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Characteristics of selected molecular methods used in identification and assessment of genetic diversity of bacteria belonging to the genus *Azotobacter*

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Abstract. Modern molecular techniques have greatly increased our knowledge concerning phylogenetic and functional diversity of microorganisms inhabiting the soil environment. Soil ecosystem is relatively complex with a high level of microbiologically diversity. The application of traditional culture-based techniques dose not reflect the total diversity of microbial community inhabiting in soil environment. On the other hand commonly used molecular methods allow for quick and accurate identification and evaluation of the genetic diversity of microorganisms inhabiting this environment. Free-living bacteria belonging to the genus *Azotobacter* commonly occurring in soil. *Azotobacter* spp. are the subject of many studies conducted both in Poland and in the world. The interest in these bacteria is largely related to their properties very useful for agriculture. Owing to their capability of fixing atmospheric nitrogen and making it available to plants and production of plant growth promotion and fungicidal substances, they are used in the production of soil bacterial inoculants. In addition, these bacteria are an excellent indicator of soil fertility, which is why they are often used as test microorganisms in many studies. The paper presents an overview of molecular microbiological techniques used to identify and evaluate the genetic diversity of *Azotobacter* spp. in studies conducted both in Poland and across the world. The ITS PCR, PCR-RFLP methods and 16S rRNA gene amplification are used to identify bacteria of the genus *Azotobacter*, and PCR MP, RAPD PCR and ARDRA are used to assess the genetic diversity of these microorganisms.

Keywords: *Azotobacter*, ITS PCR, 16S rRNA gen, PCR MP, RAPD, ARDRA

INTRODUCTION

Aerobic bacteria belonging to the genus *Azotobacter* represent a diverse group of free-living diazotrophs commonly occurring in soil. Currently, 7 species are included the genus *Azotobacter*, i.e. *A. armeniacus, A. beijerinckii, A. chroococcum, A. nigricans, A. paspali, A. salinestris* and *A. vinelandii* (Mazinani and Asgharzadeh, 2014; Tchan and New, 1984). These bacteria are extremely useful for agriculture on account of their capability to fix atmospheric nitrogen and make it available to higher plants as well as produce a range of compounds stimulating plant growth (Kumari et al., 2017; Lenart, 2008; Mrkovacki and Milic, 2001; Sivasakthi et al., 2017).

For many years, the identification of microorganisms, including *Azotobacter* spp., concerned the methods based on culture media (Kozdrój, 2013). Phenotypic identification of the bacteria belonging to the genus *Azotobacter* is a long-term and labour-intensive process, which is why various molecular methods are used to identify these bacteria quickly and accurately with increasing frequency. The availability of molecular biology methods is currently quite broad, and the selection of the appropriate research technique in genotyping often depends on: the repeatability and replicability of the method, stability, differentiating potential and the consistency of the genotyping system. The selection of the appropriate method of genotyping and genetic differentiation is also influenced by factors such as: physicochemical and biological properties of the soil, properties of the microorganism from which the genetic material is isolated and the purpose of the research conducted (Krawczyk, 2007; Łyszcz and Gałązka, 2017). Knowledge of the method which we are interested in, access to appropriate equipment and financial resources are equally important.

Currently, the taxonomy of the bacteria belonging to the genus *Azotobacter* is based on multidirectional studies, which are based on the data obtained from the analysis of phenotypic and genomic traits and from phylogenetic analysis. Over the past few decades, several methods based on bacterial DNA analysis have been proposed, which, according to the authors, enable accurate identification of these bacteria. The molecular methods used to identify and evaluate the variability of the genus *Azotobacter* are presented in Figure 1.

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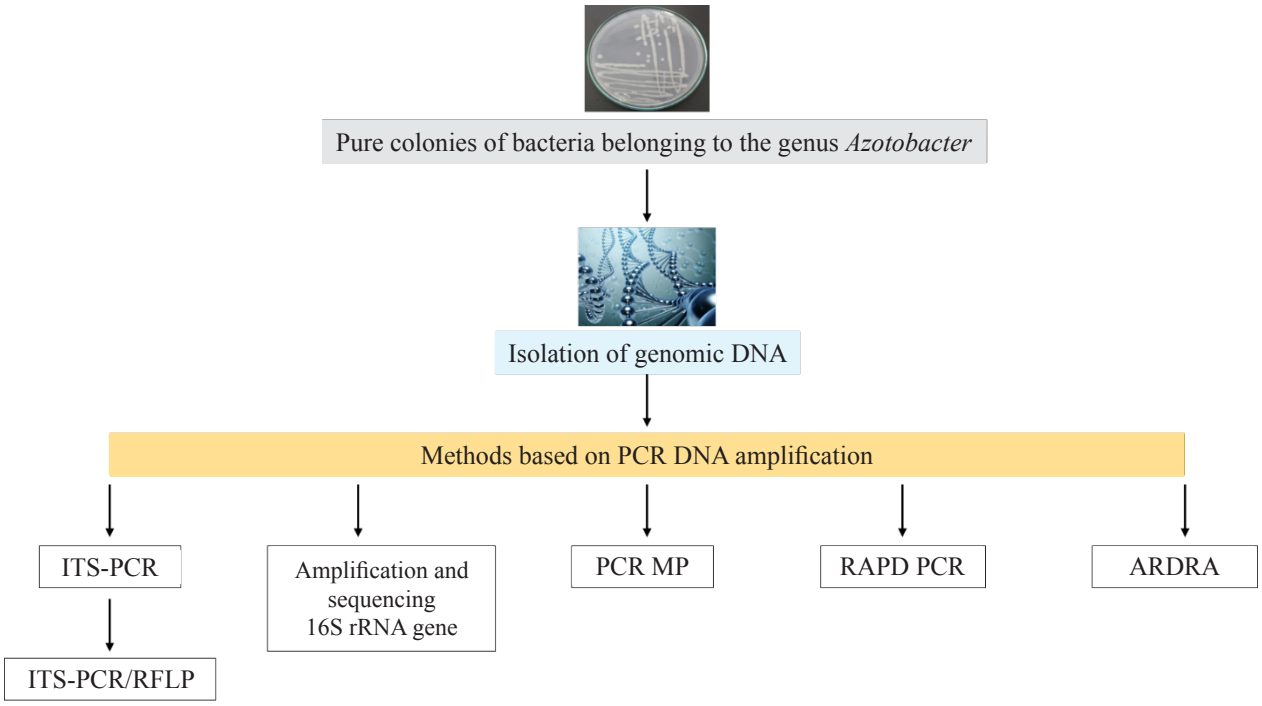

Figure 1. Molecular methods used to identify and assess the variability of bacteria belonging to the genus *Azotobacter*.
Source: Krawczyk and Kur, 2008, modified

This work presents an overview of modern molecular microbiological methods used to identify and assess the genetic diversity of *Azotobacter* spp.

MOLECULAR METHODS USED TO IDENTIFY BACTERIA OF THE GENUS *AZOTOBACTER*

ITS PCR

ITS PCR is a commonly used method of ribotyping based on the PCR technique (*PCR amplification of Intergenic rRNA Spacer Regions*). ITS PCR is one of the *fingerprinting* techniques employed for genotyping microorganisms (Jensen et al., 1993; Krawczyk and Kur, 2008). Genotyping is based on differences in the structure (length and sequence) of operons encoding ribosomal RNA (*rrn*). *rrn* operons occur in all organisms, except for viruses, and in prokaryotic organisms they are responsible for the synthesis of 16S, 23S and 5S rRNA and tRNA. Fig. 2 presents the structure of the *rrn* operon.

The number of *rrn* operons is highly varied and depends on the bacterial species, e.g. the chromosome of *Escherichia coli* contains 7 copies of the *rrn* operon, a lot more operons can be found in *Bacillus subtilis*, while the bacteria belonging to the genera *Mycoplasma*, *Mycobacterium* and *Halobacterium* have one or two copies (Krawczyk and Kur, 2008). The *rrn* operon possesses highly evolutionarily conserved areas which are separated by polymorphic regions characterised by a high degree of diversity with regard to qualitative (nucleotide sequence) and quantitative (sequence length) properties. As a result of the ITS PCR reaction, the polymorphic region located between the ge-

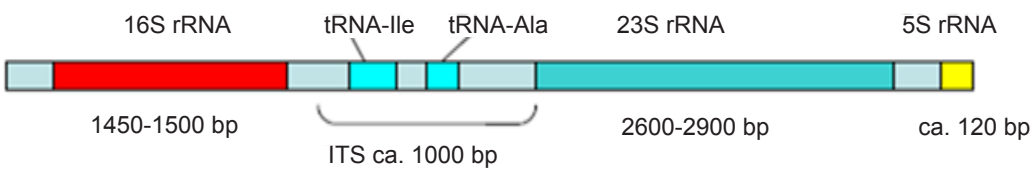

Figure 2. Structure of the operon *rrn*.
Source: Krawczyk and Kur, 2008, modified

nes encoding 16S and 23S rRNA undergoes amplification. ITS regions are characterised by varying size and a high degree of sequence diversity in particular bacterial species (Szczuka et al., 2013). Considering the fact that ITS regions are highly polymorphic, the DNA fragments obtained as a result of the PCR reaction, separated electrophoretically in agarose gel, display the band profile characteristic of the microorganism under study. In some bacterial species, the result of ITS region amplification is one product, and this is due to the fact that the 16S-23S rDNA region is undifferentiated within the genome (all *rrn* copies in the genome have the same sequence) (Wolny-Koładka, 2014).

The ITS-PCR method is used for rapid identification of species, most often it applies to pathogenic microorganisms when the detection time is of decisive importance in treatment (Khan et al., 2005; Liu et al., 2008). ITS is increasingly employed to assess biodiversity and analyse the population genetics of microorganisms (Łyszcz and Gałązka, 2017).

Lenart (2012) examined 43 strains of bacteria belonging to the genus *Azotobacter* isolated from soils used in a variety of ways, collected from Lesser Poland and Silesia provinces. The analysis aimed to confirm whether the studied strains belonged to the species *Azotobacter chroococcum*. As a result of the amplification of the 16S-23S rDNA fragment for all 43 studied strains, the author of the study obtained 1 PCR product about 630 bp in length.

PCR-RFLP – restriction fragment length polymorphism

The PCR-RFLP technique (Polymerase *Chain Reaction - Restriction Fragments Length Polymorphism*) is a combination of PCR reaction with restriction analysis. The amplification products obtained as a result of the PCR reaction are subjected to restriction enzymes which recognise and cut DNA in specific sites within the sequence. This allows for detecting the restriction fragment length polymorphism – RFLP – characteristic of the studied microorganism, which enables bacterial strains to undergo differentiation within a species. The products of restriction digestion are subjected to electrophoretic separation, on the basis of which a band pattern characteristic of a given organism is obtained – the so-called restrictive formula. Then the number and size of the resulting products are evaluated and compared with the pattern characteristic of the given species. An important factor affecting the efficiency of the PCR-RFLP technique is the selection of appropriate restriction enzymes because individual enzymes cut sequences at different places, conditioning the obtainment of different restriction patterns. Restriction enzymes are most often selected basing on the knowledge of the nucleotide sequence of the studied gene, however, if the amplicon sequence is unknown, the selection of restriction enzymes is determined by experimental results. This method is used both to identify microorganisms at the species level and intra-species genotyping (Brzeszcz et al., 2013; Krawczyk and Kur, 2008; Liu et al., 2010).

Lenart (2012) used the ITS-PCR/RFLP method to confirm that *Azotobacter* spp. strains belong to one species. As a result of the digestion of the ITS restriction fragment with *HindIII* endonuclease, two products, approximately 230 and 360 bp in length, were obtained for 43 strains tested. The conducted research showed 100% similarity between the analysed isolates, which allowed for determining that they belong to the same species. The author of the dissertation stated that the restriction analysis of the bacterial ITS region may be used as a reliable method for the rapid identification of *Azotobacter* spp. at the genus or species level, but it is an inappropriate method for testing intraspecific variation. RFLP analysis of the nifH gene was applied by Bhatia et al. (2009) to study the genetic diversity of bacterial strains belonging to the genus *Azotobacter* isolated from various soils from cotton farms in India. According to the studies, the degree of genetic similarity was determined at the level of $\geq 80\%$ among isolates coming from different places and belonging to different species.

Amplification and sequencing of the 16S rRNA gene

The 16S rRNA gene belongs to the group of evolutionarily conservative genes and is therefore considered a very good phylogenetic tool commonly used in microbiology to identify microorganisms. It occurs in all bacterial species and includes 1500 base pairs. Carl Woese and George E. Fox were the first to develop the basis for molecular identification based on 16S rRNA gene sequencing (Woese and Fox, 1977). The 16S rRNA gene consists of both highly conservative regions, where the primers are designed, as well as super-variables specific for particular species. Conservative fragments are present in all the strains of one species or type of bacteria, which allows for confirming their consanguinity and placement in the same taxonomic unit. The analysis and comparison of variable region sequences with the patterns in the database allows for finding fragments or individual nucleotides differentiating them, which enables the isolation of new subspecies or strains (Jaroszewska and Misiewicz, 2012).

The amplification of the 16S rRNA gene is very often used for the initial identification of the bacteria from the genus *Azotobacter*. Mazinani and Asgharzadeh (2014), as well as Aquilanti et al. (2004a, 2004b) amplified the 16S rRNA gene in their studies using 27f and 1495r primers. In turn, Jiménez et al. (2011) used Y1 and Y3 primers, for the amplification of this gene, whereas Tejera et al. (2005), in the research aimed to identify the strains of bacteria belonging to the genus *Azotobacter*, isolated from rhizosphere soils used for sugarcane cultivation, used 41f and 1488 primers.

MOLECULAR METHODS USED TO ASSESS THE GENETIC DIVERSITY OF *AZOTOBACTER* SPP.

PCR MP

PCR MP method (PCR Melting Profile) belongs to the group of LM PCR (Ligation-Mediated PCR) based on the selective amplification of restrictive fragments with adapters attached to them. Among the LM PCR methods, great popularity is enjoyed also by: AFLP (Amplified Fragment Length Polymorphism), IRS-PCR (Infrequent Restriction Site PCR), ADSRRS (Amplification of DNA Surrounding Rare Restriction Sites) and LM PCR/Shifter (Ligation-Mediated Shifter PCR). All of the LM PCR methods follow the same pattern, including the following stages:

- enzymatic digestion of genomic DNA,
- ligation of adapters to the ends of restrictive fragments,
- amplification of the selected fragments in a PCR reaction,
- electrophoretic separation of amplification products.

The PCR MP method was proposed in 2003 by Masny and Płucienniczak (2003). The application of the PCR MP method in epidemiological studies was first presented in 2006 in the work by Krawczyk et al. (2006) for genotyping of *E. coli* isolates. The PCR MP method is based on the phenomenon of different thermal stability of DNA fragments arising from the digestion of restrictive genomic DNA. The PCR MP method involves the digestion of genomic DNA with one restrictive enzyme (medium-cutting, yielding about 400–600 fragments and leaving 5' hanging ends), ligation of the short adapter (made of an auxiliary oligonucleotide with a sequence complementary to the 5' hanging end and the proper ligated oligonucleotide) and amplification with primers complementary to the sequence of the restrictive ends of fragments terminated with an adapter (Fig. 3). Due to the occurrence of heterogeneity in the distribution of GC base pairs in the bacterial genome, after having been digested by restrictive enzymes, fragments differing in length, base composition, as well as denaturation temperature are obtained. The use of standard denaturation temperature in PCR cycles in the range of 94–95 °C causes the amplification of all restrictive fragments terminated with adapters. In order to obtain a limited number of amplified DNA fragments for easier analysis of results, a lower denaturation temperature is used in the PCR cycles. Due to the different thermal stability of the double-stranded restrictive fragments, which depends on the composition of the nucleotide sequence and the percentage of GC pairs, it is possible to experimentally select the denaturation temperature in which only part of the restrictive fragments undergoes denaturation, becoming matrices for amplification. Appropriate selection of denaturation temperature in PCR MP cycles determines the differentiating potential and degree of complexity of the band profile. After electrophoretic separation, the resultant amplicons form a unique band pattern characteristic of the DNA of a given bacterial strain, which is then analysed after ethidium bromide staining in the light of a UV lamp. At the optimal denaturation temperature, the number of PCR products should be large enough to allow differentiation of the studied microorganisms within a group, and at the same time small enough so that the electrophoretically obtained profile of the PCR products will be legible and easy to interpret. Most commonly, the number of amplification products visible on the gel is 15-30 (Krawczyk et al., 2006; Krawczyk, 2007; Krawczyk and Kur, 2008; Krawczyk et al., 2008; Wolny-Koładka, 2014).

PCR MP is a repetitive method, characterized by high differentiating potential, which can be regulated by selecting the appropriate restriction enzyme and changing the denaturation temperature in the PCR reaction cycles. The MP PCR technique is universal, so it can also be used in the study of large genomes, including eukaryotic ones, and DNA from various sources can be used for analysis. Unlike the other techniques in the LM PCR group, PCR MP allows the differentiation of DNA fragments of identical length but with different nucleotide composition, affecting the change in the melting temperature (Krawczyk et al., 2006; Krawczyk and Kur, 2008).

Lenart (2012) used the PCR method of genotyping in her research in order to determine the genetic diversity of 43 *A. chroococcum* strains. Using this method, she obtained 43 different electrophoretic profiles, thus no strains with the same genotype were identified. Even the strains that came from areas with the same usage pattern, or from areas which were geographically close, did not exhibit similarity. Using the PCR-MP method it was demonstrated that the total genetic similarity of the isolated strains was 27%.

RAPD – polymorphism of randomly amplified DNA

RAPD method (Randomly Amplified Polymorphic DNA) was first described by Williams et al. (1990) and Welsh and McClelland (1990) in 1990. This method is based on the PCR reaction carried out on a genomic DNA of microorganisms by using one primer, or a combination of two or many primers depending on the differentiation potential of the method. Similarly, to PCR, it occurs in three successive stages – denaturation of a double-stranded DNA chain, attachment of primers and elongation of the DNA chain. The basic difference that appears between these methods is the usage of a single, short primer oligonucleotide with a length of 9–10 nucleotides in the RAPD technique. This primer containing a randomly selected sequence of nucleotides is attached at different places in genomic DNA resulting in the creation of a few to over a dozen PCR products. Next, the products of the PCR reaction are separated on an agarose or acrylamide gel (Olive and Bean, 1999; Ranjard et al., 2000; Rastogi and Sani, 2011; Robak et al.,

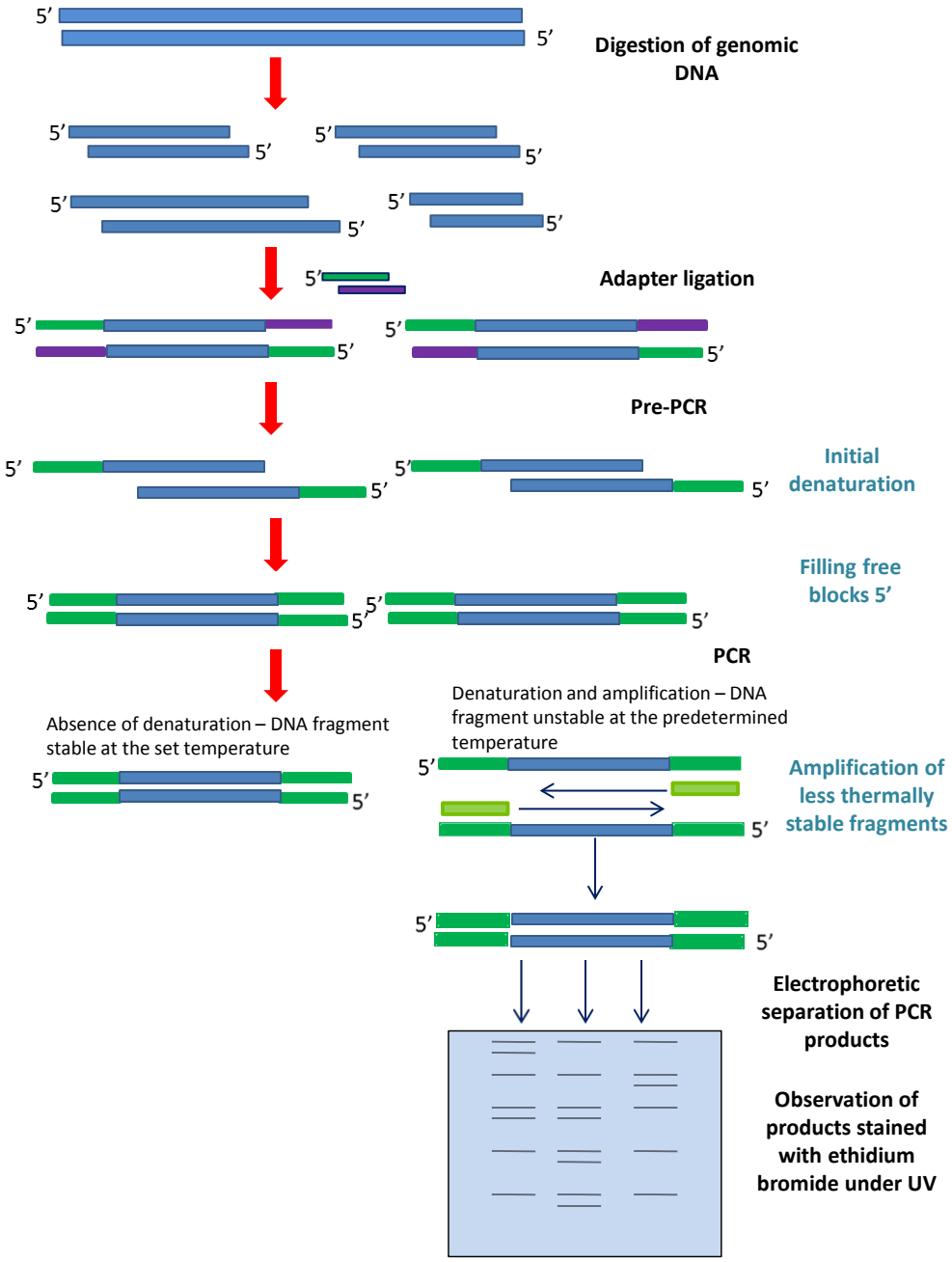

Figure 3. Diagram showing the course of the PCR MP technique.
Source: Stojowska, 2012, modified

2005) (Fig. 4). The number and location of random primer binding sites are different for different bacterial species. As a result, the band pattern obtained as a result of the separation of amplification products by electrophoresis is characteristic of individual bacterial strains (Rincon-Florez et al., 2013; Welsh and McClelland, 1990; Williams et al., 1990).

The RAPD method is used in studies determining the genetic variability of microorganisms and has found a number of applications in population genetics, molecular evolution, genetic mapping, as well as in the breeding and genetics of microorganisms, animals and plants (Devi et al., 2014; Łyszcz and Gałązka, 2017).

The RAPD technique with randomly selected primers was used by Lenart (2012) to assess the intraspecific diversity of 43 *A. chroococcum* strains. The analysis showed a high degree of differentiation among the tested isolates. The differentiation of *A. chroococcum* strains carried out with the RAPD method allowed for obtaining 43 different electrophoretic profiles, which allowed for concluding that each strain has a different genotype. The total genetic si-

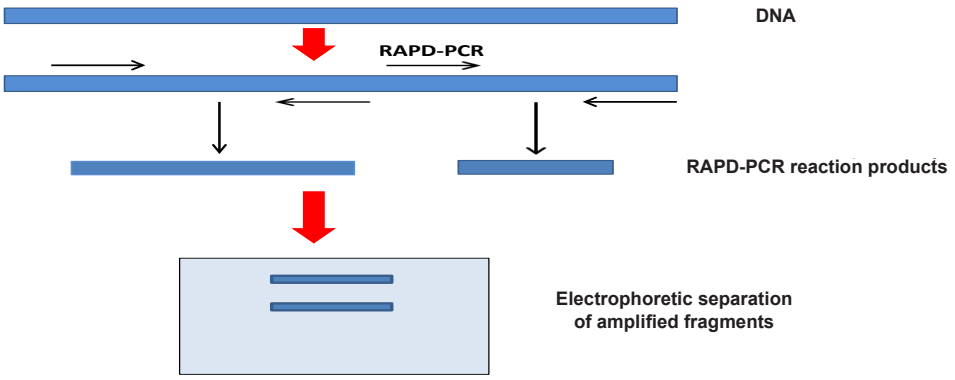

Figure 4. Diagram of RAPD-PCR technique.
Source: Arif et al., 2010, modified

milarity between the isolates established with the RAPD method was 31%.

ARDRA – restriction analysis of amplified rDNA fragments

ARDRA (Amplified Ribosomal DNA Restriction Analysis) is a restrictive analysis of amplified rDNA fragments. ARDRA is one of the fingerprinting techniques which is used to assess microbial differentiation (Kirk et al., 2004; Kumar and Joshi, 2008). In this technique, the PCR product, which is a DNA fragment encoding 16S rRNA or 18S rRNA molecules, is digested with several selected restriction endonucleases (Cetecioglu et al., 2012; Jampachaisri et al., 2005; Pandey et al., 2009; Rastogi and Sani, 2011). Before the restrictive analysis, there is an additional step of cloning a fragment of DNA obtained after the amplification of the 16S rRNA gene using an appropriate vector (Fig. 5). This additional step is supposed to prevent cross-contamination of 16S rRNA gene fragments derived from various microorganisms. Then, the obtained products of varying length undergo separation in agarose or polyacrylamide gels and are classified according to the restrictive digestion pattern. The application of this technique enables observing differences between closely related groups of microorganisms (Friedrich et al., 2002; Pandey et al., 2009). This method is widely used for the analysis of microbial populations in environmental samples, as well as to determine the structure and dynamics of microorganisms (Fernandez et al., 1999; Gich et al., 2000; Oravecz et al., 2004; Pandey et al., 2009; Slabbert et al., 2010). According to the research conducted, the ARDRA technique is considered to be the most accurate of all fingerprinting methods. A comparison of the effects of various fingerprinting methods showed that ARDRA is a more effective method than RAPD and AFLP (Dherbécourt et al., 2006; Jawad et al., 1998; Pandey et al., 2009).

Mazinani and Asgharzadeh (2014) used the ARDRA technique to identify and assess the genetic diversity of 50 strains of *Azotobacter* bacteria isolated from different rhizosphere soils in Iran. Universal primers were used to amplify the 16S rRNA gene for all 50 isolates: 27f and 1495r, obtaining 1 product with a length of 1500 bp. Restrictive analysis of the PCR product was carried out using three endonucleases: *RsaI*, *HpaII*, *HhaI*. Statistical analysis of genetic similarity allowed for distinguishing two genotypic groups: A (containing 17 strains identified as *Azotobacter vinelandii*) and B (containing 23 strains of *A. chroococcum* and 10 strains of *A. beijerinckii*). Aquilanti et al. (2004a), comparing different methods of isolation and initial identification of *Azotobacter* from soils collected in Italy, concluded that ARDRA is a suitable method for identifying these bacteria at the species level. Amplification of the 16S rRNA gene was performed using universal primers: 27f and 1495r, and the obtained product was digested with restrictive enzymes: *RsaI* and *HhaI*. The restrictive analysis of the PCR product with the *RsaI* enzyme resulted in a characteristic 5-band electrophoretic profile for all strains belonging to the genus *Azotobacter*. In addition, the authors of the study found that the use of *RsaI* endonuclease allows for distinguishing species belonging to the *Azotobacteraceae* family from other types of bacteria which bind atmospheric nitrogen, i.e. *Beijerinckia*, *Azospirillum*, *Agrobacterium*, *Rhizobium* and *Pseudomonas*. In turn, the products of restrictive digestion with the use of the *Hhal* enzyme separated in agarose gel, yielded 6 species-specific restriction profiles characteristic of only the strains belonging to the genus *Azotobacter*. The ARDRA technique was used by Aquilanti et al. (2004b) also for the identification of bacteria from the *Azotobacteraceae* family and tested on 48 soil isolates and 28 reference strains belonging to the group of free-living nitrogen-binding bacteria and most commonly found in the soil environment. The 16S rRNA gene was amplified using the universal primers 27f and

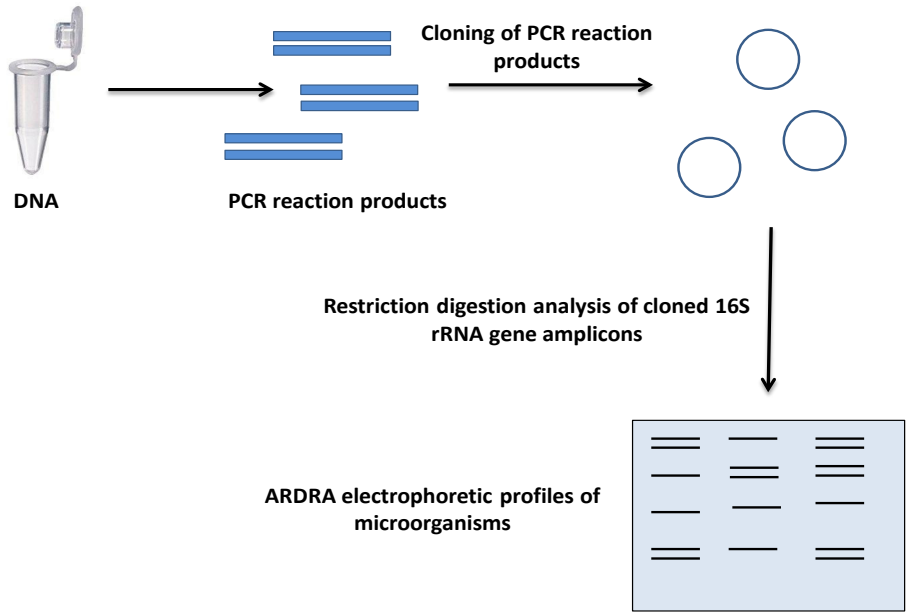

Figure 5. Diagram of ARDRA technique.
Source: Pandey i in., 2009, modified

1495 r, and then the PCR products were digested with five restrictive enzymes: *RsaI*, *HhaI*, *HpaII*, *FnuDII* and *AluI*. The analysis of the obtained restrictive profiles allowed the conclusion whereby that the specific phylotype for each species can be identified using the ARDRA method. The authors of the study demonstrated that this technique can be successfully used to identify members of the *Azotobacteraceae* family. Also, Jiménez et al. (2011) used the ARDRA technique to identify and assess the genetic variability and similarity of bacterial strains belonging to the genus *Azotobacter* isolated from the soils used for various vegetable crops in Colombia.

CONCLUSION

In recent years, methods based on molecular biology have become a highly valuable tool in studying the diversity of microorganisms in many environments, including the bacteria of the genus *Azotobacter*. These bacteria are the subject of many studies conducted both in Poland and abroad. The interest in *Azotobacter* spp. is largely related to their properties which can be used in agriculture. Their ecological role and the possibility of using these microorganisms in the production of soil-applied bioproducts has been known for a long time. For many years, the identification of these bacteria has been based on culture methods, however, the development of molecular techniques has allowed for the precise classification and proper identification of the microorganisms in question. The authors of many scientific publications, using various available molecular methods, attempt to identify and assess the genetic diversity of *Azotobacter* spp. strains isolated from environmental samples. Currently, methods of identifying bacteria of the genus *Azotobacter* based on PCR technique enjoy particular interest. Comparing the ITS-PCR / RFLP technique and the amplification and sequencing of the 16S rRNA gene with the genotyping methods – MP PCR, RAPD PCR and ARDRA, it may be concluded that the last three methods are more useful for intraspecific differentiation of *Azotobacter* spp., while the first two can be used for rapid identification of these bacteria at the species level.

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