Reclassification of the Taxonomic Framework of Orders Cellvibrionales, Oceanospirillales, Pseudomonadales, and Alteromonadales in Class Gammaproteobacteria through Phylogenomic Tree Analysis

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ABSTRACT Orders Oceanospirillales and Pseudomonadales play important roles in various ecosystems as the keystone taxa of microbiomes. However, the two orders present a close evolutionary relationship, which might have caused taxonomic misinterpretation and resulted in an incorrect understanding of their evolutionary history. In this study, first, we used the 16S rRNA gene sequences of 2,049 species of Gammaproteobacteria to build a phylogenetic tree, which demonstrated that reports regarding the evolutionary relationship of orders Cellvibrionales, Oceanospirillales, and Pseudomonadales based on a single conserved gene with a poor resolution have been conflicting; in particular, the major families Moraxellaceae and Pseudomonadaceae of order Pseudomonadales were separated from orders Cellvibrionales and Oceanospirillales. Subsequently, we constructed the bac120 trees of all representative reference genomes of class Gammaproteobacteria based on 120 ubiquitous single-copy proteins from bacteria and a phylogenomic tree based on the 119 core genes of 257 reference genomes obtained from orders Cellvibrionales, Oceanospirillales, and Pseudomonadales to cross validate and infer their intrinsic evolutionary relationships. These results indicated that two novel orders, Moraxellales ord. nov. and Kangiellales ord. nov., and three novel families, Marinobacteraceae fam. nov., Perlucidibacaceae fam. nov., and Zooshikellaceae fam. nov., should be proposed. Additionally, orders Cellvibrionales and Oceanospirillales were merged into the order Pseudomonadales except for families Moraxellaceae and Kangiellaceae in class Gammaproteobacteria, which currently includes 18 families. Our work sheds some light on the evolutionary history of class Gammaproteobacteria, which could facilitate the detection and taxonomic analysis of natural communities.

IMPORTANCE The orders Cellvibrionales, Oceanospirillales, and Pseudomonadales, as three major orders of the largest bacterial class, Gammaproteobacteria, play important roles in various ecosystems as the keystone taxa of microbiomes, but their evolutionary relationship is currently polyphyletic and chaotic. Here, we constructed a bac120 tree and core-genome tree and calculated the amino acid identity (AAI) value to explore their intrinsic evolutionary history. In this study, we proposed two novel orders and three novel families. This evolution study vastly reconstructed the taxonomic framework of class Gammaproteobacteria and could provide a more distinct perspective on global distribution and evolutionary patterns of these environmental microorganisms.

KEYWORDS class Gammaproteobacteria, core genome, Moraxellales ord. nov., Kangiellales ord. nov., Marinobacteraceae fam. nov., Zooshikellaceae fam. nov., Perlucidibacaceae fam. nov., Pseudomonadales ord. nov.
The tree of life is arguably the most important organizing principle in biology and perhaps the most widely understood depiction of the evolutionary process. It explains how we are related to other organisms and where we may have come from (1). With the continuous reduction in sequencing costs and new developments in biotechnology and bioinformatic tools, a multigene-based phylogenomic tree approach in which genomic data are used for phylogenomic analysis appears to be a better approach for defining genera or higher taxa than the use of 16S rRNA gene-derived phylogeny (2). In August 2018, Parks et al. (3) proposed a standardized bacterial taxonomy based on a genome phylogeny and substantially revised the tree of life. We believe that the new classification framework will provide important guidance for future reclassification studies.

*Gammaproteobacteria* spreads throughout global ecosystems, including marine, land, and sediment environments and animal hosts, and represents the largest bacterial class, including 19 orders, 58 families, and 381 genera according to LPSN and the website [https://www.ezbiocloud.net/taxonomy?tn=Gammaproteobacteria&depth=3](https://www.ezbiocloud.net/taxonomy?tn=Gammaproteobacteria&depth=3). To date, 78,338 *Gammaproteobacteria* genomes have been deposited in the National Center for Biotechnology Information (NCBI) database (October 2019); the representative genomes in the RefSeq category include 808 genomes. Therefore, phylogenomic tree construction based on all genomes of *Gammaproteobacteria* is difficult due to the restriction of computing resources; alternatively, the construction of an evolutionary tree based on the representative genomes in the RefSeq category is relatively easy and accurate.

*Cellvibrionales*, *Oceanospirillales*, and *Pseudomonadales* are three major orders of *Gammaproteobacteria* that play important roles in various ecosystems as the keystone taxa of microbiomes (4). For instance, order *Cellvibrionales* has a putative important function in oligotrophic marine environments (5). The order *Oceanospirillales* shows remarkable potential for the natural attenuation of spilled oil in deep-sea surface sediments (6), and almost all species of family *Endozoicomonadaceae* have been isolated from marine animals, while most members of genus *Zooshikella* can produce prodigiosin, which is an effective proapoptotic agent that can be used against various cancer cell lines while showing little or no toxicity toward normal cell lines (7). The order *Pseudomonadales* plays an important role in contaminated soil remediation and plant-associated microbiota (4), and many members of order *Pseudomonadales* present clear associations with human health as pathogens, such as *Acinetobacter baumannii*, *Moraxella catarrhalis*, and *Pseudomonas aeruginosa*.

As of the writing of the manuscript, order *Oceanospirillales* includes 11 families ([https://lpsn.dsmz.de/order/oceanospirillales](https://lpsn.dsmz.de/order/oceanospirillales)), order *Pseudomonadales* includes 3 families ([https://lpsn.dsmz.de/order/pseudomonadales](https://lpsn.dsmz.de/order/pseudomonadales)), and order *Cellvibrionales* includes 5 families (5). In the last decade, based on rapid advances in phylogenetic and molecular analyses, several revisions have been carried out in the order *Oceanospirillales*, with numerous genera being split into separate families (5); for instance, family *Endozoicomonadaceae* was split from family *Hahellaceae* in 2018 (8). Additionally, genus *Marinobacterium* was reclassified into family *Oceanospirillaceae* (9), indicating that the systematic evolution of the order is still unclear due to the discovery of increasing numbers of species. In 2017, we discovered a novel genus, *Mangrovitalea*, which is closely related to genus *Marinobacter*, and we classified this new genus into order *Alteromonadales* (10). However, in this study, we found that genera *Tamilnadubacter* (11), *Mangrovitalea*, and *Marinobacter* formed a robust clade in a phylogenetic tree and that they were distantly phylogenetically related to *Alteromonadales*; therefore, they should be allocated to higher taxonomic ranks. The major families *Moraxellaceae* and *Pseudomonadaceae* of order *Pseudomonadales* were separated by a branch containing orders *Cellvibrionales* and *Oceanospirillales* according to the 16S rRNA-based The All-Species Living Tree (LTP), release 132, which illustrated that the order *Pseudomonadales* had polyphyletic lineages. Intriguingly, in 2018, the Genome Taxonomy Database (GTDB) taxonomy proposed the transfer of the majority of the members of orders *Oceanospirillales* and *Cellvibrionales* to order *Pseudomonadales*; however, this classifi-
cation has not been proposed anywhere in the literature, and thus, the intrinsic evolutionary relationship of orders Oceanospirillales, Cellvibrionales, and Pseudomonadales is still a question worth discussing.

RESULTS AND DISCUSSION

Phylogenomic tree based on small-subunit (SSU)-rRNA of Gammaproteobacteria. Figure 1 was generated based on 16S rRNA gene sequences, and 2,049 sequences representing 2,049 species of class Gammaproteobacteria with validly published names were downloaded from the SILVA Living Tree Project v128 database and the EzBioCloud database. The results indicated that genera Marinobacter, Mangrovitalea, and Tamilnaduibacter formed a monophyletic clade with family Oleiphilaceae (Fig. 1), which was also shown by the GTDB phylogeny reconstructed from 120 ubiquitous single-copy
protein-coding genes (3), and these genera were distantly related to order Alteromonadales, implying that the monophyletic clade could represent a novel family. We refer to the clade as group 1 in the following text.

According to the phylogenetic tree based on 16S rRNA genes in group 1, Zooshikella, Endozoicomonadaceae, Pseudomonadaceae, and Cellvibrionales presented an indication of sharing a relatively close ancestor, whereas Pseudomonadaceae and Moraxellaceae were separated on different branches (Fig. 1). However, the topological structure of the branch with low bootstrap values (Fig. 1) indicated that the tree was unstable; therefore, it was also unclear what order group 1 belongs to in the tree.

**Evolutionary analysis based on the genomes.** We constructed a bac120 tree based on 120 concatenated ubiquitous single-copy proteins of bacteria (12) (Fig. 2; see also Fig. S1 in the supplemental material) from a total of 783 genomes (completeness >90% and contamination <5%) (Table S1) by using FastTree software according to the method described by Parks et al. (3). In 2018, the GTDB taxonomy proposed the transfer of the majority of the members of Oceanospirillales and Cellvibrionales and group 1 to Pseudomonadales, and Kangiellaceae was transferred to order Enterobacterales (3). Similarly, orders Pseudomonadales, Cellvibrionales, and Oceanospirillales and group 1 were also clustered on a branch with the support of the highest bootstrap value of 1.0, except for family Kangiellaceae in class Gammaproteobacteria, and according to Fig. 2a and Fig. S1, the family Kangiellaceae formed an independent branch at the order level that was different from GTDB taxonomy. In addition, the clade of family Moraxellaceae displayed the longest length in Fig. 2b and was located away from other families of order Pseudomonadales; Fig. 2b indicated they were partitioned by the branch of the order Cellvibrionales.

Then, we chose 257 reference genomes of Pseudomonadales, Cellvibrionales, Oceanospirillales, and group 1 for further analysis; the major information for these genomes is collected in Table S2. The pangenomes of the reference genomes were analyzed, and the results indicated that they shared 119 core genes, which were annotated according to the UniProt database (13); these proteins are mostly involved in DNA replication, transcription and translation, and ATP production. The sizes of the core and pangenomes were strongly dependent on the number of genomes analyzed, resulting in shrinking core genomes and expanding pangenomes with an increase in the depth of genome sampling (Fig. S2).

Subsequently, a core-genome-based phylogeny was reconstructed based on 119 concatenated single-copy core genes of the 257 genomes with optimal models by using the IQtree package. The results showed that the topological structure of the tree based on the core genome (Fig. 3 and Fig. S3) was highly similar to the bac120 tree (Fig. 2b and Fig. S1).

The amino acid identity (AAI) values between the 257 genomes were calculated as well because AAI values are used for prokaryotic taxonomic analyses (14). The AAI comparisons conducted by Luo et al. (15) indicated that related but different genera typically exhibit values ranging from 60% to 80%; thus, interfamilies typically exhibit values of less than 60%. In our study, the AAI values were clustered in a heatmap via the complete method of hclust (Fig. 4); we found that the interfamiliary AAI values were below 60% and that intrafamily AAI values were mostly greater than 60% (Fig. S4), consistent with the work of Luo et al. (15).

The order Oceanospirillales was paraphyletic (Fig. 2a and Fig. 3). The type genus Kangiella (16) of family Kangiellaceae (17) formed a stably separate clade, was positioned away from order Oceanospirillales, and was distinct from closely related orders Aeromonadales (18) and Thiotrichales (19) of class Gammaproteobacteria based on the bac120 tree with bootstrap value 100 (Fig. 2a and Fig. S1). In terms of physiological phenotypic characteristics, the genomic G+C content of family Kangiellaceae ranged from 40.1 to 44.4%, whereas that of Oceanospirillales ranged from 43.1 to 68.6%, revealing a significant difference (Wilcoxon test; \( P < 0.01 \)). Additionally, extracellular protein degradation and amino acid utilization are significant and prominent features.
**FIG 2** The unrooted maximum-likelihood tree was constructed by using FastTree with the WAG+CAT model based on 120 concatenated protein amino acid sequences of the 783 genomes. Each tip represents a species. (a) A pruned subtree from the unrooted maximum-likelihood tree. The bootstrap value of the backbone is displayed with a number. (b) Bootstrap values (from 0.9 to 1) are shown with filled circles. The tree was modified and visualized using the Interactive Tree of Life (iTOL 4.3) (itol.embl.de/).
of the type genus Kangiella of family Kangiellaceae due to the absence of a complete pathway for carbohydrate metabolism according to the description of Wang et al. (20); for instance, almost all members of genus Kangiella can hydrolyze casein and gelatin, while most of the members of order Oceanospirillales were negative for that (Table 1). In terms of fatty acid composition characteristics, the major fatty acid components of family Kangiellaceae are iso-C\textsubscript{15:0}, C\textsubscript{16:0} 10-methyl, and iso-C\textsubscript{11:0} 3-OH (21), which are obviously different from almost all other members of order Oceanospirillales, in which C\textsubscript{16:0} 10\text{Me}, C\textsubscript{16:1} 10\text{Me}, and/or C\textsubscript{16:1} \omega6c are the major fatty acid components. The major polar lipids of almost all members of family Kangiellaceae were phosphatidylglycerol (PG), phosphatidylethanolamine (PE), and phosphatidylmonomethylethanolamine (PME) (22), while those for the order Oceanospirillales were diphosphatidylglycerol (DPG), PE, and PG. Additionally, Q-8 was the predominant ubiquinone of family Kangiellaceae, while the predominant ubiquinone is Q-9 in all other members of order Oceanospirillales. A Manhattan-based principal-coordinate analysis (PCoA) of the gene presence and absence profile also showed that the members of the type genus Kangiella of family Kangiellaceae formed a cluster divided from other genera of order Oceanospirillales (Fig. S5). These evidences indicated that family Kangiellaceae should be reclassified as the novel order Kangiellales ord. nov., including the family Kangiellaceae, of which the type genus is Kangiella. Despite the GTDB classifying the order Kangiellales into the order Enterobacteriales (23, 24), however, the order Enterobacteriales was very large,
including some clades with excessive branch length based on the bac120 tree (Fig. S1 and Fig. 2a). Additionally, the major fatty acids of almost all members of order Enterobacterales were C\textsubscript{14:0}-\textit{c}, C\textsubscript{16:0}-\textit{c}, C\textsubscript{18:1} ω\textit{7c}, and C\textsubscript{17:0} (24), illustrating an obvious difference from order Kangiellales. Therefore, we inferred the classification was inaccurate in the GTDB.

Intriguingly, the shared gene blocks of Pseudomonadaceae (including genera Azotobacter, Pseudomonas, and Obliquimonas) and Moraxellaceae (including genera Acinetobacter, Alkanindiges, Moraxella, Perluclidibaca (25), and Psychrobacter) displayed obvious distinction, and the dendrogram of heatmap rows revealed that they formed an independent branch that was consistent with the topology of the bac120 core-genome tree (Fig. 3 and Fig. S3 and S6). Additionally, the type genus Ventosimonas of family Ventosimonadaceae formed a clade within family Pseudomonadaceae with a long branch length in the bac120 and core-genome tree (Fig. S1 and S5). We also observed that Alcanivoracaceae, Balneatrichiaceae, Halomonadaceae, Hahellaceae, Oleiphilaceae, Oceanospirillaceae, Saccharospirillaceae, Zooshikelleaceae, Pseudomonadaceae, Ventosimona-
monadaceae, Cellvibrionaceae, Haliaceae, Microbulbiferaceae, Portiococaceae, Spongibacteraceae, and group 1 shared more genes with each other than they shared with Moraxellaceae (Fig. S6). A Manhattan-based principal-coordinate analysis (PCoA) of the gene presence and absence profile also showed that the members of the type genus Pseudomonas of family Pseudomonadaceae and the type genus Moraxella of family Moraxellaceae clustered in different quadrants (Fig. S6). Additionally, the phenotypic characteristics between family Moraxellaceae and other families of order Pseudomonadales are notably different. First, almost all members of family Moraxellaceae contain C16:1ω9c as a major fatty acid component (26, 27), while the component was not detected in other families of Pseudomonadales (28). Second, the cell shapes of family Moraxellaceae are short rods or coccolid or coccal or may exhibit a characteristic multicellular micromorphology, and cells usually occur in pairs or short chains (29); however, other families of order Pseudomonadales have just one cell form that is rod-shaped, and cells usually occur in singles (Table 1). Third, the cells of family Moraxellaceae are nonmotile in liquid media and do not exhibit flagellation, but the other families of order Pseudomonadales typically have polar flagella (Table 1) (30). Fourth, except for some strains of Acinetobacter and Psychrobacter, no acid is produced from carbohydrates in family Moraxellaceae; however, the other families of order Pseudomonadales can produce acid from glucose and so on (30). In addition, the genome size and G+C% between Pseudomonadaceae and Moraxellaceae present significant differences (Wilcoxon test; P < 0.0001) (Fig. S7). In light of these results, it is proposed that Moraxella be reclassified as the type genus of Moraxellaceae ord. nov.

However, we found the family Moraxellaceae was paraphyletic and formed three separate clades, and the three clades clustered with genera Moraxella and Psychrobacter, Alkanindigis and Acinetobacter, and Perlucidibaca in the bac120 and core-genome tree, respectively (Fig. 3 and Fig. S1 and S5). The genus Perlucidibaca was positioned away from the other two clades in the core-genome tree (Fig. 3 and Fig. S6). The comparison of the AAI values, and shared gene blocks of the pangenome of intrafamily Moraxellaceae, also indicated that genus Perlucidibaca apparently differed from genera Acinetobacter, Alkanindigis, Moraxella, and Psychrobacter (Fig. 5 and Fig. S4). In addition, the phenotypic synapomorphies of Perlucidibaca obviously differed from those of Acinetobacter, Alkanindigis, Moraxella (25), and Psychrobacter. For example, the original description of Perlucidibaca (25) indicated that the members of this taxon are facultatively aerobic and that their anaerobic growth is similar to aerobic growth, whereas genera Acinetobacter, Alkanindigis, Psychrobacter, and Moraxella are strictly aerobic bacteria (25). Besides, the major fatty acids of genus Perlucidibaca are C16:0, C18:1ω7c, C16:1ω7c, and/or C16:1ω6c and C12:0 3-OH, while C16:1ω9c is a minor component (31), the major respiratory quinone of genus Perlucidibaca is Q-12 (32), while that of the major member of family Moraxella is Q-8, and additionally, the cells of genus Perlucidibaca usually occur as singles (Table 1) (31). In light of these results, it is proposed that

### Table 1: Phenotypic characteristics of Kangiellales, Moraxellales, Pseudomonadales, Enterobacterales, and Perlucidibacaceae

| Characteristic          | Kangiellales | Moraxellales | Pseudomonadales | Enterobacterales | Perlucidibacaceae |
|-------------------------|--------------|--------------|-----------------|------------------|------------------|
| Cell shape              | Rods         | Short rods, coccolid or coccal | Rods, spiral | Rods | Rods |
| G+C content (%)         | 40.1–44.4    | 38–48        | 43.1–68.6       | 22–60            | 55–65            |
| Fatty acids             | iso-C_{15:0}, C_{16:0} 10-methy, and iso-C_{11:0} 3-OH | C_{18:1}ω9c, C_{18:0} and C_{16:0}, and C_{16:1}ω6c/C_{16:1}ω7c | C_{16:0}ω7c, C_{16:0}ω7c and/or C_{16:1}ω6c | C_{16:0}ω7c, C_{16:1}ω6c, and C_{12:0}3-OH |
| Ubiquinone              | Q8           | Q8           | Q9              | NA               | Q12              |
| Flagellation            | +            | −            | +               | +                | +                |
| Hydrolysis of:          |              |              |                 |                  |                  |
| Casein                  | +            | NA           | V               | NA               | NA               |
| Gelatin                 | +            | NA           | V               | NA               | NA               |

*The data are from original isolation papers and/or Bergey’s Manual. References are as follows: Kangiellales, 17; Moraxellales, 29; Pseudomonadales, 30, 36; Enterobacterales, 30. The names of the orders or families were proposed in the study. NA, not applicable; +, present/tested positive; −, absent/tested negative; V, variable among strains.*
**Phylogenomic Analysis of Gammaproteobacteria**

**FIG 5** Heatmap showing the AAI values between genera Acinetobacter, Alkanindiges, Moraxella, Psychrobacter, and Perlucidibaca.

Perlutidibaca should be reclassified as the type genus of *Perlutidibaca* fam. nov., and it should be shifted out of the novel order *Moraxellales* and merged into order *Pseudomonadales*.

As shown in Fig. 3, group 1 formed a robust lineage and presented a close relationship with families *Hahellaceae* and *Olephilaceae* in the bac120 and core-genome tree (Fig. 2 and 3), and the GTDB taxonomy classified the branch as belonging to family *Olephilaceae*; however, we found that the G+C content of the genera *Marinobacter*, *Mangrovitalea*, and *Tamilnaduivibacter* ranged from 53.7 to 63.2% and that of family *Olephilaceae* ranged from 43.4 to 47.8%. Additionally, the AAI value between group 1, *Hahellaceae*, and family *Olephilaceae* was less than 60% (Fig. 4a). Furthermore, many species of group 1 can utilize various carbon sources including aliphatic and polycyclic aromatic hydrocarbons, acyclic isoprenoid compounds, and many sole carbon sources, while all strains of family *Olephilaceae* can use only aliphatic hydrocarbons and their derivatives as carbon sources for growth (33). Additionally, the cellular fatty acid patterns of most strains of group 1 were C<sub>16:0</sub>, C<sub>18:1ω9c</sub>, C<sub>16:1ω7c</sub>, and/or C<sub>16:1ω6c</sub> and C<sub>12:0</sub>-3-OH, while those of the *Olephilaceae* were C<sub>16:0</sub>, C<sub>16:1ω7c</sub>, and/or C<sub>16:1ω6c</sub> and C<sub>16:1ω9c</sub>. The major polar lipids of group 1 were dihexadecylglycerol (DPG), phosphatidylethanolamine (PE), and phosphatidylglycerol (PG), and those of the family *Olephilaceae* were PE, PG, and phosphatidylmethylethylamine (DME) (33); thus, these chemotaxonomic indices between group 1 and family *Olephilaceae* have certain differences. These results indicated that the two clades represented two different families, contradicting the GTDB taxonomy. Therefore, we designated the lineage as family *Marinobacteraceae* fam. nov. because the first valid name of the genus of this
clade was *Marinobacter*, first proposed in 1992 (34); the family comprises four genera: *Marinobacter*, *Mangrovitalea*, *Pseudohalomonas*, and *Tamilnaduibacter*. In addition, the species "*Marinobacter nanhaiticus*" (35) was transferred from genus *Marinobacter* to *Tamilnaduibacter* and named "*Tamilnaduibacter nanhaiticus*" comb. nov., which was suggested because the species "*Marinobacter nanhaiticus*" always forms a robust clade with genus *Tamilnaduibacter* in the bac120 tree and the core-genome tree (Fig. 2 and 3), and the AAI value between *Tamilnaduibacter salinus* and "*T. nanhaiticus*" was 70.99 and higher than those of *T. salinus* and other *Marinobacter* members (Fig. 4a).

*Oceanospirillaceae* (36) appeared to be polyphyletic and formed five highly supported subgroups in the bac120 and core-genome tree, implying that it should be split into five novel families. However, *Oceanospirillaceae* has been found to include 21 genera (https://www.ezbiocloud.net/taxonomy?tn=Oceanospirillaceae&depth=2) thus far, whereas only 15 genera were obtained in the core-genome tree. Hence, additional genome sequences need to be made available if these new families are to be proposed.

We also observed that the family *Endozoicomonadaceae* and the genus *Zooshikella* formed a robust clade with family *Pseudomonadaceae*, indicating that the genus *Zooshikella* presents the closest evolutionary relationship with family *Endozoicomonadaceae* (Fig. 2 and 3 and Fig. S1). The family *Endozoicomonadaceae* and genus *Zooshikella* shared more genes with each other than they shared with other families (Fig. S6). Additionally, the phenotypical synapomorphies of *Zooshikella* and family *Endozoicomonadaceae* are not obviously different; for example, they have similar major fatty acid components including C16:0, C18:1 ω7c, C16:1 ω7c, and/or C16:1 ω6c, the major quinone was Q-9, and almost all members of genus *Zooshikella* and family *Endozoicomonadaceae* were mesophilic (37, 38), except that genus *Endozoicomonas* also included C10:0 3-OH as a major hydroxyl fatty acid component; further, PE, PG, phosphatidylserine (PS), and DPG are present in the polar lipid pattern of family *Endozoicomonadaceae*, while the genus *Zooshikella* shows DPG, PE, and PG, except that PS was not detected (Table 2) (8, 39). In light of these results, the family *Endozoicomonadaceae* should be transferred to a novel family and named *Zooshikellaceae* fam. nov. because the genus *Zooshikella* was first proposed in 2003 within the clade (37), and the family comprises four genera: *Endozoicomonas*, *Kistimonas*, *Parendozoicomonas* (8), and *Zooshikella*. The family name has been given already (https://gtdb.ecogenomic.org/searches?q=%25Zooshikella%25&s=al), but the classification is different from GTDB in that family *Zooshikellaceae* includes only the genus *Zooshikella*. This proposal does not conflict with the 16S rRNA gene tree provided in the initial description of the genus, despite the tree being poorly resolved.

As indicated by the results presented in Fig. S4 and Fig. 4b, the AAI values between *Alcanivoracaceae*, *Balneatrichaceae*, *Halomonadaceae*, *Hahellaceae*, *Oleiphilaceae*, *Oceanospirillaceae*, *Saccharospirillaceae*, *Zooshikellaceae*, *Pseudomonadaceae*, *Perlucidibacaceae*, *Cellvibrionaceae*, *Halieaceae*, *Microbulbiferaceae*, *Portiococaceae*, *Spongibacteraceae*, *Ventosimonadaceae*, and *Marinobacteraceae* were significantly higher than those between the above taxa and *Moraxellaceae* (Wilcoxon test P < 0.0001). Together, the results...
from this study indicated that 17 families shared a common ancestor at the order level; therefore, these families were merged into one order, Pseudomonadales, consistent with the designation of the GTDB (3) because the first species proposed was Pseudomonas aeruginosa in 1900. The closest order to Pseudomonadales is Moraxellales. A possible AAI threshold of 54 was proposed to differentiate among orders according to Fig. 4b, but it needs further study to be used in other complex phyla such as Firmicutes or Bacteroidetes.

**DISCUSSION**

**Emendation of the order Pseudomonadales.** In this study, we proved that two novel families, Marinobacteraceae and Perlucidibacteraceae; families Alcanivoracaceae (40), Balneatrichiaceae, Halomonadaceae (41), Hahellaceae (42), Oleiphilaceae (33), Oceanospirillaceae, Saccharospirillaceae (43), and Zooshikellaceae of order Oceanospirillales; Pseudomonadales and Ventosimonadaceae (28) of order Pseudomonadales; and Cellvibrionaceae, Halieaceae, Microbulbiferaceae, Porticocaceae, and Spongiibacteraceae (5) of order Cellvibrionales shared a relatively recent ancestor and formed a robust branch based on two typical phylogenomic tree and AAI values in class Gammaproteobacteria. Therefore, orders Oceanospirillales, Cellvibrionales, and Pseudomonadales were merged into the single order Pseudomonadales with the exception of families Moraxellaceae and Kangiellaceae, including 19 families in the partial taxonomic reconstruction of class Gammaproteobacteria.

Almost all members of order Pseudomonadales are mesophilic, the major fatty acid components are C16:0, C16:1ω7c and/or C16:1ω6c, and C18:1ω7c, and the major respiratory quinone is Q-9.

Type genus: Pseudomonas; class: Gammaproteobacteria.

**Description of Moraxellales ord. nov. Moraxellales (Mo.ra.xel.la’les. N.L. fem. dim. n. Moraxella type genus of the order; suff. -ales, ending denoting an order; N.L. fem. pl. n. Moraxellales, the Moraxella order).**

The description is the same as that for family Moraxellaceae (29). Type genus: Moraxella; class: Gammaproteobacteria.

**Description of Kangiellales ord. nov. Kangiellales (Kan.gi.el.la’les. N.L. fem. dim. n. Kangiella, type genus of the order; suff. -ales, ending denoting an order; N.L. fem. pl. n. Kangiellales, the Kangiella order).**

The description is the same as that for family Kangiellaceae (17). Type genus: Kangiella; class: Gammaproteobacteria.

**Description of Marinobacteraceae fam. nov. Marinobacteraceae (Mar.i.no.bac.te-ra’ce.ae. N.L. masc. n. Marinobacter, type genus of the family; -aceae, suff. ending denoting a family; N.L. fem. pl. n. Marinobacteraceae, the Marinobacter family).**

The family belongs to order Oceanospirillales, class Gammaproteobacteria, and mainly consists of bacteria isolated from the sediments of marine environments. The cellular fatty acid patterns of most strains are C16:0, C18:1ω9c, summed features 3 and C12:0 3-OH. The G+C content of the genomic DNA ranges from 53.7 to 63.2%. At present, the family comprises genera Marinobacter, Mangrovitalea, Pseudohalomonas, and Tamilnaduibacter. The definition of the family relies mainly on the construction of phylogenetic relationships based on 16S rRNA gene sequences and phylogenomic relationships based on core genomes and concatenated 120 ubiquitous single-copy protein sequences.

Type genus: Marinobacter; order: Pseudomonadales.
Description of Perlucidibacaceae fam. nov. *Perlucidibacaceae* (Per.lu.ci.di.ba.ca’ce.ae. N.L. fem. n. *Perlucidibaca*, type genus of the family; -aceae, suff. ending denoting a family; N.L. fem. pl. n. *Perlucidibacaceae*, the *Perlucidibaca* family).

The description is the same as for genus *Perlucidibaca* (25, 32).

Type genus: *Perlucidibaca*; order: Pseudomonadales.

Description of Zooshikellaceae fam. nov. *Zooshikellaceae* (Zoo.shi’ke.la’ce.ae. N.L. fem. dim. n. *Zooshikella*, type genus of the family; -aceae, suff. ending denoting a family; N.L. fem. pl. n. *Zooshikellaceae*, the *Zooshikella* family).

The major fatty acid components were \( C_{16:0} \), \( C_{16:1}\omega7c \) and/or \( C_{16:1}\omega6c \), and \( C_{18:1}\omega7c \); the major quinone was Q-9; PE, PG, PS, and DPG are present in the major polar lipid pattern; and almost all members of the family were mesophilic. At present, the family comprises genera *Endozoicomonas*, *Kistimonas*, *Parenzoicomonas*, and *Zooshikella*. Members of this family form a stable clade in the reconstructed phylogenetic tree based on 16S rRNA gene sequences and the phylogenomic tree based on core genomes and concatenated 120 ubiquitous single-copy protein sequences. The type genus of the family is *Zooshikella*.

MATERIALS AND METHODS

SSU-rRNA-based phylogeny. Reference sequences of class *Gammaproteobacteria* with valid published names were downloaded from the SILVA Living Tree Project v128 database and the EzBioCloud database. The package MAFFT v7.402 was used for sequence alignment, and identical sequences were deleted by using RAxML before constructing the tree. Phylogenetic trees based on data sets of 16S rRNA gene sequences were constructed using RAxML (44) by applying the -f a, -p 12345, -x 12345, -# 1,000 or 200, and -m GTRGAMMA parameters. The 16S rRNA gene identity values were obtained through a rate heterogeneity (46) (+GAMMA) in FastTree to a concatenated alignment of 120 ubiquitous single-copy proteins (12) with the GTDB -tk tool (3). These trees were modified and visualized using the Interactive Tree of Life (ITOL) (itol.embl.de/).

Pangenome and phylogenomic analysis. The sequences were annotated using Prokka v1.12 (47). The pangenome was estimated using the rapid large-scale prokaryotic pangenome analysis (Roary v3.11.2) tool (48) with parameters -i 50 -cd 99. Briefly, the annotated genes from all 257 representative reference genomes of orders *Oceanospirillales*, *Cellvibrionales*, and *Pseudomonadales* and genera *Tamilnaduibacter*, *Mangrovitalea*, and *Marinobacter* (Table S2) were first filtered to remove partial sequences and iteratively preclustered with CD-HIT. These procedures resulted in a substantially reduced set of protein sequences. An all-against-all comparison of the reduced sequences with 50% sequence identity was performed with BLASTP. The sequences were then clustered with Markov clustering algorithm (MCL), and the preclustering results from CD-HIT were finally merged together with the results of MCL. Homologous clusters were divided into core, accessory, and unique genomes. The core genome comprised genes shared within at least 99% of the genomes. The cumulative sizes of the pangenome and core genome were calculated by selecting genomes with replacement in random order 500 times and then calculating the mean size of each sampling point.

A pangenome matrix was generated based on the presence or absence of all genetic loci in each individual genome produced by Roary. We selected the top 6,000 genes of the matrix to produce the heatmap with the heatmap package, species were clustered, and PCoA was performed based on the presence and absence of orthologs according to the Manhattan distance by using hclust in R.

Phylogenomic analysis of 119 concatenated single-copy core genes. The phylogenomic tree was generated based on the concatenated single-copy core genes. The core-genome sequences were accurately aligned with MAFFT v7.402. The resulting multiple sequence alignment length was 121,975 bp and retained 37,526 bp after trimming with Gblocks 0.91b (49) with default parameters. A phylogenomic tree was inferred using the IQtree package to search optimal models and further verify the morphologies and topologies of the phylogenomic tree (50) using the command -bb 1000 -m MFP -+ MERGE +R, and the RAxML program was applied with the parameters -f a, -p 12345, -x 12345, -# 200, and -m GTRGAMMAI (50), based on trimmed concatenated single-copy core genes.

Whole-genome relatedness indices. The AAI is the mean amino acid identity of orthologous genes. To validate our taxonomic proposals, we performed AAI comparisons between these genomes. The AAI indices were deduced from pairwise conserved comparisons of coding proteins and calculated using
Supplemental material is available online only.

**FIG S1** PDF file, 1.5 MB.

**FIG S2** DOCX file, 0.4 MB.

**FIG S3** PDF file, 0.4 MB.

**FIG S4** PDF file, 1.2 MB.

**FIG S5** PDF file, 0.7 MB.

**FIG S6** TIF file, 2.7 MB.

**FIG S7** DOCX file, 0.1 MB.

**TABLE S1** XLSX file, 0.1 MB.

**TABLE S2** XLSX file, 0.05 MB.

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We declare that we have no conflict of interest.

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