

Geographic Impact on Genomic Divergence as Revealed by Comparison of Nine Citromicrobial Genomes

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ABSTRACT

Aerobic anoxygenic phototrophic bacteria (AAPB) are thought to be important players in oceanic carbon and energy cycling in the euphotic zone of the ocean. The genus *Citromicrobium*, widely found in oligotrophic oceans, is a member of marine alpha-proteobacterial AAPB. Nine *Citromicrobium* strains isolated from the South China Sea, the Mediterranean Sea, or the tropical South Atlantic Ocean were found to harbor identical 16S rRNA sequences. The sequencing of their genomes revealed high synteny in major regions. Nine genetic islands (GIs) involved mainly in type IV secretion systems, flagellar biosynthesis, prophage, and integrative conjugative elements, were identified by a fine-scale comparative genomics analysis. These GIs played significant roles in genomic evolution and divergence. Interestingly, the coexistence of two different photosynthetic gene clusters (PGCs) was not only found in the analyzed genomes but also confirmed, for the first time, to our knowledge, in environmental samples. The prevalence of the coexistence of two different PGCs may suggest an adaptation mechanism for *Citromicrobium* members to survive in the oceans. Comparison of genomic characteristics (e.g., GIs, average nucleotide identity [ANI], single-nucleotide polymorphisms [SNPs], and phylogeny) revealed that strains within a marine region shared a similar evolutionary history that was distinct from that of strains isolated from other regions (South China Sea versus Mediterranean Sea). Geographic differences are partly responsible for driving the observed genomic divergences and allow microbes to evolve through local adaptation. Three *Citromicrobium* strains isolated from the Mediterranean Sea diverged millions of years ago from other strains and evolved into a novel group.

IMPORTANCE

Aerobic anoxygenic phototrophic bacteria are a widespread functional group in the upper ocean, and their abundance could be up to 15% of the total heterotrophic bacteria. To date, a great number of studies display AAPB biogeographic distribution patterns in the ocean; however, little is understood about the geographic isolation impact on the genome divergence of marine AAPB. In this study, we compare nine *Citromicrobium* genomes of strains that have identical 16S rRNA sequences but different ocean origins. Our results reveal that strains isolated from the same marine region share a similar evolutionary history that is distinct from that of strains isolated from other regions. These *Citromicrobium* strains diverged millions of years ago. In addition, the coexistence of two different PGCs is prevalent in the analyzed genomes and in environmental samples.

Aerobic anoxygenic phototrophic bacteria (AAPB) are a widespread functional microbial group in the euphotic zone of the ocean and are thought to play important roles in the cycling of marine carbon and energy (1–10). AAPB can harvest light using bacteriochlorophyll *a* (BChl *a*) and various carotenoids to form a transmembrane proton gradient for the generation of ATP (1, 11). The photosynthetic process is performed by a series of photosynthetic operons, including *bch*, *crt*, *puf*, *puh*, and some regulatory genes, forming a highly conserved 40- to 50-kb photosynthetic gene cluster (PGC) (12, 13). The heart of anoxygenic photosynthesis is the reaction center, encoded by the *puf* and *puh* operons (14).

The genus *Citromicrobium*, belonging to the order *Sphingomonadales* in the class *Alphaproteobacteria*, is a member of the marine AAPB (15–17). Since the initial isolation of the type species, *Citromicrobium bathyomarimum* strain JF-1, from deep-sea hydrothermal vent plume waters in the Juan de Fuca Ridge (Pacific Ocean), dozens of *Citromicrobium* strains were isolated from a depth of 500 to 2,379 m near this region (17, 18). Genomic analyses showed that members of the genera *Citromicrobium* and *Erythrobacter* generally contained the shortest and simplest PGC

structures among all known AAPB (16). Analysis of the *C. bathyomarimum* JL354 genome led to the discovery that two different PGCs could coexist in one bacterium, with one complete cluster and the other cluster incomplete (15, 16). Horizontal gene transfer (HGT) was detected to mediate the incomplete PGC acquisition, and multiple mechanisms mediating HGT were also found through studying its genome, including a gene transfer agent (GTA),

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TABLE 1 Genome information for the nine strains

Strain	Genome size (Mb)	No. of contigs	G+C content (%)	No. of genes	No. of CDSs ^a	No. of COGs	No. of tRNAs	Sequencing coverage (×)	Isolation source	Accession no.
JL31	3.16	22	65.1	3,092	2,960	1,969	45	180	South China Sea	LAIH00000000
JL1351	3.16	17	65.1	3,090	2,961	1,981	45	155	South China Sea	LAPR00000000
WPS32	3.16	16	64.9	3,056	2,925	1,934	44	250	South China Sea	LAPS00000000
JL477	3.26	1	65.0	3,168	3,027	2,004	45	220	South China Sea	CP011344
JL354	3.27	68	65.0	3,208	3,137	2,010	45	26	South China Sea	ADAE00000000
JL2201	3.27	22	65.1	3,250	3,105	1,975	45	245	South Atlantic Ocean	LARQ00000000
RCC1878	3.28	14	64.8	3,194	3,061	1,966	45	205	Mediterranean Sea	LBLZ00000000
RCC1885	3.28	14	64.8	3,197	3,063	1,975	45	190	Mediterranean Sea	LBLY00000000
RCC1897	3.28	17	64.8	3,192	3,113	1,978	45	440	Mediterranean Sea	LUGI01000000

^a CDSs, coding sequences.

prophage, integrative conjugative element (ICE), and the type IV secretion system (T4SS). *Citromicrobium* species may benefit from obtaining genes by HGT to compete and survive in natural environments. Additionally, genes encoding xanthorhodopsin, which is a light-driven proton pump like bacteriorhodopsin (19–21), but more effective at collecting light, are also found in citromicrobial genomes (22, 23).

A recent pyrosequencing analysis of *pufM* genes showed that *Citromicrobium*-like AAPB were mainly distributed in the oligotrophic ocean and were relatively more abundant in the upper twilight zone (150 to 200 m depth) than in the subsurface waters (5 m and 25 m) of the western Pacific Ocean (24). However, in this study, no sequences belonging to the incomplete PGC were detected, although the *Citromicrobium*-like *pufM* gene belonging to the complete PGC showed a high relative abundance.

Fortunately, a great number of citromicrobial strains have been isolated from the Mediterranean Sea, the South China Sea, and the South Atlantic Ocean (16, 25). The Mediterranean Sea is an almost completely enclosed sea connected with the eastern North Atlantic Ocean by the narrow Strait of Gibraltar. The South China Sea is the largest marginal sea in the western Pacific Ocean, extending from subtropical to tropical zones. The sampled area in the South Atlantic Ocean (13.857°S, 26.018°W) is a region of tropical open oceans. Although a great number of studies showed that microbes displayed biogeographic patterns in the ocean (26, 27), little is understood about the geographic isolation impact on the genome divergence of marine microbes.

The aims of this study were to (i) illustrate the evolutionary divergence of *Citromicrobium* genomes with identical 16S rRNA sequences but different geographical origins and (ii) demonstrate the prevalence of the coexistence of two PGCs in these strains and within the marine environment.

MATERIALS AND METHODS

Isolation of *Citromicrobium* strains. *Citromicrobium* strains JL31 (24.458°N, 118.247°E), JL354 (21.684°N, 112.918°E), JL477 (22.167°N, 115.153°E), JL1351 (17.994°N, 120.287°E), and WPS32 (17.000°N, 115.000°E) were isolated from euphotic waters from the South China Sea on plates containing rich organic (RO) medium (Table 1) (17). Strain JL2201 was isolated from South Atlantic surface water (13.857°S, 26.018°W) on an RO medium plate (Table 1). Strains RCC1878 and RCC1885 were isolated from the Ionian Sea (at the middle of the Mediterranean Sea) surface water (34.133°N, 18.450°E) on two low-strength agar medium plates, and strain RCC1897 was isolated from western Mediterranean Sea (38.633°N, 7.917°E) water (Table 1) (25).

Genome sequencing and assembly. Whole-genome sequencing of *Citromicrobium* sp. strain JL354 was performed by 454 pyrosequencing, as previously reported (15, 16). The genomes of JL31, JL477, JL1351, JL2201, WPS32, RCC1878, RCC1885, and RCC1897 were obtained by an Illumina MiSeq system. Paired-end reads of average 250-bp length were assembled using the Velvet software (version 2.8) (28). The sequencing coverages ranged from 155× (JL1351) to 440× (RCC1897). The genome of strain JL477 has been completed, and the other seven genomes each possessed 14 to 22 contigs (Table 1).

Gene prediction and annotation. The open reading frames (ORFs) were analyzed using a combination of Glimmer 3.02 (29) and GeneMark (30, 31). All predicted ORFs were then annotated using the NCBI Prokaryotic Genome Annotation Pipeline (32) and Rapid Annotations using Subsystems Technology (RAST) (33). rRNA identification was performed with the RNAMmer 1.2 software (34), and tRNAscan-SE (version 1.21) was used to identify the tRNA genes (35).

The genomic average nucleotide identity (ANI) was calculated by the JSpecies Online Service (<http://jspecies.ribohost.com/jspeciesws>) (36).

Core genome and pangenome analyses. Orthologous clusters (OCs) were assigned by grouping all protein sequences from the nine genomes using OrthoMCL based on their sequence similarity (E value <10⁻⁵, >50% coverage) (37). The core and pangenomes were analyzed according to the method described by Tettelin et al. (38). The functional proteins were classified by comparison with the COG (Clusters of Orthologous Groups) databases (39).

SNP discovery. Single-nucleotide polymorphisms (SNPs) were detected by sequence comparisons of the 9 *Citromicrobium* genomes using MUMmer (40). Because 8 out of 9 genomes were draft genomes, the positions of SNPs from all genomes relative to the sequence of strain JL477 were recorded. Paralogous genes and repeated regions were removed from our analysis. The synonymous SNPs of coding regions were used to roughly estimate the pairwise strain divergence time (41).

Sequence comparison. The genome of JL477 was compared to the other eight citromicrobial genomes *in silico* using the BLAST Ring Image Generator (BRIG) software (42). Regions with nucleotide sequence similarity above 70% are shown on the map. Nine genetic islands (GIs) were identified from the comparative map. The upstream and downstream regions of each GI were then retrieved and manually searched for the presence of conserved regions or signature genes (such as tRNA). Some GIs and their flanking genes from different genomes were chosen for pairwise comparison (see Table S1 in the supplemental material). Although there were eight draft genomes with a number of contigs involved in analyses, the completeness for each genome was more than 99% as a result of the high genome sequencing coverage. Gaps between contigs usually were intergenic regions or did not contain more than three genes (see Table S1). All gene losses (especially more than a 10-kb fragment) occurred inside contigs but not between contigs (see Table S1).

Phylogenetic analysis. All *pufM* gene sequences collected from the NCBI database, *Citromicrobium* genomes, and environmental samples were aligned using Clustal X, and phylogenetic trees were constructed using the maximum likelihood and neighbor-joining algorithms of the MEGA 6 software (43). The phylogenetic trees were supported by bootstrap for resampling test, with 100 and 1,000 replicates for the maximum likelihood and neighbor-joining algorithms, respectively.

Environmental sample collection. Seawater samples were collected on board during a western Pacific Ocean cruise in July 2011. Seawater was collected at two stations (P3 [129.00°E, 14.00°N] and P10 [130.00°E, 2.00°N]) and five depths (5 m, 25 m, 75 m, 150 m, and 200 m). For each sample, 2 to 3 liters of seawater was prefiltered through a 20- μ m-pore filter, and the microorganisms were then collected onto 0.22- μ m-pore polycarbonate filters (Millipore). Nucleic acids were extracted using hot sodium dodecyl sulfate, phenol, chloroform, and isoamyl alcohol (24, 44). The high-quality DNA was stored at -20°C for future use.

Sequence generation and processing. Considering that previous primers (2, 45) had five mismatches with *pufM* sequences belonging to the incomplete PGC in *Citromicrobium*, the following primer set was used to amplify the environmental DNA: *pufM*_Citro forward (5'-TACGGSAAY TTSTWCTAC-3') and *pufM*_Citro reverse (5'-GCRAACCAGYANGCC CA-3'). High-throughput sequencing of the *pufM* gene (~240 bp) was performed using Illumina MiSeq technology. The generated high-throughput sequence data were processed as described in Zheng et al. (24). Briefly, after quality control, all sequences were grouped into operational taxonomic units (OTUs) using a 6% cutoff. One representative sequence for each OTU was chosen to perform local BLAST against our *pufM* sequence database (for details, see Zheng et al. [24]).

Accession number(s). The complete JL477 genome sequence is available under GenBank accession no. CP011344. Whole-genome sequences of strains JL31, JL354, JL1351, WPS32, JL2201, RCC1878, RCC1885, and RCC1897 are available under GenBank accession numbers LAIH00000000, ADAE00000000, LAPR00000000, LAPS00000000, LARQ00000000, LBLZ00000000, LBLY00000000, and LUGI01000000, respectively. All *pufM* gene representative sequences of *Citromicrobium*-related OTUs and each OTU's abundance are shown in Table S2 in the supplemental material.

RESULTS AND DISCUSSION

Overview of nine *Citromicrobium* strains. Nine *Citromicrobium* strains were used to perform comparative genome analyses, which were isolated from the South China Sea (strains JL31, JL354, JL477, JL1351, and WPS32), the Mediterranean Sea (strains RCC1878, RCC1885, and RCC1897), and the tropical South Atlantic Ocean (strain JL2201) (Table 1). Although 1 to 2 base mismatches were found in the 16S rRNA sequences collected from the GenBank database (strains JL354, RCC1878, RCC1885, and RCC1897) or after Sanger sequencing (the other five strains), the 16S rRNA sequences (1,442 bp, one copy per genome) extracted from the nine genomes with high sequencing coverage were identical. The Sanger sequencing might induce some biases or mismatches during the 16S rRNA amplification and sequencing PCR steps.

The nine genomes displayed highly similar genomic characteristics in terms of genome size (from 3.16 to 3.28 Mb), G+C content (from 64.8 to 65.1%), gene number (from 3,056 to 3,250), COGs (from 1,934 to 2,010), and tRNA number (44 or 45) (Table 1).

Pan- and core genomes of the *Citromicrobium* strains. Based on the total set of genes from the 9 sequenced strains, the *Citromicrobium* pangenome consisted of 3,546 predicted orthologous clusters (OCs), with a conserved core genome of 2,691 OCs. The cumulative length of the core genome was approximately 2.50

Mbp, which covered >75% of each genome. The flexible genome comprises 853 OCs, including 362 unique OCs and 490 OCs shared by more than one strain but not all strains.

The core genome is mainly involved in housekeeping functions and central metabolism, from the Calvin cycle to the tricarboxylic acid (TCA) cycle. Approximately 80% of the predicted core genes are assigned to COG functional categories. The predicted core genes contain a relatively high percentage of genes assigned to the following COG categories: general function prediction only (R), amino acid transport and metabolism (E), unknown function (S), translation, ribosomal structure, and biogenesis (J), energy production and conversion (C), lipid transport and metabolism (I), cell wall/membrane/envelope biogenesis (M), and inorganic ion transport and metabolism (P). Due to a larger fraction of putative or hypothetical genes, only 36.8% of the flexible genes are assigned to COG functional categories. Compared to the core genes, they include an overrepresentation of genes assigned to the following COG categories: general function prediction only (R), lipid transport and metabolism (I), replication, recombination and repair (L), intracellular trafficking, secretion, and vesicular transport (U), secondary metabolite biosynthesis, transport, and catabolism (Q), cell motility (N), transcription (K), and defense mechanisms (V). Most of flexible genes were sourced from the genetic island regions.

Genomic ANI. The average nucleotide identity (ANI) shared between genome pairs ranged from 95.96% to 100% (see Table S3 in the supplemental material). Five genomes, JL31, JL1351, JL354, JL477, and JL2201, share more than 99.5% ANI between them but share lower values with three RCC strains (from 95.96 to 96.47%) and WPS32 (from 96.39 to 96.44%). The three RCC strains had the lowest percentages of all the genomes involved in pairwise comparisons (see Table S3).

Genome pairs JL31 and JL1351, JL31 and JL2201, JL1351 and JL2201, JL477 and JL354, RCC1878 and RCC1885, RCC1878 and RCC1897, and RCC1885 and RCC1897 showed strikingly high ANI (almost 100%) (see Table S3 in the supplemental material). Among them, genome pairs JL31 and JL1351, JL477 and JL354, RCC1878 and RCC1885, RCC1878 and RCC1897, and RCC1885 and RCC1897 showed high genomic percentages (>98.0%) involved in pairwise comparisons (see Table S3), indicating closer evolutionary relationships with each other.

The proposed cutoff of the ANI between two genome sequences for a species boundary is 95 to 96% (36). Five JL strains share 95.96 to ~96.47% ANI with three RCC strains; therefore, three RCC strains isolated from the almost-enclosed Mediterranean Sea have a long divergence history from other strains and tend to evolve into a novel group. However, all nine *Citromicrobium* strains have identical 16S rRNA sequences. This emphasizes that traditional diversity studies, which classify sequences into operational taxonomic units based on the nucleotide sequence similarity, underestimate real environmental microbial information. The classification and diversity results based on environmental 16S rRNA could not link to *in situ* microbial functions (46, 47).

Comparison of nine genomes. A comparison of all nine genomes (JL477 versus the others) showed high synteny of major regions and a significantly high level of sequence conservation (Fig. 1; see Table S1 in the supplemental material). DNA fragment insertions and deletions were detected in a genome comparison (one versus the other eight) (see Table S1).

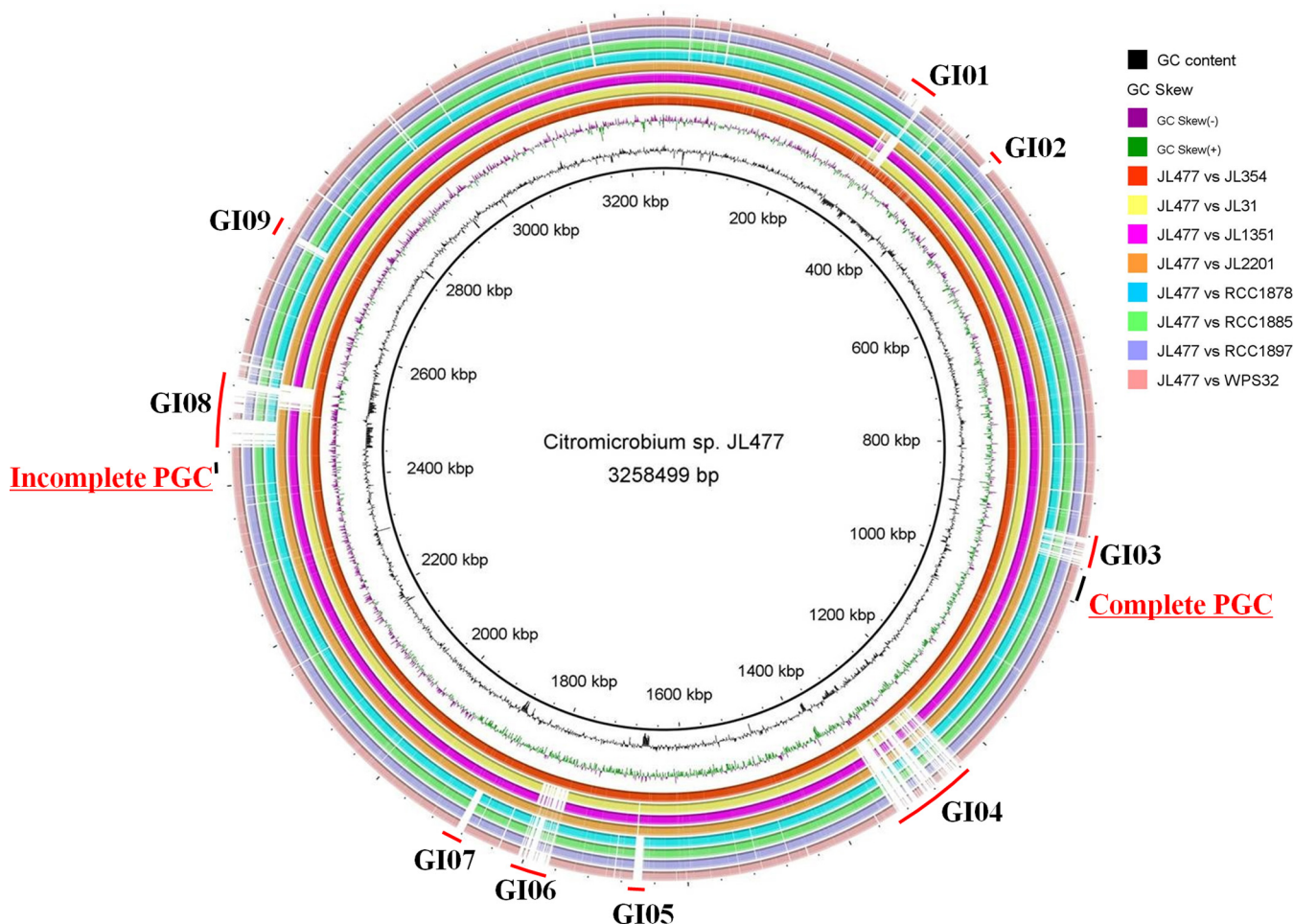


FIG 1 Whole-genome map of *Citromicrobium* sp. JL477 compared with other eight *Citromicrobium* genomes. From the inner to outer circles: G+C content plot with a gray circle representing 50%, GC skew plot, *Citromicrobium* sp. JL354, *Citromicrobium* sp. JL31, *Citromicrobium* sp. JL1351, *Citromicrobium* sp. JL2201, *Citromicrobium* sp. RCC1878, *Citromicrobium* sp. RCC1885, RCC1897, and *Citromicrobium* sp. WPS32. Genomic island regions are indicated with red line on the outermost circle from GI01 to GI09. The complete and incomplete PGCs are labeled by the black line on the outermost circle.

Horizontal gene transfer (HGT) is common in bacteria, contributing to the genomic plasticity and possibly to environmental adaptation (48). To better understand genome plasticity and unique genome characteristics, nine specific genomic regions larger than 10 kb (except GI07, with 9.7 kb) in size were identified based on the comparative genome map (Fig. 1). They were absent or different in the corresponding regions of the eight other genomes (JL477 versus the others) and are here designated genomic islands (GIs) GI01 to GI09 (Fig. 1). These nine GIs each contribute approximately 12% of the genome size. Almost all the GIs were regarded as originating from HGT (gene gain and loss) mediated by the transposases, integrases, and conjugal transfer systems, and five of them (GI03, GI04, GI07, GI08, and GI09) were flanked by a tRNA gene. Previous studies showed that GIs frequently originate from integration events associated with tRNA-encoding genes (49–51). These nine GIs are scattered throughout the genomes, and their general features and sequence information are summarized in Table 2.

GI01 and T4SS. GI01 mainly consists of a *trb* gene cluster, *trbBCDEJLFGI*, which is probably involved in the conjugal transfer of mobile genetic elements mediated by the type IV secretion

system (T4SS) (51–53). In the genomes of strains JL354, RCC1878, RCC1885, and RCC1897, the highly homologous gene cluster (here denoted T4SS-I) is detected at the same chromosome position as in strain JL477 (Fig. 2A), which is flanked by putative genes for T4SS protease (*traF*), relaxase (*virD2*), and ATPase for transfer DNA (T-DNA) transfer (*virD4*) in the upstream regions and for genes associated with amino acids metabolism, transmembrane transport, and transcriptional regulation in the downstream region.

Interestingly, the same flanking gene organization was found in the genomes of strains JL31, JL1351, and JL2201 but with gene fragment loss in the middle of two genes (genes 7 and 8) (Fig. 2B). There is a 770-bp deletion in the latter part of gene 7 and a 670-bp deletion in the front part of gene 8, which indicates a large DNA fragment deletion in these three genomes.

In the genomes of strains JL31, JL1351, and JL2201, a *trb* gene cluster (here denoted T4SS-II) that is located in an integrase-mediated foreign DNA fragment was also found (Fig. 2C). The average nucleotide identity between T4SS-I and T4SS-II was low (<50%), indicating that the T4SS-II gene cluster was acquired via HGT mediated by the integrase. In addition, a three-gene cluster

TABLE 2 Detailed information for the nine GIs

GI	Size (kb)	G+C content (%)	tRNA(s)	No. of genes	No. of transposases and integrases	No. of hypothetical proteins	Predicted function(s)
01	36.7	60.66		34	0	10	T4SS
02	11.4	66.80		16	1	4	GTA
03	35.5	65.13	tRNA-Ser-GGA	36	1	10	Flagellar biosynthesis
04	101.1	62.14	tRNA-Ser-GCT	88	1	18	Choline and betaine uptake, glycerolipid and glycerophospholipid metabolism, fatty acid metabolism, pyruvate metabolism
05	11.3	52.08		0		0	Unknown
06	38.1	65.98		58	2	30	Prophage
07	9.7	54.39	tRNA-Pro-TGG, tRNA-Met-CAT	4	1	2	Unknown
08	113.4	60.62	tRNA-Met-CAT	98	1	21	ICE
09	11.5	65.76	tRNA-Ser-CGA	3	0	0	Flagellar hook-length control

coding for the type I restriction-modification system (TIRS), which protects microbes from the foreign DNA (e.g., bacteriophage), was detected in the integrated DNA fragment. The inserted sequence is adjacent to the tRNA-CCG gene in these three

genomes. In the genomes of strains RCC1878, RCC1885, RCC1897, and WPS32, HGT derived from integration events also occurred adjacent to the tRNA-CCG gene (Fig. 2D and E). However, different inserted gene clusters were found in these three

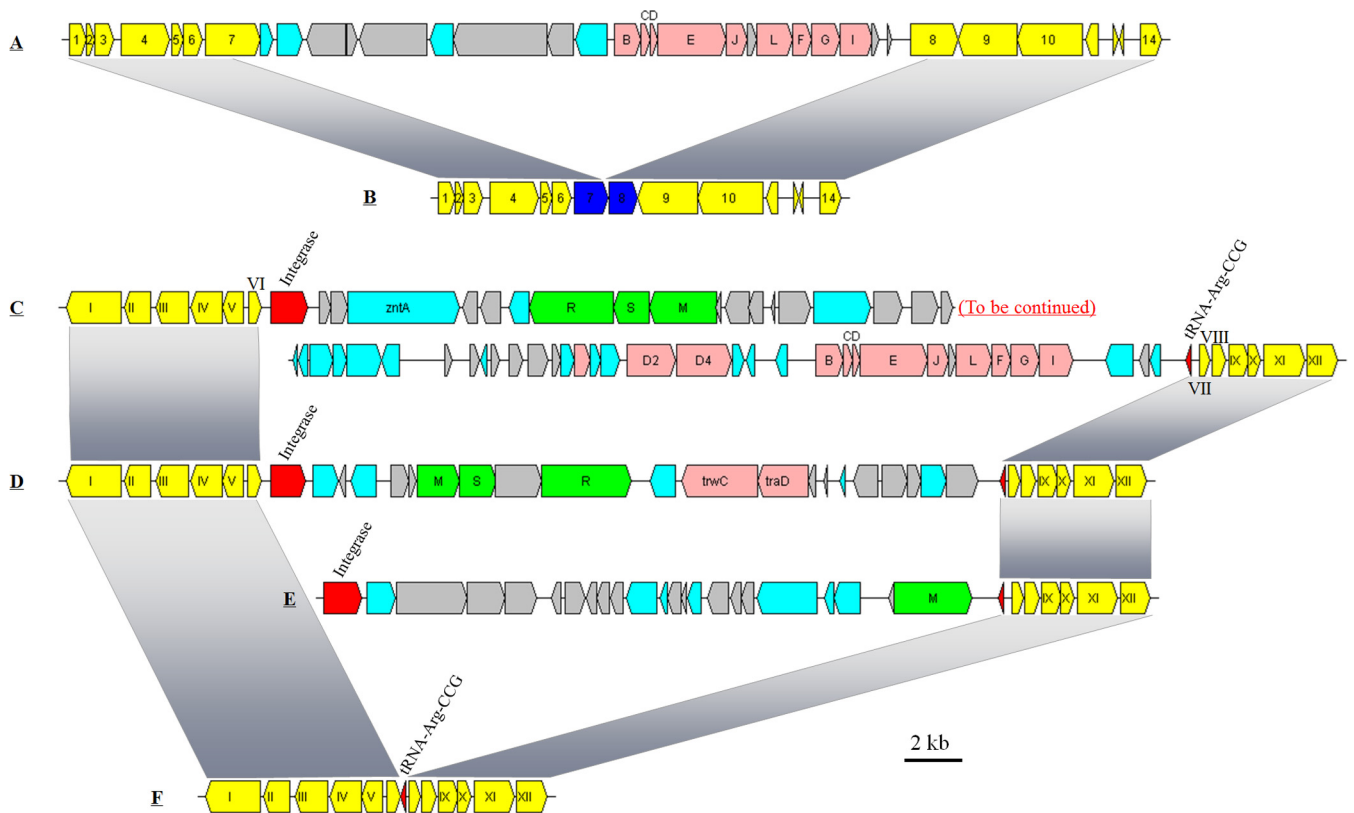


FIG 2 Organization of GI01 structural genes in *Citromicrobium* genomes. (A) Structure found in strains JL477, JL354, RCC1878, RCC1885, and RCC1897. (B) Structure found in strains JL31, JL1351, and JL2201. (C) Structure found in the three RCC strains. (D) Structure found in strain WPS32. (E) Structure found in strain WPS32. (F) Structure found in strains JL477 and JL354. Yellow, conserved upstream and downstream genes (from 1 to 14, and from I to XII) of the GI01 gene cluster in *Citromicrobium* genomes; pink, *trb* gene cluster; red, tRNA or integrase; green, type I restriction-modification system; cyan, genes with other known functions; light gray, hypothetical genes. 1, type IV secretory pathway, protease TraF; 2, hypothetical protein; 3, membrane-bound lytic murein transglycosylase C precursor; 4, type IV secretory pathway, VirD2 components (relaxase); 5, hypothetical protein; 6, hypothetical protein; 7, coupling protein VirD4, ATPase required for T-DNA transfer; 8, asparagine synthetase (glutamine-hydrolyzing); 9, acylamino-acid-releasing enzyme; 10, TonB-dependent receptor; 11, RNA polymerase sigma-70 factor, extracytoplasmic factor (ECF) subfamily; 12, hypothetical protein; 13, hypothetical protein; 14, transcriptional regulator. I, cell division protein FtsH; II, ATPase, ParA family protein; III, butyryl-CoA dehydrogenase; IV, alpha-methylacyl-CoA racemase; V, enoyl-CoA hydratase; VI, ferrichrome-iron receptor; VII, hypothetical protein; VIII, hypothetical protein; IX, sterol desaturase family protein; X, hypothetical protein; XI, hypothetical protein; XII, hypothetical protein.

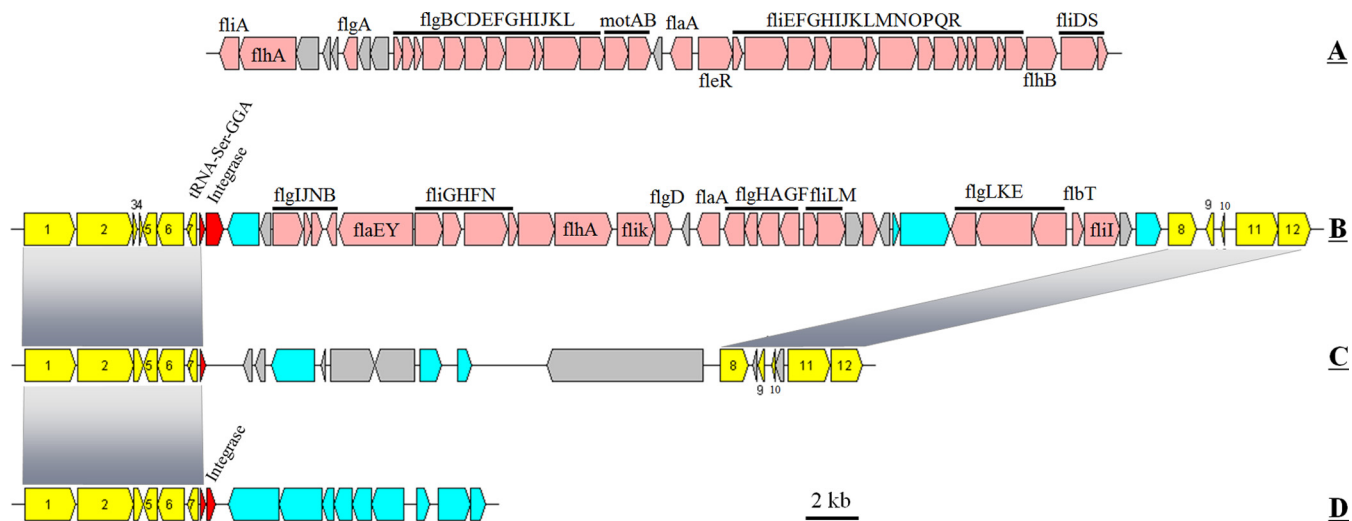


FIG 3 Organization of flagellar and GI03 structural genes in *Citromicrobium* genomes. Yellow, conserved upstream and downstream genes (from 1 to 12) of the GI03 gene cluster in *Citromicrobium* genomes; pink, flagellar gene cluster; red, tRNA or integrase; cyan, genes with other known functions; light gray, hypothetical genes.

genomes. A TIRS gene cluster was also observed in the genomes of strains RCC1878, RCC1885, and RCC1897, but TIRS gene clusters from three RCC strains share less than 50% nucleotide identity with strains JL31, JL1351, and JL2201 (Fig. 2D). Two genes homologous to *trwC* and *traD*, both involved in conjugative transfer, were found next to the TIRS in the inserted sequence of strains RCC1878, RCC1885, and RCC1897 (Fig. 2D). The inserted sequence flanking tRNA-CCG in the genome of strain WPS32 contains a few genes involved in restriction-modification and antirestriction, and several hypothetical genes (Fig. 2E). No inserted genes were found around tRNA-CCG in the genomes of JL477 and JL354 (Fig. 2F). This suggests that different foreign DNA fragments are independently integrated into the same tRNA gene, which contributes to bacterial genome evolution and species divergence (50). No *trb* gene cluster was found in the genome of strain WPS32.

GI02, containing a gene transfer agent. A gene transfer agent (GTA) is an unusual bacteriophage-like element of genetic exchange that transfers a random host genomic DNA fragment (4 to 14 kb in size) between closely related bacteria (54, 55). Analysis of citromicrobial genomes found a GTA gene cluster present in all nine genomes at the same chromosome position in GI02 (see Fig. S1 in the supplemental material). The structure and composition of the GTA gene cluster and flanking genes are identical in all the genomes, except for that of strain WPS32 (see Fig. S1B). For example, an approximately 3-kb DNA fragment mediated by transposase is inserted in the front of GTA in all genomes but was absent in strain WPS32. We speculate that in this strain, the transposase, after acquisition, mediated the gene loss of the downstream region, including the ORFs of the GTA.

GI03, involved in flagella and motility. Flagella support marine bacterial motility and allow cells to move toward favorable living conditions in the environment, e.g., nutrient-rich and light (56–58). Sometimes, flagella also contribute to adhesion (56, 59). In some special cases, flagella might also provide an advantage for bacterial competition (56, 57). Two gene clusters for flagellar biosynthesis were found in the nine genomes

(Fig. 3). The first cluster (flagella I) was common in all genomes (Fig. 3A), while the second (35.5 kb in size, flagella II) was detected in only five genomes, JL31, JL354, JL477, JL2201, and JL1351 (Fig. 3B). Flagella I mainly consists of two large gene clusters (*flgBCDEFGHIJKL* and *fliEFGHIJKLMNOPQR*), *motAB*, *fliDS*, and some regulatory genes (Fig. 3A). The organization of flagella II is irregular (Fig. 3B).

An integrase mediates the acquisition of the flagella II gene cluster, and the integration event occurs adjacent to the tRNA-GGA gene. In the other four genomes, the inserted DNA sequences were also found at the same position. In the three RCC genomes, a 17.1-kb inserted fragment was detected, and only a few genes could be annotated as encoding a known function (*traG* and DNA invertase) (Fig. 3C). In the WPS32 genome, genes involved in the serine-glyoxylate cycle and that were respiration related were found at the same position (Fig. 3D).

GI04. GI04, the longest GI, at 101.1 kb in size, is mainly involved in choline and betaine uptake as well as the metabolism of glycerolipids, glycerophospholipids, fatty acids, and pyruvate. This large DNA fragment was integrated into JL354 and JL477 chromosomes via an integrase flanked by the tRNA-GCT gene. The other genomes, except for WPS32, have the same flanking genes with no GI04 sequences. GI04 contains 88 genes, 18 of which have unknown functions. The G+C content of GI04 (62.14%), is lower than the genomic G+C content.

In the WPS32 genome, an approximately 47.9-kb inserted DNA fragment was also found at the same chromosome position adjacent to the tRNA-GCT gene, and its acquisition was mediated by the integrase. It also contains several fatty acid metabolism-related genes but with significantly lower sequence identity (or different genes) than in strains JL477 and JL354.

GI05. GI05 (approximately 11.3 kb) displayed the lowest G+C content (52.08%). Only found in the genomes of strains JL31, JL354, JL477, JL1351, and JL2201, it contains four genes, and its coding region represents less than 50% of its sequence. The only known function was a DNA polymerase of family B.

GI06, a Mu-like prophage. In a previous study, we isolated one

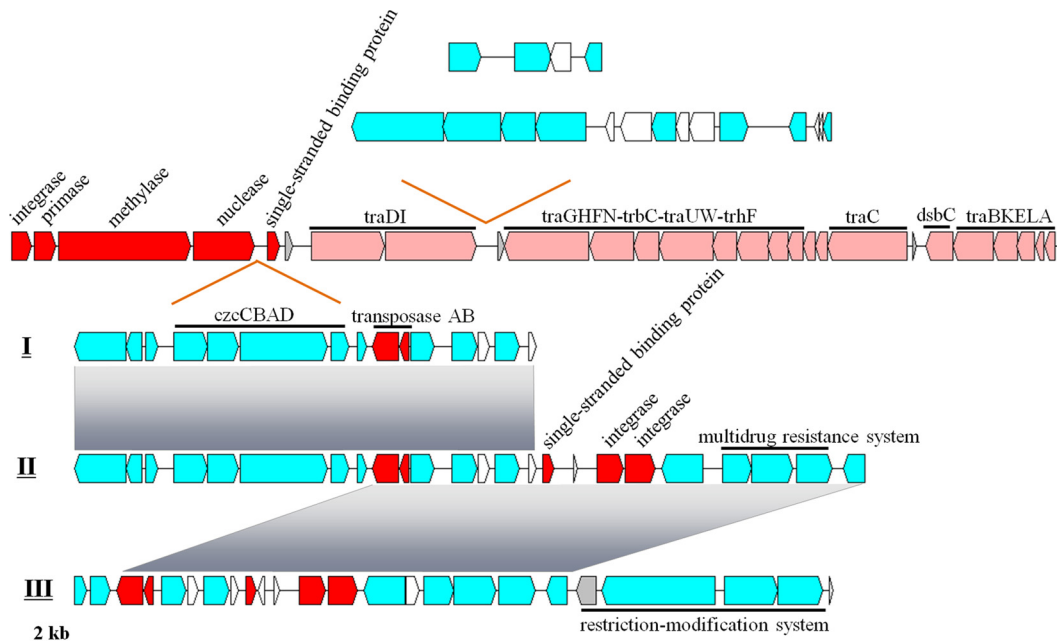


FIG 4 Structure and composition of ICEs. Two hot spots were detected in all ICEs. One contained three types of exogenous gene clusters (I, II, and III), and the other contained two types. Red, phage-related genes; pink, conjugation-related genes; cyan, genes with other known functions; white, hypothetical genes.

inducible bacteriophage from strain JL354, consisting of three parts: an early expression region and regions encoding heads and tails (60). Nearly identical prophage sequences were observed in the genomes of strains JL354, JL477, and JL2201. They share high levels of structural conservation and sequence identity and are defined here as prophage type I (see Fig. S2 in the supplemental material). In addition, another type of prophage (here defined as prophage type II) was found in strains JL2201, RCC1878, RCC1885, and RCC1897. The type II prophage has structure modules similar to those of type I (see Fig. S2), but they share significantly lower sequence identity.

All three type I prophages share the same upstream and downstream genes, and the first downstream gene is a transposase. Type II prophages integrated into the host chromosome in a different position with type I prophage. This indicates that these two types of prophages originate from different integration events.

Two types of prophages coexist in the genome of strain JL2201. The type I prophage in strain JL2201 is identical to the prophage found in strains JL354 and JL477. However, the type II prophage in strain JL2201 lost its early expression region but kept the structural genes encoding heads and tails (see Fig. S2 in the supplemental material). Interestingly, the structural genes form duplication (approximately 26 kb \times 2) centers around the last gene with less than 93% nucleotide identity (see Fig. S2). The incomplete duplicated prophage sequence might contribute to an increase in viral particle production under the control of the early expression genes of prophage I in strain JL2201.

GI07. GI07, located between the tRNA-TGG and tRNA-CAT genes, is the shortest (approximately 9.7 kb) among all GIs. Its G+C content (54.39%) is much lower than the genomic G+C content (64.8 to 65.1%). It contains three genes, an integrase, a hypothetical gene, and a reverse transcriptase, and the coding sequences represent approximately 50% of its length. The gene or-

ganization and composition of GI07 in the five JL genomes are identical.

In the three RCC strains, the inserted foreign DNA (approximately 11.3 kb with 60.67% G+C content) is after the tRNA-TGG gene. It consists of nine genes mainly involved in the type I restriction-modification system, flavodoxin reductase, and fatty acid metabolism. The corresponding region was not detected in the WPS32 genome.

GI08, involved in ICEs. Integrative and conjugative elements (ICEs) are defined as self-transmissible mobile genetic elements with the capacity to integrate into and excise from a host chromosome (61, 62). The core ICEs are made up of three typical genetic modules: ICE integration and excision, ICE conjugation, and ICE regulation modules (62–64). ICEs integrate characteristics of both temperate bacteriophages (the front part) and conjugative plasmids (the latter part) (Fig. 4) (62, 65). ICEs have been reported to contain several intergenic hot spots where a diverse range of exogenous genes can be carried, including antibiotic or heavy-metal resistance genes (65). ICEs mediate HGT among prokaryotes and greatly facilitate microbial genome evolution and ecological fitness (61, 62).

All analyzed genomes except that of strain WPS32 possess an ICE. Based on the structure and gene composition, the eight ICEs could be classified into three groups, namely, group 1 (JL477 and JL354), group 2 (JL1351, JL2201, and JL31), and group 3 (RCC1878, RCC1885, and RCC1897).

Two intergenic hot spots carrying exogenous genes were found in the eight genomes. The first one is located between genes encoding a nuclease and a single-stranded DNA binding protein. It also contains three different exogenous gene clusters (I, II, and III) corresponding to three types of ICEs (group 1, group 2, and group 3). Exogenous gene cluster I is present in the genomes of JL477 and JL354 (group 1) and mainly consists of heavy-metal resistance

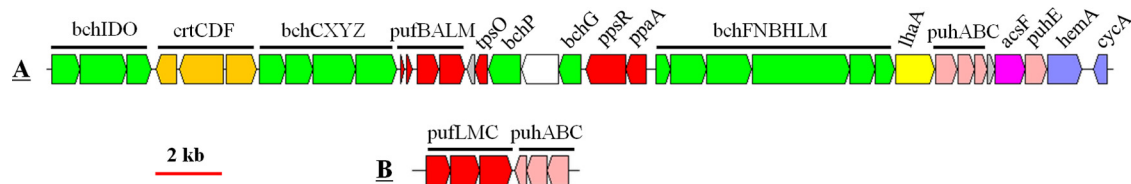


FIG 5 Structure and arrangement of two PGCs in *Citromicrobium*. (A) Complete PGC. (B) Incomplete PGC. Green, *bch* genes; red, *puf* and regulator genes; pink, *puh* genes; orange, *crt* genes; blue, *hem* and *cyc* genes; yellow, *lhaA* gene; blank, uncertain or unrelated genes; gray, hypothetical protein. The horizontal arrows represent putative transcripts.

genes (*czcCBAD*) and their transcriptional regulator (*merR*), transposase AB, copper homeostasis, and antirestriction genes. Exogenous gene cluster II, found in group 2, contains all the genes of exogenous gene cluster I and also possesses several extra multidrug resistance genes, whose acquisition is mediated by two integrases. Interestingly, although no ICE was found in the genome of WPS32, this genome contains multidrug resistance genes and two integrases. Exogenous gene cluster III lost heavy-metal resistance genes but gained restriction-modification-related genes. This hot spot might carry a limited length of foreign genes, suggesting that these microbes might carefully select foreign genes that are optimally adapted to their environment.

The second hot spot, located between gene clusters *traDI* and *traGHFN*, mostly comprises peptidase and nuclease metabolism-related genes. In ICE group 1, it contains 14 genes with known functional genes for nucleases, helicases, ATPases, peptidases, and a transcriptional regulator. The group 2 ICE lost a large part of the conjugation module (*traDI* and *traGHFN-trbC-traUW-trhF*). The second hot spot in ICE group 3 is composed of only five genes related to peptidases, pyrophosphatases, and transcriptional regulators. Downstream of ICE group 3, an approximately 27-kb gene fragment mainly involved in fatty acid metabolism, butyrate metabolism, and branched-chain amino acid biosynthesis is absent in the genomes of three RCC strains and WPS32.

GI09. GI09 is an approximately 11.5-kb fragment with a G+C content similar to that of the genome (65.76%). It contains three genes, a giant hypothetical gene (8.70 kb), a transcriptional regulator, and a histidine kinase, which together represent 97.14% of its sequence. GI09 is adjacent to a tRNA-CGA gene. The protein for the giant hypothetical gene product comprises three domains: an immunoglobulin beta-sandwich folding domain, a cadherin-like beta-sandwich domain, and an auto-transporter beta-domain. Cadherins are suggestive of adhesion molecules that mediate Ca^{2+} -dependent cell-cell junctions (66). Usually, bacteria or cells containing the same cadherins tend to preferentially aggregate.

GI09 was not detected at the same position in the genomes of strains RCC1878, RCC1885, and RCC1897. However, we found a remnant short sequence predicted to be a hypothetical gene (324 bp) that shares 91% (296/324) nucleotide identity with the giant hypothetical gene of strain JL477. This supports the hypothesis that three RCC strains lost the GI09 sequence.

Coexistence of two PGCs in genomes of *Citromicrobium* isolates. Interestingly, two different (one complete and one incomplete) PGCs were found in all nine genomes. The complete PGC consists of two conserved subclusters, *crtCDF-bchCXYZ-pufBALM* and *bchFNBHLM-lhaA-puhABC* (Fig. 5A). The complete PGC organization is identical in all nine genomes in terms of gene ar-

range and composition. The incomplete PGC contains only the *pufLMC* and *puhABC* genes (Fig. 5B). The incomplete PGC, which was proved to be obtained by HGT (15, 16), is located at the same position in all the genomes and is flanked by respiratory complex I and coenzyme A (CoA) metabolism-related genes. This indicates that the ancestral *Citromicrobium* strains obtained the incomplete PGC before divergence. Both the complete and incomplete PGCs are close to the GI regions, creating conditions for gain and loss of phototrophic genes (Fig. 1).

The *pufM* sequences from the complete PGC formed a clade close to that of *Erythrobacter* species also belonging to the order *Sphingomonadales*, alpha-IV subcluster (Fig. 6A). The *pufM* sequences from the incomplete PGC formed a distant clade branching with *Fulvimarina pelagi* HTCC 2506 (alpha-VI subcluster) (Fig. 6A). This phylogenetic placement is in agreement with our previous finding showing that the incomplete PGC genes might have been acquired from a *Fulvimarina*-related species (16).

In both *pufM* clades, the sequences could be grouped into three clusters: three RCC strains formed one cluster, WPS32 by itself was a second cluster, and the other five strains formed a third cluster (Fig. 6A).

Coexistence of two copies of *pufM* in *Citromicrobium* environmental sequences. A total of 540,022 good-quality sequence reads were obtained from two stations at five depths (5, 25, 75, 150, and 200 m) using the revised primers (Table 3). A large proportion (29.8%) of *pufM* sequences having *Citromicrobium* as the closest relative were obtained. Among them, 66,182 and 95,052 sequences were classified into the complete and incomplete PGC clades, respectively.

Eleven and 10 OTU (>10 sequences) were classified into the *Citromicrobium* complete and incomplete PGC clades, respectively (Fig. 6B and C). All the environmental sequences differed from *pufM* sequences from the isolates. Five main OTUs (with more than 1,000 sequences) were retrieved, three (denovo741, denovo766, and denovo718) in the complete PGC clade and two (denovo180 and denovo574) in the incomplete PGC clade (Table 3). Interestingly, denovo741 and denovo180 showed similar positions in their phylogenetic trees (Fig. 6B and C). Their representative sequences shared 99.1% (230/232) and 99.6% (227/228) nucleotide identity with the *pufM* sequences belonging to the complete and incomplete PGCs of strain JL477, respectively. In addition, denovo741 and denovo180 demonstrated the same depth distribution pattern (Table 3). A similar situation was observed for denovo766 and denovo574, whose representative sequences shared 91.4% (212/232) and 94.3% (217/230) nucleotide identity, respectively (Table 3).

However, our analysis did not find an OTU corresponding to a copy of denovo718 in the incomplete PGC clade (Fig. 5B). This

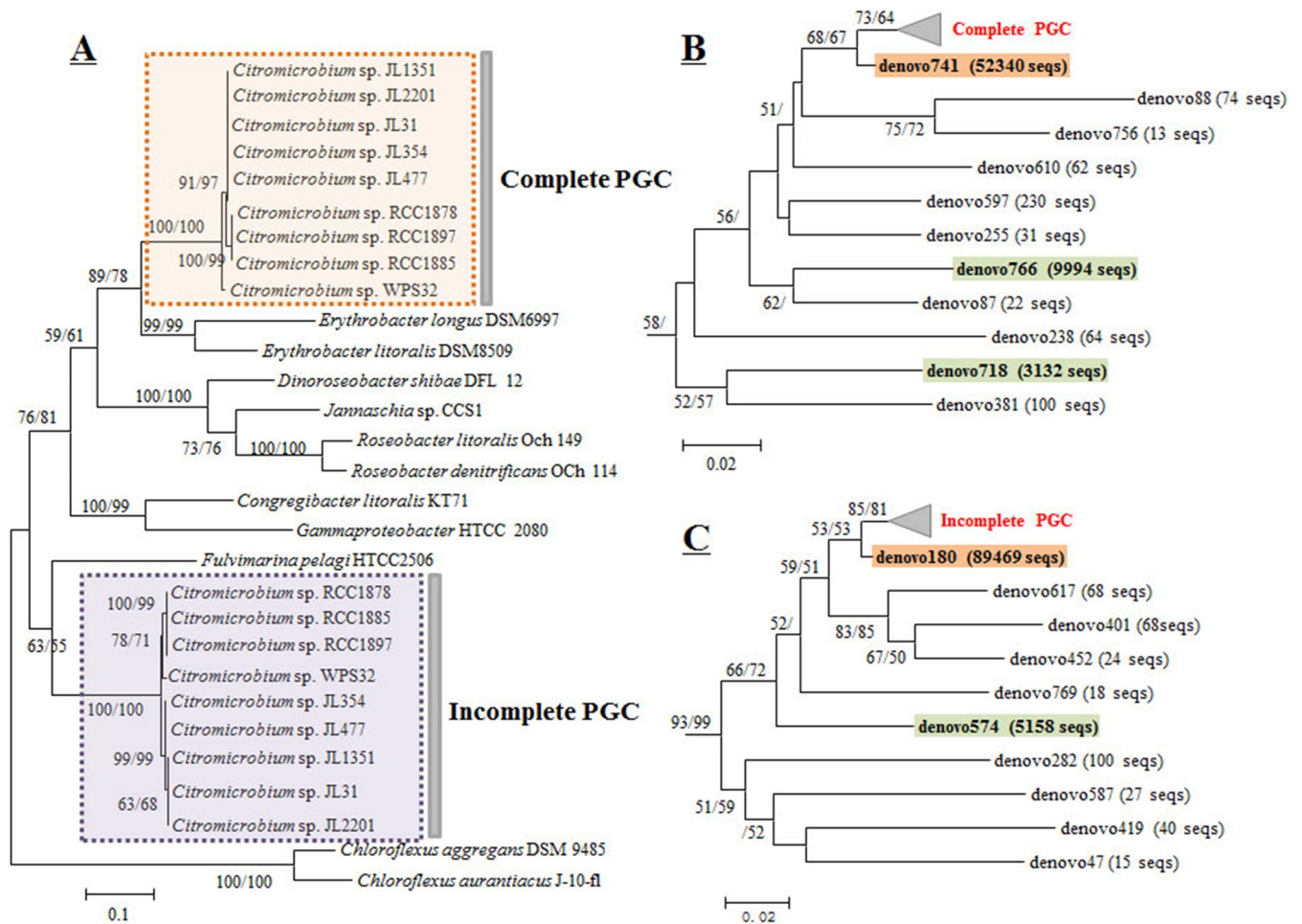


FIG 6 Neighbor-joining phylogenetic trees based on *pufM* gene sequences. (A) Phylogenetic tree containing *pufM* sequences of the nine isolates. (B) Partial tree containing environmental *pufM* sequences from the complete PGC. (C) Partial tree containing environmental *pufM* sequences from the incomplete PGC. Only bootstrap percentages (>50%) are shown (neighbor-joining/maximum likelihood).

may suggest that some citromicrobial strains have lost the incomplete PGC or that denovo718 is a novel *Citromicrobium* relative.

SNPs. The number of SNPs of the eight genomes relative to the complete genome of strain JL477 had a wide range. More than 84,000 SNPs were found in the genomes of strains RCC1878, RCC1885, RCC1897, and WPS32, while fewer than 200 SNPs were present in strains JL354, JL31, and JL1351. In the genome of JL2201, 1,603 SNPs were found, and most of them (1,379 SNPs)

originated from the prophage I sequences, suggesting that viruses had much higher evolutionary rates. Approximately 90% of all SNPs are located in coding regions and are scattered throughout the genomes except in the genetic islands.

Based on the growth rate (0.72 to 2.13 day⁻¹) of AAPB in the ocean (3), their generation time should be approximately 250 to 750 generations per year. The estimated divergence times based on the accumulation of synonymous mutations that excluded SNPs

TABLE 3 Distribution and identity of environmental *pufM* sequences retrieved from sites P3 and P10 at different depths

OTU ID	No. of environmental <i>pufM</i> sequences by site and depth (m)										Total	Identity (%)
	P10					P3						
	5	25	75	150	200	5	25	75	150	200		
denovo180	820	1,517	7,885	1,830	15,898	5,382	14,689	5,287	10,405	25,756	89,469	99.6
denovo574	17	301	1,956	15	81	2,255	126	47	308	52	5,158	94.3
denovo741	721	460	3,645	982	10,915	783	9,503	3,423	7,093	14,815	52,340	99.1
denovo766	76	586	2,950	36	147	5,586	73	419	98	9,994	9,994	91.4
denovo718	11	109	662	10	9	645	44	1,629	3	10	3,132	88.4
Total sequences	62,205	61,988	42,912	72,509	78,655	35,183	73,232	37,557	31,088	44,693	540,022	

from GIs span a long history. The divergence times among JL477, JL31, JL1351, and JL354 are in century timescales, and these four strains diverge at a millennial timescale with JL2201. The three RCC strains and the WPS32 strain diverged from the five JL strains millions of years ago.

Geographic relationship. The isolates used in the study originate from diverse geographic locations, including the Mediterranean Sea, the South China Sea, and the South Atlantic Ocean. Water from the Atlantic Ocean refilled the Mediterranean Sea through the Strait of Gibraltar 5.33 million years ago (67, 68). Before the water poured in, the Mediterranean almost entirely dried out as a result of the Messinian salinity crisis (67, 68). In other words, the modern Mediterranean Sea has an ~5.33 million-year history. Microbes in the almost-enclosed Mediterranean Sea might have evolved their unique characteristics compared to those in the other open-ocean regions. That is consistent with the divergence time between three RCC strains and five JL strains.

Both phylogenies based on marker genes and comparisons of genome sequences revealed that strains from the same region (South China Sea or Mediterranean Sea) shared a similar evolutionary history and are distinct from those originating from other regions (South China Sea versus Mediterranean Sea). Geographic differences are partly responsible for driving the observed evolutionary divergences, and they allow microbes to diverge through local adaptation to specific environmental conditions (69–71). The divergence processes within species are traditionally considered microevolutionary. However, some specific events, such as viral infection, grazing, or extreme physical events, might contribute to unusual evolutionary diversification (e.g., strain WPS32).

HGT plays an important role in *Citromicrobium* genomic plasticity. Three integration events occurred, mediated by two types of prophages (JL477 and JL354; three RCC strains; JL2201), corresponding to the three marine regions from which the strains originated. Three of the nine strains were free of viral infection. Several genes preventing viral infection are detected in their GIs, suggesting that bacterium-phage interactions are actively ongoing in their environment.

A comparison of nine *Citromicrobium* genomes that share identical 16S rRNA sequences provides new insights into bacterial microevolution and divergence under different environments. The distribution of various genetic islands plays important roles in genomic plasticity and adaptability. The information gathered by comparing *Citromicrobium* genomes sheds new light on the evolution and environmental adaptations resulting from geographic isolation in *Citromicrobium* species.

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