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Pan-Genome Analysis and Secondary Metabolic Pathway Mining of Biocontrol Bacterium *Brevibacillus brevis*

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Abstract: *Brevibacillus brevis* is one of the most common biocontrol strains with broad applications in the prevention and control of plant diseases and insect pests. In order to deepen our understanding of *B. brevis* genomes, describe their characteristics comprehensively, and mine secondary metabolites, we retrieved the genomic sequences of nine *B. brevis* strains that had been assembled into complete genomes from the NCBI database. These genomic sequences were analyzed using phylogenetic analysis software, pan-genome analysis software, and secondary metabolite mining software. Results revealed that the genome size of *B. brevis* strains ranged from 6.16 to 6.73 Mb, with GC content ranging from 47.0% to 54.0%. Phylogenetic analysis classified the nine *B. brevis* strains into three branches. The analyses of ANI and dDDH showed that *B. brevis* NEB573 had the potential to become a new species of *Brevibacillus* and needed further research in the future. The pan-genome analysis identified 10032 gene families, including 3257 core gene families, 3112 accessory gene families, and 3663 unique gene families. In addition, 123 secondary metabolite biosynthetic gene clusters of 20 classes were identified in the genomes of nine *B. brevis* strains. The major types of biosynthetic gene clusters were non-ribosomal peptide synthase (NRPS) and transAT polyketide synthase (transAT-PKS). Furthermore, a large number of untapped secondary metabolites were identified in *B. brevis*. In summary, this study elucidated the pan-genome characteristics of the biocontrol bacterium *B. brevis* and identified its secondary metabolites, providing valuable insights for its further development and utilization.

Keywords: *Brevibacillus brevis*; pan-genome; secondary metabolite; genome mining

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1. Introduction

Brevibacillus brevis is a widely distributed Gram-positive bacterium, which is environmentally friendly and contains no endotoxin [1]. *B. brevis* is extensively used in the biological control of plant disease, pollutant degradation, heavy metal remediation, and other fields [2–4]. Particularly in the biological control of plant diseases, *B. brevis* has emerged as a key player due to its capacity to produce a wide range of antimicrobial active products, such as gramicidin, tyrocidine, exopolysaccharides, chitinase, and ethylparaben [5–9]. In 1959, Edeines, the non-ribosomal peptide antimicrobial natural products, were identified in *B. brevis* Vm4, exhibiting strong antibacterial activity against bacteria, mycoplasma, and fungi, as well as an inhibitory effect on tumor cells [10]. In 2002, it was

found that *B. brevis* NO.G1 could produce chitinase with high stability, significantly inhibiting the growth of molds in vegetables [11]. In 2007, gramicidin S was identified in *B. brevis* Nagano, inhibiting parasites by rapidly and selectively lysing infected erythrocytes [12]. In 2010, the isomer of surfactin was identified in *B. brevis* HOB1, proving to be a powerful biosurfactant capable of dissolving various bacteria [13]. In 2012, tostadin, an antibacterial peptide, was identified in *B. brevis* XDH. This peptide exhibits high solubility in water, excellent thermal stability, and a potent inhibitory effect on *Escherichia coli* and *Staphylococcus aureus* when cultured in vitro [14]. In 2013, bacteriocins were identified in *B. brevis* GM100, capable of inhibiting Gram-negative bacteria, Gram-positive bacteria, *Pseudomonas aeruginosa*, *Agrobacterium*, and *Candida tropicalis* [15]. In 2016, a new antimicrobial peptide was identified in *B. brevis* MH9, showing certain antibacterial activity against *Escherichia coli* and *Salmonella typhi* [16]. In 2020, the active substance siderophore was identified from *B. brevis* GZDF3, demonstrating strong antibacterial activity against *Candida albicans* [17].

With the significant advancements in sequencing technologies, genomic data continue to grow, revealing remarkable genomic diversity in microbial genomes. Even among different strains of the same species, significant differences in DNA contents exist. These differences suggest that the entire gene pool of a single strain is much smaller than that of the given species [18]. The concept of the "pan-genome" first proposed by Tettelin in 2005, refers to all the genes present in a particular species. Pan-genome includes the core genome (genes present in all strains), accessory genome (genes present in some strains), and unique genome (genes unique to specific strains) [19]. Pan-genomic analysis is increasingly used in mining microbial functional genes [20]. Traditional methods for analyzing secondary metabolites of microorganisms may have limitations. However, by analyzing known genomic data in bacteria through pan-genomic analysis, it is possible to identify novel secondary metabolic gene clusters and potential active substances [21,22].

Although *B. brevis* has shown remarkable abilities in biological control of plant diseases, growth promotion, and pollutant degradation, most current studies focus on specific strains, which limits a full understanding of the genomic characteristics and secondary metabolite production potential of *B. brevis*. This study aims to address this gap by analyzing the pan-genome of *B. brevis* through a comparison of the genomes of nine *B. brevis* strains that have undergone whole-genome sequencing. In addition, antiSMASH was employed to identify secondary metabolic gene clusters and potential active substances in these strains. The goal of this study is to further explore the genomic features of *B. brevis* and to provide a foundation for a comprehensive understanding of its characteristics and potential applications in biological control.

2. Materials and Methods

2.1. Materials

A total of nine complete genomic sequences of *B. brevis* strains were retrieved from NCBI (https://www.ncbi.nlm.nih.gov/ (accessed on 13 November 2023)), including five strains isolated from soil, one strain from tobacco roots and one strain from cell culture. To assess the completeness and contamination of these genomes, we used the lineage-specific workflow from CheckM with default parameters [23]. A genome was included only if it had $\geq$90% completeness, $\leq$10% contamination, and an overall quality of $\geq$ 50% (defined as completeness—5 $\times$ contamination) [24]. After checking the quality, all nine genomes were kept for further analysis. The general features of the relevant strains are described in Table 1.

2.2. Average Nucleotide Identity (ANI) and Digital DNA–DNA Hybridization (dDDH) Analysis

ANI analysis was performed using the complete genome sequences. ANI values were estimated using the online software IPGA v1.09 (https://nmdc.cn/ipga/ (accessed on 20 November 2023)) [25]. Typically, the cut-offs for species delineation were 95% ANI [26]. In addition, the dDDH values were calculated using Genome-to-Genome Distance Calculator 3.0 provided by the Leibniz Institute DSMZ website (https://ggdc.dsmz.de/ggdc.php#

(accessed on 20 November 2023)) [27]. The 70% species cut-off of dDDH is usually kept in taxonomic studies of bacteria [28].

2.3. Phylogenetic Analyses Based on Genomic Sequences

The genome phylogeny of the nine *B. brevis* strains was constructed using the concatenated multiple sequence alignments of 120 bacterial single-copy marker genes with GTDB-tk v1.4.0 software [29]. The results obtained were used to reconstruct the evolutionary tree using the iTOL software (https://itol.embl.de/itol.cgi (accessed on 2 April 2024)) [30].

2.4. Pan-Genome Analysis

Pan-genome analysis was conducted using the BPGA, version 1.3, (Bacterial Pan Genome Analysis) tool [31]. The genomic sequences in GenBank formats for the nine *B. brevis* strains were uploaded to the BPGA software, and pan-genome analysis was performed. The USEARCH software, version 11, was used to construct the core genome of *B. brevis* with 50% sequence homology as the truncation value. MUSCLE software was used to perform tandem alignment of core gene families. The Gnu-plot software, version 4.6, was used to draw the pan-genome and core genome point map. Functional analysis of all core gene families, accessory gene families, and unique gene families was performed using COG annotation.

2.5. Analysis of Secondary Metabolite Biosynthetic Gene Clusters

The potential secondary metabolite biosynthetic gene clusters were investigated using the online software antiSMASH7.0 (https://antismash.secondarymetabolites.org (accessed on 23 November 2023)) [32]. The default parameters were used for the antiSMASH analysis with relaxed detection strictness. AntiSMASH could accurately identify all known secondary metabolic gene clusters when it can use a specific profile hidden Markov models [33].

3. Results

3.1. General Genomic Characteristics of B. brevis

The complete genomes of nine *B. brevis* strains, isolated from different geographic locations and sources, were sequenced. Table 1 illustrates the basic characteristics of the nine *B. brevis* strains obtained from the NCBI database. Statistical analysis of the genome data revealed that the genome size of *B. brevis* ranged from 6.16 to 6.73 Mb, the GC content ranged from 47.0% to 54.0%, the gene number ranged from 5784 to 6592, and the number of CDS ranged from 5455 to 6425.

Table 1. The general genome features of the nine *B. brevis* strains used in this study.

Strain	Size Mb	GC %	Gene	CDS	Source	Country	Accession No.	Reference
NCTC2611	6.73	47.5	6592	6425	—	—	LR134338.1	—
HK544	6.49	47.5	6133	5766	Soil	South Korea	CP042161.1	[34]
DZQ7	6.44	47.5	6087	5752	Tobacco rhizosphere soil	China	CP030117.1	[6]
NBRC 100599	6.30	47.5	6123	5949	—	—	AP008955.1	—
X23	6.64	47.0	6450	6144	Soil of vegetable field	China	CP023474.1	[35]
B011	6.16	47.5	5784	5455	Tobacco roots	China	CP041767.1	—
NEB573	6.23	54.0	6106	5856	Cell culture	—	CP134050.1	—
HNCS-1	6.35	47.0	6041	5770	Soil	China	CP128411.1	[36]
MGMM11	6.32	47.0	5893	5776	Rhizospheric soil	Russia	CP124547.1	—

3.2. Genetic Diversity of the Nine B. brevis Strains Based on ANI and dDDH Analyses

ANI and dDDH analyses are powerful tools for evaluating the genetic relationships at the genome-wide level, particularly for distinguishing closely related species. In this study, the genetic diversity of the nine *B. brevis* strains was evaluated using ANI and dDDH analyses of whole genome sequence. The ANI comparison matrix of the whole genome sequences revealed that, except for the NEB573 strain, the ANI values of pairwise

comparisons among the other eight strains were all above 92.2% (slightly less than 95% species cut-off). The ANI values of the NEB573 strain compared to other strains ranged only between 73% and 74%, indicating a slightly more distant genetic relationship between NEB573 and the other eight strains (Figure 1A). The results of the dDDH analysis aligned with those of the ANI approaches, showing that the genomes (except NEB573) together had dDDH values above 69.5% (slightly less than 70% species cut-off), and NEB573 compared to other strains had significantly low dDDH values (Figure 1B). In summary, NEB573 had the potential to become a new species and needed further attention in the future.

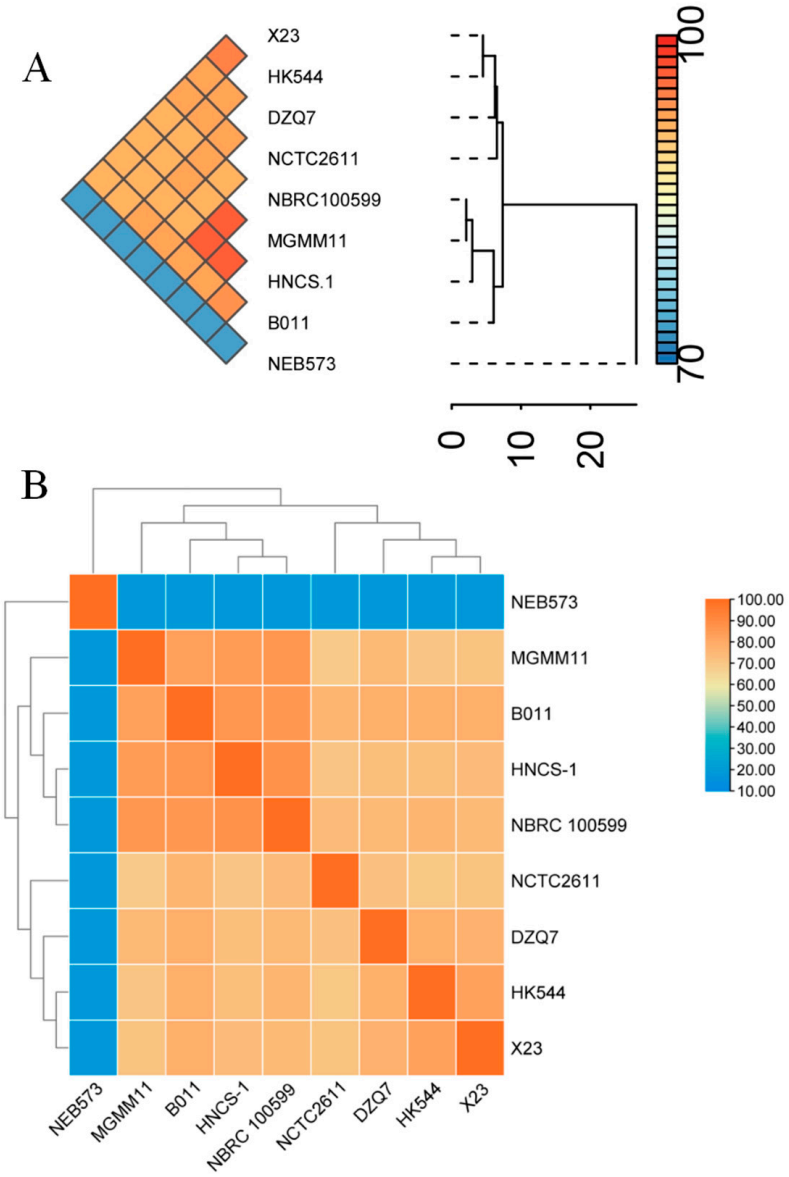

Figure 1. ANI (**A**) and dDDH (**B**) analyses of the nine *B. brevis* strains.

3.3. Phylogenetic Analysis of the Nine B. brevis Strains

The results of the phylogenetic analysis showed that the nine *B. brevis* strains were classified into three branches. One branch included NEB573, and one branch included X23 and HK544, while the other strains formed a separate branch (Figure 2). Compared with the separation source data, it was found that the evolutionary relationship of *B. brevis* strains might have a certain correlation with the separation source. NEB573 isolated from cell culture had a distant relationship with the other eight *B. brevis* strains. The results of the phylogenetic analysis were consistent with those of the ANI and dDDH analyses.

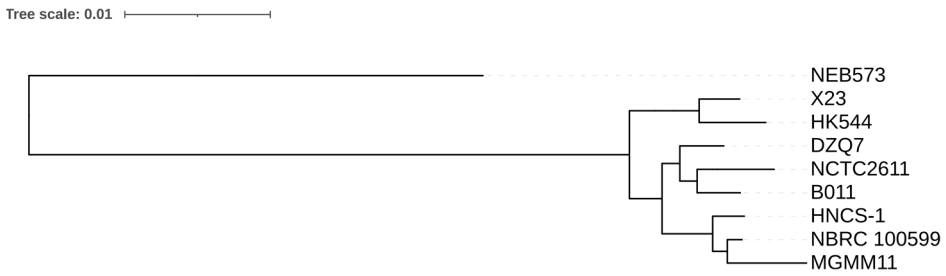

Figure 2. Phylogenetic tree of nine *B. brevis* strains based on 120 bacterial single-copy marker genes.

3.4. Pan-Genome Characteristics of B. brevis

Pan-genome analysis was conducted on the nine *B. brevis* strains with assembled complete genomes. A total of 52,449 functional genes were used for cluster analysis, leading to the identification of 10,032 gene families (Figure 3A). Each gene family represents a hypothetical homologous gene. These gene families exist in different genomes, with the conservation of a gene family increasing with the number of genomes it covers. The genes in the core genome are responsible for determining the basic biological characteristics and main phenotypic traits [37].

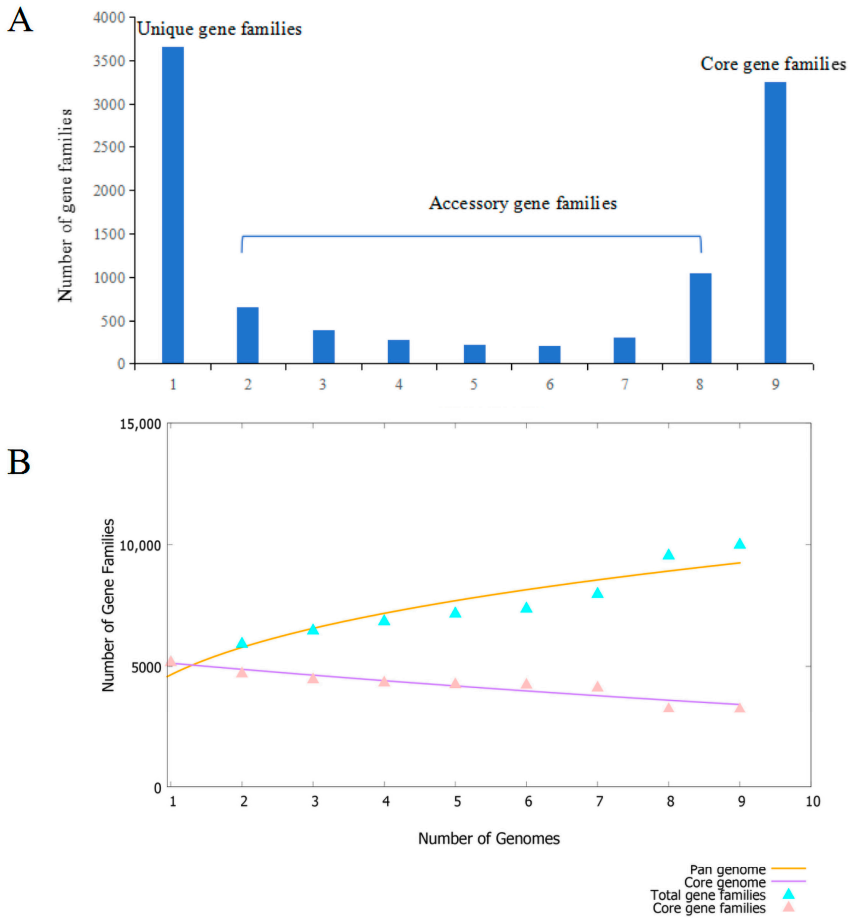

Figure 3. Pan-genome analysis of the nine *B. brevis* genomes. (**A**) Distribution of gene families. (**B**) Curve development of pan (blue color) and core (pink color) genomes.

The analysis revealed that there were 3257 core gene families shared by the nine *B. brevis* strains, accounting for 32.47% of the pan-genome of *B. brevis*. Additionally, there were 3112 accessory gene families, making up 31.02% of the pan-genome. Furthermore, there were 3663 unique gene families contained in one strain, accounting for 36.51% of the pan-genome. It is expected that the number of gene families in one or nine genomes is

larger, while the number of gene families in 2~8 genomes is smaller. This is because the more genomes analyzed, the greater the possibility of finding new genes. However, the number of core gene families is negatively affected by the addition of new strains; that is, as new strains are added, the possibility of gene sharing between strains decreases.

The relationship between the number of genomes, core genomes, and pan-genomes of *B. brevis* was analyzed and calculated using BPGA software (Figure 3B). The fitting equation for the relationship between pan-genome size (f(x)) and the genome number (x) was $f(x) = 5307.29x^{0.280145}$. The fitting equation for the relationship between core genome size (f1(x)) and the number of genomes (x) was $f1(x) = 5125.7e^{-0.0581278x}$. According to these fitting equations, it could be observed that as the number of sequenced *B. brevis* genomes increased, the pan-genomes increased, while the core genomes gradually decreased. Therefore, it can be speculated that the pan-genome of *B. brevis* remains open.

In a COG (clusters of orthologous groups of proteins) analysis of the pan-genome, the core, accessory, and unique gene families were found to be distributed across all COG categories. Figure 4 shows that genes related to amino acid transport and metabolism, carbohydrate transport and metabolism, and inorganic ion transport and metabolism were significantly enriched in the core gene families. This reflected the high ability of *B. brevis* to thrive in different environments, relying on various carbon sources, nitrogen sources, and inorganic salts. Essential genes for bacterial growth, such as those involved in transcription, translation, and signal transduction, were also significantly enriched in the core gene families. It was worth noting that a large number of core gene families were related to general functional prediction, indicating that the *B. brevis* strains might have unknown antibacterial mechanisms. In addition, the core, accessory, and unique gene families contained a large number of genes classified as "Function unknown", possibly because they had not been studied or were partially pseudogenes.

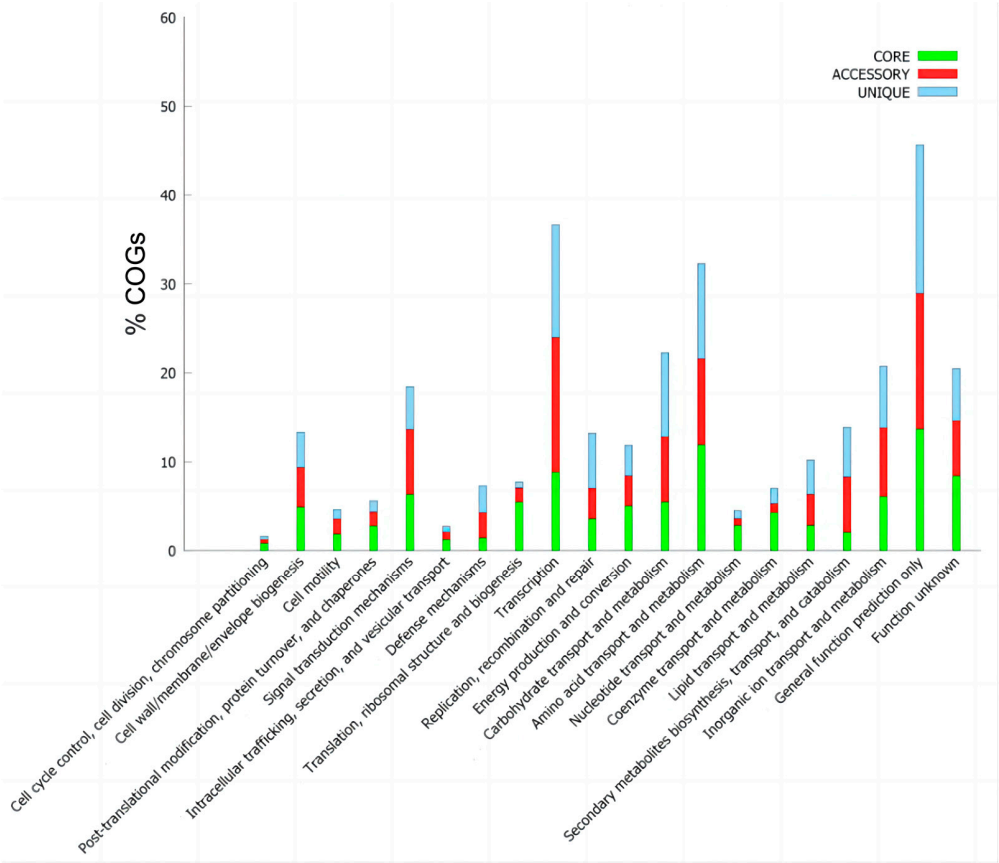

Figure 4. COG functional analysis of pan-genome. The graph shows the predicted function of proteins encoded by core, accessory, and unique gene families of the pan-genome.

3.5. Secondary Metabolite Biosynthetic Gene Cluster of B. brevis

AntiSMASH is a powerful and comprehensive bioinformatics tool used for identifying and annotating biosynthetic gene clusters of secondary metabolites. In this study, the antiSMASH 7.0 software was employed to predict the secondary metabolite synthesis gene clusters in the genomes of nine *B. brevis* strains. The results showed that *B. brevis* possessed a strong ability to synthesize secondary metabolites, with each strain predicted to produce an average of 14 secondary metabolites. Interestingly, there was no direct relationship between the number of secondary metabolic gene clusters and the genome size of the strains.

A total of 123 secondary metabolic gene clusters were predicted and classified into 20 classes across the nine *B. brevis* strains. The non-ribosomal polypeptide synthase (NRPS) gene cluster exhibited the highest occurrence, with a frequency of 51 times, followed by the transAT polyketones (transAT-PKS) gene cluster which occurred 20 times (Figure 5). These findings suggest that *B. brevis* strains primarily produce non-ribosomal peptides and transAT polyketones as their main secondary metabolites.

Among all the predicted gene clusters, 58 gene clusters showed certain homology with known gene clusters, and 32 of them had more than 70% homologies. The main products were petrobactin and tyrocidine, indicating that *B. brevis* generally has the potential ability to produce these compounds (Table 2). The presence of a large number of gene clusters with low homology also suggested the presence of numerous new secondary metabolites in *B. brevis*.

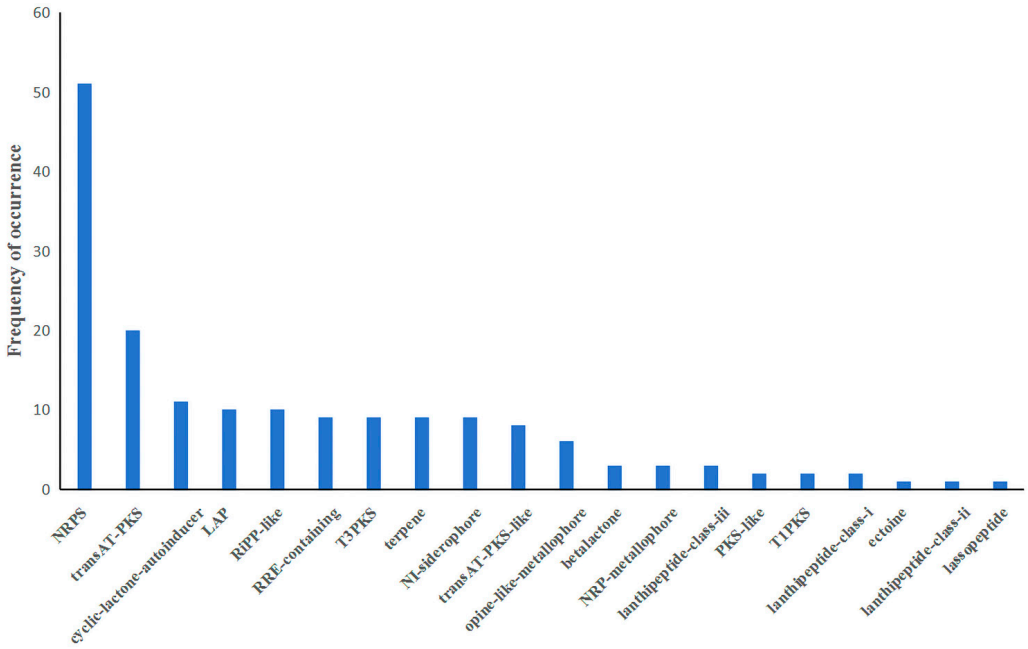

Figure 5. Occurrence frequency of secondary metabolite synthesis gene clusters.

Table 2. Annotated secondary metabolite gene clusters with similarity greater than 70%.

Strain	Accession No.	Gene Cluster	Start	End	Homolog of Known Cluster	Similarity
NCTC2611	LR134338.1	Cluster 1	1476119	1536630	ulbactin F/ulbactin G	100%
		Cluster 2	3005749	3016132	ectoine	100%
		Cluster 3	3024715	3133409	gramicidin	91%
		Cluster 4	3177936	3200077	bacillopaline	100%
		Cluster 5	3202856	3276565	tyrocidine	75%
		Cluster 6	4016684	4048395	petrobactin	83%

Table 2. *Cont.*

Strain	Accession No.	Gene Cluster	Start	End	Homolog of Known Cluster	Similarity
HK544	CP042161.1	Cluster 7	361636	422151	ulbactin F/ulbactin G	100%
		Cluster 8	1975342	2049051	tyrocidine	81%
		Cluster 9	2814515	2846223	petrobactin	83%
		Cluster 10	5799588	5988436	macrobrevin	100%
DZQ7	CP030117.1	Cluster 11	2108577	2140291	petrobactin	83%
		Cluster 12	2955322	3051077	tyrocidine	81%
		Cluster 13	3161865	3399925	marthiapeptide A	83%
		Cluster 14	5529371	5713939	macrobrevin	100%
NBRC 100599	AP008955.1	Cluster 15	2116926	2148625	petrobactin	83%
		Cluster 16	2856081	2929792	tyrocidine	100%
		Cluster 17	2932793	2954934	bacillopaline	100%
		Cluster 18	3002439	3150775	gramicidin	100%
X23	CP023474.1	Cluster 19	2255634	2287345	petrobactin	83%
		Cluster 20	3097361	3167698	tyrocidine	81%
		Cluster 21	4897981	4958493	ulbactin F/ulbactin G	100%
B011	CP041767.1	Cluster 22	2141737	2173451	petrobactin	83%
		Cluster 23	2896872	2988295	tyrocidine	81%
		Cluster 24	3019366	3143800	gramicidin	91%
HNCS-1	CP128411.1	Cluster 25	2178678	2210377	petrobactin	83%
		Cluster 26	2946955	3020665	tyrocidine	87%
		Cluster 27	3022626	3044767	bacillopaline	100%
		Cluster 28	3088959	3197575	gramicidin	91%
MGMM11	CP124547.1	Cluster 29	2772174	2803873	petrobactin	83%
		Cluster 30	3518961	3592672	tyrocidine	81%
		Cluster 31	3595633	3617774	bacillopaline	100%
		Cluster 32	3673595	3781752	gramicidin	91%

4. Discussion

B. brevis is commonly considered a biocontrol bacterium and is widespread in the soil and sediment [1]. Due to its diverse potential functions, it is widely used in agriculture and environmental remediation [38].

Whole genome sequencing helps to assemble the genome into a complete genome sequence, resulting in more accurate and in-depth genome annotation information. As an important source of biocontrol strains in nature, the abundant genomic information of *B. brevisis* is very important for agriculture in selecting suitable biocontrol strains. In the NCBI database, there are nine *B. brevis* strains with complete genomic sequences. In this study, we first analyzed the phylogenetic relationship of these nine *B. brevis* strains and the phylogenetic tree was constructed based on 120 bacterial single-copy marker genes in series. The phylogenetic analysis classified the nine *B. brevis* strains into three branches. The results showed that NEB573, which was currently classified as one strain of *Brevibacillus brevis*, had a very distant relationship with other *Brevibacillus brevis* strains. Its classification status might need to be reconsidered.

Over the past decade, whole genome sequencing has become a most common experiment as algorithms, as ANI and dDDH have arisen as reproducible, reliable, and highly informative alternatives to wet lab DDH [39]. The ANI and dDDH analyses of the nine *B. brevis* strains showed that the ANI values were more than 92.2% and the DDH values were more than 69.5% among the strains of *B. brevis* (except NEB573). It was found that NEB573 was a special heterospecies in *B. brevis* strains. NEB573 was the only *B. brevis* strain isolated from cell culture and had significantly higher GC content (54.0%). The ANI and dDHH values of the NEB573 compared to other strains were significantly lower than the species cut-off values. The significant genomic differences between NEB573 and other strains maybe because it was separated from a very special separation source. Of course, there is every indication that NEB573 had the potential to become a new species

of *Brevibacillus*, but this would require quite a lot of future research work to support the change of its classification.

We also conducted pan-genome analysis on these *B. brevis* genomes. The pan-genome of *B. brevis* consisted of 35,258 functional genes, and a total of 10,032 gene families were identified. Among these, there were 3257 core gene families, 3112 accessory gene families, and 3663 unique gene families. The pan-genome can be classified into open or closed pan-genome according to its characteristics. An open pan-genome refers to an increase in the number of genes in the pan-genome as individual genomes are added within the species. A closed pan-genome, on the other hand, describes a pan-genome where the number of genes tends to saturate with the addition of individual genomes in the species. The opening or closing of pan-genomic features reflects the species diversity in gene composition and also indicates the difference between the species' living environment and its ability to exchange genetic material with the external environment [40]. By calculating the relationship between the pan-genome, the core genome, and the number of genomes, we found that as the number of sequenced genomes increased, the total number of pan-genomes also increased, indicating that the pan-genome of *B. brevis* was open, and *B. brevis* exhibited relatively strong genetic elasticity and high genetic diversity. The frequent isolation of *B. brevis* from diverse environments and the pangenomic evidence of a bacterial species well-adapted for survival also provided a primary indication of its ecological success.

Through secondary metabolite synthesis gene cluster analysis, a total of 20 categories and 123 secondary metabolism gene clusters were found in the nine *B. brevis* genomes. On average, each strain of *B. brevis* had 14 secondary metabolism gene clusters, with the most common types being NPRS and transAT-PKS gene clusters. In addition, a total of 58 gene clusters showed certain homology with known gene clusters, with 32 gene clusters having homologies greater than 70%. These results suggest that *B. brevis* may have similar metabolite synthesis pathways, with non-ribosomal peptides and transAT polyketides being the most likely active substances.

When compared to other bacteria, such as 13 *Planctomycetes* strains (average of eight secondary metabolic gene clusters per genome) [41], 211 anaerobic bacteria (average of five secondary metabolic gene clusters per genome) [42], and 328 *Bacillus* bacteria (average of seven secondary metabolic gene clusters per genome) [43], *B. brevis* had a relatively higher number of secondary metabolic gene clusters per strain. This suggests a higher potential for the synthesis of new substances in *B. brevis*.

In recent years, the growing prevalence of plant diseases and drug-resistant pathogens has weakened the efficacy of existing chemical pesticides. It is increasingly important to develop new biological pesticides with novel mechanisms of significant activity. Numerous studies have demonstrated that *B. brevis* can produce a variety of secondary metabolites that play important roles in antagonizing pathogens, promoting plant growth, bacterial colonization, biofilm formation, and physiological metabolism [5–9]. In this study, a large number of gene clusters in the nine *B. brevis* strains showed low homology with known gene clusters, suggesting the presence of numerous new secondary metabolites in *B. brevis* that warrant further exploration.

5. Conclusions

As a branch of comparative genomics, pangenomics provides a new perspective for understanding the dynamic changes in gene composition, genome characteristics, and gene number of species. In this study, the genetic diversity of *B. brevis* was studied at the genome-wide level. We conducted pan-genome analysis on nine *B. brevis* strains with complete genomes, revealing that their pan-genomes contained 10032 functional genes, including 3663 unique gene families and 3257 core gene families. The results also showed that with the increase in genome number, the pan-genome set of *B. brevis* still showed an increasing trend, indicating that there was high genetic diversity in this species population. The comparative analysis of genomes showed that *B. brevis* NEB573 had the potential to become a new species of *Brevibacillus*. In addition, the secondary metabolic gene clusters

were identified using antiSMASH7.0 software, resulting in the annotation of 20 classes and 123 secondary metabolic gene clusters. The main secondary metabolite synthesis gene clusters were NRPS gene clusters and transAT-PKS gene clusters. The presence of a large number of untapped secondary metabolites in *B. brevis* suggests that it has great potential for applications in plant disease resistance, plant growth promotion, and other aspects. However, due to the limited number of complete genomes of *B. brevis*, the research on the relationship between separation environment and evolution could be strengthened in the future.

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Data Availability Statement: The source of all data generated or analyzed in this study is listed in this published article.

Conflicts of Interest: Author Shiyong Tan was employed by the company Hunan Tevos Ecological Technology Co., Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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