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Whole Genome Analysis by TYGS Assigns *Azotobacter* Species to the *Pseudomonas* Clade as A Distinct Group

TYGS ile Tüm Genom Analizi *Azotobacter* Türlerini *Pseudomonas* Kladına Ayrı Bir Grup Olarak Tayin Etmektedir

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WHOLE GENOME ANALYSIS BY TYGS ASSIGNS *AZOTOBACTER* SPECIES TO THE *PSEUDOMONAS* CLADE AS A DISTINCT GROUP

ABSTRACT

Azotobacter is a nitrogen-fixing soil bacterium and its close relationship with *Pseudomonas* species has been debated in several studies for the last two decades. Previous studies have focused on the partial sequences of 16S rDNA and protein-encoding gene regions but could not differentiate *Azotobacter* spp. from the genus *Pseudomonas*. Therefore, the present study examined the evolutionary relationships of *Azotobacter* and *Pseudomonas* species based on 16S rDNA gene and whole genome sequences. Whole genome sequence analysis was conducted by an automated high-throughput web server TYGS. Based on 16S rDNA gene sequences in all the phylogenetic analyses, all the analyzed *Azotobacter* species clustered within the genus *Pseudomonas* but in two separate groups. *Azotobacter chroococcum* and *Azotobacter beijerinckii* were in distinct clade within the *Pseudomonas* genera, showing that such two species are more closely related to each other than other species of the *Azotobacter* genus. As in the case of 16S rDNA-based comparisons, complete genome analysis with eight available *Azotobacter* strains demonstrated that such eight strains also grouped within the *Pseudomonas* genus but as a separate cluster. Phylogeny-based taxonomic classification of *Azotobacter* spp. needs to be re-evaluated with reliable distinguishing genes or the genus description may be changed as a *Pseudomonas*.

Keywords: TYGS, *Pseudomonadaceae*, Phylogeny, Evolutionary Relationship.

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TYGS İLE TÜM GENOM ANALİZİ *AZOTOBACTER* TÜRLERİNİ *PSEUDOMONAS* KLADİNA AYRI BİR GRUP OLARAK TAYİN ETMEKTEDİR

ÖZ

Azotobacter, azot sabitleyici bir toprak bakterisidir ve *Pseudomonas* türleriyle yakın ilişkisi son yirmi yıldır birçok çalışmada tartışılmıştır. Önceki çalışmalar, 16S rDNA'nın kısmi gen dizilerine ve protein kodlayan gen bölgelerine odaklanmıştır ancak *Azotobacter* türlerini *Pseudomonas* cinsinden ayırt edememiştir. Bu nedenle, mevcut çalışma *Azotobacter* ve *Pseudomonas* türlerinin evrimsel ilişkilerini 16S rDNA gen ve tüm genom dizilerine dayanarak incelemiştir. Tüm genom dizileme analizi otomatik yüksek verimli ağ sunucusu TYGS tarafından yapılmıştır. 16S rDNA gen dizilerine dayanarak yapılan tüm filogenetik analizlerde, analiz

edilen tüm *Azotobacter* türleri *Pseudomonas* cinsi içerisinde kümelenmiştir fakat iki ayrı grupta yer almıştır. *Azotobacter chroococcum* ve *Azotobacter beijerinckii*, *Pseudomonas* cinsi içinde farklı bir kladdaydı ve *Azotobacter* cinsinin diğer türlerine göre bu iki türün birbirlerine daha yakın olduğunu göstermiştir. 16S rDNA tabanlı karşılaştırmalarda olduğu gibi, mevcut sekiz *Azotobacter* suşlarıyla yapılan tüm genom analizi, bu sekiz suşun *Pseudomonas* cinsi içinde ayrı bir küme olarak gruplandığını göstermiştir. *Azotobacter* türlerinin filogeniye dayalı taksonomik sınıflandırmasının güvenilir ayırt edici genlerle yeniden değerlendirilmesi gerekmektedir ya da cins tanımı *Pseudomonas* olarak değiştirilebilir.

Anahtar Kelimeler: TYGS, *Pseudomonadaceae*, Filogeni, Evrimsel İlişki.

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1. INTRODUCTION

Azotobacter is a genus of the family *Pseudomonadaceae* (Lalucat et al., 2022) and has seven known species such as *Azotobacter chroococcum, Azotobacter beijerinckii, Azotobacter tropicalis, Azotobacter nigricans, Azotobacter salinestris, Azotobacter armeniacus,* and *Azotobacter vinelandii*. These species are free-living, nitrogen-fixing, Gram-negative soil bacteria residing in the rhizosphere of various plants and have been considered a plant growth-promoting microorganism that significantly improves plant health, crop productivity, and soil fertility (Aasfar et al., 2021; Çam et al., 2022). The inoculation of these agriculturally important bacteria to plant roots offers great advantages to the growth and development of a great number of plants through nitrogen fixation, phytohormone and hydrogen cyanide production, phosphorus solubilization, and siderophore production (Sumbul et al., 2020; Aasfar et al., 2021; Çam et al., 2022). Besides growth-promoting activities, *Azotobacter* spp. plays an important role in soil bioremediation, biocontrol activity against phytopathogens, biofertilizers, nutrient mobilization, and the amelioration of several abiotic stress factors in plants (Sumbul et al., 2020; Aasfar et al., 2021; Çam et al., 2022). Therefore, accurate phylogenetic classification of such agriculturally important species will be of importance for sustainable agriculture.

Azotobacter spp. belong to a phenotypically well-defined genus (Lalucat et al., 2020) but, over the past years, a number of phylogenomic studies have highlighted the close evolutionary relationships of *Azotobacter* species with the genus *Pseudomonas* based on the sequences of 16S rDNA and protein-encoding genes (Özen and Ussery, 2012; Bueno-Gonzalez et al., 2020; Lalucat et al., 2020). In those studies, the members of *Azotobacter* genera were embedded within *Pseudomonas* clade in the constructed phylogenetic trees, indicating their phylogenetically close relationships. For this reason, an amendment in the taxonomic classification of

Azotobacter species is necessary with a thorough genome-based taxonomic analysis to solve the genotypic discrepancies between the two genera (Bueno-Gonzalez et al., 2020; Lalucat et al., 2020).

Phylogenetic analysis based on 16S rDNA gene sequences has been long used for determining phylogenetic and evolutionary relationships among bacterial species, but its use does not have sufficient discriminatory power for species differentiation (Rediers et al., 2004; Lalucat et al., 2020). As in the case of 16S rDNA analysis, the use of the concatenated sequences of housekeeping genes could not also differentiate *Azotobacter* from *Pseudomonas* species (Bueno-Gonzalez et al., 2020; Lalucat et al., 2020). In recent years, whole genome sequence analysis has been accepted as a superior way over phenotypic properties for the classification and identification of bacterial species (Lalucat et al., 2020). Therefore, this study conducted 16S rDNA-based phylogenetic analyses by the most commonly used methods and a whole genome analysis by a high-throughput web server TYGS to resolve the taxonomic issue between *Azotobacter* and *Pseudomonas* species.

2. MATERIAL AND METHODS

2.1. Phylogenetic Analyses

Evolutionary analysis of the genus *Azotobacter* together with its phylogenetically closely related *Pseudomonas* genus was conducted by maximum likelihood (ML) and Bayesian phylogenetic methods based on the partial sequences of 16S rDNA gene. The FASTA sequence of all the corresponding strains used for the construction of the phylogenetic trees based on 16S rDNA gene region were manually retrieved from NCBI (National Center for Biotechnology Information)–Blast with their corresponding accession numbers given in the constructed trees. The whole genome analysis of the respective species was performed by TYGS, Type (Strain) Genome Server. The full-length sequences of the respective strains were automatically obtained from continuously growing TYGS database.

2.2. Maximum Likelihood

ML is a model-based method and uses log-likelihood nonparametric bootstrap supports when generating evolutionary trees. In this study, for ML analysis, Tamura-Nei was selected as the best model for the estimation of nucleotide substitution (Tamura and Nei, 1993). ML tree topology was generated using MEGA11 version 11.0.10 with 1000 bootstrap replicates (Tamura et al., 2021) and demonstrated with the highest log likelihood values in Figure 1.

2.3. Bayesian Analysis

Unlike ML which generates phylogenetic trees with bootstrapping, Bayesian inference of phylogeny relies on posterior probabilities for the branch support of trees. Bootstrapping and posterior probabilities are of great interest to phylogenetic research and they cannot be interchangeable (Douady et al., 2003), therefore both analyses were employed in the present study to obtain more robust and reliable results based on 16S rDNA gene sequences.

Bayesian posterior probabilities were computed with MrBayes version 3.2.6 using Markov chain Monte Carlo algorithms (Huelsenbeck and Ronquist, 2001) in Geneious Prime software version 2023.0.4 by running 4 chains for 1,000,000 generations (sampled every 200 generations). HKY85 was chosen as the substitution model with gamma rate variation. The 100,000 trees were discarded as burn-in for chain stability. The consensus tree with support values was generated with remaining trees in Geneious Prime software and the results were obtained in Newick format. The phylogenetic tree was re-constructed in MEGA11 using Newick file and shown in Figure 2.

For the construction of phylogenetic trees based on partial 16S rDNA sequences, UPGMA, Neighbor-Joining (NJ), and maximum parsimony (MP) analyses were also performed using MEGA11 (version 11.0.10) with 1000 bootstraps (Tamura et al., 2021). The results of these three methods were given in the supplementary file as Figure S1, S2, and S3, respectively. Tamura-Nei model was selected for UPGMA and NJ analysis (Tamura and Nei, 1993). Maximum parsimony method employs the Subtree-Pruning-Regrafting algorithm when building the tree (Nei and Kumar, 2000).

2.4. TYGS Analysis

TYGS is a user-friendly, automated, high-throughput web server for genome-based classification and identification of Bacteria and Archaea, and provides a large database of new genomic and taxonomic information in terms of genera, species, and subspecies (Meier-Kolthoff and Göker, 2019). This unique comprehensive platform, compared to earlier approaches, facilitates the genome-based taxonomic classification of prokaryotes by automatically determining the closest genome sequences from a continuously growing new database (Meier-Kolthoff and Göker, 2019). The whole genome classification of the genus *Azotobacter* was conducted by TYGS and displayed in Figure 3. In addition to genome-scale phylogeny, TYGS generated a phylogenetic tree based on the full-length sequences of 16S rDNA gene region (Figure S4).

3. RESULTS AND DISCUSSION

An intimate relationship between *Azotobacter* and *Pseudomonas* species has been observed in several studies (Rediers et al., 2004; Bueno-Gonzalez et al., 2020). Previously, this relationship based on the sequence of 16S rDNA gene included *Azotobacter* spp. in a family of the order *Pseudomonadales* but now in a separate genus in the family *Pseudomonadaceae* (Young and Park, 2007). Further, in the past, nitrogen fixation ability of *Azotobacter* species was a major distinguishing characteristic from *Pseudomonas* spp. but recently it has been proposed that some *Pseudomonas* species can also fix atmospheric nitrogen (Vermeiren et al., 1999; Kulakov et al., 2002; Rediers et al., 2004). These discrepancies necessitate a more comprehensive analysis of *Azotobacter* species with the members of the genus *Pseudomonas.*

In this study, analysis of the phylogenetic relationships between *Azotobacter* and *Pseudomonas* species based on partial sequences of 16S rDNA gene indicated that all the analyzed *Azotobacter* species were embedded within *Pseudomonas* genera, but partly in separate groups. According to the results of ML and Bayesian analysis, *A. chroococcum* and *A. beijerinckii* were in a distinct group within the genus *Pseudomonas,* indicating that such two species are more closely related to each other than other species of the genus *Azotobacter* (Figures 1 and 2). Similar results were also obtained from the analyses of UPGMA, NJ, and MP (Figures S1, S2, and S3). Likewise, as in the case of the findings of this study, *Azotobacter* species were found to be within *Pseudomonas* clade as two separate groups based on 16S rDNA gene sequences in the ML (Bueno-Gonzalez et al., 2020; Çam and Bicek, 2023) and Bayesian analysis (Çam and Bicek, 2023).

Similarly, *A. tropicalis, A. salinestris, A. armeniacus, A. nigricans, A. vinelandii* and *Azorhizophilus paspali* formed a separate distinct branch within *Pseudomonas* lineage with an exception for the position of *Pseudomonas thermotolerans*. *P. thermotolerans* caused a division in *Azotobacter* clade, more closely related to *A. vinelandii* in both Bayesian (Figure 2) and maximum parsimony analyses (Figure S3). The phylogenetic position of *P. thermotolerans* was unstable. According to the result of ML analysis of the present study, *P. thermotolerans* is far from the *Azotobacter* group. However, in a recent study, a close relationship of *P. thermotolerans* with such *Azotobacter* species was found in the phylogenetic tree built as a result of ML analysis (Bueno-Gonzalez et al., 2020), which may partly explain why *P. thermotolerans* is within, or very close to, such *Azotobacter* group in Bayesian and maximum parsimony analysis in this study.

In the 16S rDNA gene-based phylogenetic analyses (like ML, Bayesian, UPGMA, and NJ), *Azorhizophilus paspali* consistently clustered with *A. vinelandii* with a high bootstrap value (>90%), thus causing a division within *Azotobacter* clade. Such complication was also demonstrated by Bueno-Gonzalez et al. (2020), claiming

that *Azorhizophilus paspali* was accepted as the basonym of *Azotobacter paspali* under the *Azotobacter* genus in Bergey's Manual of Systematics in its 2005 edition. They suggested that *Azorhizophilus paspali* should be re-included in the genus *Azotobacter* due to the close phylogenetic relationships of such two species.

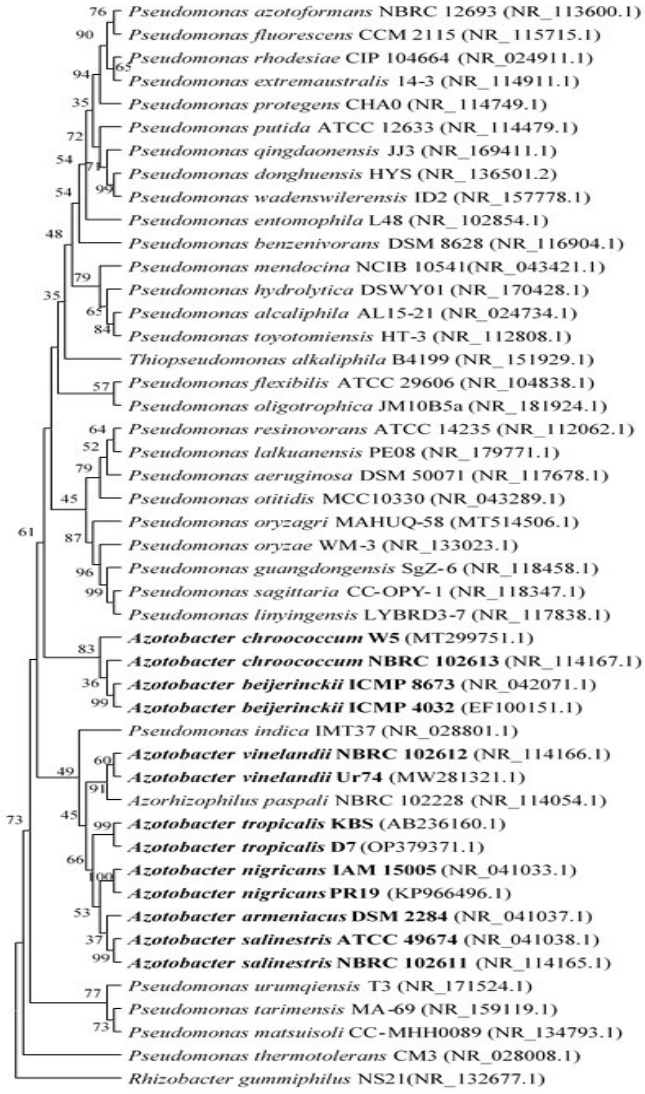

Figure 1. Phylogenetic tree of *Azotobacter* spp. together with the genus *Pseudomonas* based on 16S rDNA gene sequences by maximum likelihood analysis with 1000 bootstrap.

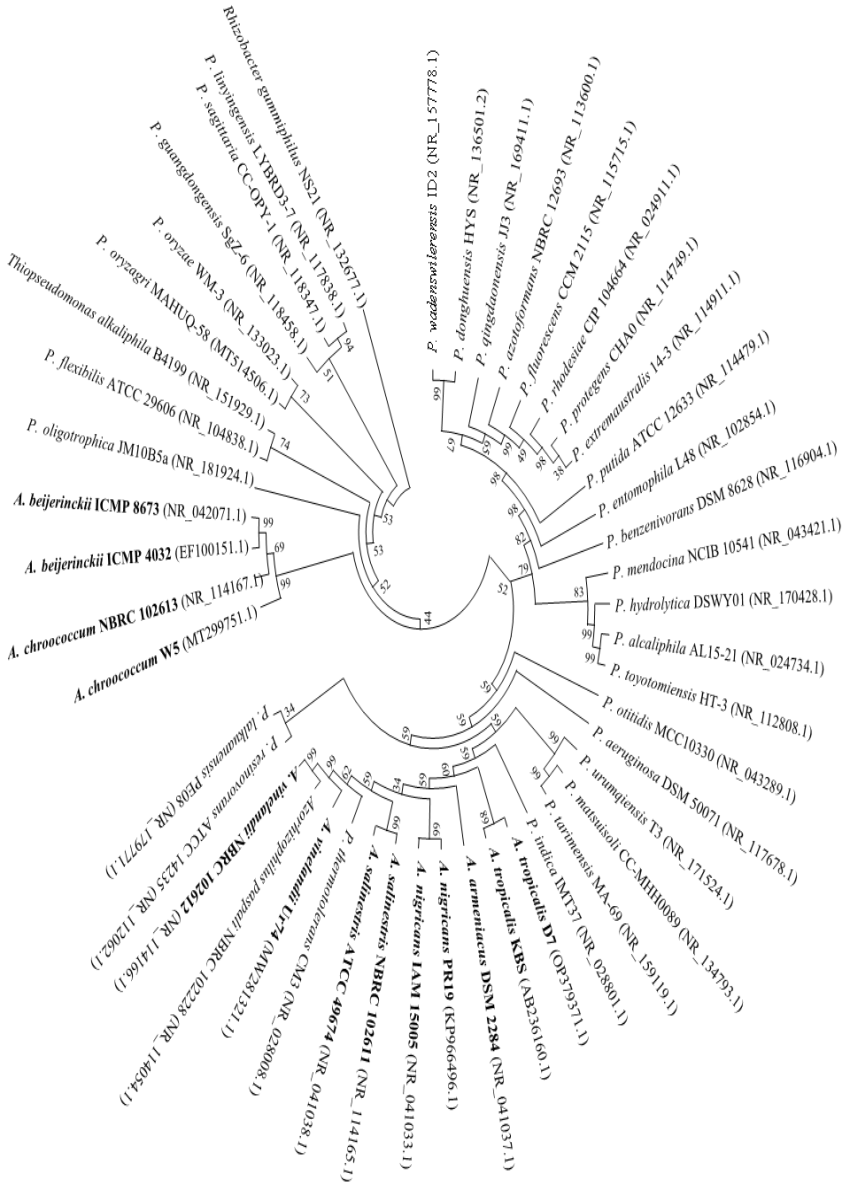

Figure 2. Phylogenetic tree of *Azotobacter* spp. together with the genus *Pseudomonas* based on 16S rDNA gene sequences by Bayesian analysis

Analysis of full-length sequences of 16S rDNA by TYGS also showed that *Azotobacter* species distinctly cluster within *Pseudomonas* but, unlike other analyses conducted in this study, *A. beijerinckii* was found to be more closely related to *A. vinelandii* rather than *A. chroococcum* (Figure S4). The position of this clade is not stable because of the limited numbers of *Azotobacter* species incorporated. TYGS is a continuously growing new platform for the genome-based identification of prokaryotes (Meier-Kolthoff and Göker, 2019), therefore could analyze only three species of the genus *Azotobacter* in its current form. The addition of new *Azotobacter* sequences to the server may change the taxonomic position of *A. beijerinckii* but not the position of *Azotobacter* clade within *Pseudomonas* genus since this clade caused a clear division in *Pseudomonas* with a 74% bootstrap support (Figure S4).

16S rDNA gene region is highly conserved, showing an extremely low evolutionary rate, thus its resolution degree is insufficient for the analysis of intrageneric relationships (Rediers et al., 2004)2004. It is also debatable whether the use of a single gene is enough to accurately determine the evolutionary relationships of microbial species (Rediers et al., 2004)2004. The use of housekeeping genes would be useful for the determination of phylogenetic relationships between bacterial species, therefore they constructed the phylogenetic trees based on the sequences of both 16S rDNA gene and 25 protein/enzyme-encoding housekeeping genes (Rediers et al., 2004)2004. Similar to the result of the present work, Rediers et al. (2004) observed that *A. vinelandii* was still within *Pseudomonas* genera, clustering with *Pseudomonas aeruginosa* PAO1 among the species used due to the higher sequence similarity of such two species with each other than other strains of *Pseudomonas* species. Interestingly, according to the results of comparative bioinformatic analysis, a big majority of essential genes in *P. aeruginosa* were extremely well-conserved in *A. vinelandii*, showing the polyphyletic origin of *A. vinelandii* from the genomic backbone of *Pseudomonas* through horizontal gene transfer (Martínez-Carranza et al., 2019).

Furthermore, Bueno-Gonzalez et al. (2020) conducted a multilocus sequence analysis with the partial sequences of three housekeeping genes (*gyrB*, *rpoB*, and *rpoD*) but the sequence analysis of these genes still assigned *Azotobacter* to the *Pseudomonas* cluster. In a more comprehensive phylogenetic analysis with 227 bacterial species/subspecies, including two *Azotobacter* species, based on the concatenated partial sequences of four housekeeping genes (16S rDNA + three protein-encoding genes mentioned above), such two *Azotobacter* species were still between the clusters of *Pseudomonas* spp. but with a distinct branch (Lalucat et al., 2020) as in the case of the results of the present study conducted based only on 16S rDNA gene sequences.

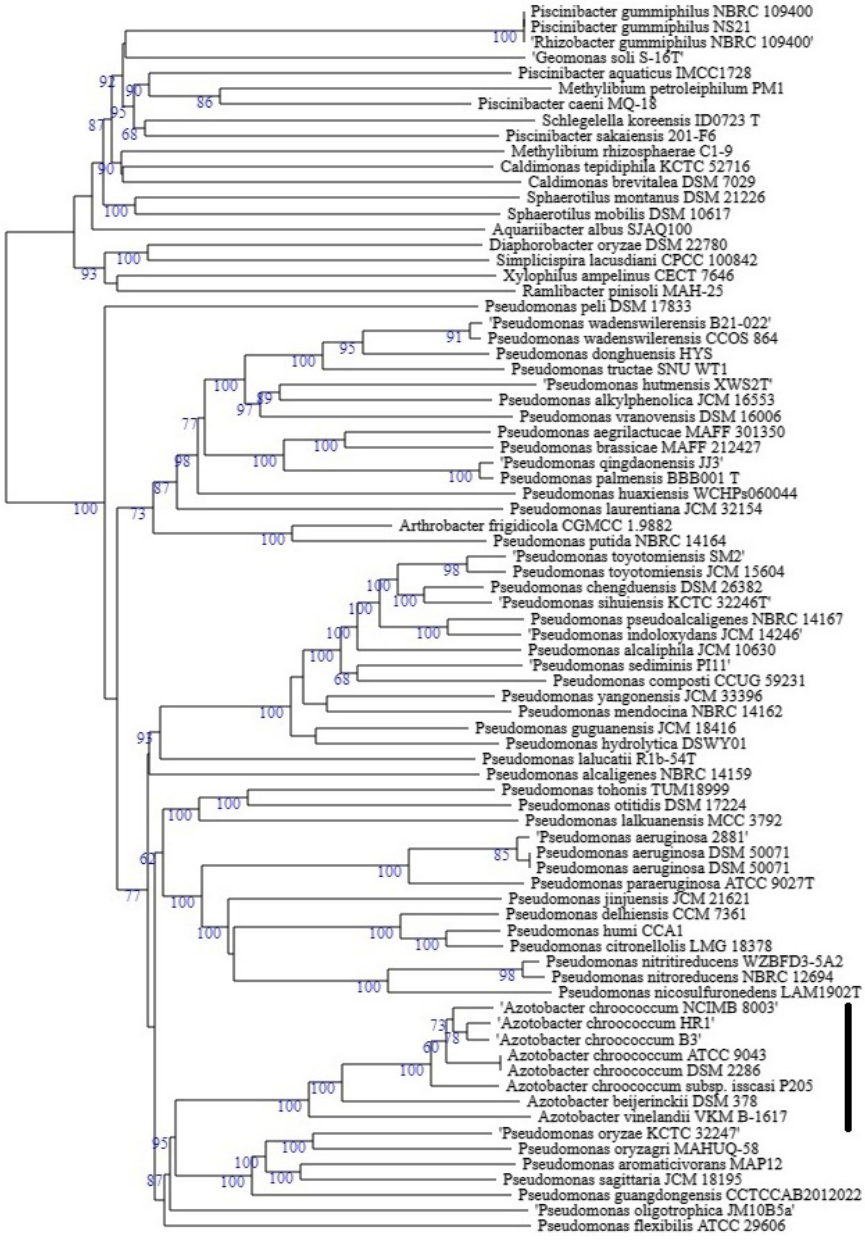

Figure 3. Phylogenetic tree of *Azotobacter* spp. together with more closely related bacterial species based on complete genome sequences by TYGS

On the other hand, Genome Taxonomy Database analysis of the concatenated amino acid sequences of 120 proteins, mainly ribosomal ones, assigned *Azotobacter* as a distinct genus but within *Pseudomonas* genera (Lalucat et al., 2020). In that study, analysis of conserved proteins of *P. aeruginosa* DSM 50071T and *A. vinelandii* DJ showed a 45% index that is slightly lower than the proposed 50% cut-off value for genus differentiation, considering *Azotobacter* as a different genus but in the lower borderline. However, in that analysis, they also observed a 48% index for *Pseudomonas syringae*. Such difficulties were attributed to the big differences in the genome size of *Pseudomonas* genera (Lalucat et al., 2020). In another study, the topology of the phylogenetic tree obtained by the analysis of the concatenates sequences of all the used proteins, consisting of over 12,000 amino acid residues, was similar to that of 16S rDNA gene (Rediers et al., 2004)2004. Additionally, the phylogenetic analysis of pan genome protein families demonstrated a much closer relationship of *A. vinelandii* to *Pseudomonas* than other related species(Özen and Ussery, 2012)2012. It appears that the use of protein-encoding genes could not contribute to distinguishing *Azotobacter* species from the genus *Pseudomonas,* necessitating a thorough genomic analysis as also suggested by Bueno-Gonzalez et al. (2020) and Lalucat et al. (2020) for the accurate classification of bacterial genera.

The comparisons of whole genome sequences are a robust taxonomic classification of bacterial species to solve the difficulties encountered during 16S rDNA-based phylogenetic analysis (Lalucat et al., 2020), therefore there is a growing interest in the use of complete genome sequences for the analysis of phylogenetic relationships between bacterial species (Rediers et al., 2004)2004. Whole genome sequence analyses focus on the phylogenetic relationships of bacterial species by the multiple alignments of homologous sequences in their core genomes, thus significantly contributing to modern bacterial taxonomic classification (Lalucat et al., 2020). However, in this study, the results of whole genome analysis of *Azotobacter* spp. with *Pseudomonas* members were partly concordant with those of 16S rDNA-based analyses. Complete inter-species genome analysis by TYGS placed all the used *Azotobacter* species in a separate distinct cluster within *Pseudomonas* lineage with 100% bootstrap support (Figure 3) as already observed with 16S rDNA comparisons. In this respect, genetic-based classification of *Azotobacter* species needs to be re-evaluated with the discovery of a set of reliable genes for the accurate differentiation of *Azotobacter* from *Pseudomonas* species, or the name of the genus *Azotobacter* may be replaced with *Pseudomonas*.

Conflict of Interest

The author declares that there is no conflict of interest.

Ethics

This study does not require ethics committee approval.

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