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# The phenotypic and genomic diversity of *Aspergillus* strains producing glucose dehydrogenase*

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**Twelve *Aspergillus* sp. strains producing glucose dehydrogenase were identified using ITS region sequencing. Based on the sequences obtained, the genomic relationship of the analyzed strains was investigated. Moreover, partial gdh gene sequences were determined and aligned. The amplified fragment length polymorphism (AFLP) method was applied for genomic fingerprinting of twelve *Aspergillus* isolates. Using one PstI restriction endonuclease and five selective primers in an AFLP assay, 556 DNA fragments were generated, including 532 polymorphic bands. The AFLP profiles were found to be highly specific for each strain and they unambiguously distinguished twelve *Aspergilli* fungi. The AFLP-based dendrogram generated by the UPGMA method grouped all the *Aspergillus* fungi studied into two major clusters. All the *Aspergillus* strains were also characterized using Biolog FF MicroPlates to obtain data on C-substrate utilization and mitochondrial activity. The ability to decompose various substrates differed among the analyzed strains up to three folds. All of the studied strains mainly decomposed carbohydrates.**

**Key words**: *Aspergillus*, glucose dehydrogenase, identification, metabolic diversity

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## INTRODUCTION

The genus *Aspergillus* belongs to the largest phylum within the fungi kingdom of Ascomycota. It comprises a large group of filamentous, anamorph fungi classified into 837 (Hawksworth, 2011) species which are human and plant pathogens (e.g. *A. fumigatus*, and *A. flavus*), as well as producers of specific, industrially important, bioproducts (e.g. *A. niger, A. terreus* and *A. oryzae*) (David *et al*., 2006). Due to the presence of various bioactive compounds and the capacity to produce specific proteins (e.g. enzymes such as α-amylase, glucose oxidase, invertase, pectinase, proteinases, and glucose dehydrogenase) and other metabolites such as organic acids (e.g. citric, gluconic and itaconic acids), the members of *Aspergillus* genus are considered as ones of the most economically important genera of microfungi (Bennett *et al*., 1998; Punt *et al*., 2002; Krijgsheld *et al*., 2013). Therefore, fast and robust methods of *Aspergillus* species identification are very important in the industrial biotechnology.

Although the biochemical and morphological features of microfungi are still being used for characterization and differentiation thereof (Shahriarin *et al*., 2011), presently molecular methods are also used for taxonomic studies. The analysis of the genomic diversity of fungi includes such methods as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), multilocus enzyme electrophoresis (MLEE), protein-coding gene sequences and internal transcribed spacers (ITS) or 25S rDNA sequencing techniques (Geiser *et al*., 2007; Perrone *et al*., 2006). In this paper, we focused on three methods used for characterization of fungal diversity at the genome level, i.e. sequence analysis of the ITS region and the gdh gene, as well as Amplified Fragment Length Polymorphism (AFLP) analysis. AFLP is a DNA fingerprinting technique described by Vos *et al*. (1995), which is based on selective PCR amplification of a subset of restriction enzyme-digested DNA fragments (Blears *et al*., 1998; Mueller *et al*., 1999). A simplified AFLP protocol has been developed for rapid identification and genetic diversity studies of organisms. A modified protocol of the AFLP technique involves the use of one restriction enzyme, one adaptor and one primer (Suazo *et al*., 1999; Tyrka, 2002; Pawlik *et al*., 2012).

Since many fungi grow relatively rapidly in pure culture, it is possible to use physiological and biochemical tests to identify and classify these organisms. Differences in the fungi phenotypic characteristics have contributed to identification of many new species (Guarro *et al*., 1999). The Biolog FF MicroPlate system has been recently introduced for rapid identification and characterization of filamentous fungi based on their abilities to utilize 95 carbon substrates (Druzhinina *et al*., 2006). This system has a wide range of research applications, including fungal species identification, investigation of different substrate utilization abilities and growth, secondary metabolite and antimicrobial profiles, optimizing

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**Abbreviations**: AFLP, amplified fragment length polymorphism; RFLP, restriction fragment length polymorphism; RAPD, random amplified polymorphic DNA; MLEE, multilocus enzyme electrophoresis; GOD, glucose oxidase; GDH, glucose dehydrogenase; AWCD, average well color development; UPGMA, unweighted pair group method with arithmetic means

culture conditions, as well as study of biological control agents (Seidl *et al*., 2006; Lakhesar *et al*., 2010; Mohammad *et al*., 2011).

Given the economic importance of many species of the *Aspergillus* genus, and their prevalence in the natural environment, these fungi have become a frequent target of scientific research (Bignell, 2010). The wide range of enzymes produced by *Aspergillus* species is of major importance to many diverse industries. For instance, hydrolyzing enzymes produced by these fungi, such as glucoamylase or pectinase, have been very often used in the food industry (Akhter *et al*., 2011; Abdalwahab *et al*., 2012). On the other hand, due to its electrochemical activity, glucose oxidase (GOD) is deployed as a part of a glucose sensor or a biofuel component (Wong *et al*., 2008). GOD has been considered as a main glucose-sensing enzyme in the electrochemical biosensors and test strips used by patients in self-monitoring of the blood glucose level. Lately, a lot of information in the literature has addressed the construction of different glucose biosensors containing enzymes as a possible alternative to GOD. One of the most often used biocatalyzers is glucose dehydrogenase (GDH), a redox enzyme also produced by *Aspergillus* strains (Zafar *et al*., 2012; Yamashita *et al*., 2013).

FAD-dependent glucose dehydrogenase (D-glucose:acceptor 1-oxidoreductase) was discovered for the first time in 1951, in *Aspergillus oryzae* (Ogura, 1951). FAD-depended GDHs have been also found in the periplasm region of Gram-negative bacteria (e.g. Burkholderia cepacia), as cytosolic enzymes in some insects (e.g. *Drosophila melanogaster, Anopheles gambiae, Apis mellifera, Tribolium castaneum*), and as extracellular enzymes in fungi (Ferri *et al*., 2011). In the *Aspergillus* genus, FAD-dependent glucose dehydrogenase was identified in *A. niger* (Muller, 1977), *A. oryzae* (Ogura, 1951) and *A. terreus* (Tsujimura *et al*., 2006).

The aim of this study was to determine the phenotypic and genomic diversity of 12 *Aspergillus* strains showing the ability to synthetize glucose dehydrogenase. For this purpose, the analysis of the ITS region and gdh gene sequences, and amplified fragment length polymorphism (AFLP), as well as the analysis of the fungi ability to utilize different carbon sources by the Biolog FF Microplate method were applied.

## MATERIALS AND METHODS

**Fungal strains and cultivation**. The *Aspergillus* strains examined in this study (Table 1) were obtained from the Department of Biotechnology, Human Nutrition and Science of Food Commodities, University of Life Sciences, Lublin, Poland. The stock cultures of the fungal strains were maintained on potato dextrose agar (PDA) slants. The slants were inoculated with mycelia and incubated at 25°C for 7 days, and then used to seeding (about 5 mm2 of the slants punched by a sterilized cutter) 100 mL Erlenmayer culture flasks containing 30 mL of liquid potato dextrose broth (PDB) medium. Next, the flasks were cultivated in a Multitron (Inforce HT, Switzerland) rotary shaker at 160 rpm, at 30°C for 3 days. Broth cultures were then harvested by centrifugation at $10\,000 \times g$ for 10 minutes and used for DNA extraction.

**Isolation of total DNA**. Mycelia from 30 mL liquid 3-day-old cultures (100–500 mg) were transferred into sterile 2.0 mL Lysing Matrix tubes containing lysing Matrix Beads (type A) and homogenized twice in Fastprep-24 (MP Biomedicals, USA), in 0.5 mL of cold spermidine-SDS buffer (4 mM spermidine, 10 mM EDTA, 0.1 M NaCl, 0.5% SDS, 10 mM β-mercaptoethanol, 40 mM Tris-HCl, pH 8.0) for 20 seconds, at the 5 m/s speed of the rotor. After the centrifugation at $10\,000 \times g$, at 4°C for 8 min, the supernatant was used for the DNA extraction as in Borges *et al*. (1990) The DNA concentration and purity was determined with a ND-1000 spectrophotometer (Thermo 142 Scientific, USA) and DNA was stored at –20°C

**PCR amplification and sequencing of the ITS region, as well as the gdh gene**. PCRs were performed in a T-personal thermal cycler (Biometra, Germany) using Thermo Scientific DreamTaq Green DNA Polymerase. The ITS region of the studied fungi was amplified with ITS1-ITS4 primers (Table 2), as described previously (White *et al*., 1990; Gardes *et al*., 1993).

To amplify fungal 500-bp-long fragments of the *gdh* gene, three pairs of primers specific to each tested species (Table 2) were used. The specific primers were designed with Lasergene v.8.0.2 software (DNASTAR Inc., USA) on the basis of the *Aspergillus* sp. glucose dehydrogenase genes published in GenBank.

**Table 1. GenBank accession numbers of ITS and partial gdh sequences of *Aspergillus* strains determined in this study**

| Strain number in FCL[a] | Strain name | GenBank Accession no. | |
|---|---|---|---|
| | | ITS region | Partial *gdh* gene |
| 358 | *Aspergillus terreus DSM 5770* | KF154418 | KF415096 |
| 359 | *Aspergillus terreus DSM 826* | KF154417 | KF415095 |
| 360 | *Aspergillus oryzae 13/5* | KF154416 | KF415094 |
| 361 | *Aspergillus oryzae 13/6* | KF154415 | KF415093 |
| 363 | *Aspergillus oryzae 13/52* | KF154414 | KF415092 |
| 364 | *Aspergillus awamori 13/51* | KF154413 | KF415091 |
| 365 | *Aspergillus niger 13/2* | KF154412 | KF415090 |
| 372 | *Aspergillus niger 13/40* | KF154410 | KF415089 |
| 373 | *Aspergillus niger 13/41* | KF154409 | KF415088 |
| 374 | *Aspergillus niger 13/48* | KF154408 | KF415087 |
| 375 | *Aspergillus terreus F 413* | KF154407 | KF415086 |
| G-13 | *Aspergillus niger G-13* | KF154406 | KF415085 |

[a]FCL, Fungal Collection of Lublin, Department of Biochemistry, Maria Curie-Skłodowska University, Lublin, Poland

**Table 2. List of oligonucleotide primers and adapters, and their annealing temperatures**

| Primer name | Primer sequence 5′-3′ | $T_m$ [°C] |
|---|---|---|
| An-gdhF | GCCTGGGTAGAAGCTTTCCAG | 50 |
| An-gdhR | GAGGATTGGATGCCTCCGATG | 50 |
| At-gdhF | ACGCCTGGAAACCATTGGC | 47 |
| At-gdhR | CAATGACTTCCTTCTCCGC | 47 |
| Ao-gdhF | CCTACCAGCTCTCAGGTTGC | 47 |
| Ao-gdhR | GTTGGGTTTCCAACTCCTGA | 47 |
| PstI_10 | GACTGCGTACATGCAGGAG | 53.3 |
| PstI_14a | GACTGCGTACATGCAGACGACGT | 55.8 |
| PstI_19 | GACTGCGTACATGCAGATT | 48.9 |
| PstI_21 | GACTGCGTACATGCAGGA | 50.3 |
| PstI_22 | GACTGCGTACATGCAGGCG | 55.4 |
| PstI_AF | CTCGTAGACTGCGTACATGCA | 51 |
| PstI_AR | TGTACGCAGTCTAC | 42 |
| ITS1 | TCCGTAGGTGAACCTGCGG | 55.4 |
| ITS2 | TCCTCCGCTTATTGATATGC | 49.7 |

The edited sequences of the ITS region and the partial gdh gene sequences were compared to the sequences deposited in the GenBank database by the BLAST version 2.2.31 search engine. The number of identities, the maximum score, and E values were taken into consideration.

**Phylogenetic analysis**. Nucleic acid sequences were analyzed using Lasergene v.8.0.2 analysis software package (DNASTAR Inc., USA). Multiple alignments of the sequences and the levels of sequence similarity were determined using the ClustalW algorithm (Larkin *et al*., 2007). Phylogenic trees were constructed via the neighbor-Joining (NJ) algorithm, using Lasergene MegAlign 8.0.2. (DNASTAR Inc., USA). Database searches were performed with the BLAST version 2.2.31 programs at the National Centre for Biotechnology Information (Bethesda, USA) (Altschul *et al*., 1990).

**Nucleotide sequence accession numbers**. The GenBank accession numbers of the studied *Aspergillus* sp. ITS region and the *gdh* gene sequences are listed in Table 1.

**AFLP analysis**. The AFLP reactions were performed as described by Vos *et al*. (Vos *et al*., 1995), with some modifications. Adapters and primers were synthesized by Genset Oligos, France, and IBB PAN, Poland. Briefly, 1 μg of genomic DNA (in a total volume of 30 μL) was digested with 20 U of the PstI restriction enzyme (Thermo Scientific, USA), and ligated for 1 hour at 37°C with double stranded PstI_AF and PstI_AR adaptors. The ligation solution containing the double-stranded adapters (1 μL), DNA digested with PstI (500 ng), 5U T4 DNA ligase (Thermo Scientific, USA) and 1 x T4 ligase buffer (40 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP, pH 7.8) was incubated for 4 hours at 37°C (25 μl final volume). The ligated DNA was then precipitated with a mixture of 3 M sodium acetate, pH 5.5, and ice cold 96% ethanol (1:25) at –18°C for 30 minutes to remove unbound adapters. DNA was harvested by centrifugation (10 000 × *g*, 4°C, 20 minutes), dried in a vacuum Concentrator Plus (Eppendorf AG, Germany), dissolved in 50 μl of sterile water, and used as a template in the amplification reaction.

**Nonselective PCR amplification**. Nonselective PCR was performed to check the digestion and ligation reactions. PCR was carried out in a 25 μL volume containing 5 μL of ligated with double-stranded adapters and purified DNA, 0.2 mM of each dNTP, 1.5 mM MgCl2, 0.4 U Taq DNA polymerase LC, recombinant (Thermo Scientific, USA), 1 x PCR buffer (75 mM Tris-HCl, pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01 % Tween 20) and 750 nM forward adaptor PstI_AF as a primer. Amplifications were carried out in a T-personal thermal cycler (Biometra, Germany) under the conditions as follows: 95°C for 2 min 30 s, followed by 45 cycles of 45 s at 94°C, 45 s at 54°C, and 45 s at 72°C. The final cycle was followed by an additional 10 min at 72°C.

**Selective PCR amplification**. PCRs were performed in a 50 μL total volume which consisted of 1 x PCR reaction buffer (Thermo Scientific, USA), 2.5 mM MgCl2, 0.2 mM of each dNTP, 1 U of Taq DNA Polymerase LC-recombinant (Thermo Scientific, USA), 10 pmol of each primer, and 0.5 μL of restriction DNA fragments. All amplification reactions were performed in a T-personal thermal cycler (Biometra, Germany) under the conditions as follows: 94ºC for 2 min 30 s, followed by seven cycles of amplification, with annealing temperature gradually decreasing by 1ºC/cycle for 7 cycles: 94ºC for 30 s, first annealing for 30 s (annealing temperature depended on primers $T_m$), 72ºC for 30 s, and next 33 amplification cycles of 94ºC for 45 s, 48–56ºC (anneal-

**Table 3. AFLP primers used in the analysis of *Aspergillus* sp. strains, number and range of length of all amplified DNA fragments, number of polymorphic fragments and percentage of polymorphism**

| Primer name | Primer's selective bases | No. of all fragments | Fragment range length [bp] | No. of polymorphic fragments | Percentage of polymorphism |
|---|---|---|---|---|---|
| PstI_10 | GAG | 147 | 310–1984 | 147 | 100 |
| PstI_14a | ACGACGT | 73 | 250–1217 | 61 | 83.6 |
| PstI_19 | ATT | 124 | 410–2860 | 112 | 90.3 |
| PstI_21 | GA | 144 | 265–2450 | 144 | 100 |
| PstI_22 | GCG | 68 | 293–1540 | 68 | 100 |
| Sum | | 556 | | 532 | |
| Average | | 111.2 | | 106.4 | 94.78 |

ing temperature depended on primers' $T_m$) for 45 s, and 72°C for 45 s. The final cycle was followed by an additional 10 min at 72°C. The PCR products were stored at 4°C until further analysis. The adapters and primers employed for AFLP are shown in Table 3.

**Electrophoresis and imaging**. The amplified products were separated by electrophoresis in a 1.5% agarose gel in 1× TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0). The electrophoresis was run at 120 V in TBE buffer, in a horizontal gel electrophoresis system (Agagel Mini, Biometra). The gels were stained with ethidium bromide and photographed under UV light using a G:BOX system (Syngene, USA).

**Data analysis**. The electropherograms were analyzed using GeneTools software (Syngene, USA). AFLP markers were manually scored as binary data for the presence or absence of fragments between 100 and 3000 bp. A table containing this binary information was used to calculate Jaccard's pairwise similarity coefficients, as implemented in the FreeTree program, version 0.9.1.50 (Hampl *et al*., 2001), and cluster analysis was performed by the UPGMA method (unweighted pair–group method with arithmetic averages). The phylogenetic tree was visualized and edited using NTSYSpc software version 2.01. (Exeter Software Co., New York).

**Biolog microplate analysis**. Carbon assimilation and mitochondrial activity were investigated using Biolog FF MicroPlates (Biolog, Inc., Hayward, CA). The inoculation procedure was based on the original FF MicroPlate (Biolog Inc., USA) technique (manufacturer supplied protocol) and the protocol modified as in Frąc (2012), with modification as in Pawlik *et al.* (2015). The most consistent readings came from 8-day old Biolog plates and these were used in the analyses.

**Biolog data treatment**. Data from all experiments were combined in a single matrix and analyzed with the STATISTICA 10.0 (StatSoft Inc., USA) software package. All data were subjected to descriptive statistical evaluations (mean, minimum, maximum, and standard deviation values) and checked for outliers, as in Druzhinina *et al.* (Druzhinina *et al*., 2006). The average well color developments (AWCDs) of the different replicates were calculated, where AWCD equals the sum of the difference between the OD of the blank well (water) and substrate wells, divided by 95 (the number of substrate wells in the FF plates), developed by the fungus after 192 h of incubation. Functional diversity was measured as substrate richness. The number of different substrates utilized by each strain (counting all positive OD readings) was calculated. Cluster analysis (Tryon, 1939; Hartigan, 1975) was used to detect groups in the data set. In most cases, the cluster-joining analysis was made with Euclidian distance, and complete linkages as the amalgamation rule, i.e. the distances between the clusters were determined by the greatest distance between any two objects in the different clusters. One-way or main-effect analyses of variance ANOVAs (confidence interval 95%) were performed to compare the growth of selected strains on individual carbon sources. ANOVA was followed by a post-hoc analysis using the Tukey's HSD (Honestly Significant Difference) *t*-test. The summed data matrixes were also evaluated following multidimensional scaling to detect additional relationships between variables.

## RESULTS

### PCR amplification and sequence determination of the ITS region and the gdh gene

All the *Aspergillus* genus fungi studied here were differentiated by the analysis of their ITS region sequences. The fungus-specific universal primer pair ITS1 and ITS4 was able to successfully amplify the ITS region of all the tested strains, and one PCR product for each strain was obtained. The sizes of the ITS amplicons varied from 517 bp (strain 363) to 611 bp (strains 375). The sequences of these products indicated 99–100% identity to the corresponding *Aspergillus* ITS sequences from the GenBank database. The GenBank accession numbers, assigned to the nucleotide ITS sequences determined in this study are listed in Table 1.

Cluster analysis of all analyzed ITS sequences performed using the unweighted pair group method with arithmetic means (UPGMA), separated the studied *Aspergillus* strains into three main clusters and two separate lineages occupied by strains 358 and 363 (Fig. 1). Cluster I included five strains of *Aspergillus* niger (365, 372, 373, 374, G-13), and *Aspergillus* awamori strain 364. Cluster II grouped two *Aspergillus* terreus strains i.e. 359 and 375. Cluster III was comprised of *Aspergillus* oryzae strains 360 and 361. *Aspergillus* oryzae strain 363 was located on the outskirt of the tree, and the other *Aspergillus* strains included in the analysis displayed ITS sequence similarity in the range from 25 to 27%.

Moreover, sequence analysis of the partial gdh gene of all studied strains, performed by PCR, yielded a single band for all strains, and the sizes of the bands varied from 443 bp (strain G-13) to 578 bp (strains: 374 and 365).

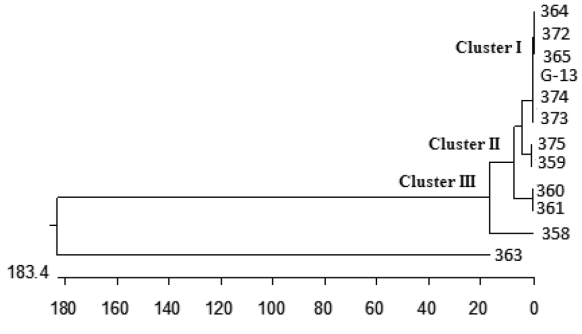


**Figure 1. Phylogenetic tree constructed with NJ method based on ITS region sequences for the 12 *Aspergillus* strains.**
The numbers in parentheses are strain numbers in FCL; scale bar indicates base substitutions per 100 bases; strains grouping into three main clusters (I, II and III) have been indicated

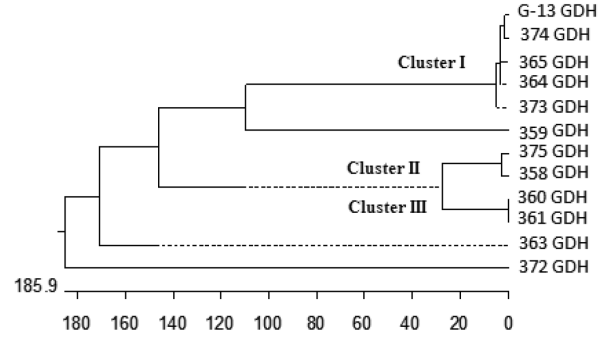


**Figure 2. Phylogenetic tree constructed with NJ method based on ITS region sequences for the 12 *gdh* partial sequences of *Aspergillus* strains.**
The numbers in parentheses are strain numbers in FCL; scale bar indicates base substitutions per 100 bases; strains grouping into three main clusters (I, II and III) have been indicated

**Table 4. Jaccard's pairwise similarities between the analyzed *Aspergillus* sp. strains calculated on the basis of the polymorphic bands**

| Fungal strain | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 358 | 1.000 | | | | | | | | | | | |
| 2 | 359 | 0.315 | 1.000 | | | | | | | | | | |
| 3 | 360 | 0.198 | 0.120 | 1.000 | | | | | | | | | |
| 4 | 361 | 0.200 | 0.109 | 0.982 | 1.000 | | | | | | | | |
| 5 | 363 | 0.300 | 0.213 | 0.140 | 0.141 | 1.000 | | | | | | | |
| 6 | 364 | 0.309 | 0.236 | 0.157 | 0.159 | 0.787 | 1.000 | | | | | | |
| 7 | 365 | 0.304 | 0.216 | 0.141 | 0.143 | 0.977 | 0.804 | 1.000 | | | | | |
| 8 | 372 | 0.328 | 0.203 | 0.157 | 0.159 | 0.867 | 0.822 | 0.886 | 1.000 | | | | |
| 9 | 373 | 0.304 | 0.216 | 0.141 | 0.143 | 0.977 | 0.804 | 1.000 | 0.886 | 1.000 | | | |
| 10 | 374 | 0.333 | 0.211 | 0.138 | 0.140 | 0.673 | 0.735 | 0.686 | 0.735 | 0.686 | 1.000 | | |
| 11 | 375 | 0.282 | 0.724 | 0.115 | 0.116 | 0.267 | 0.310 | 0.270 | 0.257 | 0.270 | 0.280 | 1.000 | |
| 12 | G-13 | 0.306 | 0.221 | 0.161 | 0.163 | 0.618 | 0.673 | 0.630 | 0.673 | 0.630 | 0.875 | 0.289 | 1.000 |

The phylogenetic relationship between the 12 studied *Aspergillus* strains based on the sequences of the partial *gdh* genes are presented in the dendrogram constructed by UPMGA clustering (Fig. 2). The strains were separated into three distinct clusters, each with strains grouped as in the ITS sequence dendrogram, except for strain 372. Outside the main clusters, two separate lineages were found to be occupied by strains 363 and 372. *Aspergillus* niger strain 372 and *Aspergillus* oryzae strain 363 were located on the outskirt of the tree, and the other *Aspergillus* strains used in the analysis showed a partial gdh sequence similarity level in the range from 24.6 to 29.5%, and 28.5 to 50.9%, respectively.

## AFLP analysis

The rare cutting restriction endonuclease PstI and five selective primers were used in the AFLP analysis to fingerprint the genomes of 12 fungal strains belonging to four species of the *Aspergillus* genus (*A. terreus, A. oryzae, A. awamori*, and *A. niger*) (Table 1).

Smears obtained in the nonselective PCR amplification proved efficient degradation of DNA by PstI endonuclease (data not shown). In the selective amplification reactions, all five selective primers successfully amplified the restriction fragments in all of the 12 analyzed fungi. The AFLP method applied, has provided characteristic genomic markers to differentiate all the *Aspergillus* sp. strains except two. Although a variable number of amplified bands were obtained in the PCR reaction with each primer, all of them generated polymorphic and unambiguously scored fragments. A total of two to seven selective bases were found to provide a sufficient complex pattern for DNA fragment analysis (Table 3). The number of legible bands with the primer sets used was in the range from 4 to 17 per strain. In total, 556 well defined amplicons were formed in the AFLP reactions with five primer pairs. The large number of DNA bands observed demonstrates that AFLP analysis is a robust and efficient method to differentiate the *Aspergillus* strains. PstI_10 and PstI_21 primers amplified the highest number of restriction fragments (147 and 144 respectively), while the fewest amplicons (68 and 73) were obtained with PstI_22 and PstI_14a primers. On average, 111 AFLP markers in the size range from 250 to 2861 bp were amplified per primer. In total, 556 robust and reliable restriction fragments, including 24 monomorphic (about 5%) and 532 polymorphic (about 95%) ones were observed across all twelve isolates, which corresponded to an average of about 106 polymorphic bands per primer combination. Among the primer sets used, the AFLP reaction with PstI_10 primer produced the most polymorphic (147) band sets. With the PstI_14a primer, the lowest (61) number of polymorphic bands was found. The primers used in the AFLP reactions are characterized in Table 3.

A binary matrix was used to compute genome similarity between *Aspergillus* strains based on AFLP polymorphism (Table 4). Average Jaccard's similarity coefficient (42) among the studied strains was relatively high, i.e. 0.504. The highest similarity coefficient, 1.000, was found between strains 365 and 373 (both classified as *A. niger*) and the lowest (0.109) between *A. terreus* 359 and *A. oryzae* 361 strains.

The genomic relationship between the studied *Aspergillus* strains is presented on the dendrogram constructed with an unweighted pair group method with arithmetic means (UPGMA) cluster analysis (Fig. 3). The analysis showed that the analyzed strains were separated into two main clusters. Out of the 12 fungi analyzed in this study, 10 were classified as group A, and 2 as group B, at the DNA profile similarity of 14%. The first group

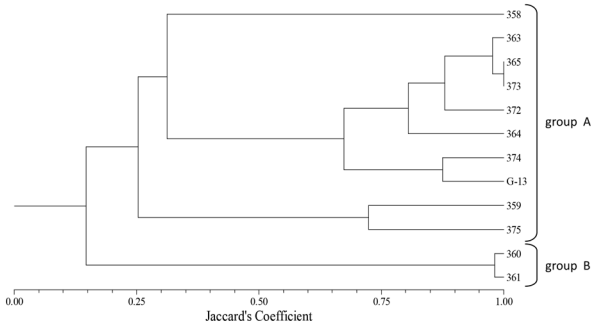


**Figure 3. Phylogenetic tree of the 12 *Aspergillus* sp. strains generated by UPGMA clustering based on Nei and Li's genetic distance (Nei *et al*., 1979).**

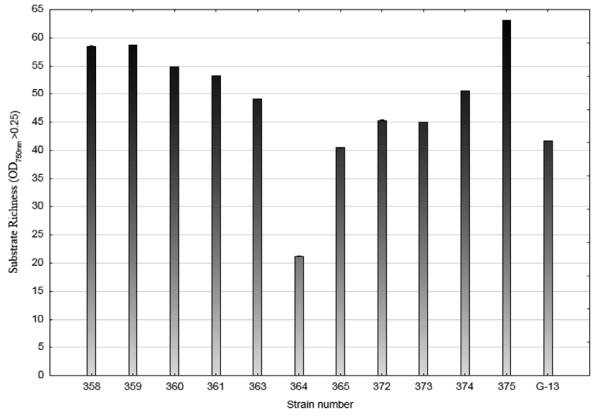


**Figure 4. Substrate richness utilized by *Aspergillus* strains.**

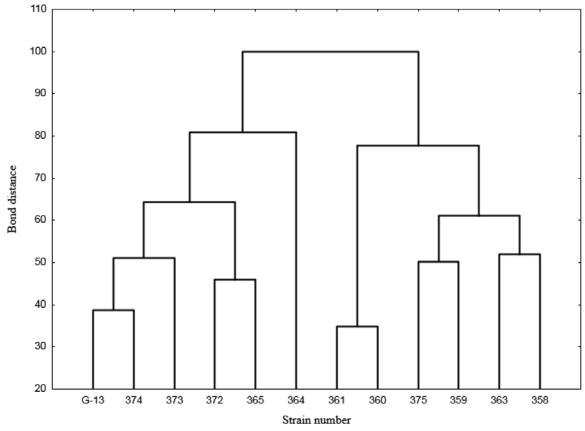


**Figure 5. Cluster analysis-based dendrogram showing diversity of studied *Aspergillus* strains with respect to utilization of carbon sources from an FF-MicroPlate (Biolog Inc.).**

was comprised of only two strains, i.e.: *A. oryzae* 360 and 361. The second cluster combined strains identified as *A. terreus* (3 strains), *A. oryzae* (1 strain), *A. niger* (5 strains), and *A. awamori* (1 strain). The analysis revealed existence of several subgroups within group A, and a clear separation of the *A. terreus* 358 strain from the remaining cluster structure. The highest genetic similarity, 1.00, was exhibited by *A. niger* 365 and 373. Strain 358 classified outside of any subgroup, and the other *Aspergillus* sp. strains included in the analysis displayed an AFLP profile similarity level in a range from 19.8 to 100%.

### Biolog microplate analysis

Biolog FF Microplate tests complement traditional genomic analysis by offering quantitative assessment of the organism's physiological response to different substrates and environments. The Biolog systems are used for rapid identification of microorganisms using a principle of the "metabolic fingerprint". Due to assimilation of simple or complex carbon substrates, different species of fungi are characterized by a specific metabolic profile, which can be used in identification thereof.

We used the Biolog system to distinguish the 12 studied *Aspergillus* strains, as well as to understand their metabolic differences. The FF MicroPlate test panel contains 95 wells, each with a different carbon compound, and 1 well with water (control). The metabolic activity of the analyzed strains (AWCD — average well color development) was calculated based on absorbance at 750 nm (Fig. 4). The highest catabolic activities were shown by *A. terreus* 375 strain with capabilities to decompose 63 carbon sources, whereas *A. awamori* 364 strain was able to assimilate only 21 C-sources. Higher catabolic activities were also displayed by two other *A. terreus* strains: 358 and 359, which were able to decompose more than 55 carbon sources.

The growth rate of the twelve studied *Aspergillus* sp. strains on the 95 carbon sources was varied (Fig. 5). Carbohydrates were the most frequently metabolized carbon and energy sources by all the analyzed strains (Fig. 6). Among them, N-acetyl-D-glucosamine was the best source of carbon and energy for most studied fungi. Furthermore, D-trehalose, D-fructose, sucrose and D-sorbitol were found among the most assimilated carbon sources (Fig. 6).The results of the Biolog test concerning the metabolic activities of the studied *Aspergillus* sp. strains were subjected to UPMGA clustering analysis. The dendrogram presented in Fig. 5 shows diversification of *Aspergillus* sp. strains with respect to the analyzed metabolic features. Two large subgroups were distinguished based on utilization of carbon sources using the FF MicroPlate. The first subgroup includes five *A. niger* strains (G-13, 374, 373, 372, 365) and *A. awamori* 364 strain. The second subgroup comprises three *A. terreus* (375, 359, 358) and three *A. oryzae* (361, 360, 363) strains. Each subgroup contains six strains with >81% and >78% similarity of the metabolic activity studied.

## DISCUSSION

Fungal taxonomy is a dynamic, progressive discipline that consequently requires changes in nomenclature. This poses difficulties for microbiologists inexperienced in mycology; therefore, fungi are mostly classified on the basis of their appearance rather than on the nutritional and biochemical differences which are of such importance in bacterial classification (Guarro *et al.*, 1999). Nowadays, techniques used for classification and taxonomical studies of fungi may be divided into three groups based on: I — the morphological features (mycelium and spore color and shape, sexual development), II — genome analysis (AFLP, RAPD, gene sequences etc.), and III - physiological, nutritional and biochemical characterization. Despite of the growing number of techniques used in the taxonomy of microorganisms, it is still hard to define what a fungal species is (Xu, 2005). This seems especially difficult for the *Aspergillus* genus, which comprises up to 837 species classified to approximately ten different teleomorph genera (Samson *et al.*, 2009; Geiser *et al.*, 2007; Hawksworth, 2011; Krijgsheld *et al.*, 2013).

The *Aspergillus* genus is a diverse group that comprises *A. nidulans* and *A. fumigatus* with genome sequences as different as those of fish and humans. The large differences in genomic sequences have been proposed to be a result of an accelerated evolutionary rate, which in the *Aspergillus* genus may last for 200 million years (Galagan *et al.*, 2005; Cai *et al.*, 2006; Hawksworth, 2011). It is known that each year brings to light new the *Aspergillus* strains which are of great importance in fermentation industry, and their proper taxonomic classification, as well as simple and reliable identification, are very important (Perrone *et al.*, 2007). In this paper, four different techniques were used to differentiate 12 strains of the *Aspergillus* genus, classified to four species: *A. niger, A. oryzae, A. terreus* and *A. awamori*, from which the last one

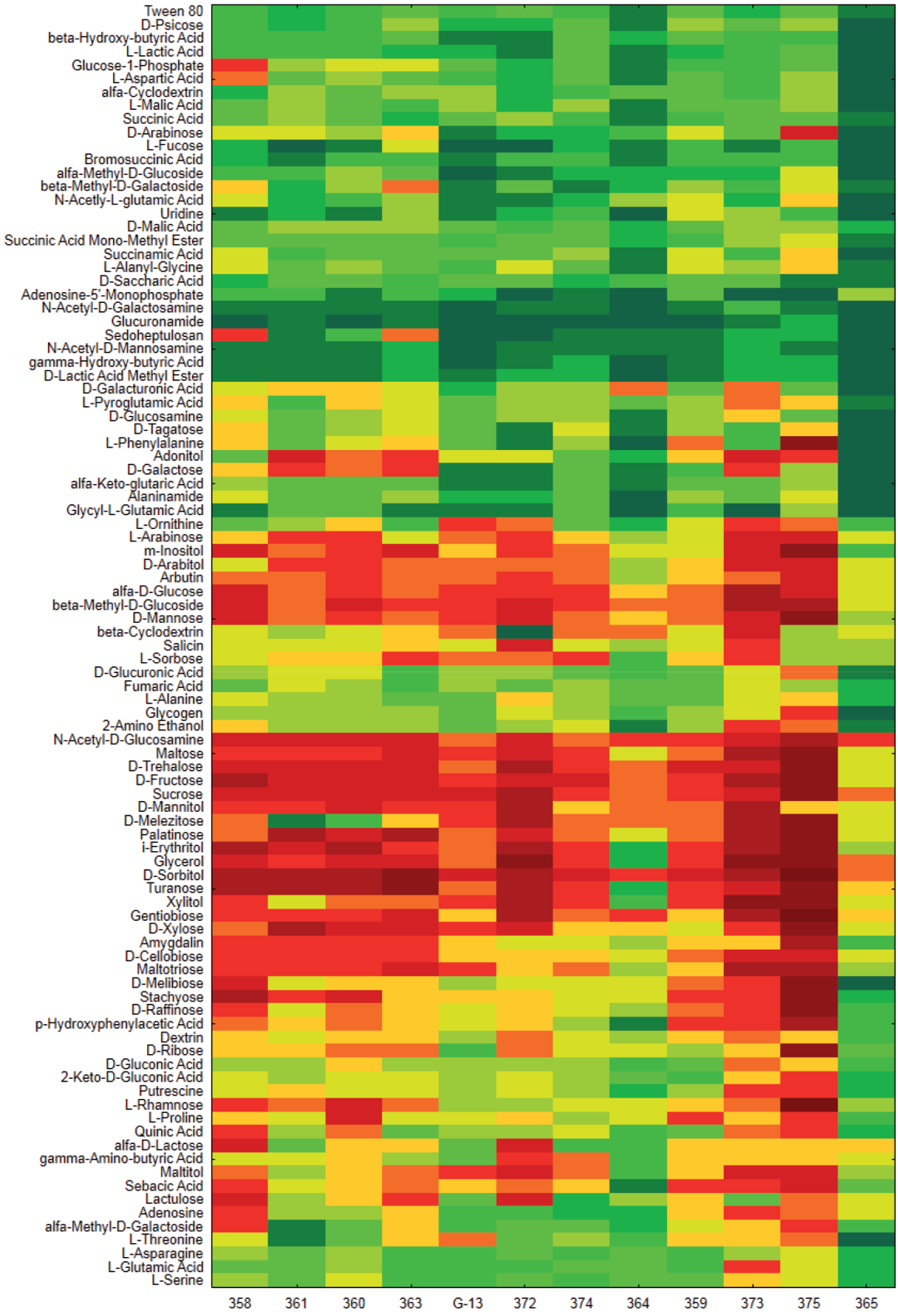


**Figure 6. Phenotype profiles of *Aspergillus* strains generated from FF MicroPlate using Biolog System; color scale of the heat maps indicates the growth of an organism (mycelial density A750 nm) on particular substrate**

may be easily mistaken with *A. niger*. All the analyzed strains were identified at the species level and differentiated based on the comparative ITS region sequences presented in the dendrogram. Partial sequences of the glucose dehydrogenase gene were analyzed in the same way. Geiser *et al.* (Geiser *et al.*, 2007) suggested that the genealogy inferred from a single gene may not reflect organism phylogeny because of the stochastic effects of the segregation of variation during speciation and recombination. Considering the fact that analysis of a single gene may be insufficient to differentiate strains of the same species, AFLP analysis was performed, which successfully differentiated the *A. niger* (Perrone *et al.*, 2006), *A. carbonarius* (Botton *et al.*, 2008) or *A. ochraceus* strains (Schmidt *et al.*, 2003). Designation of the affiliation of fungal species should include data from as many sources as possible, and should comprise not only morphological, physiological, and molecular features of these microorganism, but also their biochemical characteristics which may be useful in future biotechnological processes (Geiser *et al.*, 2007). Therefore, the ability of the analyzed strains to decompose 95 carbon sources was studied using Biolog FF Microplates, which proved to be useful in differentiation of *Trichoderma atroviride* (Seidl *et al.*, 2006) and other Trichoderma sp. strains (Lopes *et al.*, 2012). We confirmed that the ITS region was very useful in classification of fungal strains at the species level. The dendrogram proved that the ITS sequences grouped the analyzed strains into species well, except for *A. terreus* 358 and *A. oryzae* 363 which formed a separate lineage, and *A. awamori* which clustered with the *A. niger* strains. The results from our experiment agreed with those obtained by Henry *et al.* (Henry *et al.*, 2000), who indicated that intraspecies variations among the *Aspergillus* strains are minimal. It is worth to underline that participants of the All Fungi DNA Barcoding Planning Workshop (Smithsonian Conservation and Research Center, Front Royal, Virginia, 13–15 May, 2007) stated the ITS region as the first choice for DNA barcoding of the Fungal Kingdom (www.allfungi.org) and that they also found that ITS region sequences do not always resolve closely related phylogenetic species (Bruns, 2001; Geiser *et al.*, 2007).

AFLP seems to be a very good technique, which was successfully used for identification and differentiation of various *A. fumigatus, A. ochraceus* and *A. carbonarius* strains (Schmidt *et al.*, 2003; Warris *et al.*, 2003; Schmidt *et al.*, 2004), and seems to be a very good technique of differentiation of fungi. However, although AFLP appears to be rather better for differentiation of fungi than the ITS analysis, both of these techniques give similar strain grouping, except for *A. oryzae* 363. Additionally, the clustering of the studied strains in the dendrogram based on the partial *gdh* gene sequence analysis corresponded almost perfectly to that of ITS and AFLP dendrograms, except for *A. niger* 372 and *A. oryzae* 363, respectively. Surprisingly, the numerical analysis of the phenotypic profiles of the studied strains very well clustered the aspergilla strains according to their species affiliation, except for *A. oryzae* 363 strain, which grouped together with *A. terreus* 358 fungus. Kubicek *et al.* (Kubicek *et al.*, 2003) indicated that Biolog analysis may not only be used as an identification tool, but also highlights intraspecific variation not apparent from analyses of sequence data and, therefore, it may be potentially important in understanding variation at the level of evolving species. Therefore, the Biolog test is important in better understanding of the history of the evolving species. Additionally, our results proved that *A. awamori* 364, commonly classified as black *Aspergillus*, was closely related to *A. niger* and clustered with them in all three phylograms. It should also be pointed out that *A. awamori* 364 strain was able to assimilate the lowest number of substrates (mainly carbohydrates), and this property can be treated as evolutionary evolved high metabolic strain specialization. Assuming that all the analyzed strains mainly decompose sugars as carbon sources, and *A. awamori* is not an exception in this matter, the ability to decompose a fewer number of carbon sources (mainly sugars) may even seem to be a strain specialization. Similar results were obtained by Druzhinina *et al.* (Druzhinina *et al.*, 2010), Kubicek *et al.* (Kubicek *et al.*, 2003) and Mohammad *et al.* (Mohammad *et al.*, 2011), who found that strains of Trichoderma sp. and *A. niger* assimilate mainly carbohydrates as a sole carbon source. In conclusion, it should be pointed out that to identify and differentiate fungi, and to determine their relationship at the species level, different techniques should be applied, including those characterizing organisms in terms of phenotypic and genomic features.

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