Paracrocinitomix mangrovi gen. nov., sp. nov., isolated from a mangrove sediment and phylogeny of the family Cryomorphaceae revisited: proposal of two new families, Phaeocystidibacteraceae fam. nov. and Owenweeksiaceae fam. nov

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Abstract

A Gram-stain-negative and rod-shaped bacterial strain designated GM2-3-6-6^T was obtained from a mangrove sediment. Cells were light yellow-pigmented, catalase-positive and oxidase-positive. Carotenoid pigment was produced. Phylogeny of the 16S rRNA gene showed that strain GM2-3-6-6^T was affiliated to the family *Crocinitomicaceae*, sharing maximum sequence similarities with *Crocinitomix algicola* 0182^T, *C. catalasitica* IFO 15977^T, and *Putridiphycobacter roseus* SM1701^T of 93.8%, 93.6%, and 92.5%, respectively. The average nucleotide identity (ANI) values, digital DNA-DNA hybridization (dDDH) estimates and average amino acid identity (AAI) values between strain GM2-3-6-6^T and the three close relatives were 68.55–68.80%, 18.50–19.20%, and 59.01–62.33%, respectively. The complete circular genome of strain GM2-3-6-6^T was 4,365,762 bp in length with a DNA G + C content of 34.98%. The respiratory quinone was MK-7. The major polar lipids consisted of phosphatidylethanolamine, two unidentified phospholipids, one unidentified aminoglycolipid, one unidentified aminolipid and four other unidentified lipids. The major fatty acids were iso-$C_{15:0}$, iso-$C_{15:1}$ G, summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$), and iso-$C_{17:0}$ 3-OH. Based on genomic, phenotypic, and chemotaxonomic characterizations, strain GM2-3-6-6^T represents a novel species of a novel genus, for which the name *Paracrocinitomix mangrovi* gen. nov., sp. nov. is proposed. The type strain is GM2-3-6-6^T (= MCCC 1K04831^T = KCTC 82931^T). Additionally, phylogenomic analysis of the family *Cryomorphaceae* related members, including type strains and uncultivated bacteria, was performed using the Genome Taxonomic Database toolkit (GTDB-Tk). Based on 16S rRNA gene phylogeny and genomic features, two novel families, *Phaeocystidibacteraceae* fam. nov. and *Owenweeksiaceae* fam. nov. are proposed.

Introduction

Members of *Flavobacteriales* within the class *Flavobacteriia* of the phylum *Bacteroidota* are ubiquitous in various habitats, for instance, soil, fresh water, brackish water or seawater, and marine macroalgae. Seven families with validly published names were proposed in the order *Flavobacteriales* (https://lpsn.dsmz.de/order/flavobacteriales), the largest number of species (> 150) being classified in the family *Flavobacteriaceae* and a few species in six other families: the *Blattabacteriaceae*, *Crocinitomicaceae*, *Cryomorphaceae*, *Ichthyobacteriaceae*, *Schleiferiaceae*, and *Weeksellaceae*.

The family *Crocinitomicaceae* was proposed in 2016 based on the phylogenetic analysis of 16S rRNA genes, 23 rRNA genes and concatenated orthologous proteins (Munoz et al. 2016). It currently consists of seven genera with validly published names, including *Crocinitomix* (type genus), *Brumimicrobium*, *Fluviicola*, *Lishizhenia*, *Wandonia*, *Salinirepens* (Munoz et al. 2016) and *Putridiphycobacter* (Wang et al. 2020). Members of this family were monophyletically clustered based on the rRNA gene and with DNA G + C content of 31.4–49.5% (Bowman 2020; Munoz et al. 2016). The family *Cryomorphaceae* was first proposed to accommodate three psychrophilic genera, *Cryomorpha* (type genus), *Brumimicrobium* and *Crocinitomix* (Bowman et al. 2003). By means of Genome Taxonomic Database (GTDB) based phylogenomic inference, it only included one genus *Cryomorpha* and one species *Cryomorpha ignava* till now (Bowman 2020). GTDB, using 120 conserved concatenated proteins (Bac120 sets), is considered to be a reliable tool to define bacterial taxonomic ranks (Parks et al. 2018), and proved a useful tool in resolving the taxonomy of the family *Cryomorphaceae*, including not only the cultivated species but also uncultivated organisms characterized by single cell amplified genomes and metagenome assembled genomes (Bowman 2020). Five genera, *Owenweeksia*, *Phaeocystidibacter*, *Luteibaculum*, *Salibacter*, and *Vicingus*, previously affiliated to the family *Cryomorphaceae*, were excluded and separated into three families: *Luteibaculaceae* (type genus *Luteibaculum*), *Salibacteraceae* (type genus *Salibacter*), and *Vicingaceae* (type genus *Vicingus*) (Bowman 2020). The family *Schleiferiaceae* was emended by using GTDB, and currently encompasses five genera *Schleiferia*, *Owenweeksia*, *Phaeocystidibacter*, *Thermaurantimonas* (Bowman 2020) and *Croceimicrobium* (Liu et al. 2021).

In the classification of the family *Cryomorphaceae* related members, phylogeny of 16S rRNA gene demonstrated disparate topology with the phylogenomic tree based on concatenated core genes, which was possibly due to sporadic description of the species within this group. For instance, based on phylogeny of its 16S rRNA gene, *Phaeocystidibacter* did not form a monophyletic cluster with *Owenweeksia* (Zheng et al. 2015). *Croceimicrobium* and *Owenweeksia* are tightly clustered, but

were separated from *Phaeocystidibacter* by *Salibacter* members (Liu et al. 2021). *Schleiferia* and *Thermaurantimonas* demonstrated a deep monophyletic branch, distant from other *Flavobacteriales* members (Liu et al. 2021). This inconsistent topology made us question the classification of the genera *Schleiferia*, *Owenweeksia*, *Phaeocystidibacter*, *Thermaurantimonas* and *Croceimicrobium* into the same family.

In this study, a light-yellow pigmented strain designated GM2-3-6-6^T was isolated from mangrove sediment collected in Luoyang Mangrove Preservation in Quanzhou Bay (Huang et al. 2021). The 16S rRNA gene of strain GM2-3-6-6^T shared low sequence similarities (< 94.5%) with validly published species within the family *Crocinitomicaceae*. This study aimed to confirm the taxonomic position of strain GM2-3-6-6^T using a polyphasic taxonomic method. Additionally, the phylogeny of the family *Cryomorphaceae* and related members was re-evaluated based on the genome sequences.

Materials And Methods

Strain isolation and cultivation

Strain GM2-3-6-6^T was isolated from a mangrove sediment sample collected from the Luoyang mangrove preservation area (24°59' N, 118°42' E), Quanzhou Bay, China, in September 2019 (Huang et al. 2021). The sediment sample was serially diluted with sterile natural seawater, and an aliquot (100 µl) of each dilution was spread onto Marine Broth 2216 (MB, BD) agar plate (Huang et al. 2021). Strain GM2-3-6-6^T was picked and purified on MB agar plates. It was routinely cultured on MB agar medium at 25-30^{o}C and stored at -80^{o}C in MB containing 20% glycerol (v/v).

Phylogeny of 16S rRNA gene

The 16S rRNA gene sequence of strain GM2-3-6-6^T was obtained by using Sanger sequencing with the universal primers 27F and 1492R (DeLong 1992), and deposited in GenBank under the accession number MT829391 (Huang et al. 2021). The sequence was used to search against the *nr* database in GenBank and SILVA SSU 132 database (Quast et al. 2013) using BLAST to find the close relatives. The type stains of closest relatives were identified by using the EzBioCloud server (Yoon et al. 2017a). All the sequences were aligned using ClustalW with default parameters. The phylogenetic trees were reconstructed using MEGA 7.0 (Kumar et al. 2016). *Chitinophaga pinensis* DSM 2588^T affiliated to the class *Chitinophagia* was selected as an outgroup. A neighbor-joining tree was constructed using Kimura-two parameter model based on 1,000 bootstrap replications. A maximum-likelihood tree was constructed using the best model determined using MEGA 7.0, and 1,000 bootstrap replications and 95% partial deletion site were used.

Genome Sequencing

A culture of strain GM2-3-6-6^T in the exponential growth phase centrifuged at 13,000 rpm for 1 min. Genomic DNA was extracted from the pellet using a bacterial DNA extraction kit (SBS, Shanghai, China) according to the manufacturer's instruction. The draft genome sequence of strain GM2-3-6-6^T was determined by using the Illumina HiSeq X-Ten platform (Shanghai Majorbio Bio-Pharm Technology Co., Ltd, Shanghai, China) according to the manufacturer's instructions. Paired end reads of 151 bp (PE151) in length were obtained. The PE151 reads were firstly quality checked to remove the low base of quality <20 and length <50 bp using sickle (https://github.com/najoshi/sickle). Then, clean reads were assembled into contigs using SPAdes v 3.8.0 with a serial of *k* values of 21, 33, 55, 77, 99, 127 and –*careful* flag (Bankevich et al. 2012). Assembled sequences shorter than 1 kb were removed. The genome quality was evaluated using QUAST (Gurevich et al. 2013). Then, complete genome sequencing was conducted using PacBio sequencing with one Sequel SMRT cell. A 10-kb DNA fragment library was prepared using PacBio Sequel sequencing platform according to the manufacturer's instruction (Tianjin Biochip Corporation, Tianjian, China). Sequencing reads were assembled with PacBio long reads using the pipeline of HGAP 4 in SMRT Link (V6.0.0.47841) to obtain the complete genome sequence.

Genome completeness and contamination were determined by using CheckM v1.0.1 (Parks et al. 2015). The *rrn* operon (16S-23S-5S) was identified using RNAmmer (Lagesen et al. 2007). Functional genes were predicated using Prodigal (Hyatt et al. 2010) with default parameters. Gene annotation were carried out using the blast+ program with e-value cutoff of 1e-5 (Camacho et al. 2009) with nr database and the Rapid Annotation using Subsystems Technology (RAST) server (Aziz et al. 2008). The secondary metabolite biosynthetic gene clusters were predicted from the genome sequences by antiSMASH (Medema et al. 2011).

The average nucleotide identity (ANI) values were estimated using OrthoANI computation on the EzBioCloud Database (Yoon et al. 2017b). Average amino acids identities were calculated using CompareM v0.1.2 (https://github.com/dparks1134/CompareM). The percentage of conserved proteins (POCP) values were also calculated for genomic comparison (Qin et al. 2014).

Phylogenomic tree

The bacterial genomes affiliated to the families *Crocinitomicaceae*, *Cryomorphaceae*, *Ichthyobacteriaceae*, *Schleiferiaceae*, *Luteibaculaceae*, *Salibacteraceae*, and *Vicingaceae* were obtained from the genome portal in GenBank (https://www.ncbi.nlm.nih.gov/genome/). Then, the quality of the genome sequences was determined by using CheckM v1.0.1 (Parks et al. 2015). Genomes with <80% completeness and >5% contamination were removed from the phylogenomic analysis. The phylogenomic tree was constructed using a concatenated alignment of 120 bacterial conserved concatenated proteins (Bac120 sets) with GTDB-tk v. 0.3.2 by using FastTree (Parks et al. 2018). Then, the phylogenetic tree was modified using the online Interactive Tree of Life (iTOL) (Letunic and Bork 2007).

Phenotypic characteristics

The cell morphology of strain GM2-3-6-6^T was observed by using transmission electron microscope after negatively stained. The fresh cells grown on MB agar plate was spread on copper grids and then stained with 2% phosphotungstic acid. The cell morphology was observed with transmission electron microscope (Hitachi HT-7800). Colonies on MB agar plates were observed after incubating the plates at 30oC for 3 days. Catalase activity was detected by bubble production in 3% H_2O_2 (v/v). Oxidase activity was detected using the oxidase reagent (bioMérieux) to observe the color change. The hydrolysis ability of Tween 40, 60, 80, starch, agar, skimmed milk, and carboxymethyl cellulose (CMC) were detected by inoculating the strains on MB agar plates. Motility was observed by puncturing the cells into MB containing 0.5% agar. The growth temperature range, NaCl tolerance range and pH range were determined as described in our previous study (Huang et al. 2020). The additional physiological and biochemical properties were determined using the three API strips, API ZYM, API 20E and API 20NE (bioMérieux) according to the manufacturer's instructions. Three closely related type strains, *C. catalasitica* IFO 15977^T (= DSM 4133^T), *C. algicola* 0182^T (=MCCC 1H00128^T) and *Putridiphycobacter roseus* SM1701^T (=KCTC 62302^T) were used as reference strains. The tested strain and reference strains were maintained under the same laboratory conditions.

Chemotaxonomic characteristics

The fatty acids composition, respiratory quinone system, and polar lipid profile of strain GM2-3-6-6^T were determined as follows. For the analysis of fatty acids, strain GM2-3-6-6^T and three reference strains were cultured in 150 ml MB shaking for 150 rpm at 25^{o}C for 5 days. The cells were collected by centrifugation at 8,000 rpm for 10 min at room temperature. The cellular fatty acids were saponified, methylated and extracted, and analyzed using gas chromatograph following the standard MIDI protocol (Sherlock Microbial Identification System, version 6B). The polar lipids were extracted from the cells using a chloroform/methanol system and analyzed using two-dimensional TLC with Merck silica gel plates. The appropriate spraying reagents were used to detect the total lipids (10% ethanolic molybdophosphoric acid), aminolipids (ninhydrin), phosphorus-containing lipids (molybdenum blue). The respiratory quinone was extracted using chloroform/methanol (2:1, v/v) and detected using reversed-phase HPLC.

Results And Discussion

Phylogenetic analysis of 16S rRNA gene sequence

The 16S rRNA gene sequence of strain GM2-3-6-6^T (1,384 bp in length, accession number: MT829391) obtained by Sanger sequencing had 100% identity with the complete sequence (1,513 bp in length) identified and extracted from the genome sequence. Two ribosomal RNA (*rrn*) operons (16S-23S-5S) were found in the complete genome, and they had 100% sequence similarity. Comparison of 16S rRNA gene sequence of strain GM2-3-6-6^T with nucleotide sequences in GenBank and SSU reference sequences in SILVA (Release 138) showed top hits with uncultured bacterium clones of <95.8% sequence similarities. Comparison with type strain sequences in the EzBioCloud server showed that strain GM2-3-6-6^T had maximum sequence similarities with *C. algicola* 0182^T, *C. catalasitica* IFO 15977^T, and *P. roseus* SM1701^T of 93.8%, 93.6%, and 92.5%, respectively. These values are below the recommended taxonomic threshold of a genus delineation with sequence identity of 94.5% (Yarza et al. 2014), indicating that strain GM2-3-6-6^T may represent a novel species of a novel genus.

Phylogeny of 16S rRNA gene in the neighbor-joining tree (NJ) and maximum likelihood tree (ML) indicated that strain GM2-3-6-6^T was well clustered with the members of the family *Crocinitomicaceae*, and formed an independent branch closely related with *Crocinitomix* members and *P. roseus* (**Supplementary Figure S1 and S2**). Phylogeny of the 16S rRNA gene supported that strain GM2-3-6-6^T represents a novel genus lineage within the family *Crocinitomicaceae*. Members of the family *Crocinitomicaceae* were well clustered and shared 16S rRNA gene sequence similarities of 86.74-98.49%.

Phylogeny of the 16S rRNA gene also found that a small number of members related to *Cryomorpha ignava* ACAM 647^T formed multi-family lineages with low bootstrap values (**Supplementary Figure S1 and S2**). Firstly, the type species *Schleiferia thermophila* formed a strongly supported clade with *Thermaurantimonas aggregans* (sharing 93.91% sequence similarity), belonging to the family *Schleiferiaceae*. Secondly, two species within the genus *Phaeocystidibacter* sharing 95.42% similarity formed an independent clade. Here we proposed a novel family *Phaeocystidibacteraceae* fam. nov. to accommodate the genus. Thirdly, the species *Owenweeksia hongkongensis* and *Croceimicrobium hydrocarbonivorans* were tightly clustered (bootstrap value of 100%), and they should be considered a distinct family from the families *Salibacteraceae*, *Schleiferiaceae*, and *Phaeocystidibacteraceae*. Thus, a novel family designated *Owenweeksiaceae* fam. nov. is proposed, with *Owenweeksia* as the type genus. Fourthly, *Vicingus serpentipes* ANORD5^T and *Acidiluteibacter ferrifornacis* S-15^T, sharing 89.83% of 16S rRNA gene sequence similarity, formed a highly supported clade, affiliating to the family *Vicingaceae*.

Genomic features and genomic relatedness

The draft genome size of strain GM2-3-6-6^T determined using Illumina sequencing was 4,338,207 bp on 15 contigs (>1 kb) with the two largest sequences of 1,394,190 bp and 1,261,120 bp in length. The complete genome size of strain GM2-3-6-6^T determined using PacBio sequencing was 4,365,762 bp with one circular chromosome (**Table 1**). The genome size of strain GM2-3-6-6^T was similar to close relatives *C. catalasitica* IFO 15977^T, *C. algicola* 0182^T, and *P. roseus* SM1701^T of 4,622,888 bp, 3,719,297 bp, and 4,042,952 bp, respectively. The DNA G+C content of strain GM2-3-6-6^T was 34.98%, which was also similar to three closely related species (**Table 1**).

The antiSMASH server revealed a carotenoid biosynthetic gene cluster (BGC, ~20 kb) in the genome sequence of strain GM2-3-6-6^T, which encoded the key enzymes that synthesize the carotenoid, including phytoene desaturase, phytoene synthase, and phytoene dehydrogenase (**Figure S3**). The carotenoid BGCs were also found in the genomes of *C. catalasitica* IFO 15977^T, *C. algicola* 0182^T, and *P. roseus* SM1701^T, while they demonstrated a different gene arrangement, indicating that the carotenoid BGCs were very diverse in the *Crocinitomicaceae* members.

The ANI values of strain GM2-3-6-6^T compared to *C. catalasitica* IFO 15977^T, *C. algicola* 0182^T, and *P. roseus* SM1701^T were 68.80%, 68.55%, and 68.79%, respectively. These values were lower than the suggested threshold of species delineation (95-96%) (Yoon et al. 2017a), suggesting that strain GM2-3-6-6^T represents a novel species. The digital DNA-DNA hybridization (dDDH) values of strain GM2-3-6-6^T compared to *C. catalasitica* IFO 15977^T, *C. algicola* 0182^T, and *P. roseus* SM1701^T were 19.00%, 18.50%, and 19.20%, which were also below the threshold value of species delineation (70%) (Meier-Kolthoff et al. 2013), indicating that strain GM2-3-6-6^T represented a novel species. The average amino acid identities of strain GM2-3-6-6^T compared to *C. catalasitica* IFO 15977^T, *C. algicola* 0182^T, and *P. roseus* SM1701^T were 61.21%, 62.33%, and 59.01%, respectively. These values were below the threshold of a new genus (65%) (Konstantinidis et al. 2017), supporting that strain GM2-3-6-6^T represented a novel genus. POCP values calculated between strain GM2-3-6-6^T and *C. catalasitica* IFO 15977^T, *C. algicola* 0182^T, and *P. roseus* SM1701^T were 0.488, 0.542, and 0.465, respectively. These values also supported strain GM2-3-6-6^T to be classified in a novel genus (Qin et al. 2014).

Phylogenomic analysis

Compared to 16S rRNA gene sequences, phylogeny based on genome sequences enable more accurate classification of bacterial and archaeal groups. The GTDB tools (Chaumeil et al. 2019) were used in this study to determine the taxonomic position of strain GM2-3-6-6^T and closely related members of the order *Flavobacteriales*. A total of 126 genomes including an outgroup *Chitinophaga pinensis* DSM 2588^T were used in the phylogenomic analysis (**Figure 1**). The majority of the phylogenetic lineages in the tree were obtained from uncultivated bacteria, indicating that most lineages are still awaiting to be cultivated.

Phylogenomic analysis based on Bac120 sets showed that strain GM2-3-6-6^T was affiliated to the family *Crocinitomicaceae* and formed a clade with an uncultivated bacterium Bin_13 (accession number: JABURP000000000), which was obtained from a marine sediment core in northeastern Brazil. This clade was neighbored by the species *C. algicola*, *C. catalasitica*, and *P. roseus*, a topology congruent with the phylogeny of the 16S rRNA gene (**Supplementary Figure S1 and S2**).

Phylogenomic analysis based on Bac120 sets strongly supported classification of the family *Cryomorphaceae*-related members in multiple-family-level clades (**Figure 1**). Firstly, the type species *Cryomorpha ignava* was placed in an independent clade as a family-level taxon *Cryomorphaceae*, which was clearly separated from the genera *Luteibaculum*, *Salibacter*, *Phaeocystidibacter*, *Owenweeksia*, and *Croceimicrobium*. This result agreed with the analysis of the family *Cryomorphaceae* by Bowman (2020). Secondly, the species *Luteibaculum oceani* and *Salibacter halophilus* formed two separate monophyletic clades, affiliated to the *Luteibaculaceae* and *Salibacteraceae*, respectively. Thirdly, the phylogenomic tree strongly supported that *Phaeocystidibacter marisrubri* and *Phaeocystidibacter luteus* belonged to a novel family, closely related to two novel clusters containing uncultivated bacteria. Fourthly, the clade including *Schleiferia thermophila* and *Thermaurantimonas aggregans* formed a node with the clade containing *Owenweeksia hongkongensis* and *Croceimicrobium hydrocarbonivorans*, which is inconsistent with the 16S rRNA gene phylogeny, where the two clades were assigned to different families. The family, *Schleiferiaceae*, including the genera *Schleiferia* and *Thermaurantimonas*, and *Owenweeksiaceae*, including the genera *Owenweeksia* and *Croceimicrobium* were proposed according to the principle of priority. Lastly, *A. ferrifornacis* and *V. serpentipes* formed a distinct lineage regarded as a novel family, the topology being congruent with the phylogeny of 16S rRNA gene sequences.

Phenotypic characteristics

Cells of strain GM2-3-6-6^T were Gram-strain-negative, ovoid or short rod-shaped, 1-1.5 μm long and 0.7 μm, and non-motile (**Figure 2**). Colonies grown on MB agar plate at 25 ^oC for 3 days were light yellow-colored, a color distinct of that of its close relatives (**Table 1**). Catalase activity and oxidase activity were positive, as in close relatives. Growth was observed at 15-40^oC (optimum, 25^oC), at pH 6-8 (optimum, 7) and in the presence of 0.5-4.0% NaCl (optimum, 2.0%, w/v). Strain GM2-3-6-6^T cannot degrade starch, cellulose (CMC), skimmed milk and Tween 40, Tween 60 and Tween 80, similar to *C. catalasitica* IFO

15977^T and *P. roseus* SM1701^T, but different from *C. algicola* 0182^T, which degraded agar and CMC. Additional physiological and biochemical properties are given in **Table 1** and in the species description.

Chemotaxonomic Characteristics

The respiratory quinone determined in strain GM2-3-6-6^T was MK-7, which was consistent with close relatives, *Crocinitomix* members (Shi et al. 2017), *P. roseus* (Wang et al. 2020), and *Wandonia*, while MK-6 was present as major quinone in other members of the family *Crocinitomicaceae* (**Table 2**). The major fatty acids (>5%) of strain GM2-3-6-6^T were iso-$C_{15:0}$ (55.3%), summed feature 3 ($C_{16:1}\omega7c$ and $C_{16:1}\omega6c$) (10.1%), iso-$C_{15:1}$ G (9.1%), and iso-$C_{17:0}$ 3-OH (7.9%) (**Table S1**). The percentage of iso-$C_{15:1}$ G in strain GM2-3-6-6^T was lower than in *C. catalasitica* IFO 15977^T (28.4%) and *P. roseus* SM1701^T (23.8%), which can differentiate the close relatives.

The polar lipid profiles of strain GM2-3-6-6^T included phosphatidylethanolamine (PE), two unidentified phospholipids (PL), one unidentified aminolipid (AL), one unidentified aminoglycolipid (AGL), and four other unidentified lipids (**Figure S4**). GL, present in the close relatives (**Table 2**), was not identified in strain GM2-3-6-6^T.

Proposal of *Owenweeksiaceae* and *Phaeocystidibacteraceae*

Phylogenomic analysis placed the *Vicingaceae*, *Ichthyobacteriaceae*, and *Salibacteraceae* into separate clusters with genomic size of 2.9-3.5, 1.2-3.6, and 2.3-4.5 Mbp, respectively (**Table S2**). *Phaeocystidibacteraceae*, *Owenweeksiaceae, Schleiferiaceae*, and two additional clusters containing uncultivated organisms clustered together, but the genomic characterizations, including 16S rRNA gene sequences similarities and ANI and AAI values, showed a certain distinctiveness (**Supplementary Figure S1** and **S2**). The 16S rRNA gene sequence similarities compared among these three families were lower than 90.35% (**Table 3**), nearly approaching the threshold identity of a family (86.5%) (Yarza et al. 2014). The ANI values and AAI values are close to the family boundary, 73.7-74.5%, and 52.3-57.1%, respectively (**Table 4**).

The genomic size of *Owenweeksiaceae* calculated from the draft genomes was 3.86-4.46 Mbp (DNA G+C content of 40.2-46.0%), which was much larger than the 2.0-2.7 Mbp of members of the *Schleiferiaceae* (DNA G+C content of 42.6-45.3%). The *Phaeocystidibacteraceae* have genome sizes of 3.2-3.4 Mbp, intermediate between the *Owenweeksiaceae* and the *Schleiferiaceae* (**Table 5** and **Table S2**). The genomic size could be used as a useful feature to differentiate the three families. Also, catalase was positive in *Owenweeksiaceaea* and *Phaeocystidibacteraceae*, but negative in *Schleiferiaceae*. In addition, the polar lipids of *Schleiferiaceae* did not contain glycolipid (GL), which was present in the *Phaeocystidibacteraceae* and *Owenweeksiaceae* (**Table 5**).

Conclusion

Based on the phylogenetic, genomic, phenotypic, and chemotaxonomic characteristics, strain GM2-3-6-6^T represents a novel species of a novel genus, for which the name *Paracrocinitomix mangrovi* gen. nov., sp. nov. is proposed. The type strain is GM2-3-6^T (= MCCC 1K04381^T = KCTC 82931^T). Based on the phylogenetic analysis and genomic features, two novel families *Phaeocystidibacteraceae* fam. nov. and *Owenweeksiaceae* fam. nov. are proposed. Our study provided a taxonomic framework for the family *Cryomorphaceae* based on the genomic data.

Description of the genus Paracrocinitomix

Paracrocinitomix (Pa.ra.cro.ci.ni.to'mix. Gr. pref. *para*, beside; N.L. fem. n. *Crocinitomix*, a bacterial genus; N.L. fem. n. *Paracrocinitomix*, a genus next to *Crocinitomix*).

Cells are Gram-stain-negative, strictly aerobic, rod-shaped, non-flagellated, and non- motile. NaCl is required for growth. The respiratory quinone is MK-7. The major fatty acids are iso-$C_{15:0}$, summed feature 3 ($C_{16:1}\omega7c$ and $C_{16:1}\omega6c$), iso-$C_{15:1}$ G and

iso-$C_{17:0}$ 3-OH. The major polar lipids are phosphatidylethanolamine, unidentified phospholipids, unidentified aminolipids, an unidentified aminoglycolipid, and other unidentified lipids. The type species is *Paracrocinitomix mangrovi*.

Description of Paracrocinitomix mangrovi

Paracrocinitomix mangrovi (man.gro'vi. N.L. gen. n. *mangrove*, of a mangrove).

In addition to those given in the genus description, the species has the following characteristics. Colonies on MB agar plates cultured for 3 days at 30 ^{o}C are round, light-yellow pigmented and convex. Cells are Gram-stain-negative and rod-shaped. Growth occurs between 15 and 40 ^{o}C with an optimum at 25 ^{o}C, at 0.5-4% NaCl (w/v) with an optimum of 2%, and a pH range of 6.0–8.0. Catalase-positive and oxidase-positive. Caroteonid pigment was produced. Nitrate is not reduced to nitrite. H_2S is not produced. Hydrolysis of gelatin is positive. Hydrolysis of urea and aesculin is negative.

The major polar lipids include phosphatidylethanolamine (PE), two unidentified phospholipids (PL), one unidentified aminolipid (AL), one unidentified aminoglycolipid (AGL), and four other unidentified lipids. The genome size is 4.3 Mbp with DNA G + C content of 35.0%.

The type strain is GM2-3-6-6^T (= MCCC 1K01284^T = KCTC 82931^T), isolated from mangrove sediment. The GenBank/EMBL/DDBJ accession number of 16S rRNA gene sequence and whole genome sequences of strain GM2-3-6-6^T are MT829391 and JAHXZQ000000000 (draft genome) and CP091819 (complete genome), respectively.

Description of Phaeocystidibacteraceae **fam. nov.**

Phaeocystidibacteraceae (Phae.o.cys.ti.di.bac.te.ra.ce'ae. N.L. masc. n. *Phaeocystidibacter*, a bacterial genus; -*aecae*, ending to denote a family; N.L. fem. pl. n. *Phaeocystidibacteraceae*, the *Phaeocystidibacter* family).

The description is as that for *Phaeocystidibacter* (Zhou et al. 2013), which is the type genus. The family has been separated from *Schleiferiaceae* members based on phylogenetic analysis of 16S rRNA gene and genome sequences.

Description of Owenweeksiaceae **fam. nov.**

Owenweeksiaceae (Ow.en.week.si.a.ce'ae. N.L. fem. n. *Owenweeksia*, a bacterial genus; -*aecae*, ending to denote a family; N.L. fem. pl. n. *Owenweeksiaceae*, the *Owenweeksia* family).

The description is as that for *Owenweeksia* (Lau et al. 2005), which is the type genus. The family has been separated from *Schleiferiaceae* members based on phylogenetic analyses of 16S rRNA gene and genome sequences. The family includes the genera *Owenweeksia* and *Croceimicrobium*. Genome size is about 3.9–4.5 Mbp, and DNA G + C content is 40.2–46.0%.

Declarations

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Conflicts of interest

No conflicts of interest are declared by the authors.

Availability of data

The GenBank/EMBL/DDBJ accession numbers of the 16S rRNA gene sequence and genome sequence of strain GM2-3-6-6^T are MT829391, and JAHXZQ000000000 (draft genome) and CP091819 (complete genome), respectively.

Ethics approval

This article does not contain any studies with animals.

Consent for publication

The authors approved for the publication.

Authors' contributions

Z. Huang conceived the study and wrote the manuscript. Y. Huang, Q. Lai and W. Wang conducted the experiments. A. Oren proposed names, wrote, and checked etymologies, and edited and corrected the manuscript. All authors reviewed the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

Supplementary Materials

The supplementary tables and figures are available with the online version of this article.

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Tables

Table 1. Differential characteristics of strain GM2-3-6-6^T compared to close relatives.

1. Strain GM2-3-6-6^T, 2. *C. catalasitica* IFO 15977^T 3. *C. algicola* 0182^T, 4. *P. roseus* SM1701^T.

All strains were positive for catalase, leucine arylamidase, weak positive for esterase (C4), valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase. Hydrolysis of aesculin were weak positive. CMC, carboxymethyl cellulose, +, positive, w, weak positive, -, negative, ND, no data.

Characteristics	1	2	3	4
Colony color	Light-yellow	Light-yellow	Yellow	Orange
Oxidase	+	+	-	+
Growth temperature (°C)	15-40 (25)	0-30 (25)[a]	10-37 (30)[a]	4-30 (20)[b]
pH	6-8	ND	6-8.5	6-8[b]
NaCl tolerance (%, w/v)	0.5-4 (2)	0.5-6[a]	0.5-10 (2-3)[a]	0.5-5 (2)[b]
Degradation of agar and CMC	-	-	+	-
Esterase lipase (C8)	+	+	w	+
Lipase(C14)	+	+	w	w
Cystine arylamidase	+	w	-	w
Trypsin, α-chymotrypsin	+	-	-	-
Naphthol-AS-BI-phosphohydrolase	+	+	w	+
α-Galactosidase	-	-	+	-
β-Glucosidase and α-mannosidase	-	-	w	-
N-Acetyl-β-glucosaminidase	-	w	+	-
Urease	-	-	+	-
Hydrolysis of aesculin	-	-	+	-
Hydrolysis of gelatin	+	-	-	-
Genome size (bp)	4,365,762	4,622,888	3,719,297	4,042,952
DNA G+C content (%)	34.98	34.08	35.35	34.10
Isolation source	Mangrove sediment	Sand of a bay	A red alga	An alga

a. Data was taken from (Shi et al. 2017). b. Data was taken from (Wang et al. 2020)

Table 2. Differential characteristics of the genus *Paracrocinitomix* compared to its closely related members in the family *Crocinitomicaceae*.

Genera: 1. *Paracrocinitomix* (this study), 2. *Crocinitomix* (Shi et al. 2017), 3. *Putridiphycobacter* (Wang et al. 2020), 4. *Lishizhenia* (Lau et al. 2006, Wang et al. 2020, Chen et al. 2009), 5. *Brumimicrobium* (Luo et al. 2018), 6, *Wandonia* (Lee et al. 2010, Muramatsu et al. 2012), 7, *Salinirepens* (Muramatsu et al. 2012), 8, *Fluviicola* (Akter and Huq 2020).

+, Positive, -, negative, v, variable, ND, no data, SF3, summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$).

Genera	1	2	3	4	5	6	7	8
Number of species	1	2	1	2	4	1	1	4
Colony color	Light-yellow	Yellow	Orange	Orange	Orange	Yellow	Yellow-orange	Yellow, orange
Flexirubin-pigment	-	-	-	-	-	+	-	+
NaCl requirement	+	+	+		+			-
Catalase	+	+	+	+	+	+	+	-
Oxidase	+	+	+	-	+	+	+	-
Major Fatty acids	iso-$C_{15:0}$, SF3, iso-$C_{15:1}$ G, iso-$C_{17:0}$ 3-OH	iso-$C_{15:0}$, $C_{15:0}$ and iso-$C_{17:0}$ 3-OH	iso-$C_{15:0}$, iso-$C_{15:1}$ G and iso-$C_{16:1}$ G	iso-$C_{15:0}$, iso-$C_{15:1}$ and iso-$C_{17:0}$ 3-OH	iso-$C_{15:0}$, iso-$C_{15:1}$ G and iso-$C_{17:0}$ 3-OH	iso-$C_{15:0}$, $C_{15:0}$ and $C_{15:0}$ 2-OH	iso-$C_{15:0}$	iso-$C_{15:0}$
Major Polar lipids	PE, PL, AGL, AL, L	PE, GL, AGL, APL, PL	PE, GL, AGL, AL	PE, GL, AGL, APL	PE, GL, AL, L	PE, GL, L	PL, GL, L	PE, GL, PL, L
Quinone	MK-7	MK-7	MK-7	MK-6	MK-6	Mk-7	MK-6	MK-6
Genome Size (Mbp)	4.37	3.72-4.62	4.04	~3.57	3.41-4.27	ND	ND	4.27-4.63
Genomic G+C content (%)	34.98	34.08-35.35	34.10	34.6-37.40	33.59-34.32	38.1	36.4	36.49-41.69
Isolation source	Mangrove sediment	Sand and Marine alga	Marine alga	seawater	Sea ice, coastal sediment and saltern	abalone	seawater	Water or soil

Table 3. The 16S rRNA gene sequence similarities compared among the close relatives.

1. Owenweeksiaceae, 2. *Phaeocystidibacteraceae*, 3. *Schleiferiaceae* 4. *Salibacteraceae* 5. *Cryomorphaceae*, 6. *Vicingaceae*, 7. *Luteibaculaceae*,

	1	2	3	4	5	6
2	88.70-90.35					
3	85.53-86.80	85.70-86.75				
4	88.38-89.22	88.19-88.41	84.75-85.93			
5	86.84-86.86	85.43-85.55	83.88-83.89	86.35		
6	87.69-89.49	85.86-87.31	83.00-84.89	85.03-87.72	85.12-87.74	
7	88.81-89.36	87.68-88.19	83.67-84.83	86.74	86.79	86.02-88.05

Note: Data was calculated based on the type strains.

Table 4. ANI and AAI compared among the close relatives.

1. Owenweeksiaceae, 2. *Phaeocystidibacteraceae*, 3. *Schleiferiaceae* 4. *Salibacteraceae* 5. *Cryomorphaceae*, 6. *Vicingaceae*, 7. *Luteibaculaceae*,

ANI/AAI	1	2	3	4	5	6
2	73.77-74.48/55.67-57.13					
3	73.67-74.00/54-56.67	73.76-74.12/54.18-56.24				
4	73.68-74.17/52.33-54.3	73.59-74.00/53.01-53.75	73.62-73.84/51.71-53.62			
5	73.61-73.86/49.95-53.59	73.69-73.87/51.07-53.65	73.59-73.86/50.17-53.63	73.60-73.89/51.05-54.5		
6	73.63-73.92/52.98-54.86	73.83-74.00/53.48-54.07	73.64-73.82/52.06-53.93	73.68-73.95/53.74-55.24	73.64-73.95/51.03-54.48	
7	73.72-73.98/51.7-52.88	73.96-74.00/52.44-52.45	73.76-73.88/51.04-52.62	73.69-73.93/53.24-54.06	73.72-73.96/51.79-54.39	73.96-73.97/53.68-54.06

Note: Data was calculated based on the type strains and uncultivated bacteria.

Table 5. Differential characteristics of the close relatives, *Owenweeksiaceae*, *Phaeocystidibacteraceae* and *Schleiferiaceae*.

Family	*Owenweeksiaceae*[a]		*Phaeocystidibacteraceae*		*Schleiferiaceae*	
Genus	*Owenweeksia*	*Croceimicrobium*	*Phaeocystidibacter*[b]		*Schleiferia*[c]	*Thermaurantimonas*[c]
Species	*O. hongkongensis*	*C. hydrocarbonivorans*	*P. luteus*	*P. marisrubri*	*S. thermophila*	*T. aggregans*
Colony color	Orange	Orange	Orange	Yellow	Orange	Orange
Flexirubin	-	-	-	+	ND	ND
Catalase	+	+	+	+	-	-
Oxidase	+	-	-	+	+	+
Major Fatty acids	iso-$C_{15:0}$, SF3, and iso-$C_{15:1}$ G	iso-$C_{15:0}$, SF3, and iso-$C_{16:0}$ 3-OH	iso-$C_{15:0}$, SF3, and iso-$C_{15:1}$ G	iso-$C_{15:0}$, SF3, and iso-$C_{15:1}$ G, iso-$C_{17:0}$ 3-OH	iso-$C_{15:0}$, SF3, iso-$C_{17:0}$ 3-OH	iso-$C_{15:0}$, iso-$C_{17:0}$ 3-OH, and $C_{15:0}$
Major Polar lipids	PE, PN, AL, GL, L	DPG, PE, PG, PC, APL, GL, L	PE, AL, GL, PL, L	PE, AL, GL, PL, L	APL, AL, L	PE, AL, L
Quinone	MK-6	MK-7	MK-6	MK-6	MK-6	MK-6
Genome Size (bp)	4,000,057	4,035,598	3,213,381	3,354,239	2,599,013	2,671,880
DNA G+C content (%)	40.23	43.23	46.47	44.74	42.93	42.60
Isolation source	Seawater	Deep-sea sediment	A marine alga	Red sea sediment	A hot spring	A hot spring

a, Data was taken from (Liu et al. 2021), b, Data was taken from (Zheng et al. 2015), c, Data was taken from (Iino et al. 2020), ND, no data, SF3, summed feature 3 ($C_{16:1}\omega7c$ and $C_{16:1}\omega6c$).

Figures

Figure 1

Maximum-likelihood phylogenomic tree was constructed based on 120 bacterial conserved proteins by using FastTree.

The tree was rooted by *Chitinophaga pinensis* DSM 2588^T. Bootstrap values of the node greater than 0.7 are displayed. Family names were shown around the tree. Lineages without family names indicated are not type strains. The families *Owenweeksiaceae* and *Phaeocystidibacteraceae* were marked bold.

Figure 2

Transmission electron microscopy of the grown cells of strain GM2-3-6-6^T.

Supplementary Files

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- FigureS3.Genecluster.pdf
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- TableS2.xlsx