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Phylogenomics as baseline for taxonomy description: *Amphibiibacter pelophylacis* gen. nov., sp. nov., a novel taxon of the family *Sphaerotilaceae*, class *Betaproteobacteria*, isolated from the skin microbiota of *Pelophylax perezi* from different populations

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Abstract

Bacterial strain SL12-8^T was characterized and isolated from the skin microbiota of *Pelophylax perezi*, the Perez's frog. Strain SL12-8^T stained Gram-negative and formed rod-shaped cells that grew optimally at 25 °C and pH 7.0–7.5. The G+C content of the DNA was 66.2 mol%. Ubiquinone 8 was the respiratory quinone identified in the studied strain and the most closely related taxon. The major fatty acids were summed in feature 3 and summed in feature 8 and $C_{16:0}$, representing 84% of the total fatty acids. Phylogenetic analyses based on the 16S rRNA gene sequence placed strain SL12-8^T within the order *Burkholderiales* in a distinct lineage. The 16S rRNA gene sequence similarities of strain SL12-8^T to that of *Rubrivivax albus*, *Scleromatobacter humisilvae*, *Piscinibacter aquaticus*, *Azohydromonas caseinilytica* and *Aquincola agrisoli* were 94.41, 94.08, 93.72, 93.72 and 93.64%, respectively. The draft genome sequence of strain SL12-8^T comprises 3,115,197 bases with a 313-fold mapped genome coverage. The assembled genome consists of 53 large contigs with more than 500 bp, and the genome encodes 2,814 putative coding sequences. The analysis of the available genomes from the closest genera showed 124 core genes that reveal a novel genus-level clade including the strain SL12-8^T. Analysis of the SL12-8^T genome revealed the presence of the beta-lactone and terpene biosynthetic gene clusters. The phylogenomic, phylogenetic, phenotypic and chemotaxonomic data showed that strain SL12-8^T (=UCCCB 131^T=CECT 30762^T) represents the type of a novel species and genus, for which we propose the name *Amphibiibacter pelophylacis* gen. nov., sp. nov.

INTRODUCTION

The amphibian's skin microbiome has been intensely explored in the last decades due to the amphibian mass decline and the emerging infectious disease perspective [1–4]. Antifungal microbes were isolated from the skin of every host species sampled, which suggests that they are widely present in this taxon and confirms their important role in protecting against infectious diseases like *Batrachochytrium dendrobatidis* (*Bd*) [5–8]. In addition, amphibian skin microbiome studies allowed

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Keywords: *Amphibiibacter pelophylacis*; amphibian skin microbiota; *Burkholderiales*; genome; *Sphaerotilaceae*.
Abbreviations: AAI, average amino acid identity; ANI, average nucleotide identity; CDSs, coding sequences; COG, Clusters of Orthologous Group; dDDH, digital DNA–DNA hybridization; GBDP, genome BLAST distance phylogeny; KEGG, Kyoto Encyclopedia of Genes and Genomes; NA, nutrient agar; NCBI, National Center for Biotechnology Information; R2A, Reasoner's 2A; RAST, Rapid Annotation using Subsystem Technology; SEM, scanning electron microscopy; TSA, tryptic soy agar; TYGS, Type (Strain) Genome Server; UQ-8, ubiquinones 8.
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Nucleotide sequence data for the 16S rRNA gene of strains SL12-8^T, LB13-6, LB1–7 and 20M2A are available in the GenBank/DDBL/EMBL databases under the accession numbers KT720394, KT720383, KT720386 and KY611676, respectively. The Whole Genome Shotgun project of strains SL12-8^T has been deposited at DDBJ/ENA/GenBank under the accession JAWDIE000000000. The version described in this paper is version JAWDIE010000000. NCBI BioProject accession number is PRJNA1018472.
A supplementary figure is available with the online version of this article.

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us to understand patterns of diversity, the specificity of the host species and its relation to the surrounding environment and, mostly, the function and complexity of biomolecules produced by these symbiotic bacteria [7–9].

The *Burkholderiales* order (class *Betaproteobacteria*) includes common bacteria isolated from soil [10], freshwater [11, 12], drinking water [12] and hot spring water [13]. It is frequently reported as one of the dominant orders represented in the amphibian skin microbiome studies [7–9, 14, 15]. It comprises bacteria with high importance in the defence against pathogens and probiotic application, e.g. *Janthinobacterium lividum* (family *Oxalobacteraceae*) [14]. Currently, the *Burkholderiales* order (https://lpsn.dsmz.de/order/burkholderiales, accessed on 1 July 2024, and from the 'List of new names and new combinations previously effectively, but not validly, published' issues of this journal) includes the families *Alcaligenaceae*, *Burkholderiaceae*, *Sutterellaceae*, *Oxalobacteraceae*, *Comamonadaceae* and *Sphaerotilaceae*. A total of 10 genera have been recently reclassified within the family *Sphaerotilaceae* [16], and a total of 40 genera have been classified within the family *Comamonadaceae* (https://lpsn.dsmz.de/family/comamonadaceae, accessed on 1 July 2024). Most of these genera stain Gram-negative and are rod-shaped, and their major respiratory lipoquinones are ubiquinones 8 (UQ-8).

In previous studies, we assessed and characterized the microbiome diversity associated with the skin of the amphibian species *Pelophylax perezi* inhabiting reference freshwater, brackish water and metal-contaminated freshwater habitats [7, 9]. In this study, four bacterial strains from three sampling sites were isolated and characterized, and one new species and a new genus were described.

METHODS

Sampling and bacterial isolation

Bacterial strains belong to a collection of isolates sampled from the skin microbiome of adults of the Perez's frog, *Pelophylax perezi*. These strains were isolated during two studies that aimed to characterize the *Pelophylax perezi* skin's microbiome between populations and under different environmental conditions [7, 9].

The four isolates considered in this work belong to organisms collected at three sampling sites along the Portuguese territory (Fig. 1). Strain SL12-8^T was isolated from Salreu, a water system characterized by fluctuations in salinity, located in Ria de Aveiro. Isolates LB1-7 and LB13-6 were sampled from Lagoa das Braças, which is a freshwater coastal lagoon situated in Figueira da Foz, Coimbra district. Strain 20M2A was isolated at the lagoon located far away from villages or anthropogenic influences called Barragem de Reguengo located in the south of the country, situated in Messejana, near to Aljustrel, Beja district. Conductivity (µS.cm^{-1}), pH and dissolved oxygen (mg.l^{-1}) values were measured on site before water sampling using an LF 330/SET conductivity metre, a WTW330/SET-2 pH and OXI 330/SET dissolved oxygen metres, and physical and chemical characteristics of the sampling sites are given by [7, 9].

The animals were handled using nitrile gloves that were disinfected with 70% ethanol. Prior to collecting the cultivable microbiota from the skin, each frog was rinsed thoroughly with sterile distilled water on both the dorsal and ventral sides to ensure that only skin-associated microbes were collected rather than transient microbes associated with water and then swabbed along the entire body [3, 17]. Samples were then plated on R2A (Reasoner's 2A) agar (Difco Laboratories, Detroit, MI) and incubated at 22 °C for 5 days. After subculture and purification, strains were preserved at −80 °C in nutrient broth supplemented with 15% (v/v) glycerol. *Piscinibacter aquaticus* DSM 19915^T and *Aquabacterium parvum* DSM 11968^T were obtained from Leibniz-Institut DSMZ and used as a reference in all the tests.

Phylogenetic analyses based on 16S rRNA gene sequence

Genomic DNA was extracted using the extraction and purification E.Z.N.A. Bacterial DNA Extraction Kit (Omega Bio-Tek, Inc, USA). The 16S rRNA gene was amplified by PCR, using the universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) followed by sequencing [18]. The near-complete 16S rRNA gene of the strains was aligned with those of the type species most closely related corresponding to the order *Burkholderiales*, as described by Proença *et al.* [19]. Evolutionary distances were calculated [20], and phylogenetic dendrograms were reconstructed using the neighbour-joining [21] and maximum likelihood [22] algorithms included in the ARB software package [23] and MEGA software [24]. Tree topologies were evaluated by performing bootstrap analysis [25] of 1,000 datasets by using the ARB and MEGA software packages.

Phylogenomic and genomic analyses

Libraries of total genomic DNA were prepared using the Nextera XT Preparation Kit (Illumina, San Diego, CA, USA) following the manufacturer's instructions. The libraries were purified using HighPrep PCR Clean-up beads (MagBio Genomics, Inc.). Fragment Analyzer 5200 (Agilent NGS Fragment 1–6,000 pb methods) was used to check the fragment size distribution and

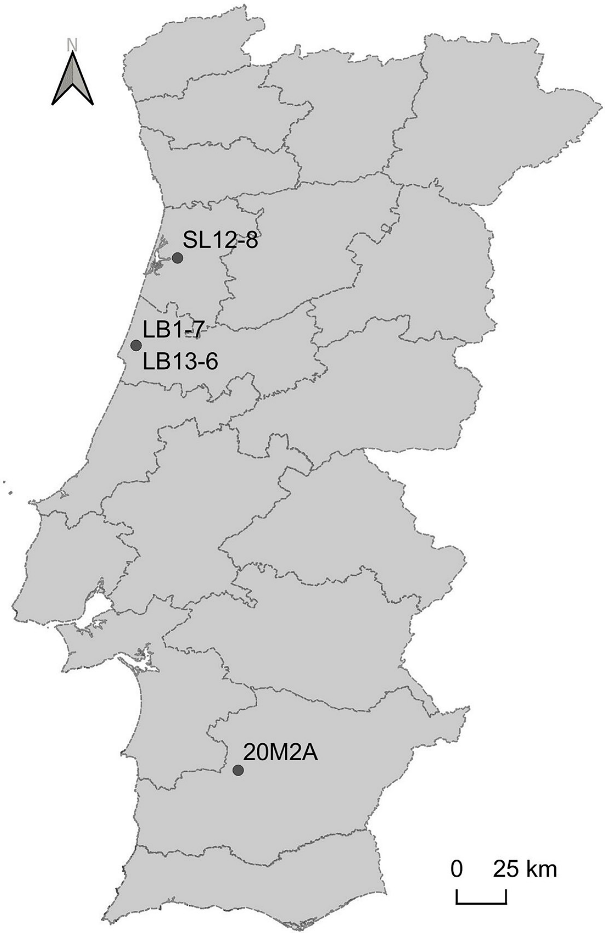

Fig. 1. Geographical location of the isolates described in this work. Strain SL12-8^T was isolated from sampling site Salreu (40° 43′ 57″ N/8° 34′ 20″ W); strains LB1-7 and LB13-6 were isolated from sampling site Lagoa das Braças (40° 14′ 32″ N/8° 48′ 17″ W); and strain 20M2A was isolated from sampling site Barragem de Reguengo (37° 51′ 38″ N/8° 13′ 56″ W).

molarity of each library. Nine picomolar libraries were sequenced on an Illumina MiSeq System with 2×300 bp chemistry (MiSeq Reagent Kit v3). Pairing, trimming and assembly based on Bruijn graphs were performed using CLC Genomics Workbench v9.5.4 (Qiagen) using default parameters.

The draft or complete genome sequences of the closest validly described species of the class *Betaproteobacteria* were downloaded from the NCBI database (https://www.ncbi.nlm.nih.gov/) to be included in this analysis. In total, 52 genomes of type species of the genera belonging to the families *Sphaerotilaceae* and *Comamonadaceae,* including strain SL12-8^T, and *Methylophilus methylotrophus* as root were used to analyse the core genome by using BPGA software [26], and a phylogenomic tree based on the core genome was performed by using the maximum likelihood algorithm included in MEGA software [24]. The genomes were subjected to the UBCG2 software and pipeline to generate phylogenetic trees from sequences of 81 concatenated core genes [27]. The genome sequence of strain SL12-8^T was also analysed by using the Type (Strain) Genome Server (TYGS), including all dependencies in the web tool, for additional phylogenomic analysis [28]. Genome distances were determined by calculating the average nucleotide identity (ANI) [29], average amino acid identity (AAI) [30] and digital DNA–DNA hybridization (dDDH) [31]. The G+C content of the genome was determined by HPLC as previously described [32] and based on the genome sequence of strain SL12-8^T.

Genome sequences were annotated using the Rapid Annotation using Subsystem Technology (RAST) server [33], National Center for Biotechnology Information (NCBI) Prokaryotic Genome Annotation Pipeline (PGAP) [34] and EggNOG tool v5.0 [35]. The genome of strain SL12-8^T was subjected to secondary metabolite gene cluster analysis by using the platform antiSMASH 3.0 [36]. Therefore, genome mining was performed for proteins that directly or their products have been recognized in amphibian-skin bacteria roles such as metabolic pathways involved in the production of indole-3-carboxaldehyde and violacein [37], indole-3-ethanol (tryptophol) [38], 2,4-diacetylphloroglucinol [39] and prodigiosin [6] by using NCBI

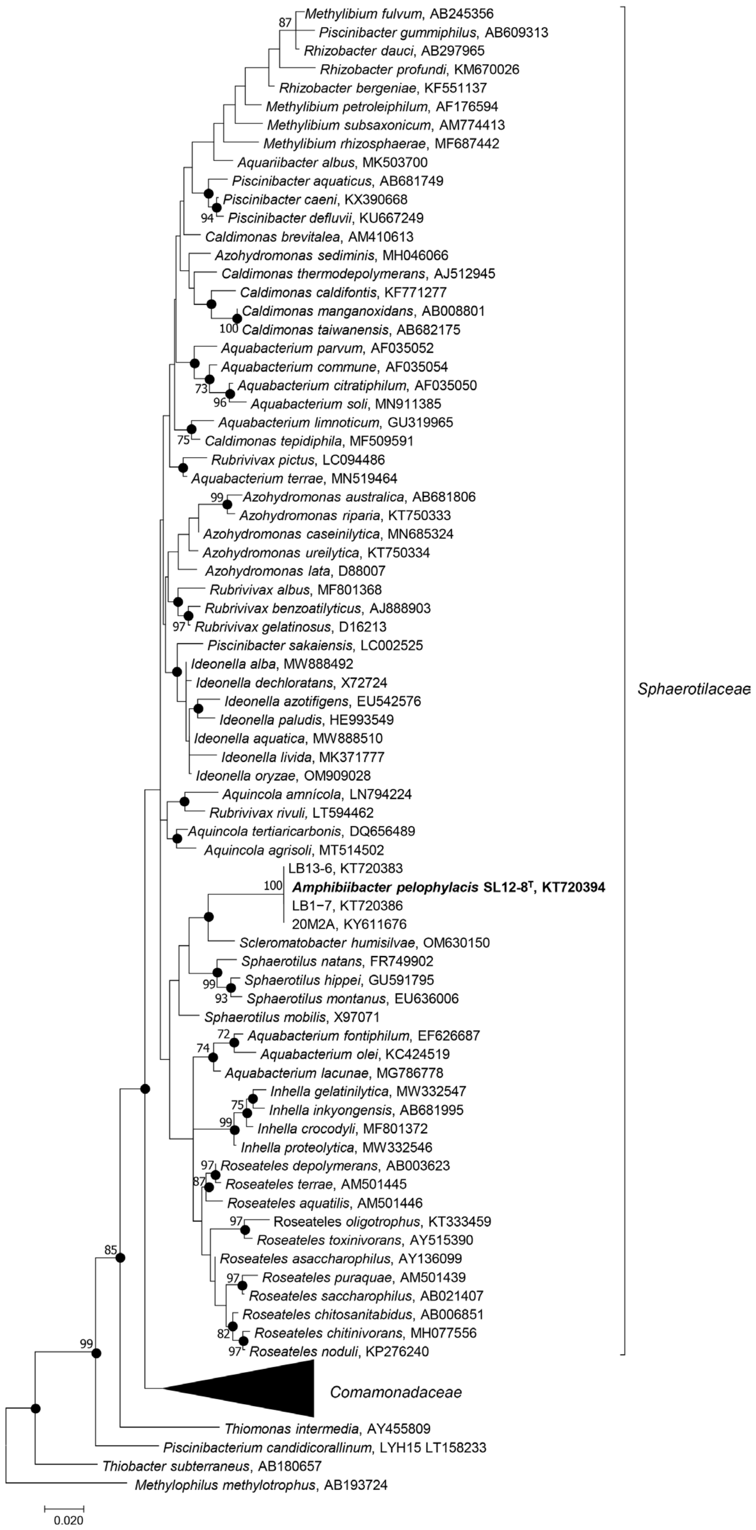

Fig. 2. Phylogenetic dendrogram based on a comparison of the 16S rRNA gene sequence of strain SL12-8^T and other *Betaproteobacteria* type strains of the family *Sphaerotilaceae*. The tree was created using the maximum likelihood method. The numbers on the tree indicate the percentages of bootstrap sampling, derived from 1,000 replications; values below 70% are not shown. The symbol () indicates node branches conserved when the tree was reconstructed using the neighbour-joining algorithm. The isolate characterized in this study is indicated in bold. Strain *Methylophilus methylotrophus* DSM46235^T, AB193724, was used as root. Scale bar, 2 inferred nucleotide substitutions per 100 nt.

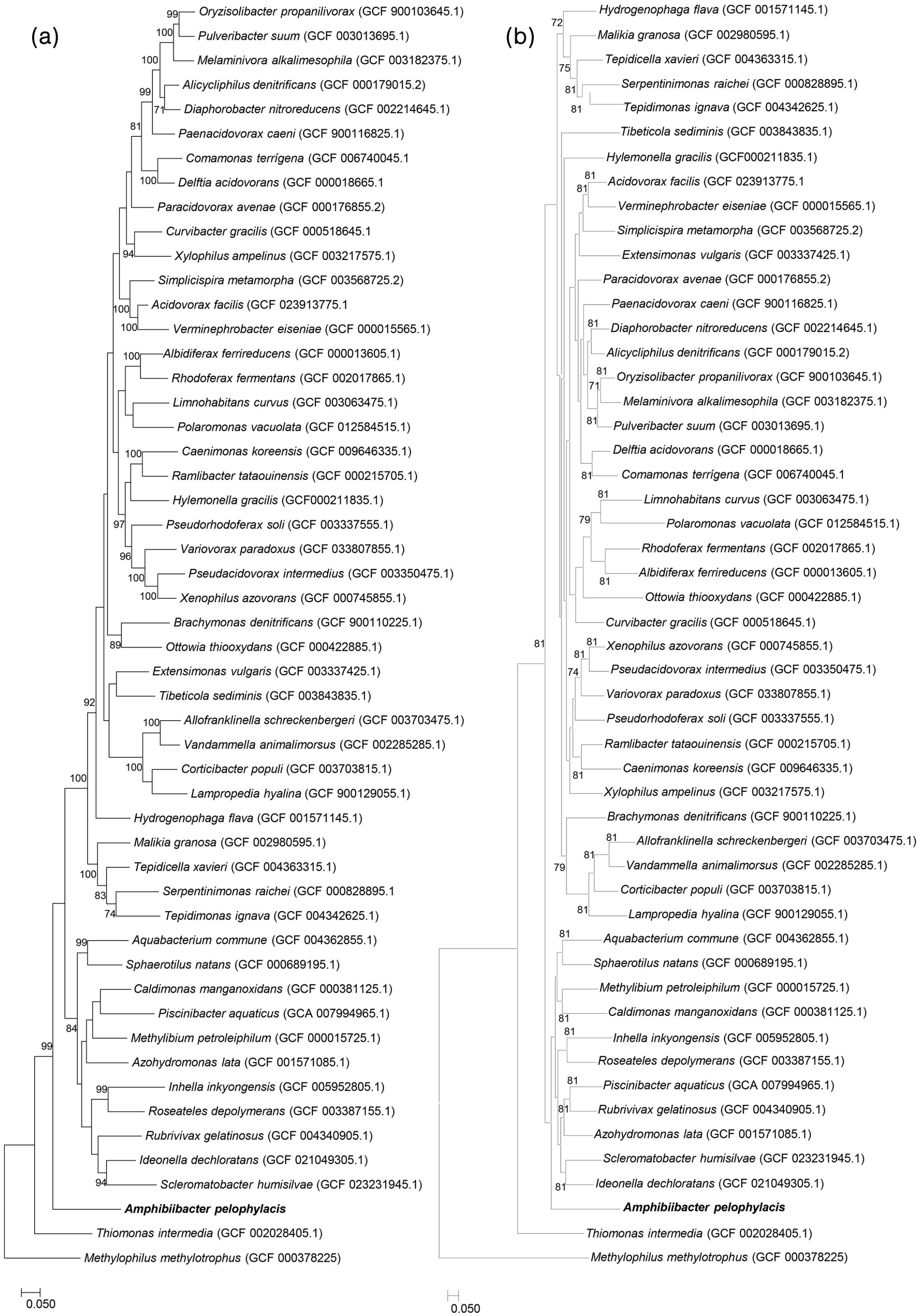

Fig. 3. The phylogenomic trees based on 124 concatenated core genes obtained by using BPGA (a) and based on 81 concatenated core genes obtained by using the UBCG2 pipeline (b).

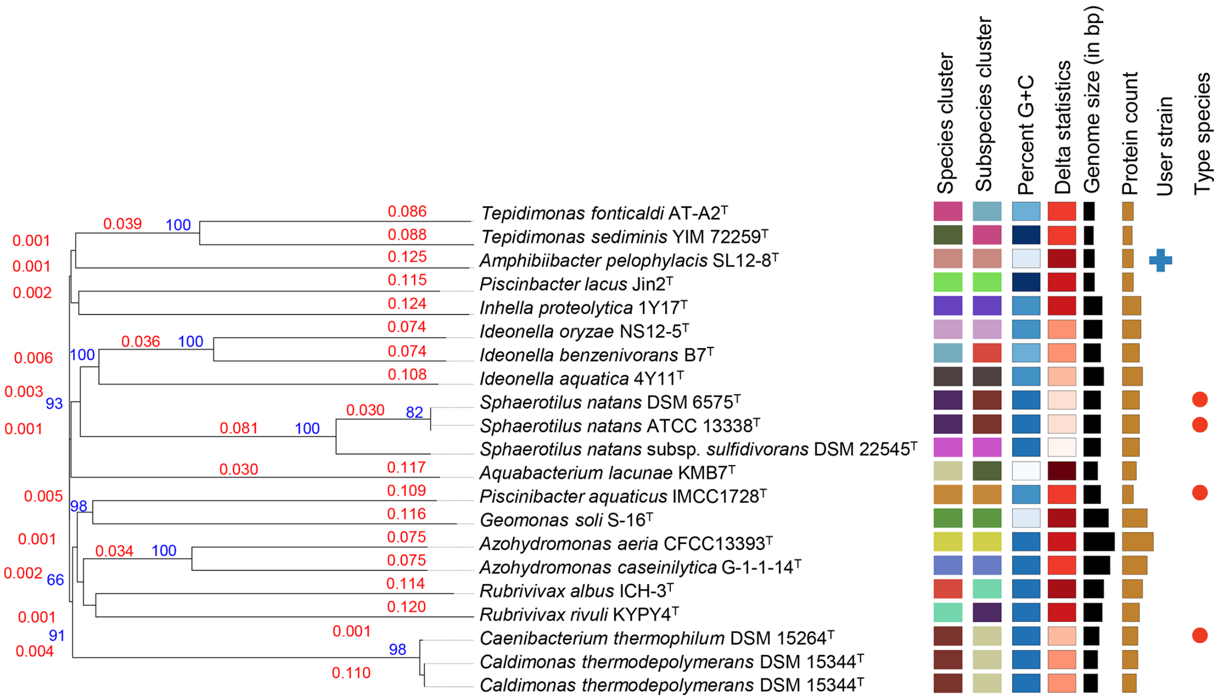

Fig. 4. The phylogenomic tree based on GBDP distances calculated from genome sequences through the TYGS server.

[40] and UniProtKB/Swiss-Prot and Uni-ProtKB/TrEMBL [41] databases, and Kyoto Encyclopedia of Genes and Genomes (KEGG) map included in RAST server. The draft genome of strain SL12-8^T was deposited at DDBJ/ENA/GenBank under the accession JAWDIE000000000. The version described in this paper is version JAWDIE010000000. NCBI BioProject accession number is PRJNA1018472.

Morphological, physiological and biochemical analyses

Cell morphology was evaluated by phase-contrast microscopy and scanning electron microscopy (SEM) after growing on R2A agar at 25 °C for 72 h. The presence of motility was observed under a microscope in a fresh broth culture.

Growth was tested on tryptic soy agar (TSA) (Difco, Laboratories, Detroit, USA), nutrient agar (NA) (Difco), R2A (Difco) and R2A without starch and supplemented with 1% Tween 80%, incubated at 25 °C for 5 days. The temperature range (18, 20, 25, 30, 37 and 45 °C) and optimum temperature for growth were assessed on R2A agar incubated up to 5 days. Salt tolerance was examined on R2A agar supplemented with sodium chloride (NaCl) within the interval 0 up to 3% (w/v) following increments of 0.5% at 25 °C for 5 days. To determine the optimal pH, cultures were tested in R2A with pH ranging from 5.5 to 8.5 (increments of 0.5 pH unit), adjusted by using 50 mM MES (pH 5–7), HEPES (pH 6–8), TAPS (pH 8–9) and CAPSO (3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid, pH 10) and incubated at 25 °C for 5 days.

Anaerobic growth was assessed on R2A agar (or modified media as referred to above for *Aquabacterium parvum* 11968^T), incubated in anaerobic chambers at 25 °C for 5 days (GENbox anaer; bioMérieux). Physiological properties and enzyme activities were assessed using API ZYM and API 20NE test strips (bioMérieux) at 37 °C and 25 °C, respectively, following the manufacturer's instructions. Oxidase activity was determined using 1% (*w/v*) tetra-methyl-p-phenylenediamine dihydrochloride. Catalase activity was assessed using 3% (*v/v*) hydrogen peroxide (H_2O_2).

Chemotaxonomic analyses

Cells were cultured on R2A (except for *Aquabacterium parvum* DSM 11968^T that was grown on modified R2A) for 48 h at 25 °C, harvested and lyophilized and then used for polar lipids and lipoquinones. Lipoquinones were extracted from freeze-dried cells, purified by TLC and then separated by HPLC to identify the type present in our strains [42]. Cells for fatty acid analysis were grown on R2A agar (except *Aquabacterium parvum* 11968^T) at 25 °C in sealed plastic plates for 48 h [18], and the fatty acid profiles were determined. Fatty acid methyl esters were obtained from the fresh wet biomass and were

Table 1. Summary of genetic characteristics of SL12-8^{T} and its closest strains

Analyses of ANI and AAI by using the available sequences from NCBI through *EzBioCloud and †dDDH values through TYGS online tools.

Strain	RefSeq assembly accession	Contig	Total length (bp)	Protein-coding gene (CDS)*	G+C content (mol%)*	dDDH†	OrthoANIu value (%)*	AAI (%)*
***Amphibiibacter pelophylacis* SL12-8^{T}**	–	53	3,115,197	2,278	66.24	–	100	100
Piscinibacter aquaticus NBRC 102349	GCA_007994965.1	3	4,565,648	2,624	69.37	19.4	73.52	63.12
Rubrivivax albus ICH-3	GCF_004016515.1	74	5,310,361	4,892	70.27	19.1	73.42	62.89
I. benzenivorans B7	GCF_020387415.1	21	4,490,200	4,061	68.8	19.5	73.80	63.31
S. humisilvae BS-T2-15	GCF_023231945.1	28	6,273,566	5,712	69.56	19.7	72.87	61.74
Aquincola rivuli KYPY4	GCF_004016505.1	31	4,929,098	4,252	70.05	19.3	73.11	62.94
Aquincola tertiaricarbonis L10	GCF_023573145.1	4	7,110,107	6,400	69.31	19.7	73.69	62.83
I. dechloratans CCUG 30977	GCF_021049305.1	2	4,544,016	4,131	69.23	20.1	73.94	63.31
Aquabacterium parvum B6	GCF_001447195.1	619	4,592,999	4,036	65.29	19.4	72.64	62.05
Rubrivivax gelatinosus ATCC 17011	GCF_004340905.1	36	5,075,359	4,703	71.44	19.5	73.51	62.81
Roseateles chitosanitabidus NBRC 102408	GCF_001598255.1	56	5,819,413	5,052	69.94	19.8	73.26	62.32
Caldimonas caldifontis BCRC 80649	GCF_002930615.1	33	3,643,199	3,404	67.68	19.4	73.06	62.97

separated, identified and quantified using the standard MIS Library Generation Software (Sherlock Microbial ID System, RTSBA 6 database, version 6.5) as previously described [43].

RESULTS AND DISCUSSION

Phylogenetics: 16S rRNA gene sequence-based phylogeny

Phylogenetic analyses of the 16S rRNA gene sequence of strain SL12-8^{T} (1,412 bp) with those of the type species of all recognized species of all the genera of the order *Burkholderiales* indicated that this organism belonged to a novel genus and species. According to the maximum likelihood and neighbour-joining phylogenetic trees, the closest relatives to strain SL12-8^{T} were *Rubrivivax albus* (94.41%), *Scleromatobacter humisilvae* (94.08%), *Piscinibacter aquaticus* (93.72%), *Azohydromonas caseinilytica* (93.72%), *Aquincola agrisoli* (93.64%), *Ideonella aquatica* (93.59%), *Aquincola rivuli* (93.56%) and *Aquabacterium parvum* (93.50%) (Fig. 2).

Phylogenomic analyses

The pangenome analysis of all the genomes available related to the closest type species of the families *Sphaerotilaceae* and *Comamonadaceae* showed that strain SL12-8^{T} has 1,596 accessory genes, 1,061 unique genes and 124 core genes (core genome). The phylogenomic trees based on 124 concatenated core genes obtained by using BPGA (Fig. 3a), based on 81 concatenated core genes obtained by using the UBCG2 pipeline (Fig. 3b) and based on Genome BLAST Distance Phylogeny (GBDP) calculated from genome sequences through the TYGS server (Fig. 4) showed that strain SL12-8^{T} was placed in a monophyletic clade (Fig. 3) and within the cluster comprising *Tepidimonas* spp., *Piscinibacter lacus* and *Inhella proteolytica* (Fig. 4), supporting the proposal of new genera and new species represented by strain SL12-8^{T}.

The analyses of ANI, AAI and dDDH between strain SL12-8^{T} and closest relatives by using the available sequences from NCBI through EzBioCloud and TYGS online tools are summarized in Table 1. In all of these *in silico* analyses, the closest member of SL12-8^{T} was *Ideonella dechloratans* CCUG 30977^{T} (73.9%, 63.3% and 20.1%, respectively), followed by *Ideonella benzenivorans* B7^{T}, *Aquincola tertiaricarbonis* L10^{T}, *Piscinibacter aquaticus* NBRC 102349^{T} and *Caldimonas caldifontis* YIM 78140^{T}, with some differences in closest ranking for each analysis, with values lower than the cutoff defined to establish a new species [44].

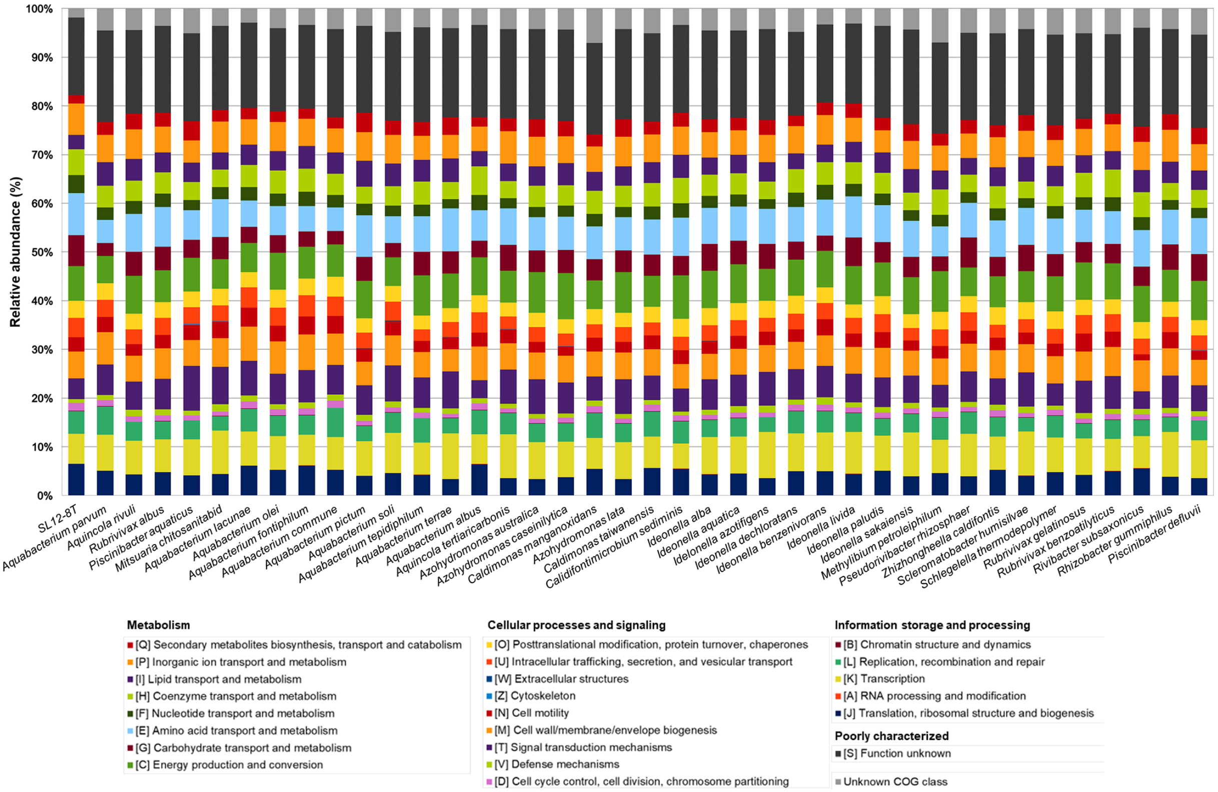

Fig. 5. Distribution and comparison of COG functional categories in the analysed genomes. Relative abundances (%) of the 22 different COG functional categories in each genome according to broad functional groups.

Genomic annotations and comparisons

The draft genome sequence of SL12-8^T was assembled into 53 contigs with more than 500 bp, totalling 3,115,197 bp, with a mapped coverage of 313-fold of the genome. The G+C content of the DNA was 66.2 mol% (comparatively with 66.0 mol% determined by HPLC). The genome encoded a total of 2,865 genes and 2,781 putative coding sequences (CDSs) (with protein). The draft genome sequence contained loci for 3 rRNAs, 44 tRNAs, 4 ncRNAs and 33 pseudogenes.

The Clusters of Orthologous Group (COG) annotation of the SL12-8^T genome showed that 96.6% of the CDS were matched to putative proteins with known functions and assigned to 22 of the COG categories (Fig. 5). The most abundant COG broad functional category was 'Metabolism' with 42.3% of the genes involved in this function. Among this COG category, the '[E] amino acid transport and metabolism' was the most predominant category (8.6%), followed by '[C] energy production and conversion' (7.1%) and '[P] inorganic ion transport and metabolism' (6.5%) which were also well-represented functional

Table 2. Gene abundance of metabolic pathways of strains through KEGG metabolic pathways in RAST server

1, *Amphibiibacter pelophylacis* SL12-8^T; 2, *Piscinibacter aquaticus* NBRC 102349^T; 3, *I. dechloratans* CCUG 30977^T; 4, *I. benzenivorans* B7^T; 5, *Rubrivivax albus* ICH-3^T.

Metabolic pathways	Distinct EC (Enzyme Commission number)	1	2	3	4	5
Benzoate degradation via hydroxylation	50	9 (18.0%)	8 (16.0%)	7 (14.0%)	10 (20.0%)	14 (28.0%)
Glycolysis/gluconeogenesis	41	17 (41.5%)	22 (53.7%)	19 (46.3%)	19 (46.3%)	21 (51.2%)
Nicotinate and nicotinamide metabolism	47	11 (23.4%)	11 (23.4%)	11 (23.4%)	11 (23.4%)	13 (27.7%)
Phenylalanine, tyrosine and tryptophan biosynthesis	31	21 (67.7%)	20 (64.5%)	20 (64.5%)	21 (67.7%)	20 (64.5%)
Tryptophan metabolism	67	9 (13.4%)	14 (20.9%)	12 (17.9%)	9 (13.4%)	13 (19.4%)

categories (Fig. 5). The other two COG broad functional categories were 'Cellular processes and signalling' and 'Information storage and processing' representing 22.6% and 17.4%, respectively (Fig. 5).

Additionally, 15.9% of the coding sequences in the SL12-8^{T} genome are classified as '[S] function unknown'.

The genome of SL12-8^{T} did not contain proteins that directly or by their products have been implicated in the roles of frog's skin bacteria such as metabolic pathways involved in the production of indole-3-carboxaldehyde and violacein, indole-3-ethanol (tryptophol), 2,4-diacetylphloroglucinol and prodigiosin. The metabolic potential of the genomes under study was also investigated by functional annotation and pathway mapping in the KEGG map metabolic pathways included in the RAST server. In all strains analysed, the phenylalanine, tyrosine and tryptophan biosynthesis pathway were the most relevant, and the strain SL12-8^{T} had 67.7% of the proteins present in this metabolic pathway, followed by glycolysis/gluconeogenesis (41.5%), nicotinate and nicotinamide metabolism (23.4%), benzoate degradation via hydroxylation (18.8%) and tryptophan metabolism (13.4%) (Table 2).

By using the web tool antiSMASH, it was possible to identify the gene clusters for secondary metabolites in the strain SL12-8^{T} and its closest strains (Table 3). Gene clusters involved beta-lactone containing a protease inhibitor, more specifically fengycin synthesis (13% of genes show similarity). Fengycin is a cyclic lipopeptide that directly attacks fungi by binding to their cell membrane, causing leakage and lysis [45]. Gene clusters involved in fengycin synthesis were also identified on *I. dechloratans* and *I. benzenivorans* genomes. Terpene-type clusters are present in all strains analysed, including SL12-8^{T}, and these broad classes of molecules are structurally and functionally diverse and include antimicrobial compounds towards some bacteria, including possible functions against amphibian pathogens [46].

Phenotypic and chemotaxonomic characteristics

Colonies of the strains SL12-8^{T}, LB13-6, LB1-7 and 20M2A were circular and white-beige. The cells of the strain SL12-8^{T} were rod-shaped (Fig. 6), Gram-stain-negative as well as the most closely related taxa, *Piscinibacter aquaticus* DSM 19915^{T} and *Aquabacterium parvum* DSM 11968^{T}, were tested in this study (Table 4). All the cells tested in this study are anaerobic facultative. The range of optimum temperatures, pH and NaCl concentrations for the growth of the four strains were similar to those of reference type strains. For example, the temperature ranges between 14 and 42 °C (optimum around 20–30 °C), pH preferentially 7 (ranging from 5 to 11) and NaCl range 0–5.0% (optimum 0–1.5%). Multiple physiological and biochemical characteristics such as a positive reaction for urease and the enzyme activity of alkaline phosphatase, β-galactosidase, α-glucosidase and β-glucosidase were observed. The profile of polar lipids of strain SL12-8^{T} consisted of three aminophospholipids, five phospholipids and one aminolipid. No glycolipids were detected (Fig. S1, available in the online Supplementary Material). The UQ-8 was the respiratory quinone identified on strains studied and in the most closely related taxa, *Piscinibacter aquaticus* DSM 19915^{T} and *Aquabacterium parvum* DSM 11968^{T}. The G+C content of the DNA of the strains was also determined by HPLC and ranges from 66.0 to 66.4 mol%, values within the range for species belonging to the closely related members of the order.

The fatty acid profiles of strain SL12-8^{T} and related strains are shown in Table 5. The major fatty acids identified among all the strains were summed feature 3 ($C_{16:1}$ ω7c and/or $C_{16:1}$ ω6c), summed feature 8 ($C_{18:1}$ *ω7*c and/or $C_{18:1}$ *ω6*c) and $C_{16:0}$ which were identified as the major fatty acids (>10%), except *Aquabacterium parvum* DSM 11968^{T} and *I. aquatica* JCM 34285^{T} for summed feature 8. However, the proportions of several fatty acids varied greatly in some strains, such as $C_{16:1}$ *ω9*c, $C_{18:1}$ *ω9c*, summed feature 5 ($C_{18:0}$ ante and/or $C_{18:2}$ *ω6,9c*) and summed feature 8 ($C_{18:1}$ *ω7*c and/or $C_{18:1}$ *ω6*c) in *Aquabacterium parvum* DSM 11968^{T} or $C_{10:0}$ 3-OH and feature 8 ($C_{18:1}$ ω7c and/or $C_{18:1}$ ω6c) in *I. aquatica* JCM 34285^{T}, among others (Table 5).

The phenotypic characterization, enzymatic activities, biochemical and physiological characteristics, fatty acid profile and phylogenetic, phylogenomic and genomic evidence indicate that strain SL12-8^{T} represents a novel species of a new genus, of the family *Sphaerotilaceae*, order *Burkholderiales* and class *Betaproteobacteria*, for which we propose the name *Amphibiibacter pelophylacis* gen. nov., sp. nov.

DESCRIPTION OF *AMPHIBIIBACTER* GEN. NOV.

Amphibiibacter (Am.phi.bi.i.bac'ter. N.L. neut. pl. n. Amphibia, a zoological class; N.L. masc. n. *bacter*, a small rod from amphibian; N.L. masc. n. *Amphibiibacter*, a rod from the amphibian).

According to 16S rRNA gene sequence analysis, it belongs to the class *Betaproteobacteria*. Cells are Gram-stain-negative, non-motile, facultatively aerobic rods. Predominant cellular fatty acids are $C_{16:0}$ summed features 3 and 8. Ubiquinone 8 is the respiratory quinone. The G+C% molar content of the DNA of the known strain of the type species is 66.2 mol%. The type species is *Amphibiibacter pelophylacis*.

Table 3. Secondary metabolite clusters identified in strain SL12-8^{T} and its closest strains with the antiSMASH web tool.

Strain	Region	Type	Start	Stop	Most similar known cluster		Similarity (%)
***Amphibiibacter pelophylacis* SL12-8^{T}**	Region 1.1	RiPP-like	23,491	245,795			
	Region 5.1	Terpene	37,589	59,404			
	Region 11.1	Beta-lactone	85,914	108,642	Fengycin	NRP	13
***Piscinibacter aquaticus* NBRC 102349^{T}**	Region 1.1	Ranthipeptide	1,176,164	1,197,557			
	Region 1.2	Terpene	1,503,222	1,523,524			
	Region 1.3	NRPS-like, NRPS, T1PKS	1,842,892	1,930,059	BE-43547A1 BE-43547A2 BE-43547B1 BE-43547B2 BE-43547B3 BE-43547C1 BE-43547C2	NRP: Cyclic depsipeptide+Polyketide: Modular type I polyketide	30
	Region 1.4	Terpene	2,462,934	2,483,968	Carotenoid	Terpene	100
	Region 1.5	NRPS-like, T1PKS	2,544,338	2,596,241	Tubulysin A	NRP+Polyketide	9
	Region 1.6	RiPP-like	2,777,852	2,788,694			
***Rubrivivax albus* ICH-3^{T}**	Region 2.1	acyl_amino_ acids	22,198	74,124			
	Region 11.1	acyl_amino_ acids	177,897	238,685			
	Region 11.2	Terpene	966,396	988,092			
	Region 11.3	hglE-KS, T1PKS	1,238,708	1,291,774			
	Region 30.1	acyl_amino_ acids	85,500	146,816	TP-1161	RiPP: Thiopeptide	25
	Region 31.1	Terpene	41,222	62,328	Carotenoid	Terpene	100
	Region 31.2	NAGGN	249,896	264,564			
	Region 32.1	Lasso peptide	311,922	334,529	Rubrivinodin	RiPP: Lasso peptide	80
	Region 34.1	RiPP-like	59,438	69,369			
	Region 34.2	RiPP-like	193,892	204,749			
***I. dechloratans* CCUG 30977^{T}**	Region 1.1	RiPP-like	1,277,534	1,287,327			
	Region 1.2	Beta-lactone	1,350,680	1,378,015	Fengycin	NRP	13
	Region 1.3	hglE-KS, T1PKS	1,461,073	1,514,228			
	Region 1.4	RiPP-like	1,710,502	1,722,217			
	Region 1.5	Terpene	2,411,633	2,433,320			
	Region 1.6	Redox cofactor	3,278,323	3,300,610			
***I. benzenivorans* B7^{T}**	Region 9.1	Arylpolyene	39,566	80,759	TVA-YJ-2	RiPP	4
	Region 9.2	RiPP-like	99,916	110,815			
	Region 9.3	Redox cofactor	708,804	731,072			
	Region 13.1	Terpene	235,308	256,986			
	Region 13.2	Beta-lactone	36,103	38,814	Fengycin	NRP	13
	Region 16.1	RiPP-like	10,771	119,398			

Continued

Table 3. Continued

Strain	Region	Type	Start	Stop	Most similar known cluster	Similarity (%)
	Region 20.1	hglE-KS, T1PKS	26,128	79,253		

acyl_amino_acids, *N*-acyl amino acid; hglE-KS, heterocyst glycolipid synthase-like PKS; NAGGN, *N*-acetylglutaminylglutamine amide; NRPS, non-ribosomal peptide synthetase; NRPS-like, NRPS-like fragment; RiPP-like, other unspecified ribosomally synthesized and post-translationally modified peptide product (RiPP); T1PKS, type I PKS (polyketide synthase).

DESCRIPTION OF *AMPHIBIIBACTER PELOPHYLACIS* SP. NOV.

Amphibiibacter pelophylacis (pe.lo.phy.la'cis. N.L. gen. n. *pelophylacis*, of *Pelophylax perezi*, Perez's frog) was isolated as part of the skin microbiota of the anuran species *Pelophylax perezi*.

Cells are Gram-staining-negative, non-motile, rods, 1–1.4 µm after 48 h incubation on R2A agar, non-spore-forming and facultative aerobic. Colonies on R2A agar are small, smooth and circular with regular and light white-beige. Cells grow on R2A, tryptic soy and nutrient agars. Growth occurs at 18–30 °C (optimum, 25 °C), at pH 6.0–8.5 (optimum, pH 7.0–7.5) and on R2A agar supplemented with 0–1.5% (w/v) NaCl (optimum, <1.5%). Catalase-positive and oxidase-negative. Negative for indole production, glucose fermentation and arginine dihydrolase (API 20NE). Positive for alkaline phosphatase, esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, *β*-galactosidase, *α*-glucosidase and *β*-glucosidase and weakly positive for esterase (C4) and *α*-galactosidase. Negative for lipase (C14), cystine arylamidase, trypsin, *α*-chymotrypsin, *β*-glucuronidase, *N*-acetyl-*β*-glucosaminidase, *α*-mannosidase and *α*-fucosidase (API ZYM). Positive for nitrate reduction, urease, aesculin hydrolysis and *β*-galactosidase, weakly gelatin hydrolysis (API 20NE). Positively assimilates the following carbon sources (API 20NE):*α*-D-glucose, D-mannose, D-mannitol and weakly malic acid. The other organic substrates included in API 20NE are not assimilated. The major fatty acids (>25%) are summed in feature 3 (contains $C_{16:1}$ *ω7*c and/or $C_{16:1}$ *ω6*c) and summed in feature 8 (consisting of $C_{18:1}$ ω7c or $C_{18:1}$ ω6c). Significant amounts (≥5%) of $C_{16:0}$ are also present. The respiratory lipoquinone is UQ-8. The

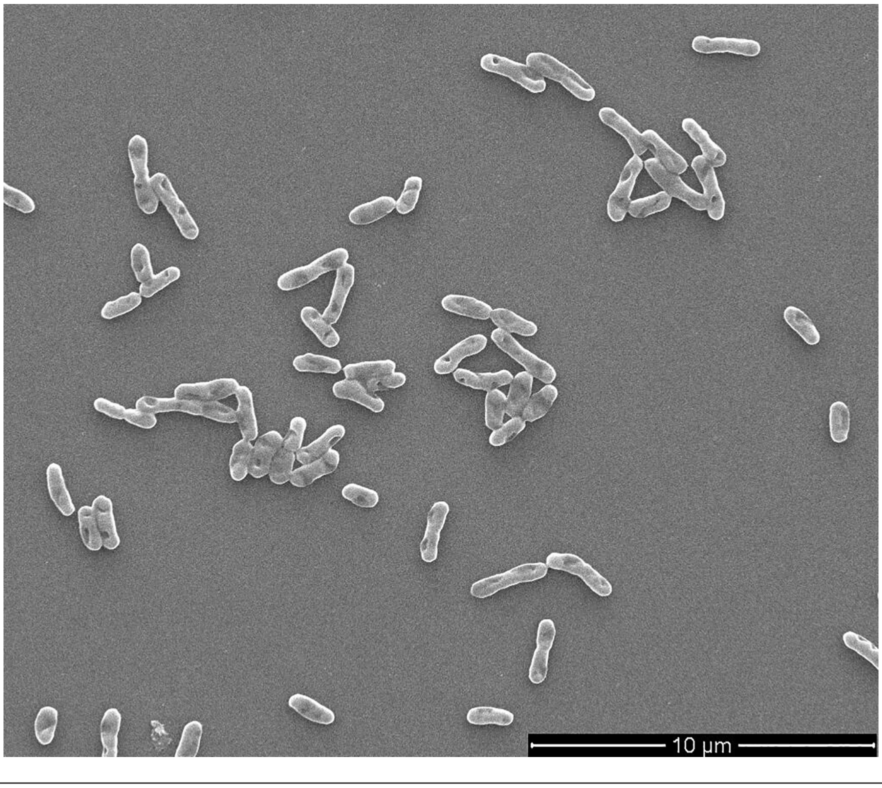

Fig. 6. SEM micrograph of strain SL12-8^T. Mag 10,000×, HV 15.00 kV, det ETD, mode SE, WD 10.4 mm. The scale bar represents 10 µm.

Table 4. Characteristics of strain SL12-8^T and the type strains of related species within the family *Sphaerotilaceae*

Strains: 1, SL12-8^T; 2, LB13-6; 3, LB1-7; 4, 20M2B; 5, *Aquabacterium parvum* DSM 11968^T; 6, *Piscinibacter aquaticus* DSM 19915^T; 7, *Rubrivivax albus* LMG 30930^T; 8, *Azohydromonas caseinilytica* NBRC 114390^T; 9, *I. aquatica* JCM 34285^T; 10, *I. dechloratans* CCUG 30977^T. All data were obtained from this study except those for *Rubrivivax albus* LMG 30930^T [47]; *Azohydromonas caseinilytica* NBRC 114390^T [48]; *I. aquatica* JCM 34285^T [49]; *I. dechloratans* CCUG 30977^T [49]. None of the strains possessed *β*-glucuronidase, *N*-acetyl-b-glucosaminidase, α-mannosidase, α-fucosidase activity, L-tryptophan, D-glucose, L-arginine di-hydrolase and phenylacetic acid. All strains showed positive activity for D-glucose, alkaline phosphatase, esterase (C4), esterase lipase (C8) and leucine arylamidase; +, Positive; w, weakly positive; –, negative. FA, facultatively anaerobic; A, aerobic; ND, no data.

Characteristic	1	2	3	4	5	6	7	8	9	10
Isolation source	Amphibian skin	Amphibian skin	Amphibian skin	Amphibian skin	Drinking water†	Freshwater pond‡	Freshwater pond	Forest soil	Aquaculture water	Sludge
Cell shape	Rod	Rod	Rod	Rod	Rod	Oblong rods	Rod	Rod		Curved rods
Cell (µm)	0.5×1–10.4	ND	ND	ND	0.5×1–2	0.4–0.7×0.8–0.9	0.4–0.8×1.2–2.0	1.2–1.7×2.6–2.9	0.6–0.8×1.3–1.6	0.7–1.0×2.5–5.0
Motility	Non-motile	Non-motile	Non-motile	Non-motile	Motile	Non-motile	Non-motile	Motile	Motile	Motile
Colony	Beige	Beige	Beige	Beige	White centre transparent edges	Beige-milkish	White	White	White	White
Temperature range (optimum) °C	18–30 (25)	18–37 (25)	18–30 (25)	18–37 (25)	14–34 (20)	15–42 (30)	25–37 (30)	15–45 (25–35)	10–40 (28-30)	15–35 (30)
pH range	6–8.5	6–8.5	6–8.5	6–8.5	6.5–10.0	6–10.0	6–8.5	5.0–11.0	5.0–8.0	6.0–8.0
Optimum pH	7–7.5	7–7.5	7–7.5	7–7.5	6.5–10.0	7	7.5–8.0	6.0–8.0	7.0	6.0–7.0
NaCl range	0–1.5%	0–1.5%	0–2.0%	0–2.0%	0–1.0%	0–1.0%	0–4.0%	0–4.0%	0–5.0%	ND
NaCl (optimum)	0–1.5%	0–1.5%	0–1.5%	0–1.5%	up to 0.8%	0–1.0%	0%	0%	0%	ND
Media	R2A/TSA/NA	R2A/ TSA	R2A/ TSA	R2A/TSA/NA	R2A replaced starch 0.1% to Tween 80	R2A	R2A/LB/TSA	R2A, TSA, veal infusion	R2A	ND
Relation to O_2	FA	FA	FA	FA	FA	FA	A	A	FA	FA
G+C DNA (mol%)	66.2* | 66.0	66.2	66.3	66.4	65.0	66.2	70.3	69.9	69.7	68.1
API 20NE										
NO_3	+	+	+	+	–	–	+	ND	+	–
N_2	+	+	+	w	–	–	+	ND	+	–
Urease	+	+	+	+	–	–	–	ND	–	–
Aesculin hydrolysis	+	+	+	+	–	–	–	ND	+	–
Gelatin hydrolysis	w	w	w	w	w	w	–	ND	+	+
β-Galactosidase	+	+	+	+	–	–	–	ND	+	–
Assimilation										

Continued

Table 4. Continued

Characteristic	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
L-Arabinose	–	–	–	–	–	–	–	–	+	–
D-Mannose	+	+	+	+	–	w	+	ND	+	–
D-Mannitol	+	+	+	+	w	–	+	–	+	–
N-Acetyl-glucosamine	–	–	–	–	w	–	–	+	+	–
Maltose	+	+	+	+	w	+	–	–	w	+
Potassium gluconate	–	–	+	+	w	–	+	+	w	–
Capric acid	–	–	–	–	–	–	–	ND	–	+
Adipic acid	–	–	–	w	w	–	–	ND	–	
Malic acid	w	–	–	w	w	–	–	–	+	+
Trisodium citrate	–	–	–	–	w	–	–	–	+	–
API ZYM										
Lipase (C14)	–	–	–	–	–	–	+	–	–	ND
Valine arylamidase	+	–	+	–	w^{+}	w^{+}	–	+	+	ND
Cystine arylamidase	–	–	–	–	–	–	–	+	+	+
Trypsin	–	–	–	–	–	+	–	+	–	–
α-Chymotrypsin	–	w^{+}	+	w^{+}	–	+	+	+	+	–
Acid phosphatase	++	+	++	++	w^{+}	–	+	ND	+	–
Naphthol-AS-BI-phosphohydrolase	++	++	++	++	w^{+}	+	+	w^{+}	+	–
α-Galactosidase	w^{+}	–	w^{+}	–	–	–	–	+	–	–
β-Galactosidase	+	w^{+}	+	w^{+}	–	–	–	–	–	–
β-Glucuronidase	–	–	–	–	–	–	–	–	–	–
α-Glucosidase	++	++	++	++	–	–	+	–	+	–
β-Glucosidase	++	++	++	++	–	–	–	+	+	–

*Data obtained from genome information.
†Data from [12].
‡Data from [50].

Table 5. Cellular fatty acid profiles of four novel strains and the reference type strains

Strains: 1, SL12-8^{T}; 2, LB13-6; 3, LB1-7; 4, 20M2B; 5, *Aquabacterium parvum* DSM 11968^{T}; 6, *Piscinibacter aquaticus* DSM 19915^{T}; 7, *Rubrivivax albus* LMG 30930^{T}; 8, *Azohydromonas caseinilytica* NBRC 114390^{T}; 9, *I. aquatica* JCM 34285^{T}; 10, *I. dechloratans* CCUG 30977^{T}. All data were obtained from this study except those for *Rubrivivax albus* LMG 30930^{T} [47], *Azohydromonas caseinilytica* NBRC 114390^{T} [48], *I. aquatica* JCM 34285^{T} [49], *I. dechloratans* CCUG 30977^{T} [49]. The major cellular fatty acids are in bold. TR, trace amount (fatty acids amounting to <1% and > 0.45%); –, not detected (or fatty acids amounting to <0.45%).

Fatty acid	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
$C_{9:0}$	–	–	–	–	–	–	–	–	1.10	TR
$C_{10:0}$	–	TR	TR	TR	–	–	–	–	TR	–
$C_{12:0}$	4.62±0.54	4.64±0.42	5.82±0.05	4.32±0.62	4.09±0.04	5.63±0.04	4.50	1.90	10.3	3.20
$C_{13:0}$	–	–	–	–	–	–	–	–	TR	–
$C_{14:0}$	2.32±0.14	2.40±0.05	2.72±0.07	2.43±0.13	TR	TR	TR	4.10	2.10	2.20
$C_{16:0}$	**15.6±0.50**	**15.3±1.15**	**13.9±0.76**	**14.3±1.27**	**22.7±0.21**	**17.9±0.43**	**22.6**	**26.1**	**18.0**	**23.9**
$C_{17:0}$	–	–	–	–	–	1.64±0.15	5.60	TR	2.00	TR
$C_{18:0}$	TR	TR	TR	TR	TR	TR	–	TR	1.60	TR
$C_{8:0}$ 3-OH	–	–	–	–	–	TR	–	–	TR	–
$C_{10:0}$ 3-OH	4.88±0.5	5.04±0.57	6.50±0.15	4.36±0.62	6.53±0.15	3.32±0.00	3.20	4.60	**10.9**	2.10
$C_{12:0}$ 2-OH	–	–	–	–	2.59±0.18	–	–	TR	–	1.20
$C_{12:0}$ 3-OH	–	–	–	–	–	–	–	–	–	3.50
$C_{14:0}$ 2-OH	–	–	–	–	–	–	–	–	–	1.80
$C_{15:0}$ 3-OH	–	–	–	–	–	–	–	TR	–	–
$C_{16:0}$ N alcohol	–	–	–	–	–	–	–	–	TR	–
$C_{14:1}$ $\omega 5c$	TR	TR	TR	TR	–	TR	–	–	TR	–
$C_{15:1}$ $\omega 6c$	–	–	–	–	–	1.36±0.13	3.90	–	1.40	TR
$C_{16:1}$ $\omega 5c$	–	–	–	–	–	–	–	–	TR	TR
$C_{16:1}$ $\omega 7c$ alcohol	–	–	–	–	–	–	–	–	TR	–
$C_{16:1}$ $\omega 9c$	–	–	–	–	**11.8±0.19**	–	–	–	–	–
$C_{17:1}$ $\omega 6c$	–	–	–	TR	–	1.26±0.17	–	–	TR	TR
$C_{17:1}$ $\omega 8c$	–	–	–	–	TR	–	–	–	TR	TR
cyclo-$C_{17:0}$	–	–	–	–	–	–	9.30	**12.7**	–	TR
$C_{18:1}$ $\omega 5c$	TR	TR	TR	TR	–	TR	–	–	–	–
$C_{18:1}$ $\omega 7c$ 11-methyl	TR	TR	TR	TR	–	–	–	–	–	TR
$C_{18:1}\omega 7c$	–	–	–	–	–	–	**13.5**	–	–	–
$C_{18:1}$ $\omega 9c$	–	–	–	–	**16.5±1.39**	–	–	–	–	–
$C_{18:3}$ $\omega 6c$ (6,9,12)	–	–	–	–	–	–	–	–	TR	–
$C_{18:1}$ $\omega 5c$	TR	TR	TR	TR	–	TR	–	–	–	–
$C_{18:1}$ $\omega 7c$ 11-methyl	TR	TR	TR	TR	–	–	–	–	–	TR
anteiso-$C_{16:0}$	–	–	–	–	–	–	–	–	TR	TR
anteiso-$C_{17:0}$	–	–	–	–	–	–	–	–	TR	TR
anteiso-$C_{17:1}$ $\omega 9c$	–	–	–	–	–	–	–	–	TR	TR

Continued

Table 5. Continued

Fatty acid	1	2	3	4	5	6	7	8	9	10
iso-$C_{10:0}$	–	–	–	–	–	–	–	–	TR	TR
iso-$C_{16:0}$	–	–	–	–	–	–	–	TR	–	TR
iso-$C_{17:1}$ $\omega 5c$	–	–	–	–	–	–	–	–	TR	–
iso-$C_{17:1}$ $\omega 10c$	–	–	–	–	–	–	–	–	TR	–
iso-$C_{17:0}$ 3-OH	–	–	–	–	–	–	–	–	–	TR
iso-$C_{19:0}$	–	–	–	–	–	–	–	–	TR	–
Summed features*										
3	**40.5±0.37**	**39.5±1.28**	**40.6±0.86**	**39.9±0.42**	**20.3±0.15**	**48.5±0.04**	**32.9**	**30.6**	**38.6**	**45.7**
5	–	–	–	–	**10.4±1.11**	–	–	–	–	–
7	–	TR	TR	TR	–	TR	1.60	–	2.3	TR
8	**27.9±1.50**	**28.3±1.54**	**24.9±0.18**	**29.1±1.23**	**2.14±0.61**	**15.5±0.09**	–	**14.5**	**2.50**	**12.2**

*Summed features are fatty acids that cannot be resolved reliably from another fatty acid using the chromatographic conditions chosen. The MIDI system groups these fatty acids together as one feature with a single percentage of the total. Summed feature 3 contains $C_{16:1}$ $\omega 7c$ and/or $C_{16:1}$ $\omega 6c$. Summed feature 5 contains $C_{18:0}$ ante and/or $C_{18:2}$ $\omega 6,9c$. Summed feature 7 contains $C_{19:1}$ $\omega 7c$ and/or $C_{19:1}$ $\omega 6c$. Summed feature 8 contains $C_{18:1}$ $\omega 7c$ and/or $C_{18:1}$ $\omega 6c$.

DNA G+C content of the is 66.2 mol%. The type strain SL12-8^T (=UCCCB 131^T=CECT 30762^T) was isolated from the skin microbiota of the Perez's frog, *Pelophylax perezi*.

Nucleotide sequence data for the 16S rRNA gene of strains SL12-8^T, LB13-6, LB1–7 and 20M2A are available in the GenBank/DDBL/EMBL databases under the accession numbers KT720394, KT720383, KT720386 and KY611676, respectively. The Whole Genome Shotgun project of strains SL12-8^T has been deposited at DDBJ/ENA/GenBank under the accession JAWDIE000000000. The version described in this paper is version JAWDIE010000000. NCBI BioProject accession number is PRJNA1018472.

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Author contributions

D.N.P. and P.V.M. designed the study. S.C. and D.N.P. performed all the experiments. S.C. and D.N.P. analyzed the data. P.V.M., I.L. and D.N.P. supported the research. S.C. and D.N.P. wrote the manuscript. All authors read, improved and approved the manuscript.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

This work was carried out under the approval for research ethics of Direcção Geral de Veterinária-DGAV (the Portuguese institution responsible for authorizing animal experimentation research). The certificate of approval is provided within this submission.

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