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Genus-specific PCR for molecular identification of novel isolates of the genus *Nesterenkonia*

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Abstract: [Objective and Method]: For rapid identification of *Nesterenkonia* species, one set of genus-specific primers was designed and synthesized for polymerase chain reaction based on the 16S rRNA gene sequences. **[Results and Conclusion]**: The genus specificity of these primers was validated with reference strains as well as with wild-type isolates. Partial sequencing results of 16S rRNA gene of the wild-type isolates confirmed that they are members of the genus *Nesterenkonia*.

Keywords: *Nesterenkonia*; Genus-specific primers; Molecular identification

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1 INTRODUCTION

A phylogenetic and chemotaxonomic re-analysis of the genus *Micrococcus* resulted in the proposal of the *Nesterenkonia* gen. nov. and the reclassification of *Micrococcus halobius* Onishi and Kamekura 1972 as *Nesterenkonia halobia*[1]. *N. halobia* is a moderately halophilic Gram-positive, non-motile coccus and can produce an extracellular amylase that depends on divalent cations and a high concentration of NaCl or KCl for activity and stability[2]. Recently, the species *N. aethiopica*, isolated from an Ethiopian soda lake[3], *N. sandarakina*, from a soil in the eastern desert of Egypt, and *N. lutea*, from a saline soil in China[4], *N. xinjiangensis* and *N. halotolerans*, from hypersaline soils in China[5], *N. lacusekhoensis*, from a hypersaline lake in eastern Antarctica[6], *N. jeotgali*, from jeotgal (a traditional Korean fermented seafood) [7], have been described. All *Nesterenkonia* species are Gram-positive, strictly aerobic and moderately halophilic or halotolerant (and some species are alkaliphilic or alkalitolerant), contain peptidoglycan of the A4α type and have DNA G+C contents in the range 64–72 mol%[4]. The remarkable adaptability to saline environment makes the *Nesterenkonia* an important group in the microbial community of saline habitat. During a biodiversity and taxonomic study on halophilic and halotolerant actinobacteria, numerous new strains were isolated from dry hypersaline soil samples collected from Xinjiang Municipality in western China.

The well-known fact is that the identification of each novel isolate by using polyphasic taxonomy method is time-consuming, which cannot ever be undertaken when handling large numbers of new isolates, as in the case in a biodiversity study or in a natural product screening program, when we were dealing with non-filamentous actinobacteria or bacteria especially. Consequently, the development of a rapid and effective method for discriminating different isolates is the crucial work in biodiversity and taxonomic study. Advances in nucleic acid techniques, based on data derived from the comparative analysis of 16S rRNA gene sequences, have allowed the development of novel and powerful tools for studying microorganisms[8]. One important application is to develop specific probes that can be applied at different taxonomic levels, including family, genus, and even species. The specific probes as selective amplification primers offer an alternative approach for the rapid identification of large numbers of strains[9,10]. If some region of DNA sequences is present in only one microbial group, but not the others,

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that region can be used as a genetic marker to detect a certain microbe. Relatively, the 16S rRNA gene sequences of different genera have more dissimilarity. Therefore, genus-level differentiation could be more easily accomplished. The application and usefulness of the genus-specific primers has already been reported for different members of the families *Pseudonocardiaceae*[11,12] and *Nocardiopsaceae* [13,14].

This study developed the genus-specific primers based on 16S rRNA genes for PCR identification of the Nesterenkonia.

2 MATERIALS AND METHODS

2.1 Bacterial strains

The bacterial reference strains used in this study are listed in Table 1. Wild-type isolates were obtained from the laboratory culture collection. All strains were grown at 28°C on the GYM (0.4% glucose, 0.4% yeast extract, 1.0% malt extract and 0.2% $CaCO_3$) agar plates, or on the saline GYM agar with 5–10% (w/v) NaCl in case of some halophilic or halotolerant strains.

Table 1 Reference strains used in this study and results obtained from selective PCR amplification with Nes1 and Nes2

Reference strains	Nes1/Nes2
	56°C
Nesterenkonia lacusekhoensis DSM 12544^T	1
Nesterenkonia halobia DSM 20541^T	1
Nesterenkonia sandarakina YIM 70009^T	1
Nesterenkonia lutea YIM 70081^T	1
Nesterenkonia xinjiangensis YIM 70097^T	1
Nesterenkonia halotolerans YIM 70084^T	1
Micrococcus lylae IFO15355^T	0
Micrococcus luteus DSM 20030^T	0
Bogoriella caseolytica DSM 11294^T	0
Kocuria polaris DSM 14382^T	0
Kocuria rosea DSM 20447^T	0
Georgenia muralis DSM 14418^T	0
Georgenia ruanii YIM 004^T	0
Isoptericola hypogeus DSM 16849^T	0
Jonesia denitrificans DSM 20603^T	0
Verrucosispora gifhornensis DSM 44337^T	0
Myceligenerans xiligouense DSM 15700^T	0
Agromyces aurantiacus YIM 21741^T	0
Kribbella alba YIM 31075^T	0
Kribbella antibiotica YIM 31530^T	0
Nocardia alba YIM 30243^T	0
Nocardia lijiangensis YIM 33378^T	0
Citricoccus alkalitolerans YIM 70010^T	0
Yania halotolerans YIM 70085^T	0
Corynebacterium halotolerans YIM 70093^T	0
Microbacterium halotolerans YIM 70130^T	0
Rhodococcus fascians DSM 20669^T	0
Jiangella gansuensis YIM 002^T	0
Glycomyces harbinensis DSM 46494^T	0
Halobacillus halophilus DSM 2266^T	0
Virgibacillus pantothenticus DSM 26^T	0
Bacillus subtilis subsp. *subtilis* DSM 10^T	0

Amplification results: 1, band of the expected size; 0, absence of amplification products.

2.2 DNA extraction

Total genomic DNA from the reference strains and all wild-type isolates were extracted as the method described by Xu *et al*. [15]

2.3 Design of oligonucleotide primers

The 16S rRNA gene sequences of reference strains were obtained from the GenBank. Alignment of multiple sequences was carried out by using the alignment program CLUSTAL_X [16] to determine the regions conserved only among *Nesterenkonia* species, from which the genus-specific primers were derived. We used PRIMER PREMIER (version 5.0) to search PCR primers in these conserved regions. The genus specificity of oligonucleotides was tested against all DNA sequences available in the EMBL database with the FASTA program[17] and in BLAST program [18], respectively. The melting temperature (T_m) was estimated according to Thomas and Dancis and the Lathe's formulae[19]. Relative T_m values obtained using 0.3 M as a standard salt concentration helped with the design of a pair of primers with similarly high melting temperatures. The probabilities of primer- dimer formation, auto-folding and false priming were also predicted by using the OLIGO program (version 6.0) in order to keep them as low as possible. The oligonucleotides were customly synthesized by the Sangon Biotech (China).

2.4 PCR amplification and 16S rRNA gene sequencing

PCR with primers Nes1/Nes2 was performed in a final volume of 25 μL containing 0.2 mM each of the four dNTPs (TaKaRa), 0.1 μM each primer, 1μL extracted DNA (including reference strains and wild-type isolates) and 0.5 U *Taq* DNA polymerase (TaKaRa) with its appropriate reaction buffer. Amplification was carried out in a Biometera Tpersonal Thermocycler, according to the following hot-start PCR profile: 4 min at 94°C for denaturartion, and then 30 cycles of 30 s at 94°C, 30 s at 56°C and 2 min at 72°C, followed by 10 min at 72°C. Negative controls without template DNA were included for each PCR experiment. Amplification products were analyzed by electrophoresis ($5V \cdot cm^{-1}$) in 1.5% (w/v) agarose gels stained with ethidium bromide. The PCR experiment with Nes1/Nes2 was repeated twice.

16S rRNA genes of 25 wild-type isolates were amplified by PCR using universal primers. The amplified 16S rRNA gene fragments were purified by using TaKaRa DNA fragment purification kit (version 2.0). The partial sequence of 16S rRNA gene was obtained automatically using a DNA sequencer (model 377; Applied Biosystems) to confirm the phylogenetic position of wild-type strains. The sequences of all wild-type strains were submitted to the GenBank. The accession numbers are listed in Table 2.

Table 2 Natural isolates used in PCR experiments with genus-specific primers Nes1/Nes2

Strains	Accession number	Nes1/Nes2
Nesterenkonia spp. YIM 90711*, YIM 90713*, YIM 90714*, YIM 90719*, YIM 90720*, YIM 90721*, YIM 90725*, YIM 90726*, YIM 90735*, YIM 91072*, YIM 91091*, YIM 91101*	EF151504-EF151515	1
Bacillus spp. YIM 90710*, YIM 90712*, YIM 91061*, YIM 91067*, YIM 91075*, YIM 91077*	EF151519-EF151524	0
Arthrobacter spp. YIM 90728*, YIM 90830*, YIM 90835*	EF151516-EF151518	0
Halomonas spp. YIM 90759*, YIM 90831*, YIM 90832*, YIM 91105*	EF151525-EF151528	0
Unidentified bacteria YIM 90722, YIM 90727, YIM 90731, YIM 90736, YIM 90833, YIM 90834, YIM 90836, YIM 91107 , YIM 91110, YIM 91113, YIM 91117		0

*Sequenced strain. Amplification results: 1, band of the expected size; 0, absence of amplification products.

2.5 Phylogenetic analysis

The phylogenetic analysis was performed by using the MEGA (version 3.1) software packages [20] after multiple alignments of data by CLUSTAL_X[16], with gaps treated as missing data. Clustering was performed by using the neighbour-joining method[21]. Bootstrap analysis was used to evaluate the tree topology of the neighbour-joining data by performing 1000 resamplings[22].

3 RESULTS AND DISCUSSION

3.1 Design of genus-specific primers

The design of one set of genus-specific primers for the selective amplification of the 16S rRNA gene regions of the genus *Nesterenkonia* has been based on the search for conserved sequences within this genus after a comparative analysis of the variable regions of the known 16S rRNA gene sequences. An alignment of the 16S rRNA gene sequences that were used for the design of the primers is shown in Fig. 1. In this alignment, two regions, covering nucleotides 190–220 and 1300–1330, corresponding to nucleotide positions of 16S rRNA gene of the *Escherichia coli* (GenBank accession number J01695), were found to be conserved among the different species of the genus. These sub-sequence homologies were high enough to consider the design of genus-specific primers. The two primers, Nes1 (5′-CGC ATA GGG TGC TGG TGG AAA G-3′) and Nes2 (5′-GAG GTC GGG TTG CAG ACT TCG-3′), were designed for the selective amplification corresponding to the region 194–215 and 1308–1328 of the 16S rRNA gene, respectively. The proposed primers might be useful for particular purposes, e.g., for identification or detection of certain actinobacteria belonging or closely related to the species *N. aethiopica*, *N. xinjiangensis*, *N. halobia*, *N. halotolerans*, *N. sandarakina*, *N. lutea* and *N. lacusekhoensis*. More distant members of the genus, which have not isolated and described from nature, seem to be hardly detected by this approach. Although this is a remarkable and considerable question, the feasibility of this method would not be denied absolutely. On the basis of the analysis of alignment and BLAST search, we can infer that these two regions are conservative in all *Nesterenkonia* species, even though only seven species were discovered until now (At the time of writing, another novel species, *Nesterenkonia jeotgali*, has been described validly by Yoon *et al.* [7]). The whole polyphasic taxonomic characterization had not been carried out on these wild-type isolates. Whereas, the results of sequencing basically showed that the isolates with absence of amplification products do not belong to the genus *Nesterenkonia*. According to our primary experiment results, it cannot absolutely be demonstrated that this pair of primers can be used for tentative assignment of new isolates to *N. aethiopica*, *N. xinjiangensis*, *N. halobia*, *N. halotolerans*, *N. sandarakina*, *N. lutea*, *N. lacusekhoensis* and closely related species. It is reasonable, however, that this pair of primers could be used for tentative assignment of new isolates to the genus *Nesterenkonia*.

The characterization of Nes1 and Nes2 for origination of the expect PCR reaction was evaluated by using PRIMER PREMIER (version 5.0) and OLIGO program (version 6.0). The only difference between Nes1 and template matching with Nes1 came from purposive manual modification. This modification can eliminate the risk of false priming, dimers forming effectively and diminish the T_m value of Nes1 simultaneously. If guanine was replaced by cytosine, the dimer would be eliminated from the analysis of software. However, the problem of false priming would not be resolved. For the same reason, there had two sites that were modified in the sequence of Nes2. Nevertheless, the possibility of dimer forming still exists. The purpose of increasing the G+C content of Nes2 was to make Nes1 and Nes2 have close T_m values, so as to make the forward primer and reverse primer anneal simultaneously. In the PCR experiment, the primer dimers did not appear. The elevated annealing temperature maybe caused this result. And the quantity

	5’ 194 . . 215 3’	5’ 1308 . . 1328 3’
	Nes1 CGCATAGGGTGCTGGTGGAAA G	Nes2 GAGGTCGGGTTGCAGACTTCG
Nesterenkonia aethiopica DSM 17733^{T} (AY574575)	ACCAGTCCTCGCATGAGGTGCTGGTGGAAA-GATTT	CAACTTCACGAAGTCGAGTTGCAGACTTCGATCCG
Nesterenkonia xinjiangensis YIM 70097^{T} (AY226510)	ACCAGTCCCCGCATGGGGTGCTGGTGGAAA-GATTT	CAACTTCACGAAGTCGAGTTGCAGACTTCGATCCG
Nesterenkonia halobia DSM 20541 (X80747)	ACCAGTCCTCGCATGGGGTGCTGGTGGAAA-GATTT	CAACTTCACGAAGTCGAGTTGCAGACTTCGATCCG
Nesterenkonia halotolerans YIM 70084^{T} (AY226508)	ACCAGTCCTCGCATGGGGTGCTGGTGGAAA-GATTT	CAACTTCACGAAGTCGAGTTGCAGACTTCGATCCG
Nesterenkonia sandarakina YIM 70009^{T} (AY588277)	ACCAGTCCTCGCATGGGGTGCTGGTGGAAA-GATTT	CAACTTCACGAAGTCGAGTTGCAGACTTCGATCCG
Nesterenkonia lutea YIM 70081^{T} (AY588278)	ACCAGTCCTCGCATGGGGTGCTGGTGGAAA-GATTT	CAACTTCACGAAGTCGAGTTGCAGACTTCGATCCG
Nesterenkonia lacusekhoensis EL-30 (AJ290397)	ACCAGTCACCGCATGGTGTGCTGGTGGAAA-GCTTT	CAACTTCACGAAGTCGAGTTGCAGACTTCGATCCG
Arthrobacter histidinolovorans DSM 20115 (X83406)	ACTCCTCATCGCATGGTGGGG-GGTGGAAA-GCTTT	CAACTTCATGGGGTCGAGTTGCAGACCCCAATCCG
Arthrobacter ilicis DSM 20138 (X83407)	ACCATCTGGCGCATGTCATGGTGGTGGAAA-GCTTT	CGACTTCACGGGGTCGAGTTGCAGACCCCGATCCG
Micrococcus antarcticus T2^{T} (AJ005932)	AACGTCTACCGCATGGTG-GGTGTTGGAAA-GAATT	CGACTTCACGGGGTCGAGTTGCAGACCCCGATCCG
Micrococcus lylae DSM 20315^{T} (X80750)	AGCATTCACCGCATGGTG-GGTGTTGGAAA-GAATT	CGACTTCATGAGGTCGAGTTGCAGACCTCAATCCG
Citricoccus alkalitolerans YIM 70010^{T} (AY376164)	ACCTCCTATCGCATGGTATGGGGGTGGAAA-GATTT	CGACTTCATGGGGTCGAGTTGCAGACCCCAATCCG
Kocuria rhizophila DSM 11926^{T} (Y16264)	ACATGTCACCGCATGGTG-GTGTGTGGAAA-GGGTT	CGACTTCACGTAGTCGAGTTGCAGACTACGATCCG
Kocuria aegyptia YIM 70003^{T} (DQ059617)	ACTGTCCACCGCATGGTG-GGTGGTGGAAA-GGGTT	CGACTTCACGTAGTCGAGTTGCAGACTACGATCCG
Rothia aeria GTC 867^{T} (AB071952)	ACCAATCTCTGCATGGGGTGTTGGTGGAAA-GCGTT	CGACTTCACGTAGTCGAATTGCAGACTACGATCCG
Rothia nasimurium CCUG 35957 (AJ131121)	ACCAGTTCCCGCATGGGATGCTGGTGGAAA-GGGAT	CGACTTCATGGGGTCGAGTTGCAGACCCCAATCCG
Intrasporangium calvum DSM 43043^{T} (AJ566282)	ACCCTCGTACGCATGTGCTGGGGGTGGAAA-GTTTT	CGACTTCATGGGGTCGAGTTGCAGACCCCAATCCG
Janibacter limosus DSM 11141^{T} (Y08540)	AGACCAACCTGCATGGGTATGGTTTGGAAA-GTTTT	CGACTTCATGGGGTCGAGTTGCAGACCCCAATCCG
Dermatophilus chelonae DSM 44178^{T} (AJ243919)	ACCAATCTCCGCATGGAGTGTTGGTGGAAA-GCTTT	CGACTTCACAGGGTCGAGTTGCAGACCCTGATCCG
Dermacoccus nishinomiyaensis DSM 20448^{T} (X87757)	ACCGATTTCCGCATGGAGTGTTGGTGGAAA-GTTTT	CGACTTCACGGGGCCGAGTTGCAGACCCCGATCCG
Dermabacter hominis DSM 7083 (X91034)	ACCTTGGCCCGCATGG-TTTTTGGTGGAAA-GTTTT	CGACTTSACGGGGTCGAGTTGCAGACCCCGATCCG
Promicromonospora aerolata V-54A^{T} (AJ487303)	ACGCT-TGCCGCATGGTGGGTGT-TGGAAA-GTTTT	CGACTTCATAGGGTCGAGTTGCAGACCCCAATCCG
Myceligenerans xiligouense XLG9A10.2 (AY354285)	AGTCTCTGCCGCATGGTGGGGGT-TGGAAA-GATTT	CGACTTCATGGGGTCGAGTTGCAGACCCCAATCCG
Bogoriella caseolytica HKI0088^{T} (Y09911)	CCTACCT-CCGCATGGTGTGTGGGTGGAAA-GCTTG	CGACTTCATGGGGTCGAGTTGCAGACCCCAATCCG
Rarobacter faecitabidus DSM 4813 (Y17870)	CCTTG-GGACACATGTTTTGGG-GTGGAAA-GTTTT	CGACTTCATGGGGTCGAGTTGCAGACCCCAATCCG
Sanguibacter marinus 1-19^{T} (AJ783958)	GATGCCT-CCGCATGGGGAGTGTCTGGAAA-GATTT	CGACTTCATGGGGTCGAGTTGCAGACCCCAATCCG
Cellulomonas persica I (AF064701)	GACGCAC-GGGCATCTGTAGCGTCTGGAAA-GATTT	CGACTTCATGGGGTCGAGTTGCAGACCCCAATCCG
Brevibacterium casei DSM 20657 (AJ251418)	ACTGCCGGACGCATG-TCTGGTGGTGGGAAAGTTTT	CGACTTCATGGGGTCGAGTTGCAGACCCCAATCCG
Brevibacterium otitidis A37/73 (X93593)	ACTACTGGCCGCATGGTCTGGTGGTGGAAA-GGGTT	CGACTTCATGAGGTCGAGTTGCAGACCTCAATCCG
Microbacterium halotolerans YIM 70130^{T} (AY376165)	CACTGTAGCCGCATGGTTTG-CAGTGGGAAAGATTT	CGACTTCACGTGGTCGAGTTGCAGACCACGATCCG
Streptomyces yunnanensis YIM 41004^{T} (AF346818)	ACACGGGGTCGCATGATCTCCGTGTGGAAA-GCTCC	CGACTTCATGGGGTCGAGTTGCAGACCCCAATCCG
Corynebacterium atypicum CCUG 45804^{T} (AJ441057)	AC--TGCGCTGTGGGTGGTG-TGGTGGAAA-GCTTT	CAACTTCATGGGGTCGAGTTGCAGACCCCAATCCG
Mycobacterium alvei CIP 103464 (AF023664)	ACCATGCACTTCCTGGTGTG-TGGTGGAAA-GCTTT	CGACTTCATGGGGTCGAGTTGCAGACCCCAATCCG
Micromonospora mirobrigensis WA201^{T} (AJ626950)	ACTGCTGGACGCATGTCTGG-TGGTGGAAA-GTTTT	CGACTTCATGGGGTCGAGTTGCAGACCCCAATCCG
Pseudonocardia halophobica IMSNU 21327^{T} (AJ252827)	ACCTTCTCCTGCATGGGAGG-GGGTGGAAA-GTTTT	CGACTTCATGGGGTCGAGTTGCAGACCCCAATCCG

Fig. 1 lignment of GenBank 16S rRNA gene sequences of members of actinobacteria taxa used to evaluate the specificity of the *Nesterenkonia*-specific primers Nes1/Nes2 Shaded boxes indicate conserved positions.

of primers used in PCR reaction volume affects the appearance of primer dimers specially. The crucial differentia, between the design of group-specific PCR primers and that of general PCR primers, is the specificity of DNA fragment matching with primers. The designer must consider this issue in entire process. In this case, the contradiction emerged between the searching for appropriate PCR primers and the guarantee of specificity of primers. When we were searching for one pair of primers with close T_m value, it was particularly conspicuous contradictions. So, the purposive manual modification was applied. Certainly, the specificity of primers would not been depressed. The BLAST and FASTA search results showed that. It must be avoided that the modifications appear at 3 ′end of primers for reducing the impact of the primer annealing.

3.2 Specificity of the primers for the genus

The alignment with the sequences of other genera showed three to nine no-match positions with the Nes1 sequence. And most of sequences had one oligonucleotide deletion. That would be good for enhancing the specificity of Nes1 for *Nesterenkonia*. The Blast result showed that 37 sequences deposited in GenBank were hit. In 37 Blast hits, the expect value of 14 Blast hits was 3.7. In the 23 Blast hit whose expect value equaled to 0.94, 15 sequences belonged to *Nesterenkonia*. The alignment with the sequences of genera showed four to five no-match positions with the Nes2 sequence. And at 3 ′end of primer, there had two or three no-match positions.

When the primer pair Nes1/Nes2 was tested in PCR using an annealing temperature of 56°C, defined as optimal for them, we obtained the expected amplification product of 1120 bp from all type strains of the genus *Nesterenkonia*. Meanwhile, no amplification products were obtained with DNA from reference strains except the *Nesterenkonia* species (Fig. 2). The same results were obtained when amplification was performed at different annealing temperature (54°C to 68°C). When

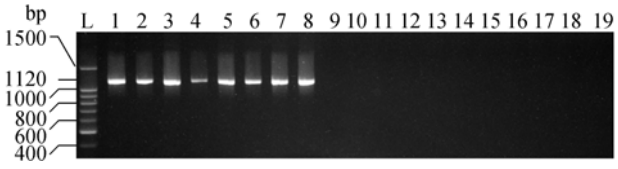

Fig. 2 Agarose gel electrophoresis of PCR products from DNA. Selective amplification at 56 °C of an 1120 bp fragment using primers Stmp1/Stmp2 specific for the genus *Nesterenkonia*. Lanes: L, DNA size ladder; 1, *Nesterenkonia lacusekhoensis* DSM 12544^T; 2, *Nesterenkonia halobia* DSM 20541^T; 3, *Nesterenkonia sandarakina* YIM 70009^T; 4, *Nesterenkonia lutea* YIM 70081^T; 5, *Nesterenkonia xinjiangensis* YIM 70097^T; 6, *Nesterenkonia halotolerans* YIM 70084^T; 7, YIM 90711; 8, YIM 90713; 9, *Micrococcus lylae* IFO15355^T; 10, *Bogoriella caseolytica* DSM 11294^T; 11, *Kocuria polaris* DSM 14382^T; 12, *Georgenia ruanii* YIM 004^T; 13, *Jonesia denitrificans* DSM 20603^T; 14, *Agromyces aurantiacus* YIM 21741^T; 15, *Kribbella antibiotica* YIM 31530^T; 16, *Nocardia lijiangensis* YIM 33378^T; 17, *Citricoccus alkalitolerans* YIM 70010^T; 18, *Halobacillus halophilus* DSM 2266^T; 19, control reaction without DNA.

we used an annealing temperature below 52°C for PCR reaction, *Micrococcus lylae* AS1.2300, *Kocuria rosea* DSM 20447 and *Citricoccus alkalitolerans* YIM 70010 were amplified. All these species above-mentioned and the genus *Nesterenkonia* belong to the family *Micrococcaceae*. So they have relativeness genetically. However, the optimization of PCR reaction system can relieve the interference of pseudo-positive results. To elevate the annealing temperature can avoid non-specific amplification effectively.

3.3 Identification of wild-type isolates

In this work, we selected a group of 36 wild-type isolates from our culture collection, which have never been definitively assigned to any genera because of the absence of required chemotaxonomic and morphological study. DNA was extracted from each of the 36 wild-type isolates and amplified with Nes1/Nes2. A positive amplification was only obtained in 12 isolates. A phylogenetic tree based on 16S rRNA gene sequences was constructed using the neighbor-joining method (Fig. 3), showing the inter- and intra-specific relationships of the wild-type strains to reference strains. The topology of the tree further confirmed that our previous studies had determined the taxonomic relationships of the wild-type strains by PCR amplification. The results obtained here with reference strains, as well as the high degree of conservation observed in most of the sequences of the analyzed wild-type isolates, validate the specificity of this new primer pair for 16S rRNA gene sequences of the genus *Nesterenkonia*. These data support the usefulness of this primer pair for the tentative assignment of new isolates to this genus from the large numbers of strains that are normally obtained from the environment.

In addition to tentative assignment of novel isolates to specific genus, genus-specific PCR combined with amplified rDNA restriction analysis technique can be used to differentiate differ species belonging to the same genus. This evolutional approach had applied in the identification of *Bacillus* species[23], *Bifidobacterium* species[24], *Staphylococcus* species[25], and *Weissella* species[26]. The specific amplicons obtained by group-specific PCR were digested by restriction enzymes. The ARDRA profiles can be used to differentiate among strains in which a positive amplification was obtained. However, the polymorphism of restriction fragments must be obtained for differentiation. Furthermore, real-time PCR combined with group-specific primers design promote the development of microbial ecology from a methodological perspective. This method had been used for the detection and quantification of *Geodermatophilaceae* from stone samples and identification of new members of the genus *Blastococcus* by Salazar *et al*.[27]. And in the field of food quality testing, specific real-time PCR also plays an important role[28,29]. Although no pathogenic *Nesteren-konia* species have been discovered in nature, *Nesterenkonia*-specific PCR primers are still very helpful to our study of its quantitative distribution and species diversity.

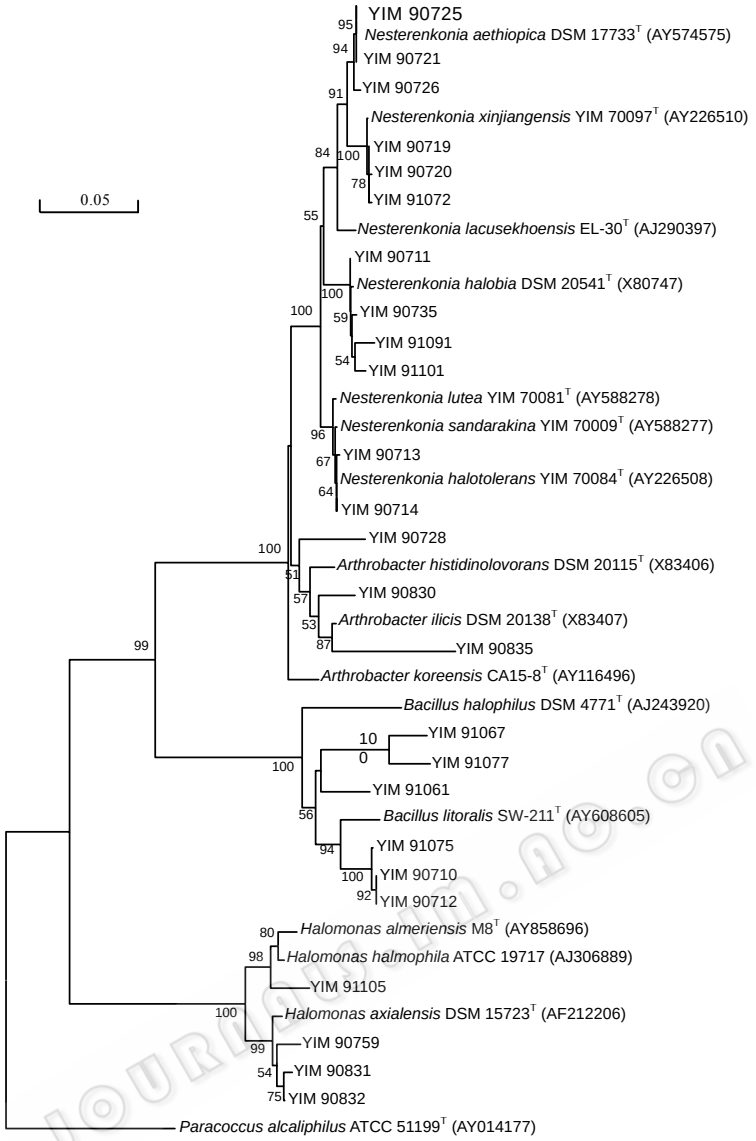

Fig. 3 Diversity of natural isolates identified with Nes1/Nes2 and their relationships to reference strains. The phylogenetic tree was constructed by the neighbor-joining method. Bootstrap values (>50%) from 1000 analyses are shown at the nodes of the tree. The scale bar represents 2 inferred nucleotides changing per 100 nucleotides.

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涅斯捷连科氏菌特异性引物 PCR 分子鉴定

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摘要：涅斯捷连科氏菌属的建立基于对 *Micrococcus halobius* 的再分类，这是一类广泛分布于高盐土壤环境的革兰氏阳性细菌，属放线菌类。在对我国西部盐湖环境放线菌的生物多样性及分类学研究中，大量类似菌株被分离。【目的】为了对其进行快速鉴定，特别是筛选出属于优势类群的涅斯捷连科氏菌，【方法】本研究根据前人以报道的方法设计了一对针对其 16S rRNA 基因的特异性 PCR 引物（Nes1/Nes2），【结果】并通过部分典型菌和野生菌株的 PCR 实验验证，结合 16S rRNA 基因的测序验证，证明了 Nes1/Nes2 的 PCR 反应有效性及其对涅斯捷连科氏菌的特异性。【结论】利用该引物可以快速准确的对涅斯捷连科氏菌进行鉴定。

关键词：涅斯捷连科氏菌；属特异性引物；分子鉴定

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