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# Genetic diversity of *Azotobacter* strains isolated from various soils of Poland

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**Abstract.** Free-living nitrogen-fixing bacteria belonging to the genus *Azotobacter* are microorganisms commonly occurring in soil. The genus *Azotobacter* includes 8 species, with *Azotobacter chroococcum* most commonly inhabiting many soils all over the world. This study aims to isolate and to identify the *Azotobacter* bacteria from various soils of Poland using RFLP analysis of the bacterial Internal Transcribed Spacer (ITS) region and 16S rRNA gene sequence analysis. 56 strains were isolated from tested soils, using the dilution-pour plates method. Restriction analysis of the bacterial ITS region was used for the characterization and differentiation of the isolated strains. It indicated that the studied isolates were not identical. Among the 56 tested strains, 36 were characterized by the same genotype in ITS-PCR/RFLP analyzes as the reference strains *A. chroococcum* DSM281 and DSM2286. For molecular identification of the isolates, the 16S rRNA gene was amplified using 27f and 1492r primers and PCR products were sequenced. These results indicate that the analyzed isolates from Polish soils belong to one species, namely *Azotobacter chroococcum*.

**Keywords:** *Azotobacter chroococcum*, soil, nitrogen fixation, genetic diversity, 16S rRNA gene

## INTRODUCTION

The genus *Azotobacter* comprises free-living, aerobic, heterotrophic Gram-negative bacteria with the ability to fix atmospheric nitrogen. This genus belongs to the family *Pseudomonadaceae* from the subclass γ-*Proteobacteria* (Rubio et al., 2013; Robson et al., 2015; Chen et al., 2018; Khosravi, Dolatabad, 2020). Eight species and two subspecies have been validly named in this genus:

– *Azotobacter armeniacus* (Thomson, Skerman, 1979),
– *Azotobacter beijerinckii* (Lipman, 1904),
– *Azotobacter bryophylli* (Liu et al., 2019),
– *Azotobacter chroococcum* (Beijerinck, 1901) (subsp. *chroococcum* (Jin et al., 2020) and subsp. *isscasi* (Jin et al., 2020),
– *Azotobacter nigricans* (Krasil'nikov, 1949) (subsp. *achromogenes* (Thomson, Skerman, 1979) and subsp. *nigricans* (Howey et al., 1990),
– *Azotobacter paspali* (Döbereiner, 1966),
– *Azotobacter salinestris* (Page, Shivprasad, 1991),
– *Azotobacter vinelandii* (Lipman, 1903).

*Azotobacter* representatives can commonly be found in soil, water, sediments, rhizosphere and phyllosphere environments (Aquilanti et al., 2004; Mazinani, Asgharzadeh, 2014; Zhang et al., 2019; Aasfar et al., 2021). Among the above-listed species, *Azotobacter chroococcum* is the most widely distributed in soils all over the world, including Poland (Ziemięcka, 1923: Martyniuk, Martyniuk, 2003; Lenart, 2012; Kozieł, Gałązka, 2021). *Azotobacter* spp. prefers neutral and slightly alkaline soils and populations of these bacteria rarely exceed several thousand cells per gram of soil. In acid soils (pH < 6.0) these bacteria are generally absent or occur in very low numbers (Martyniuk, 2008; Mazinani, Asgharzadeh, 2014; Andjelković et al., 2018). In addition, the occurrence and population size of this group of bacteria are influenced by many other environmental factors, such as soil physicochemical (organic matter content, moisture, fertility, C/N ratio) and microbiological properties or climatic conditions. However, the abundance varies as per the depth of the soil profile (Tejera et al., 2005; Bag et al., 2017; Mahato, Kafle, 2018).

*Azotobacter* spp. is considered an important fertilizing agent that contributes to the N availability and substitutes chemical fertilizers and produces secondary metabolites that are not present in artificial fertilizers (Subedi et al., 2019). Among biofertilizers, bacteria belonging to the genus *Azotobacter* play a key role in the nitrogen cycle in nature that binds atmospheric nitrogen inaccessible to plants and releases it in the form of ammonium ions available to

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plants in the soil fixing an average 10–20 kg N $ha^{-1}$ per year (Sivasakthi et al., 2017; Mahato, Kafle, 2018). The other beneficial effects include the ability to produce of growth hormones like gibberellin, auxin, cytokinin, vitamins, siderophores and growth substances responsible for seed germination, protection against root pathogens, stimulation of beneficial rhizospheric microorganisms and enhancement of plant yield (Mahato, Kafle, 2018; Sumbul et al., 2020; Wakarera et al., 2022).

The analysis of soil microbial diversity is relevant to define soil quality. Molecular methods based on PCR techniques such as restriction analysis of the internal transcribed spacer (ITS) region, 16S-18S rDNA, and restriction fragment length polymorphism (RFLP) analysis have been used to identify numerous microorganisms. The 16S rRNA gene is approximately 1500 bp long and consists of variable regions interspaced with more conserved regions (Winand et al., 2019). Sequencing of the 16S rRNA gene was used to confirm the systematic position of numerous bacterial isolates (Obele et al., 2019; Jin et al., 2020; Wakarera et al., 2022).

The present study was performed in order to analyze the genetic diversity of *Azotobacter* strain isolated from soil samples taken in various regions of Poland and to identify these isolates using RLFP analysis of the bacterial Internal Transcribed Spacer (ITS) region and 16S rRNA gene sequence analysis.

## MATERIALS AND METHODS

### Soils and isolation of *Azotobacter* spp.

In this study, 35 soils collected in 12 voivodeships of Poland were used (Table 1). Number of colony forming units (CFU) of *Azotobacter* spp. in the examined soils were assessed by the dilution-pour plates method (Fenglerowa, 1965) on N-free agar medium containing: $K_2HPO_4$ 0.5 g, $MgSO_4$ 0.2 g, NaCl 0.2 g, $CaCO_3$ 5 g, sucrose 10 g, agar 12 g, $H_2O$ distilled 1000 ml and traces of Mn, Fe and Mo. Bacteria of the *Azotobacter* genus exhibited robust growth on this medium, developing substantial, lustrous, and mucilaginous colonies. Following an incubation period of 5–7 days, these colonies underwent a distinctive transformation, adopting a rich, dark brown hue. This phenomenon highlights the unique characteristics and dynamic behavior of *Azotobacter* representatives in response to the provided environment. Randomly selected colonies were transferred to fresh N-free agar medium and purified. Subsequently, all isolates were inoculated on agar slants containing 20 g glucose, 12 g agar, 0.8 g $K_2HPO_4$, 0.2 g $KH_2PO_4$, 0.5 g $MgSO_4 \cdot 7\ H_2O$, 0.05 g $CaCl_2$, 0.025 g $FeCl_3 \cdot 6\ H_2O$ and 0.005 g $Na_2MoO_4 \cdot 2\ H_2O$ in 1000 $cm^3$ of distilled water (Thomson, Skerman, 1979) and stored in a refrigerator at 4 °C. In total 56 bacterial isolates were obtained. In the molecular analyses the following reference species were also used: *Azotobacter armeniacus* DSM2284, *Azotobacter chroococcum* DSM281, DSM2286, *Azotobacter salinestris* DSM11553, *Azotobacter vinelandii* DSM2289, *Azomonas agilis* DSM375, *Azomonas macrocytogenes* DSM721, which were provided by the Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures GmbH.

### The DNA extraction procedures

The DNA isolation was carried out using the Master Pure Complete DNA and RNA purification Kit. 10 ml of 48 hours-culture of the bacteria grown in liquid N-free medium was centrifuged at 12.000 rpm for 2 min. The pellet was resuspended in 300 µl of a lysis buffer containing 1 µl of proteinase K and incubated at 65 ºC for 15 minutes, followed by cooling down for 5–10 minutes. 1 µl RNase A was added to the mixture, vortexed vigorously for 30–60 sec., and incubated at 37 ºC for 30 minutes. The mixture was placed in an ice bath for 5 minutes and then 150 µl MPC Protein Precipitation Reagent was added and mixed and then centrifuged at 4 ºC for 10 minutes at 10.000 rpm. The supernatant was transferred to a 2 ml tube and 500 µl isopropanol was added, mixed thoroughly, and centrifuged again (10 min, 10.000 rpm). The pellet was then washed twice with 70% ethyl alcohol (2 × 150 µl), each time followed by centrifugation (5 minutes, 10.000 rpm). The DNA pellet was suspended in 75 µl of sterile Milli-Q water and stored at -20 ºC (Obele et al., 2019).

### Restriction Analysis of the ITS Region

PCR amplification of the rDNA ITS region was performed according to the procedure proposed by Kwon et al. (2005) with two primers: R16-1 (5'-CTTGTACACACCGCCCGTCA-3') and R23-3R (5'-GGTACTTAGATGTTTCAGTTC-3'). Each PCR mixture contained the following: 0.25 µl each primer, 0.4 µl dNTPs, 5 µl PCR buffer 10x (Thermo Scientific), 0.75 µl DNA polymerase (Thermo Scientific), and 1 µl genomic DNA in a final reaction volume of 50 µl. The DNA thermal cycler (Biometra) used for PCR amplification was programmed as follows: initial denaturation at 94 ºC for 5 min., 35 cycles of denaturation at 94 ºC for 1 min., annealing at 58 ºC for 1 min. and elongation at 72 ºC for 2 min., and final elongation at 72 ºC for 10 min. The PCR products (5 µl) were electrophoresed in 1.5% agarose gel in 1×TAE buffer for 1.5 h at 80 V. The gel was analyzed with UV–transilluminator (Transluminator Quantum ST4) and BIO-1D++. The PCR products were digested with 5U of restriction enzymes *Hae*III and *Msp*I at 37 ºC for 5 h in a final volume of 20 µl, as recommended by the manufacturer. The digests were run by electrophoresis on 2% agarose gels in 1×TAE buffer for 2 h at 80 V.

Table 1. Soils used for isolation of *Azotobacter* spp.

| Soil number[#] | Soil type or granulometric group | Voivodeship | pH | Number of *Azotobacter* spp. [cfu in 1 g of soil d.m.] | Symbol of the *Azotobacter* strain isolated |
|---|---|---|---|---|---|
| *1* | *2* | *3* | *4* | *5* | *6* |
| 143 | clayey silt | małopolskie | 5.3 | 98 | A143-1<br>A143-2 |
| 284 | loess | małopolskie | 5.4 | 218 | A284-1<br>A284-2 |
| 340 | weakly loamy sand | podkarpackie | 5.2 | 460 | A340-1<br>A340-2<br>A340-3 |
| 374 | loess | małopolskie | 7.3 | 5125 | A374-1<br>A374-2 |
| 384 | sandy loam | podkarpackie | 6.2 | 12925 | A384-1<br>A384-2 |
| 415 | loamy sand | śląskie | 6.3 | 29975 | A415 |
| 484 | rendzina | świętokrzyskie | 6.8 | 11000 | A484-1<br>A484-2 |
| 597 | loess | świętokrzyskie | 6.6 | 14525 | A597-1<br>A597-2 |
| 600 | loamy sand | podkarpackie | 6.9 | 1418 | A600-1<br>A600-2 |
| 663 | silt | podkarpackie | 7.5 | 3 | A663 |
| 725 | silt | podkarpackie | 7.0 | 120 | A725 |
| 727 | silt | podkarpackie | 6.4 | 1540 | A727-1<br>A727-2<br>A727-3 |
| 784 | loess | świętokrzyskie | 5.7 | 19600 | A784 |
| 786 | sandy loam | świętokrzyskie | 7.8 | 1208 | A786-1<br>A786-2 |
| 787 | loamy sand | świętokrzyskie | 7.7 | 1820 | A787 |
| 819 | clayey silt | dolnośląskie | 6.1 | 4980 | A819-1<br>A819-2<br>A819-3 |
| 850 | silt | świętokrzyskie | 6.7 | 2150 | A850 |
| 894 | sandy loam | opolskie | 7.0 | 25 | A894 |
| 1820 | weakly loamy sand | lubuskie | 6.5 | 148 | A1820-1<br>A1820-2 |
| 1940 | loamy sand | lubuskie | 6.9 | 14875 | A1940 |
| 2070 | luvisol | lubuskie | 6.9 | 10 | A2070 |
| 2684 | sandy loam | kujawsko-pomorskie | 6.7 | 5525 | A2684 |
| 3112 | cambisol | pomorskie | 6.9 | 8500 | A3112 |
| 4065 | clay | pomorskie | 6.8 | 3070 | A4065-1<br>A4065-2<br>A4065-3 |
| 4165 | silt | kujawsko-pomorskie | 6.6 | 45800 | A4165 |
| 4424 | clayey silt | pomorskie | 6.5 | 4825 | A4424 |
| 4539 | clay | warmińsko-mazurskie | 6.6 | 6325 | A4539-1<br>A4539-2 |
| 4604 | clayey silts | dolnośląskie | 6.4 | 4850 | A4604-1<br>A4604-2 |
| 4625 | luvisol | dolnośląskie | 6.4 | 1740 | A4625 |
| 4733 | loamy sand | świętokrzyskie | 6.3 | 40000 | A4733-1<br>A4733-2 |

Table 1 continuation

| *1* | *2* | *3* | *4* | *5* | *6* |
|---|---|---|---|---|---|
| 4761 | silt | świętokrzyskie | 6.9 | 16000 | A4761 |
| 4791 | silt | lubelskie | 6.7 | 100 | A4791-1<br>A4791-2 |
| 4803 | light loam | lubelskie | 7.4 | 1925 | A4803 |
| 4811 | silt | lubelskie | 7.3 | 25 | A4811 |
| 4813 | light loam | lubelskie | 6.2 | 470 | A4813-1 |

# acc. to sample site localization number

### PCR amplification of the 16S rRNA gene of the bacterial species

Genomic 16S rRNA was amplified using polymerase chain reaction amplification processes by mixing a set of universal primers 27f (5'-AGAGTTTGATCATGGCTCAG-3') as forward primer and 1492r (5'-GGTTACCTTGTTACGACTT-3') as the reverse primer (Lane, 1991; Gauri et al., 2009). A typical PCR reaction mixture was prepared by mixing 12.5 µl Dream Taq Green PCR Master Mix, 1 µl primer 27f, 1 µl primer 1492r, 8.5 µl of MilliQ water and 2 µl of the template DNA. The entire reaction mixture was made up to 25 µl in total volume. The PCR reaction mixture tubes were placed on Biometra thermo cycler machine to run 35 cycles program under a given PCR conditions for an initial denaturation at 95 ºC in 2 minutes (1 cycle), followed by denaturation at 95 ºC in 30 seconds, annealing at 52 ºC for 1 minute, extension at 72 ºC for 1.5 minutes (35 cycles) and finally extension at 72 ºC for 10 minutes (1 cycle) and hold at 4 ºC (Obele et al., 2019; Khosravi, Dolatabad, 2020). The amplified 16S rRNA gene products were run on a 1.5% agarose gel electrophoresis after staining with ethidium bromide solution for 1 h 30 minutes at 80 V. The 50–2000 Perfect Plus ™ 2 kb DNA fragment size marker was used as a standard for the size of the DNA bands.

### Sequencing of the amplified 16S rRNA gene

The 16S rRNA gene amplification products were sequenced. The obtained nucleotide sequences were compared with the published nucleotide sequences of closely related bacteria-type strains deposited in the GenBank National Centre for Biotechnology Information (NCBI) database and Ribosomal Database Project *via* BLAST searches.

### Statistical analysis

Pearson's correlation coefficient (r) was used to compare the relationship between isolates genotype and the soil properties. The statistical analysis was performed by Statistica v. 13.3 software. The coefficients are significant, with $p<0.05$.

## RESULTS AND DISCUSSION

Bacteria belonging to the genus *Azotobacter* form on selective N-free agar media represented by large, glistening, and slimy colonies, and when colonies turn dark brown after 5–7 days of incubation this indicates that such colonies were formed by *A. chroococcum* (Martyniuk, Martyniuk, 2003; Lenart, 2012; Hindersah et al., 2020). Most colonies in plates inoculated with the studied soils had such an appearance, therefore one to three randomly selected colonies were re-isolated and purified to gain 56 isolates for further molecular studies (Table 1).

Restriction analysis of the bacterial Internal Transcribed Spacer (ITS) region was used to assess the genetic diversity of the isolates. This technique is often used for the assessment of biodiversity, and genetic analysis of microbial populations, as well as for the preliminary identification of microbial species (Khan et al., 2005; Liu et al., 2008; Łyszcz, Gałązka, 2017). As a result of the amplification of the ITS region one PCR product ~1000 bp was obtained for all 56 isolated strains and 7 reference strains. The endonucleases of *Hae*III and *Msp*I were used for restriction of PCR products. The obtained products of restriction digestion gave banding patterns characteristic for individual isolates, which were compared by analyzing their number and distribution. The restriction patterns obtained for each enzyme were marked with big letters (Table 2) and the examples of these patterns are shown in Fig. 1 and 2. The presence of a large number of polymorphic bands, when using the same endonucleases demonstrates that there are differences within the sequence of the 16S-23S rDNA fragments and therefore, there is a large genetic diversity in the tested bacterial strains. As a result of digestion of the ITS fragment with the *Hae*III enzyme, 2 electrophoretic profiles (A, B) were obtained for the tested strains (Fig. 1) and 4 electrophoretic profiles (A, B, C, D) for the reference strains. Using the digestion of the ITS fragment with the

Table 2. Restriction patterns obtained for reference strains and *Azotobacter* strains isolated from soil by ITS-PCR, using the restriction enzymes *Hae*III and *Msp*I.

| Strain | Restriction patterns | | Genotype | Genotypic group |
|---|---|---|---|---|
| | HaeIII | MspI | | |
| *1* | *2* | *3* | *4* | *5* |
| *Azotobacter armeniacus* DSM2284 | B | F | BF | VII |
| *Azotobacter chroococcum* DSM281 | B | B | BB | IV |
| *Azotobacter chroococcum* DSM2286 | A | C | AC | III |
| *Azotobacter salinestris* DSM11553 | B | F | BF | VII |
| *Azotobacter vinelandii* DSM2289 | D | F | DF | VIII |
| *Azomonas agilis* DSM375 | C | D | CD | V |
| *Azomonas macrocytogenes* DSM721 | C | E | CE | VI |
| A143-1 | A | A | AA | I |
| A143-2 | A | A | AA | I |
| A284-1 | A | A | AA | I |
| A284-2 | A | A | AA | I |
| A340-1 | A | A | AA | I |
| A340-2 | A | A | AA | I |
| A340-3 | A | A | AA | I |
| A374-1 | A | B | AB | II |
| A374-2 | B | B | BB | IV |
| A384-1 | A | C | AC | III |
| A384-2 | B | B | BB | IV |
| A415 | A | A | AA | I |
| A484-1 | B | B | BB | IV |
| A484-2 | A | A | AA | I |
| A597-1 | A | A | AA | I |
| A597-2 | A | A | AA | I |
| A600-1 | A | B | AB | II |
| A600-2 | B | B | BB | IV |
| A663 | A | B | AB | II |
| A725 | B | B | BB | IV |
| A727-1 | B | B | BB | IV |
| A727-2 | A | B | AB | II |
| A727-3 | A | B | AB | II |
| A784 | B | B | BB | IV |
| A786-1 | B | B | BB | IV |
| A786-2 | B | B | BB | IV |
| A787 | A | C | AC | III |
| A819-1 | B | B | BB | IV |
| A819-2 | A | A | AA | I |
| A819-3 | B | B | BB | IV |
| A850 | B | B | BB | IV |
| A894 | B | B | BB | IV |
| A1820-1 | A | C | AC | III |
| A1820-2 | A | C | AC | III |
| A1940 | B | B | BB | IV |
| A2070 | B | B | BB | IV |
| A2684 | B | B | BB | IV |
| A3112 | B | B | BB | IV |
| A4065-1 | B | B | BB | IV |
| A4065-2 | A | A | AA | I |
| A4065-3 | A | A | AA | I |
| A4165 | A | A | AA | I |
| A4424 | B | B | BB | IV |

Table 2 continuation

| *1* | *2* | *3* | *4* | *5* |
|---|---|---|---|---|
| A4539-1 | B | B | BB | IV |
| A4539-2 | B | B | BB | IV |
| A4604-1 | A | C | AC | III |
| A4604-2 | B | B | BB | IV |
| A4625 | B | B | BB | IV |
| A4733-1 | B | B | BB | IV |
| A4733-2 | B | B | BB | IV |
| A4761 | B | B | BB | IV |
| A4791-1 | B | B | BB | IV |
| A4791-2 | B | B | BB | IV |
| A4803 | B | B | BB | IV |
| A4811 | A | C | AC | III |
| A4813 | B | B | BB | IV |

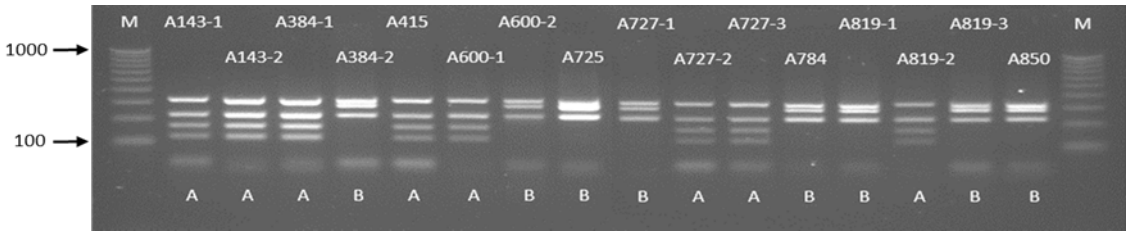


Figure 1. Agarose gel electrophoresis of restriction fragments obtained from *Hae*III digestion of the 16S-23S rDNA for some *Azotobacter* strains. M – molecular weight ladder (1000–100 bp).

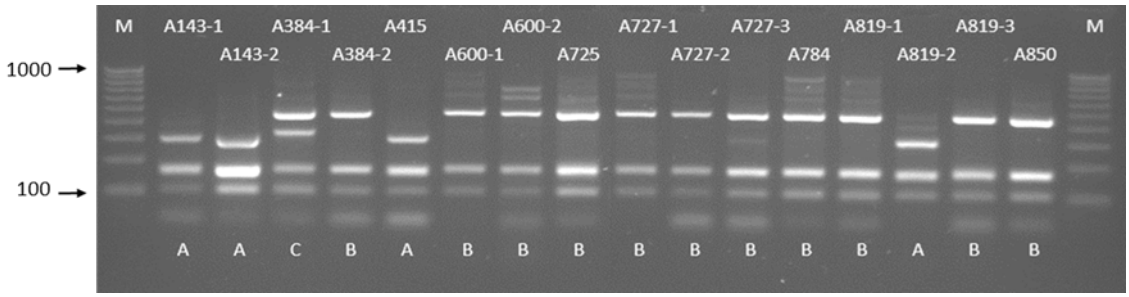


Figure 2. Agarose gel electrophoresis of restriction fragments obtained from *Msp*I digestion of the 16S-23S rDNA for some *Azotobacter* strains. M – molecular weight ladder (1000–100 bp).

*Msp*I enzyme, 3 different electrophoretic profiles (A, B, C) were obtained for the tested strains (Fig. 2), and 5 profiles for the reference strains (B, C, D, E, F).

The combined analysis of the ITS restriction patterns (*Hae*III + *Msp*I) of the tested isolates and reference strains of the genus *Azotobacter* and *Azomonas* allowed the assignment of these bacteria to eight different genotypes. These analyzes showed that the tested isolates belong to four different genotypes, while the reference strains included in the analysis form six other unique genotypes (Table 2). Among the 56 analyzed *Azotobacter* spp. isolates, as many as 30 of them showed the same genotype IV – BB, 15 strains represented the genotype I – AA, 6 isolates were characterized by the genotype III – AC, while the other 5 isolates represented the common genotype II – AB. The percentage share of the tested isolates within the individual genotype groups is shown in Fig. 3. Among the four ITS groups, 2 main groups were distinguished: IV – constituting 53% of all strains and I – constituting 27% of all strains. The percentage share of the other two ITS groups was much lower and amounted to: group III (11%) and group II (9%).

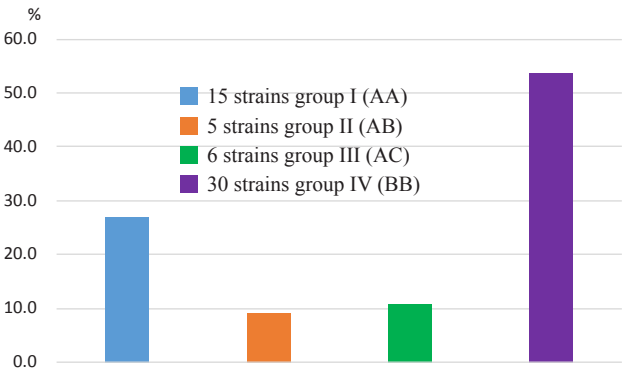


Figure 3. Percentage of tested *Azotobacter* spp. strains within particular genotype groups.

Only two of the seven reference strains were characterized by genotypes similar to the tested isolates, and they were *A. chroococcum* DSM281 representing the genotype group IV – BB, and *A. chroococcum* DSM2286 included in the group III – AC. The remaining reference strains were characterized by a different arrangement of bands in the agarose gel and on this basis they were classified into four different genotypic groups, namely V – CD (*Azomonas agilis* DSM375), VI – CE (*Azomonas macrocytogenes* DSM721), VII – BF (*Azotobacter armeniacus* DSM2284, *Azotobacter salinestris* DSM11553) and VIII – DF (*Azotobacter vinelandii* DSM2289).

The results of ITS-PCR/RFLP genotyping presented above clearly indicate that the 36 tested strains had genotypes very similar to the two reference strains *Azotobacter chroococcum* DSM281 and DSM2286 (Table 2).

Lenart (2012) using the ITS-PCR/RFLP technique with *Hind*III restrictase showed the same band pattern for all examined strains. In our study, two restriction enzymes (*Hae*III and *Msp*I) were used, and 2 or 3 restriction patterns (genetic profiles) were obtained respectively for the examined *Azotobacter* isolates. Even greater genetic diversity of these isolates (4 genetic profiles) was demonstrated after combining both restriction patterns obtained after digestion with *Hae*III and *Msp*I endonucleases. The results of the analysis of genetic variability of the tested *Azotobacter* spp. isolates presented above indicate that they are very similar to the results of research by various authors dealing with the genetic diversity of the discussed group of bacteria, also using other molecular methods. Using the ARDRA technique for 24 *Azotobacter* isolates from Indian soils, Jain et al. (2021) obtained 2 genetic profiles while 3 genetic clusters were distinguished for 13 isolates from Columbia soils (2011). Greater genetic diversity (6 profiles) of 31 strains belonging to the genus of *Azotobacter* isolated from Argentine soils was demonstrated by Rubio et al. (2013) using Rep-PCR (BOX-AIR) technique. Khosravi and Dolatabad (2020) studied the molecular differentiation and diversity analysis of *Azotobacter* species and reported that the ARDRA technique with *Hpa*II, BOX, and

Table 3. Strains selected for 16S rRNA gene sequencing.

| Genotype group/Genotype | Strain |
|---|---|
| I / AA | A143-1, A284-2, A340-1, A415, A597-1, A4065-2, A4165 |
| II / AB | A374-1, A600-1, A663 |
| III / AC | A384-1, A1820-1, A4604-1 |
| IV / BB | A384-2, A484-1, A725, A727-1, A784, A819-1, A850, A1940, A2684, A3112, A4424, A4539-2, A4625, A4733-1, A4761, A4791-1, A4803 |

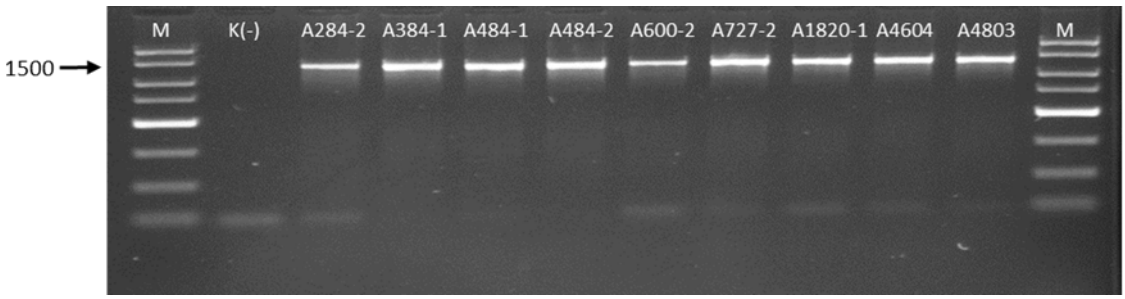


Figure 4. Agarose gel electrophoresis of amplified 16S rRNA products (product size 1500 bp) of selected *Azotobacter* spp. strains. M – molecular weight ladder (DNA Perfect Plus™ 2 kb (2000–50 bp)); K(-) – negative control.

REP PCR based markers was able to differentiate between *A. chroococcum* and *A. salinestris*. Swapna et al. (2018) confirmed the genetic diversity of *Azotobacter* spp. isolated from rhizosphere soil of chilli using RAPD.

Of the 56 tested strains, 36 were characterized by the same genotype in ITS-PCR/RFLP analyzes as the reference strains *A. chroococcum* DSM281 and DSM2286, which clearly indicated their belonging to the species *Azotobacter chroococcum* and these were AC and BB profiles. The remaining isolates with restriction patterns AA and AB showed no similarity to any of the reference strains used (Table 2). Then the phylogenetic analyzes based on the sequencing of the gene encoding 16S rRNA were performed and results for 20 isolates of the group with patterns AC and BB and 10 isolates from the other two groups (Table 3) were used in the paper. Sequencing of the 16S rRNA gene is one of the most commonly methods used for identifying and checking the degree of genetic similarity between strains of *Azotobacter* spp. Janda and Abbott (2007) showed that 90% of bacteria at the genus level and 86% at the species level could be reliably identified using 16S rRNA gene sequencing.

As a result of amplification of 16S rRNA genes one PCR product a 1500 bp was obtained for all 30 examined strains and an example of this analyze is shown in Fig. 4.

Based on the sequencing of the 16S rRNA gene, the tested isolates were identified as *Azotobacter chroococcum* species. All isolates showed similarity >90% (from 98% to 100%) to the species *Azotobacter chroococcum*. 100% similarity to the *Azotobacter chroococcum* showed two strains named A727-1 and A3112. Based on the literature data, it can be concluded that the comparative analysis of the 16S rRNA gene sequence is currently the most frequently used for phylogenetic studies of *Azotobacter* spp. at the genus and species level. Obele et al. (2019) in their research obtained a 96% degree of similarity of the 16S rRNA gene sequence of isolated strains belonging to the genus *Azotobacter* with the reference strain *Azotobacter chroococcum* deposited in the RDP Gen Bank database. Isolates used for research by Kizilkaya et al. (2010) was also identified by sequencing the 16S rRNA gene to the species *Azotobacter chroococcum*. These isolates were closely related to the reference strain *A. chroococcum* DSM2286T, and the level of 16S rRNA sequence similarity between these strains and the reference strain ranged from 99.3% to 100%. Wakarera et al. (2022) used 16S rRNA gene sequencing to identify strains isolated from semi-arid areas of Eastern Kenya. The Basic Local Alignment Search Tool (BLASTn) analysis of their sequences revealed the presence of three main *Azotobacte*r species like: *Azotobacter vinelandii*, *Azotobacter salinestris* and *Azotobacter tropicalis*. Similar research was conducted by Chen et al. (2018). Ninety-eight *Azotobacter* strains were isolated from 27 paddy fields, and 16S rRNA gene sequences were used to identify *Azotobacter* species. Of these isolates, 50 isolates were identified as *A. chroococcum*, which was the dominant species in this study; 30 isolates could be assigned to *A. beijerinckii*, 16 isolates were identified as *A. tropicalis* and only two isolates of *A. vinelandii* were obtained.

The results of molecular identification of the *Azotobacter* strains isolates from Polish soils clearly indicate that they belong to the *Azotobacter chroococcum* species. Similar results were obtained by Lenart (2012) which were based on the physiological and morphological analyses, all 43 isolated strains from Małopolskie and Śląskie voivodeships in southern Poland were defined as *Azotobacter chroococcum*. It can be stated that this species is the most abundant in Polish soils. In other studies, Lenart-Boroń et al. (2014) identified 59 *Azotobacter* spp. strains isolated from contaminated soils at the steelworks in Nowa Huta and from agricultural and forest soils in this area, using physiological tests and microscopic observations. The vast majority of the tested isolates were identified to the species *A. chroococcum*, but the presence of the other two species, *A. vinelandii* and *A. salinestris*, in the tested soils was also demonstrated. The results presented above have shown that *Azotobacter chroococcum* is the most common species representing free-living diazotrophic bacteria in Polish soils, however further studies are needed on environmental factors affecting the occurrence and diversity of these bacteria in soils of Poland.

An attempt was also made to found relationships between the genetic diversity of these isolates and their origin. It seems that there relationships between the genetic diversity of strains and their origin from a specific voivodenships may exist. For example, four out of five *Azotobacter* strains of the AB genotype (A600-1, A663, A727-2 and A727-3) were isolated from the soils of the Podkarpackie voivodeship, and one (A374-1) from the neighboring voivodeship (Table 1). Moreover, all isolates from acidic soils (A143-1 and -2, A284-1 and -2, A340-1, -2 and -3) were included in the group with the AA genetic profile, but this group also included isolates with neutral pH soils, which indicates that these relationships are not exact. From the above-mentioned three acidic soils, two or three isolates were isolated and all of them were characterized by the same genetic profile (AA), which would indicate the lack of genetic diversity of *Azotobacter* spp. bacteria in acidic soils. This interesting phenomenon requires further and broader molecular studies with larger populations of isolates of the discussed bacteria. Genetic profile tested *Azotobacter* isolates were not correlated ($p<0.05$) with soil type or granulometric group (Table 1).

## CONCLUSIONS

1. Based on the performed diagnostic analyses, all isolated strains were defined as *Azotobacter chroococcum* – it can be stated that this species is the most abundant in Polish soils.

2. Restriction analysis of ITS region has shown that the tested isolates were not identical. Only 36 strains were characterized by the same genotype in ITS-PCR/RFLP analyzes as the reference strains *A. chroococcum* DSM281 and DSM2286.

3. Based on the sequencing of the 16S rRNA gene, the tested isolates were identified as *Azotobacter chroococcum* species. All isolates showed similarity >90% (from 98% to 100%) to the *Azotobacter chroococcum* of the reference type strains deposited in Gen Bank database.

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