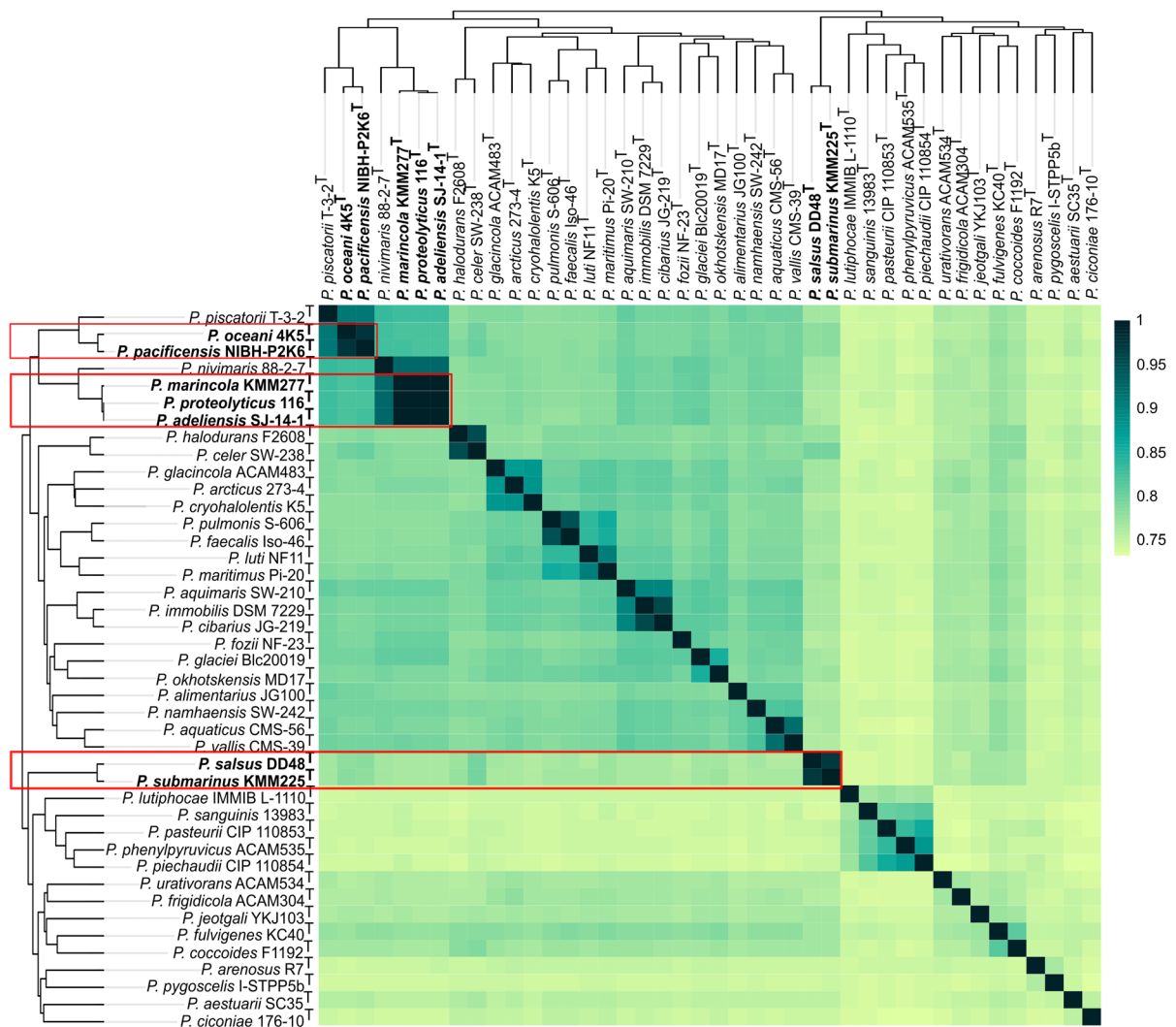


Fig. 2 Heatmap with a dual dendrogram based on the average nucleotide identity (ANI) values of 42 members of the genus *Psychrobacter* for which complete genomic information was available. The strains were compared using ANIb with

default parameters (Supplementary excel file 2). The ANIb value matrix was then plotted as a dual dendrogram in R using pheatmap package (Kolde and Kolde 2015)

The whole-genome sequence accession number of *Psychrobacter submarinus* DSM 14161^T is CAJHBQ000000000. The emended DNA G+C content (mol %) and the genome size (Mbp) of the species are 44.8 (WGS) and 2.89–3.01, respectively.

Emended description of *Psychrobacter pacificensis*

The description is as before (Maruyama et al. 2000) with the following modifications:

Psychrobacter oceani Matsuyama et al. (2015) is a later heterotypic synonym of *Psychrobacter pacificensis* Maruyama et al. (2000). The type strain is NIBH P2K6^T (= DSM 23406^T = IFO 16270^T = NBRC 103191^T), and strain 4k5 (= JCM 30235 = NCIMB 14948) is an additional strain of *Psychrobacter pacificensis*. The additional strain has optimum growth between 25 and 30 °C. NIBH P2K6^T isolates have growth yields at 4 °C comparable to those at 20 °C. However, optimal growth occurs at about 25 °C, with the maximum growth temperature being

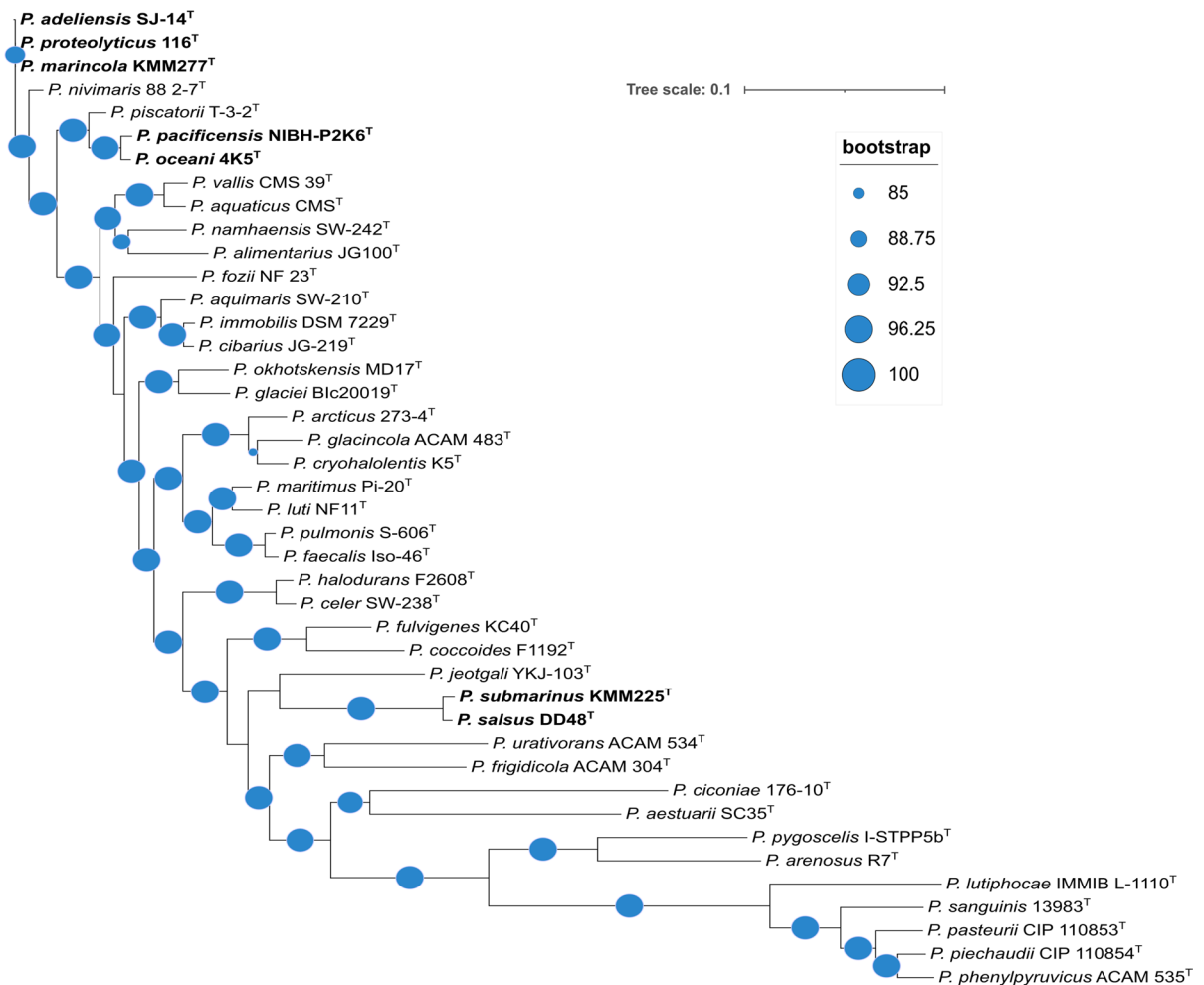


Fig. 3 Phylogenomic analysis of *Psychrobacter* species ($n=42$) using core-genome alignments. The alignment was performed using MAFFT (Katoh et al. 2002) and maximum-likelihood phylogenetic tree was reconstructed using IQ tree

v2.0.3 (Minh et al. 2020) with default parameters. The test of bootstrap was performed at 1000 re-samplings and resulting tree was visualized in iTOL (Letunic and Bork 2007)

38 °C. The whole-genome sequence accession number of *Psychrobacter pacificensis* NIBH P2K6^T is CAJHAO000000000. The emended DNA G+C content (mol %) and the genome size (Mbp) of the species are 44 (WGS) and 2.98–3.17, respectively.

Emended description of *Psychrobacter proteolyticus*

The description is as before (Romanenko et al. 2002; Shivaji et al. 2004) with the following modifications:

Psychrobacter marincola Romanenko et al. (2002) and *Psychrobacter adeliensis* Shivaji et al.

(2004) are later heterotypic synonyms of *Psychrobacter proteolyticus* Denner et al. (2001). The type strain is 116^T (=CIP 106830^T=DSM 13887^T), and strains KMM 277 (=DSM 14160) and SJ14 (=DSM 15333=MTCC 4825) are additional strains of *Psychrobacter proteolyticus*. The additional strains grow to a maximum temperature of 37 °C, and the optimum range is 25–30 °C. Additionally, strain 116^T is routinely cultivated at room temperature (22 °C±3 °C). The whole-genome sequence accession number of *Psychrobacter proteolyticus* 116^T is CAJHAS000000000. The emended DNA G+C content (mol %) and the genome size (Mbp)

of the species are 42.8–42.9 (WGS) and 3.01–3.09, respectively.

Acknowledgements J.K. thanks Gargi College, University of Delhi for providing infrastructural support. U.S. thanks Kirori Mal College, University of Delhi for providing infrastructural support. RL acknowledges Indian National Science Academy for support under the INSA Senior Scientist Programme and Alexander von Humboldt Foundation for the award of Fellowship under its Renewed Research Program.

Author contribution US and RL conceived and designed the study. US and JK performed the analysis. US, JK, and CT analyzed the data. WBW and RL provided critical input regarding the international code for nomenclature of prokaryotes. All authors wrote and approved the manuscript.

Funding The authors received no specific grant from any funding agency.

Declarations

Conflict of interest The authors declare there are no conflicts of interest.

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