Genome-based reclassification of Anoxybacillus karvacharensis Panosyan et al. as a later heterotypic synonym of Anoxybacillus kestanbolensis Dulger et al. 2004

Kadriye INAN BEKTAS ( kadriyensis@gmail.com )
Karadeniz Technical University

Halil İbrahim GULER
Karadeniz Technical University

Sabriye CANAKCI
Karadeniz Technical University

Ali Osman BELDUZ
Karadeniz Technical University

Research Article

Keywords: Anoxybacillus karvacharensis, Anoxybacillus kestanbolensis, genome-based reclassification

Posted Date: May 3rd, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1603963/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License
Abstract

In the present study, we attempted to clarify the relationship between *Anoxybacillus karvacharensis* K1\(^T\) and *Anoxybacillus kestanbolensis* NCIMB13971\(^T\) by using whole-genome phylogenetic analysis. The genome sequence of *A. kestanbolensis* NCIMB13971\(^T\) was not available in any database, so it was sequenced in this study. The 16S rRNA gene sequence obtained from the genome of *A. kestanbolensis* NCIMB13971\(^T\) had 99.93% similarity with *A. karvacharensis* K1\(^T\). Also, *A. karvacharensis* K1\(^T\) and *A. kestanbolensis* NCIMB 13971\(^T\) formed a robust branch different from other type strains of *Anoxybacillus* in the phylogenetic trees based on whole-genome sequences and 16S rRNA gene sequences.

The average nucleotide identity (ANI), average amino acid identity (AAI) and digital DNA–DNA hybridization (DDH) values between *A. karvacharensis* K1\(^T\) and *A. kestanbolensis* NCIMB13971\(^T\) were greater than the threshold values for species demarcation. The present results indicate that both strains were considered to belong to the same species and *A. karvacharensis* K1\(^T\) is later heterotypic synonym of *A. kestanbolensis* NCIMB13971\(^T\).

Introduction

The genus *Anoxybacillus*, a member of the phylum Firmucutes, was first described by Pikuta et al. (2000) describing the type species of this genus *Anoxybacillus pushchinoensis*. According to the list of LPSN (https://lpsn.dsmz.de/genus/bacillus), the genus *Anoxybacillus* includes 24 species with validly published names. The taxonomy of *Anoxybacillus* members was predominantly based on 16S rRNA gene sequence analysis and DNA-DNA hybridization (DDH). However, it is widely known that the resolving power of 16S rRNA gene analysis is generally limited and DDH is difficult to reproduce and sometimes varies depending on the particular method of the laboratory used. Phylogeny using whole-genome sequence based metrics such as average nucleotide identity (ANI), digital DDH and average amino acid identity (AAI) has become an important tool for the delineation of prokaryotic taxa (Orata et al. 2018) and is being used for the reclassification of several bacterial taxa (Liu et al., 2019; Rao et al., 2022).

*Anoxybacillus. kestanbolensis* NCIMB 13971\(^T\) was isolated from hot spring in Turkey by Dulger et al. in 2004 and described as validly named species based on a polyphasic taxonomic approach. *Anoxybacillus karvacharensis* K1\(^T\) was isolated from the Karvachar geothermal spring in Nagorno-Karabakh by Panosyan et al. in 2021 and proposed as validly named species based mainly on genomic average nucleotide identity (ANI) value and pairwise digital DNA–DNA hybridization values between the *A. karvacharensis* K1\(^T\) and *A. flavithermus*, *A. flavithermus* subsp. *yunnanensis*, *A. tengchongensis*, *A. mongoliensis*, *A. pushchinoensis*, *A. thermarum*, *A. ayderensis*, *A. kamchatkensis*, *A. eryuanensis*, *A. gonensis*, *A. rupiensis*. Phylogenetic tree based on 16S rRNA gene sequences in the original article showed that *A. karvacharensis* K1\(^T\), *A. flavithermus* DSM 2641\(^T\) and *A. kestanbolensis* NCIMB 13971\(^T\) clustered together. However, in the original article Panosyan et al. determined only *A. flavithermus* DSM
as the nearest neighbour based on 16S rRNA gene sequences similarity and phylogenetic tree, they did not identify *A. kestanbolensis* NCIMB 13971 as the nearest neighbour. The genome sequence of *A. kestanbolensis* NCIMB 13971 was not available in any database, so Panosyan et al. did not determine (ANI) value and pairwise digital DNA–DNA hybridization values between the *A. karvacharensis* K1 and *A. kestanbolensis* NCIMB 13971. Hence, in the present work *A. kestanbolensis* NCIMB 13971 was sequenced and we attempted to clarify, employing genomics-based methods, the relationship between the type strains of *A. karvacharensis* K1 and *A. kestanbolensis* NCIMB 13971. The data presented in this study provides evidence that *A. karvacharensis* K1 is later heterotypic synonym of *A. kestanbolensis* NCIMB 13971.

**Materials And Methods**

*A. kestanbolensis* NCIMB 13971 was purchased from the National Collection of Industrial Food and Marine Bacteria, and it was grown on Nutrient Agar medium incubated at 50°C for 24 h. The genome sequencing of *A. kestanbolensis* NCIMB 13971 was performed in this study, while genome sequence of *A. karvacharensis* K1 (MQAD00000000) was downloaded from GenBank database. For whole genome sequencing, genomic DNA was isolated from culture of *A. kestanbolensis* NCIMB 13971 by using the QIAamp DNA Mini Kit according to the manufacturer's instructions (Qiagen, Hilden-Germany). Whole-genome sequencing of *A. kestanbolensis* NCIMB 13971 was performed on an Illumina HiSeq 2500 platform using 2 × 250 bp paired-end reads by MicrobesNG (University of Birmingham, United Kingdom). The reads were assembled using the full SPAdes assembly strategy on the patric web server (https://patricbrc.org/) (Wattam et al. 2017). The genome annotation was performed by using the Rapid Annotations Using Subsystems Technology (RAST) server (Aziz et al. 2008). The obtained draft genome sequence was deposited in the National Centre for Biotechnology Information (NCBI) database under accession number JALLIW000000000. The 16S rRNA gene sequence was extracted from the draft genome sequence of *A. kestanbolensis* NCIMB 13971 using RNAmer (version 1.2) (Lagesen et al. 2007). The 16S rRNA gene sequence similarity values between *A. karvacharensis* K1 and *A. kestanbolensis* NCIMB 13971 were calculated using the pairwise alignment feature implemented on the EZBioCloud server (https://www.ezbiocloud.net/tools/pairA).

The 16S rRNA gene sequences of closely related type strains were downloaded from EzBioCloud server (https://www.ezbiocloud.net/) (Yoon et al. 2017a) and edited by using the BioEdit program (Hall, 1999). Multiple sequence alignment of 16S rRNA gene sequences was performed using the clustal_w (Thompson et al. 1994). Phylogenetic trees based on 16S rRNA gene sequences were reconstructed by three algorithms with the neighbor-joining (Saitou and Nei 1987), maximum-likelihood (Felsenstein 1981) and maximum-parsimony (Fitch 1971) methods, using MEGA version 7.0 (Kumar et al. 2016). Evolutionary distance matrix was calculated according to Kimura’s two-parameter model (Kimura, 1980). Bootstrap analysis based on 1000 replicates was also conducted in order to obtain confidence levels for the branches (Felsenstein, 1985).
The phylogenetic analysis of *A. karvacharensis* K1\(^T\) and *A. kestanbolensis* NCIMB 13971\(^T\) was carried out using the type strain genomes server pipeline (TYGS, https://tygs.dsmz.de/) (Meier-Kolthoff and Göker 2019). The digital DNA-DNA hybridization (dDDH) value between the draft genome sequences of *A. karvacharensis* K1\(^T\) and *A. kestanbolensis* NCIMB 13971\(^T\) was calculated using Formula 2 of the online Genome-to-Genome Distance Calculator (http://ggdc.dsmz.de/distcalc2.php) (Meier-Kolthoff et al., 2013). Average nucleotide identity (ANI) values were calculated for evaluating the genetic relationship between *A. karvacharensis* K1\(^T\) and *A. kestanbolensis* NCIMB 13971\(^T\) by using the orthoANIu algorithm and an online ANI calculator (www.ezbiocloud.net/tools/ani) (Lee et al., 2016; Yoon et al., 2017b). A phylogenetic tree based on whole-genome sequences was constructed using the TYGS web server (https://tygs.dsmz.de/) (Meier-Kolthoff and Göker 2019). The amino acid identity (AAI) value was calculated with CompareM (https://github.com/dparks1134/CompareM).

**Results And Discussion**

The phylogenetic analysis based on whole genome sequences has clarified the taxonomic inconsistence of prokaryotic taxa (Orata et al. 2018). In this study, the taxonomic relationship of *A. karvacharensis* K1\(^T\) and *A. kestanbolensis* NCIMB 13971\(^T\) was re-evaluated by using whole-genome phylogenetic analysis. *A. karvacharensis* K1\(^T\) and *A. kestanbolensis* NCIMB 13971\(^T\) were isolated from hot spring in Karvachar Nagorno-Karabakh and Turkey, respectively.

In the original article, Panosyan et al. (2021) stated that the 16S rRNA sequence of *A. karvacharensis* K1\(^T\) showed the highest similarity to *A. flavithermus* DSM 2641\(^T\) (99.81%) and less similarity to other species of the genus *Anoxybacillus*. Phylogenetic tree based on 16S rRNA gene sequences in the original article showed that *A. karvacharensis* K1\(^T\), *A. flavithermus* DSM 2641\(^T\) and *A. kestanbolensis* NCIMB 13971\(^T\) clustered together. However, in the original article Panosyan et al. determined only *A. flavithermus* DSM 2641\(^T\) as the nearest neighbor based on 16S rRNA gene sequences similarity and phylogenetic tree, they did not determine the taxonomic relationship between *A. karvacharensis* K1\(^T\) and *A. kestanbolensis* NCIMB 13971\(^T\).

In the present study, we determined whole-genome sequencing of *A. kestanbolensis* NCIMB 13971\(^T\) and obtained the 16S rRNA gene sequence from the genome of *A. kestanbolensis* NCIMB 13971\(^T\) (deposited under accession number ON331912). We noticed that the 16S rRNA gene sequence obtained from the genome of *A. kestanbolensis* NCIMB 13971\(^T\) had 99.93% similarity with *A. karvacharensis* K1\(^T\). Also, in the present study, we reconstructed phylogenetic trees based on 16S rRNA gene sequences by using the obtained the 16S rRNA gene sequence from the genome of *A. kestanbolensis* NCIMB 13971\(^T\) (Fig. 1, 2, 3). We determined that *A. karvacharensis* K1\(^T\) formed a cluster with *A. kestanbolensis* NCIMB 13971\(^T\) in the phylogenetic trees.

Further, in the phylogenomic tree (Fig. 4) *A. karvacharensis* K1\(^T\) and *A. kestanbolensis* NCIMB 13971\(^T\) formed a robust branch different from other type strains of this genus with high bootstrap resampling
values of 100%. The ANI value between *A. karvacharensis* K1<sup>T</sup> and *A. kestanbolensis* NCIMB 13971<sup>T</sup> was 96.95% which was greater than the threshold value (95–96%) for species demarcation (Richter and Rosselló-Móra 2009), confirming that *A. karvacharensis* K1<sup>T</sup> and *A. kestanbolensis* NCIMB 13971<sup>T</sup> were highly phylogenetically closely related. The calculated AAI value between the *A. karvacharensis* K1<sup>T</sup> and *A. kestanbolensis* NCIMB 13971<sup>T</sup> was 97.6% and this value is also clearly above the suggested cut-offs for species delineation (AAI > 95%) (Luo et al., 2014), confirming that they belong to the same species. Meanwhile, digital DNA–DNA hybridization (DDH) analyses indicated that *A. karvacharensis* K1<sup>T</sup> and *A. kestanbolensis* NCIMB 13971<sup>T</sup> exhibited 98.8% dDDH value which is higher than the cut-off (70%) used to classify bacterial strains to the same species (Wayne et al., 1987).

Taken together, the present results indicate that *A. karvacharensis* K1<sup>T</sup> and *A. kestanbolensis* NCIMB 13971<sup>T</sup> were considered to belong to the same species. Thus, based on the phylogenetic analysis based on whole genome sequences and rule 42 of the Bacteriological Code (Parker et al., 2019), we propose that *A. karvacharensis* K1<sup>T</sup> Panosyan et al. 2021 should be reclassified as a later heterotypic synonym of *A. kestanbolensis* Dulger et al. 2004. The type strain is NCIMB 13971<sup>T</sup> (= K4<sup>T</sup> = NCCB 100051<sup>T</sup>) and K1<sup>T</sup> (= DSM 106524<sup>T</sup> = KCTC 15807<sup>T</sup>) is an additional strain of *A. kestanbolensis*.

**Declarations**

**Acknowledgements** This study was supported by Karadeniz Technical University (KTU BAP FAT-2019-7822).

**Author Contributions** KIB designed the study. KIB, HIB and SC performed genome analysis. KIB, HIB, AOB analysed the data and wrote the manuscript. All authors read and approved the final manuscript.

**Conflict of interest** The authors declare that there is no conflict of interest.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

**References**


Figures
Figure 1

Neighbour-joining (NJ) tree constructed based on 16S rRNA gene sequences available from the GenBank database. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at branch points. Bar, 0.01 represents substitutions per nucleotide position. *Paenibacillus polymyxa* DSM 36<sup>T</sup> used as the outgroup.
Figure 2

Maximum-likelihood (ML) tree constructed based on 16S rRNA gene sequences available from the GenBank database. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at branch points. Bar, 0.02 represents substitutions per nucleotide position. *Paenibacillus polymyxa* DSM 36\textsuperscript{T} used as the outgroup.
Figure 3

Maximum-Parsimony (MP) tree constructed based on 16S rRNA gene sequences available from the GenBank database. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at branch points. Bar, 0.02 represents substitutions per nucleotide position. *Paenibacillus polymyxa* DSM 36\(^T\) used as the outgroup.
Figure 4

Phylogenetic tree based on whole-genome sequences of *A. karvacharensis* K1\(^T\) and *A. kestanbolensis* NCIMB 13971\(^T\) and related reference strains. The tree was inferred with FastME 2.1.6.1 (Lefort et al. 2015) from genome blast distance phylogeny (GBDP) distances calculated from genome sequences using the TYGS server (https://tygs.dsmz.de) (Meier-Kolthoff and Göker M 2019) The branch lengths are scaled in terms of GBDP distance formula d5. The numbers at branches are GBDP pseudo-bootstrap support values ≥64% from 100 replications with an average branch support of 97.7%. The tree was rooted at the midpoint (Farris, 1972)