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Comparative Genome Analysis of a Novel Alkaliphilic Actinobacterial Species *Nesterenkonia haasae*

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Abstract

In the present study, a comparative genome analysis of the novel alkaliphilic actinobacterial *Nesterenkonia haasae* with other members of the genus *Nesterenkonia* was performed. The genome size of *Nesterenkonia* members ranged from 2,188,008 to 3,676,111 bp. *N. haasae* and *Nesterenkonia* members of the present study encode the essential glycolysis and pentose phosphate pathway genes. In addition, some *Nesterenkonia* members encode the crucial genes for Entner-Doudoroff pathways. Some *Nesterenkonia* members possess the genes responsible for sulfate/thiosulfate transport system permease protein/ATP-binding protein and conversion of sulfate to sulfite. *Nesterenkonia* members also encode the genes for assimilatory nitrate reduction, nitrite reductase, and the urea cycle. All *Nesterenkonia* members have the genes to overcome environmental stress and produce secondary metabolites. The present study helps to understand *N. haasae* and *Nesterenkonia* members' environmental adaptation and niches specificity based on their specific metabolic properties. Further, based on genome analysis, we propose reclassifying *Nesterenkonia jeotgali* as a later heterotypic synonym of *Nesterenkonia sandarakina*.

K e y w o r d s: *Nesterenkonia haasae*, genome comparison, salt stress, pan-genome analysis, reclassification of *Nesterenkonia jeotgali*

Introduction

The phylum Actinobacteria is one of the most dominant phyla in the bacteria domain and represents one of the most primitive lineages among prokaryotes (Koch 2003; Shivlata and Satyanarayana 2015). They are prolific sources of antibiotics, beneficial bioactive compounds, and industrially important enzymes (Mehta et al. 2006; Thumar et al. 2010; Shivlata and Satyanarayana 2015). They not only occur in typical environments but also in extreme environments, which are characterized by high salinity, high/low pH, high/low temperature, and pressure (Sarethy et al. 2011; Prabhu et al. 2015; Wang et al. 2021; Chole et al. 2022; Kaari et al. 2022). Alkaliphilic bacteria are a significant source of novel chemicals, such as antimicrobials, bioactive molecules, and stable enzymes. (Sarethy et al. 2011; Preiss et al. 2015). Alkaliphilic actinobacteria were first isolated by Taber (1960). The immense potential of alkaliphiles has been recognized due to the pioneering work of Horikoshi and his coworker (Horikoshi 1971; Horikoshi and Akiba 1982). Various actinobacterial members have been reported to be alkaliphilic (Jones et al. 2005; Narsing Rao et al. 2020).

The genus *Nesterenkonia* was proposed by Stackebrandt et al. (1995) as a member of the family *Micrococcaceae*. Members of this genus were halotolerant and/or halophilic, with some being alkaliphilic or alkalitolerant (Machin et al. 2019). Members of this genus were isolated from diverse environments such as soda lakes (Delgado et al. 2006), hypersaline lakes (Collins et al. 2002), saline and alkaline soils (Li et al. 2008),

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salt pans (Govender et al. 2013), Antarctic soil (Finore et al. 2016), desert soil (Wang et al. 2014), and also from humans (Edouard et al. 2014).

Currently, the genus includes 24 validly published species (Parte et al. 2020). *Nesterenkonia* members were reported to be organic-solvent-tolerant (Shafiei et al. 2011; 2012). They possess a large number of carbohydrate-related genes, as well as genes involved in butanol fermentation and monosaccharide/polysaccharide utilization. They have been reported to produce amylase (Shafiei et al. 2012), acetone, butanol, and ethanol (Amiri et al. 2016). They were also reported to be multi-resistant, especially to cold stress, UV radiation, drought, and copper (Aliyu et al. 2016; Dai et al. 2022). Many comparative genome analyses were performed to understand pathogenomic and adaptive strategies of *Nesterenkonia* members for survival under multiple stress conditions (Aliyu et al. 2016; Chander et al. 2017; Dai et al. 2022).

Recently, a novel alkaliphilic species *Nesterenkonia haasae* was reported by our group (Wang et al. 2021). It can tolerate high pH (10.5), NaCl (25% w/v), and temperature (50°C). Owing to the vast application of alkaliphilic actinobacteria, the present study was performed to understand an in-depth genome insight, metabolic functions, and mechanism to overcome stress conditions of *N. haasae*. In addition, we performed the comparative genome analysis of *N. haasae* with other *Nesterenkonia* members.

Experimental

Materials and Methods

A total of twenty type strains (including *N. haasae*) and one "*Candidatus*" genome of *Nesterenkonia* were available in the National Center for Biotechnology Information (NCBI) database. The genomes were downloaded, and their quality was determined using CheckM v.1.0.7 (Parks et al. 2015). A graphical circular map of the genomes was performed using the CGview comparison tool (Grant et al. 2012). Functional annotation was performed by KofamKOALA (Aramaki et al. 2020) using the anvi-run-kegg-kofams program (Eren et al. 2015) and by Rapid Annotations using the Subsystems Technology (RAST) server (Aziz et al. 2008). The genomes were analyzed for the presence of secondary metabolite using antiSMASH v.6.0 (Blin et al. 2021).

The phylogenomic tree was reconstructed using the Anvi'o tool (Eren et al. 2015). All common genes in HMM source 'Bacteria_71' (which contained 71 bacterial single-copy genes) were taken and aligned using MUSCLE (Edgar 2004). The resulting tree was visualized using MEGA version 7.0 (Kumar et al. 2016). The tRNAs were predicted using tRNAscan-SE (Lowe and Eddy 1997).

Pan-genome analysis was carried out via the Anvi'o tool (Eren et al. 2015; Delmont and Eren 2018) using NCBI blast and MCL flag (van Dongen and Abreu-Goodger 2012). The average nucleotide identity (ANI) value was calculated using the pyani with the ANIb parameter (Pritchard et al. 2016).

Results and Discussion

Genome attributes. The genome size of *N. haasae* was 343,306 bp. The genome completeness and contamination of *N. haasae* were 99.1 and 1.2, respectively. A total of 21 *Nesterenkonia* genomes were included in this study. The genome completeness and contamination of *Nesterenkonia* members (Table I) were > 80% and < 5%, respectively, indicating well-curated genomes (Parks et al. 2015). The genome size and G + C content of *Nesterenkonia* members ranged from 2,188,008–3,676,111 bp and 65.8–71.7%, respectively. The detected tRNA ranged from 31–55. Detailed genome attributes of the present study *Nesterenkonia* members are listed in Table I. A graphical circular map of the genomes (using *N. haasae* as a reference genome and it's top nine closely related species) was plotted to show the presence and absence of genes (Fig. 1).

Metabolic potentials. Functional annotation was performed by KofamKOALA using the anvi-run-kegg-kofams program and by the RAST server. The RAST analysis showed that *N. haasae* encodes the highest genes for amino acids and derivatives and carbohydrate metabolism (Fig. 2). Further, genes related to phosphorus, sulfur, and nitrogen metabolism were also noticed (Fig. 2), which will be discussed in the later sections.

Breakdown of glucose is essential as it provides crucial building blocks and ATP/NAD(P)H, which is necessary for cell growth and bio-production (Hollinshead et al. 2016). The most common glycolytic routes are the Embden-Meyerhof-Parnas, the pentose phosphate, and the Entner-Doudoroff pathways (Patra et al. 2012).

The pentose phosphate pathway is composed of two branches, an oxidative and a non-oxidative branch (Zheng et al. 2017; Rytter et al. 2021). In addition to glucose catabolism, the pentose phosphate pathway also contributes to bacterial metabolic adaptation (Zheng et al. 2017; Rytter et al. 2021). The oxidative pentose phosphate pathway was reported to provide essential material for synthesizing osmolytes like glycerol. Similarly, glycolysis, pentose phosphate, and tricarboxylic acid cycle were reported to provide OAA, acetyl-coASH, and $NADPH_2$ required for ectoine production (Frikha-Dammak et al. 2021). In the present study, *N. haasae* and other *Nesterenkonia* members of the present study encode essential genes for glycolysis and pentose phosphate pathway (Table SI). In addition,

Table I
Genome attributes of present study *Nesterenkonia* members.

Nesterenkonia members (accession numbers)	Genome completeness (%)	Genome contamination (%)	Genome size (bp)	Genomic DNA G+C (%)	rRNAs	tRNAs
"*Candidatus* Nesterenkonia stercoripullorum" (DXGD00000000)	84.3	3.7	2,634,134	65.8	0	34
N. alba (ATXP00000000)	98.3	0	2,591,866	63.7	6	49
N. alkaliphile (BMFX00000000)	98.8	1.4	3,397,286	64.7	5	49
N. aurantiaca (SOAN00000000)	99.1	0	2,947,649	67.5	3	48
N. cremea (BMIS00000000)	99.5	0.8	3,083,451	66.8	5	50
N. haasae (VFIE00000000)	99.1	1.2	3,433,063	60.8	7	48
N. halophila (WIAX00000000)	75.9	0.07	2,188,008	71.7	4	31
N. halotolerans (JADBEE000000000)	99.1	1.2	2,966,101	66.2	6	47
N. jeotgali (JACJIH000000000)	98.5	3.2	3,002,985	67.4	6	50
N. lacusekhoensis (JAGINX000000000)	100	0.9	2,742,649	66.6	6	55
N. lutea (JADBED000000000)	99.5	0.07	2,958,123	66.7	6	47
N. massiliensis (CBLL000000000)	97.7	0.4	2,641,000	62.8	3	46
N. muleiensis (QWLD00000000)	97.7	0.8	3,676,111	63.5	2	46
N. natronophila (QYZP00000000)	98.3	0.07	2,524,489	61.8	5	47
N. pannonica (CP080575)	70.4	0.8	2,699,453	66	6	48
N. populi (VOIL00000000)	98.1	0.8	2,551,278	66.8	6	49
N. salmonea (VAVZ00000000)	99.1	0.3	3,283,675	61.1	3	51
N. sandarakina (JACCFQ000000000)	98.5	1	3,017,448	67.5	6	47
N. sedimenti (JABAHY000000000)	98.8	1.6	3,113,980	63	3	49
N. sphaerica (VAWA00000000)	99	1.4	2,791,176	64.2	5	47
N. xinjiangensis (JACCFY000000000)	99.7	0.6	3,569,370	68.8	6	49

N. haasae, *Nesterenkonia populi*, *Nesterenkonia xinjiangensis*, *Nesterenkonia alba*, *Nesterenkonia halophila*, *Nesterenkonia massiliensis*, *Nesterenkonia cremea* and *Nesterenkonia sphaerica* encodes key genes for Entner-Doudoroff pathways (Table SI). Studies suggest that the Entner-Doudoroff pathway alleviates oxidative stress (Chavarría et al. 2013; He et al. 2014; Hollinshead et al. 2016). The results showed that *Nesterenkonia* members might cope with stress conditions.

Sulfur is an essential element widely required by living organisms because it serves multiple critical roles in cells (Aguilar-Barajas et al. 2011). Sulfate is the preferred sulfur source for most organisms (Silver and Walderhaug 1992). The genes *cysNCD* responsible for the conversation of sulfate to adenylyl sulfate were noticed in *N. haasae*. Sulfate uptake is carried out by sulfate permeases (Aguilar-Barajas et al. 2011), and in *Nesterenkonia aurantiaca*, *N. cremea*, *Nesterenkonia lacusekhoensis*, *N. populi* and *N. alba*, genes responsible for sulfate/thiosulfate transport system permease protein/ATP-binding protein (cysUWA) were detected. The genes (*cysND*, *cysH* and *sir*) responsible for the conversion of sulfate to sulfite were also detected in *N. aurantiaca*, *N. cremea*, *N. lacusekhoensis*, *N. populi* and *N. alba* suggesting they may reduce sulfate to sulfite.

The microbial nitrogen cycle comprises nitrogen fixation, assimilatory and dissimilatory nitrate reduction, denitrification, nitrification, and anammox (Chen and Wang 2015). Nitrate reduction occurs with three different purposes: it serves as a nitrogen supply for growth (nitrate assimilation), it generates metabolic energy using nitrate as a terminal electron acceptor (nitrate respiration), and it dissipates excess reducing power (nitrate dissimilation) (Martínez-Espinosa et al. 2001). *Nesterenkonia lutea*, *N. populi*, *N. xinjiangensis*, *N. halophila*, *N. aurantiaca* and *Nesterenkonia jeotgali* encodes genes for assimilatory nitrate reduction (*nasAB*). The above results suggest that these *Nesterenkonia* members may use nitrogen for growth. *N. sphaerica*, *N. lutea*, *N. populi*, *N. xinjiangensis*, *N. halophila*, *N. massiliensis*, *Nesterenkonia salmonea*, *N. aurantiaca*, *N. jeotgali*, *Nesterenkonia natronophila*, *Nesterenkonia sediment*, *N. cremea*, *N. lacusekhoensis* and *N. haasae* encode genes for nitrite reductase (*nirBD*), suggesting they may reduce nitrite to ammonia. Further, *Nesterenkonia alkaliphila* and "*Candidatus* Nesterenkonia stercoripullorum" were found to encode genes for the urea cycle (Table SI). Further, detailed metabolic potentials of *Nesterenkonia* members are listed in Table SI.

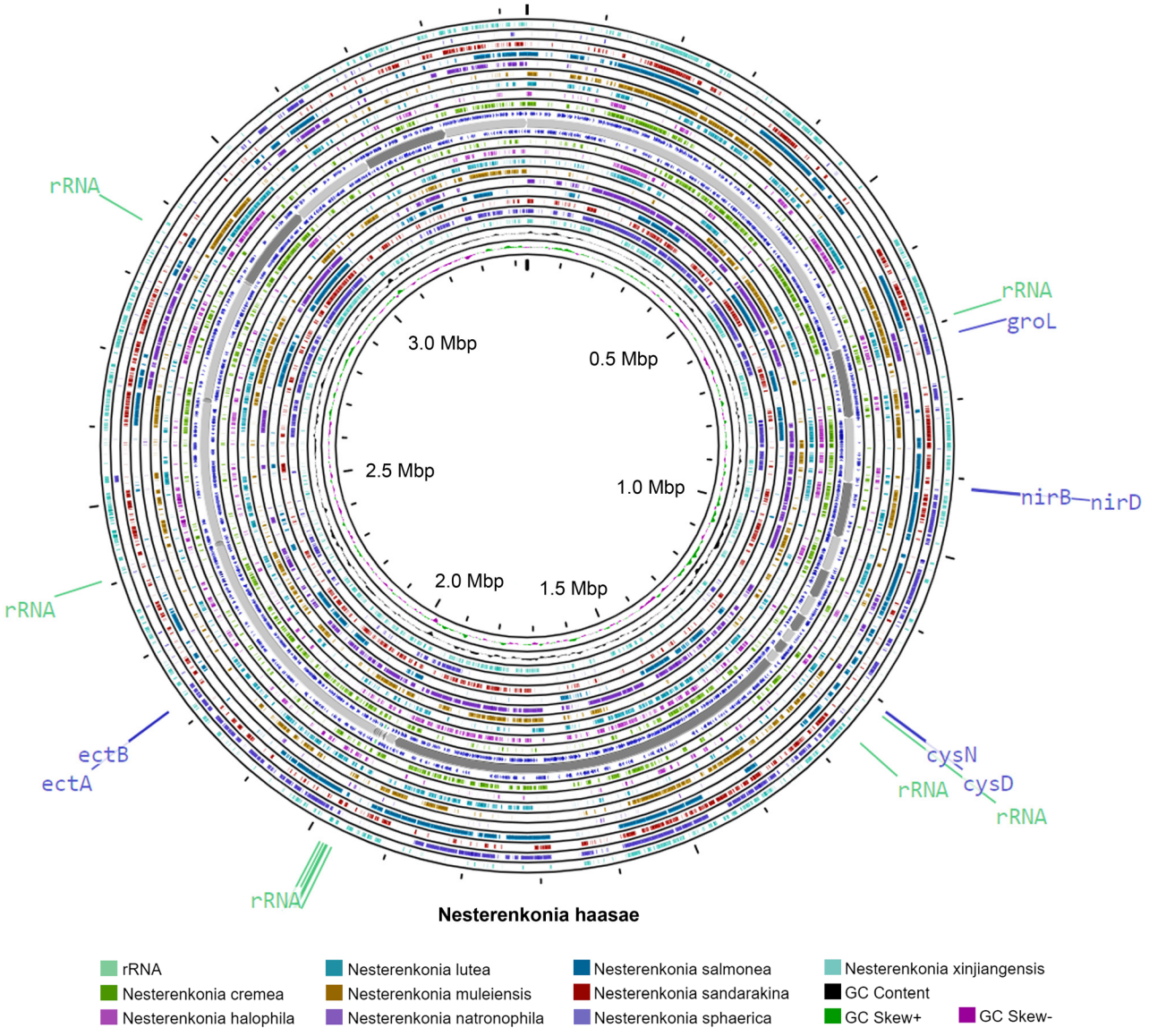

Fig. 1. Graphical circular map of the genomes showing the presence and absence of the gene. The important genes were highlighted.

Stress-related genes. Members of the genus *Nesterenkonia* exert tremendous environmental stress as most of them were isolated from alkaliphilic, or halophilic environments (Collins et al. 2002; Delgado et al. 2006; Li et al. 2008; Finore et al. 2016).

Members of the phylum *Actinobacteria* cope with osmotic stress by accumulating or synthesizing low molecular weight, highly water-soluble organic solutes, so-called compatible solutes, or osmolytes (Sadeghi et al. 2014).

Ectoine is the most commonly found osmolytes in *Streptomyces* (Bursy et al. 2008), and the genes involved in its biosynthesis were identified on the chromosome in the order *ectABC* (Zhu et al. 2014). *N. haasae* encodes genes for *ectABC* and except *Nesterenkonia pannonica* and *N. halophila,* all other *Nesterenkonia* members encode genes for ectoine biosynthesis. Glycine and betaine were also reported as important osmoprotectants (Boch et al. 1996). The genes encoding for glycine betaine synthesis were observed in all *Nesterenkonia* members of the present study (Table SI).

Oxidative stress is also associated with osmotic stress (Yaakop et al. 2016). The genes to overcome such stress were present in all *Nesterenkonia* members of the present study (Table SI).

Secondary metabolites and pangenome analysis. Actinobacteria members remain of significant interest in discovering biologically active secondary metabolites (Guerrero-Garzón et al. 2020). Actinobacteria member's genomes were reported to include many biosynthetic gene clusters producing diverse secondary metabolites (Guerrero-Garzón et al. 2020). In the present study, 16 different gene clusters for secondary metabolites production were noticed (Table II). *N. haasae* showed

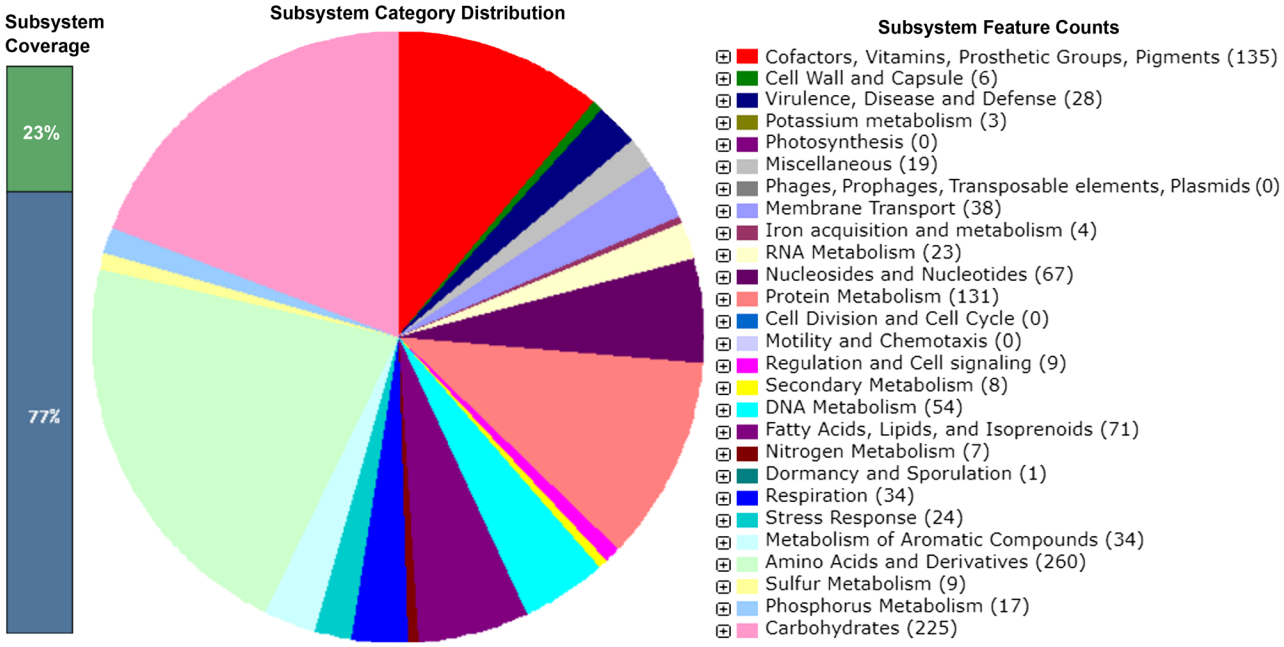

Fig. 2. Functional annotation of *Nesterenkonia haasae* using RAST server.

genes for ectoine, terpenes, and tetronasin production, while most *Nesterenkonia* members of the present study showed the presence of ectoine and terpenes.

Ectoine, as mentioned earlier, used as an osmolyte, is one of the most extensively found compatible solutes throughout different halotolerant and halophilic

Table II
antiSMASH results of *Nesterenkonia* members.

Nesterenkonia members	Most similar known cluster	Similarity
N. alba	Ectoine	75%
N. alkaliphila	Kocurin	88%
	Ectoine	75%
	T3pks	3%
	Redox-cofactor	100%
	Livipeptin	66%
N. aurantiaca	Terpenes	28%
	Ectoine	50%
N. haasae	Chejuenolide A/Chejuenolide B	7%
	Terpenes	28%
	Foxicins A-D	4%
	Ectoine	75%
N. halotolerans	Terpenes	28%
	Tetronasin	3%
	Ectoine	50%
N. jeotgali	Terpenes	28%
	Ectoine	50%
N. lacusekhoensis	Glycopeptidolipid	20%
	Terpenes	21%
	Ectoine	75%
N. lute	Terpenes	28%
	Ectoine	75%
N. massiliensis	Ectoine	50%
	Terpenes	28%

Table II.
Continued

Nesterenkonia members	Most similar known cluster	Similarity
N. muleiensis	Ectoine	75%
	Terpenes	50%
N. natronophila	Ectoine	75%
	Terpenes	28%
N. pannonica	Ectoine	50%
	Terpenes	21%
N. populi	Salinichelins	23%
	Ectoine	75%
	Terpenes	50%
N. salmonea	Terpenes	28%
	Tetronasin	3%
	Ectoine	75%
N. sandarakina	Pentalenolactone	15%
	Ectoine	50%
	Ishigamide	11%
	Terpenes	28%
N. sedimenti	Ectoine	75%
	Lankacidin C	13%
N. sphaerica	Terpenes	50%
	Ectoine	50%
N. xinjiangensis	Terpenes	28%
	Pentostatine/Vidarabine	9%
	Ectoine	75%
	Desferrioxamin B	60%

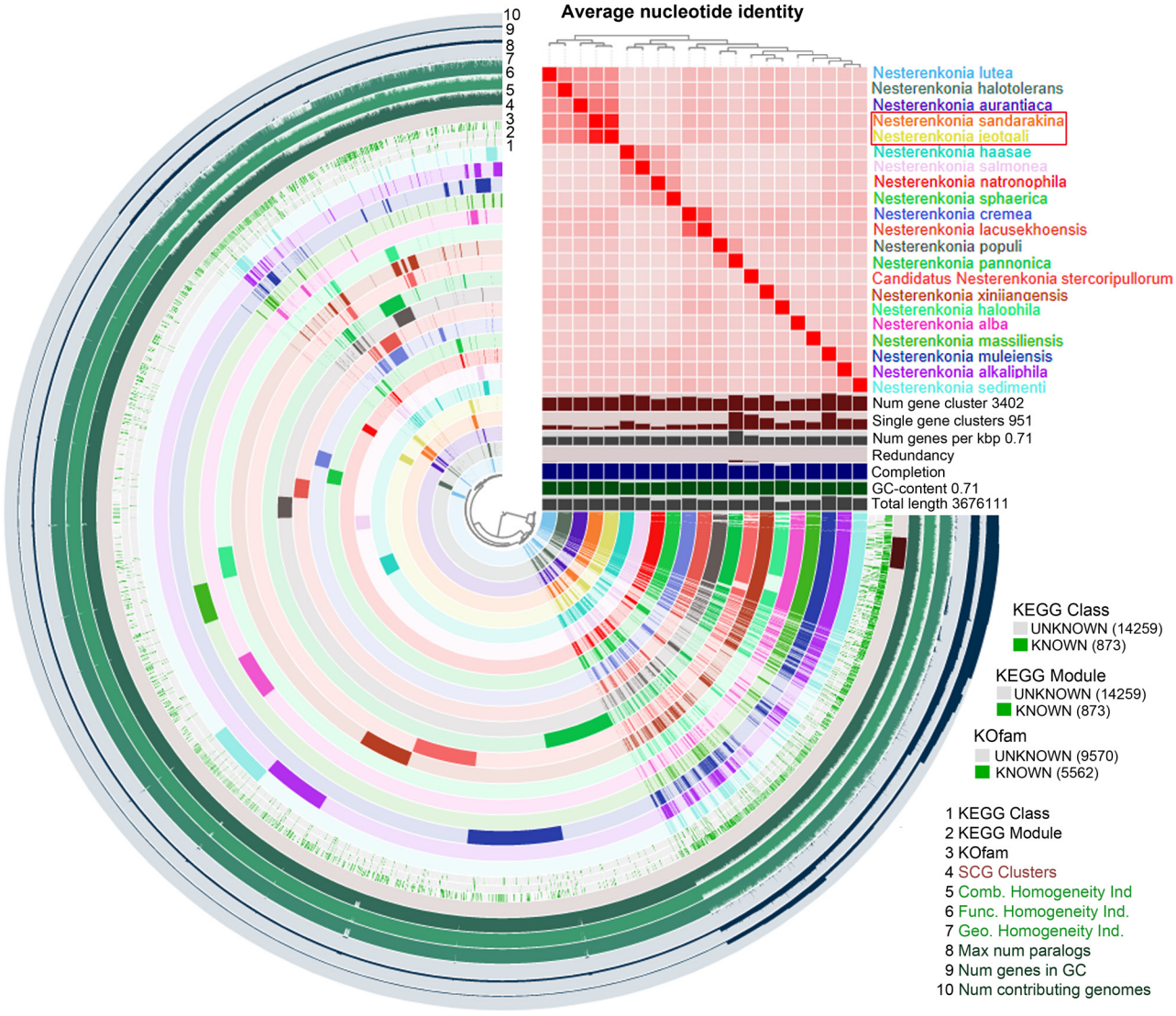

Fig. 3. Pangenome analysis of *Nesterenkonia* members.

microorganisms, including actinobacteria (Pastor et al. 2010). Ectoine has been reported as a skin protectant for anti-inflammatory treatment and a potential candidate for anti-amyloid therapeutics (Pastor et al. 2010). Thus, the discovery of ectoine in *Nesterenkonia* members may indicate significant applications for therapeutic uses, in addition to osmolyte. *N. alba* gene cluster showed high similarity to kocurin (Table II). Kocurin has been reported to be active against methicillin-resistant *Staphylococcus aureus* (Martín et al. 2013). Further, some gene clusters showed low similarity with known natural products (Table II), suggesting that these pathways may encode natural products; however, further studies are required.

ANI, phylogenomic, and pangenome analysis. Phylogeny using whole-genome sequences has become an important tool for delineating of prokaryotic taxa (Liu et al. 2019), and ANI has emerged as a robust method to compare genetic relatedness among prokaryotic strains (Jain et al. 2018). Pangenomes provide extensive characterizations of core and accessory genes in a collection of closely related microbial genomes by grouping genes based on sequence homology (Delmont and Eren 2018).

In the present study, except for *Nesterenkonia sandarakina* and *N. jeotgali*, the ANI values between *Nesterenkonia* members were <96% (Table SII, Fig. 3). The ANI value between *N. sandarakina* and *N. jeotgali* was 96.8%, above the threshold value (95–96%) for bacterial species delineation (Richter and Rosselló-Móra 2009). In the phylogenomic tree (Fig. 4), *N. sandarakina* and *N. jeotgali* clade together. The above results suggest that *N. sandarakina* and *N. jeotgali* were similar species. Fig. 3 shows the pangenome analysis of *Nesterenkonia* members. The number of singleton gene clusters, functional homogeneity index, and genome homogeneity index among *Nesterenkonia* vary. Although *N. sandarakina* and *N. jeotgali* were closely related, there was variation in the core and pangenome. The highest number of singletons was observed in *N. pannonica* and

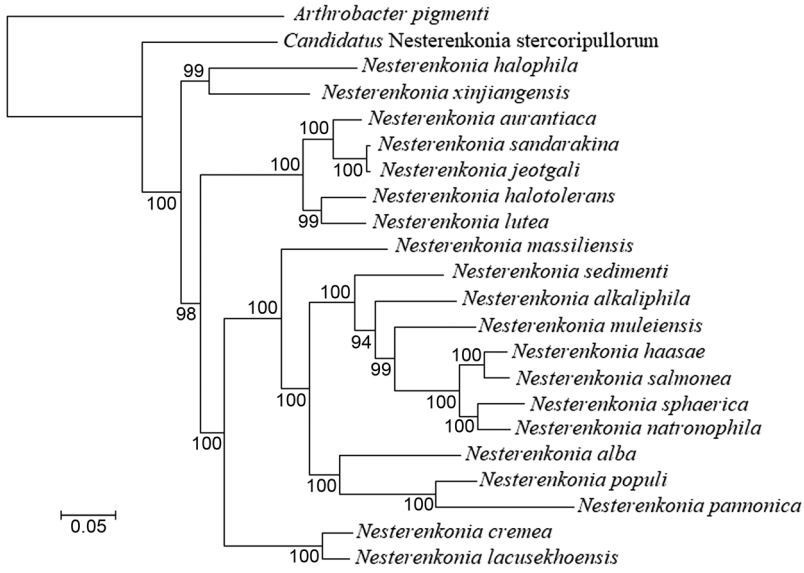

Fig. 4. Phylogenomic tree based on 71 bacterial single-copy genes showing the position of *Nesterenkonia* members. Bootstrap values (expressed as percentages of 1,000 replications) greater than 50% are shown at branch points. Bar, 0.05 represents substitution per nucleotide position. *Arthrobacter pigmenti* was used as an out-group.

Nesterenkonia muleiensis. The number of gene clusters was also found more in *N. muleiensis*.

Based on the above results, we propose reclassifying *Nesterenkonia jeotgali* (Yoon et al. 2006) as a later heterotypic synonym of *Nesterenkonia sandarakina* (Li et al. 2005).

Emended description of *Nesterenkonia sandarakina*. The description is the same as that given by Li et al. (2005) with the following modification. The genomic DNA G + C content of the type strain is 67.5%. The type strain is YIM 70009^T (= CCTCC AA 203007^T = DSM 15664^T = KCTC 19011^T). Strains JG-241 (= KCTC 19053 = JCM 12610) are other strains of this species.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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