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Intraspecific functional and genetic diversity of *Petriella setifera*

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Petriella setifera is poorly-known species with only fragmentary information in literature and with published partial genome about the fungal species. Therefore, the aim of the study was an analysis of the intraspecific genetic and functional diversity of new isolated fungal species of *P. setifera*. From the molecular biological viewpoint, the intraspecific variability was found through the Amplified Fragment Length Polymorphism analysis (AFLP). The analysis showed a good variability among the analysed isolates, which was demonstrated by the clear subdivision of all the isolates into two clusters with 51% and 62% of similarity, respectively. For the metabolic diversity, the BIOLOG system was used and this analysis revealed clear different patterns on the carbon substrates utilization between the isolates bringing a clear separation of the five isolates into three clusters with 0%, 42% and 54% of similarity, respectively. These two techniques allowed estimation of the intraspecific variability within the five isolates of *P. setifera* strains. Both the methodologies are two easy and rapid techniques to indicate the genetic and functional variability at the intraspecific level within the species, especially, if a biological and functional information about the analysed fungal strains are limited.

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Abstract

***Petriella setifera* is poorly-known species with only fragmentary information in literature and with published partial genome about the fungal species. Therefore, the aim of the study was an analysis of the intraspecific genetic and functional diversity of new isolated fungal species of *P. setifera*. From the molecular biological viewpoint, the intraspecific variability was found through the Amplified Fragment Length Polymorphism analysis (AFLP). The analysis showed a good variability among the analysed isolates, which was demonstrated by the clear subdivision of all the isolates into two clusters with 51% and 62% of similarity, respectively. For the metabolic diversity, the BIOLOG system was used and this analysis revealed clear different patterns on the carbon substrates utilization between the isolates bringing a clear**

separation of the five isolates into three clusters with 0%, 42% and 54% of similarity, respectively. These two techniques allowed estimation of the intraspecific variability within the five isolates of *P. setifera* strains. Both the methodologies are two easy and rapid techniques to indicate the genetic and functional variability at the intraspecific level within the species, especially, if a biological and functional information about the analysed fungal strains are limited.

Keywords: *Petriella setifera*, genetic fingerprinting, metabolic diversity, soft rot fungi

1. Introduction

The species of *Petriella setifera* (Alf. Schmidt) Curzi belongs to the family Microascaceae of the division Ascomycota, Kingdom Fungi, and is found especially in enriched soil (for example dung, manure, or composts) (Danon, Chen & Hadar, 2010; Lackner & De Hoog, 2011). The family Microascaceae consists of 20 genera and 200 species. In this family, a limited number of Fungi potentially harmful or infectious for humans can be found (which are *Pseudallescheria* and *Scedosporium* genus) (Rainer & De Hoog, 2006). The information about this family come from the medical field and the first morphological identification and DNA sequence analysis of *P. setifera* was performed by Kwaśna et al. (2005). The presented work includes analysis of the genetic diversity and metabolic profile of fungal species due to the lack of published genome or other information about the intraspecific diversity and functionality of this species in the soil and organic waste.

To identify the isolated fungal species, we have used the Large Subunit Ribosomal (LSU) sequencing. According to many authors (Schoch et al., 2012; Pawlik et al., 2015a,b), to identify a

Fungus, it is possible to apply the Internal Transcribed Spacer (ITS) sequencing because it is a standard barcode maker for Fungi. Zhao et al. (2013) claimed that the identification of Fungi performed with a highly-conserved region was not phylogenetically informative within family level. In fact, Issakainen et al. (1999, 2003) developed a taxonomic classification using the LSU and the Small Subunit Ribosomal (SSU) rRNA gene, and they confirmed that both these two regions can be used for phylogenetic analysis. Particularly, the SSU rRNA is better to use to analyse the higher taxonomic level, whereas the LSU rRNA is better for analysing closely related genera (Issakainen et al., 1999).

To analyse the genetic diversity of *Petriella setifera*, the Amplified Fragment Length Polymorphisms (AFLP) analysis was used. The AFLP analysis was first described by Vos et al. (1995) and it was used to analyse a fungal community at taxonomic level by Majer et al. (1996). This fingerprinting analysis consists of three principal steps: restriction of the total genomic DNA and ligation to oligonucleotide adapters, selective amplification of restricted fragments, and the analysis of the amplified fragments through vertical electrophoresis in a polyacrylamide gel or using the capillary sequencing approach in a genetic analyser. The AFLP facilitates estimation of the genetic diversity (Mueller & Wolfenbarger, 1999) and the levels of intraspecific variation (Tooley et al., 2000) between and within a species owing to its taxonomic range, discriminatory power, reproducibility, lack of the need for knowledge of the nucleotide sequence, and ease of interpretation and standardization (Savelkoul et al., 1999; Perrone et al., 2006a). This was confirmed by the recent studies of Perrone et al. (2006a,b), in which the AFLP was used to clarify the relationship within or between closely related species. The application of AFLP analysis for fungal studies has also been demonstrated by other authors (Bakkeren, Kronstad & Lévesque,

2000; Tooley et al., 2000; Abdel-Satar et al., 2003; Radišek et al., 2003; Schmidt, Niessen & Vogel, 2004; Perrone et al., 2006a,b, Pawlik et al., 2015a,b; Rola et al., 2015).

The functional diversity, i.e. the fungal ability to use different carbon sources, is assessed with the used of the BIOLOG FF MicroPlates™ method. This system is a rapid method for analysis of the catabolic potential of a fungal community or fungal strain pure culture based on their abilities to utilize 95 carbon substrates. Based on the results of catabolic profiles, we can determine two ecological indices (i.e. substrate richness (R) and Average Well Density Development (AWDD)) that can help to understand and know the role of Fungi. These indices are especially sensitive indicators that reveal the differences between the strains (Frąc, Oszust & Lipiec, 2012). In the last year, the BIOLOG system was introduced for rapid characterization of the fungal community (Stefanowicz, 2006; Singh, 2009; Janusz et al., 2015; Pawlik et al., 2015a,b; Rola et al., 2015).

Recently, three studies on fungal species have been carried out using BIOLOG FF Plate™ and AFLP fingerprinting analysis; in the first one, Rola et al. (2015) used these two methodologies to analyse the phenotypic and genetic diversities of *Aspergillus* strains which synthesize glucose dehydrogenase. The other two studies have estimated the genetic and metabolic biodiversities in *Ganoderma lucidum* strains (Pawlik et al., 2015a) and in *Coprinus comatus* (Pawlik et al., 2015b). In the present work, since there is little information about the species composition and genetic variability of *Petriella* sp., we want to use these analyses that will allow us to evaluate the genetic and functional diversities between *Petriella setifera* strains isolated from compost with the final aim to find an intraspecific difference among these isolates without possessing any genetic information about the analysed species. To determine the genomic variability, we propose the analysis of the AFLP fingerprinting; in turn, we propose the analysis of the fungal ability to use different carbon sources using the BIOLOG FF MicroPlates™ system to determine the metabolic

92 potential. In this paper, we have demonstrated for first time a combination of genomic and
93 functional diversity assays in *P. setifera* and the development of the first protocol on the AFLP
94 fingerprinting analysis applied to this species. The results showed for the first time that the use of
95 molecular biology techniques (such as AFLP and BIOLOG analyses) can allow the identification
96 of intraspecific diversity without knowing a lot of information on the analysed fungal species.
97

98 ## 2. Materials and methods

99

100 ### 2.1 *Petriella setifera* isolates

101

102 Five strains of *P. setifera* (G11/16; G14/16; G16/16; G17/16; G18/16) were isolated from
103 industrial compost with the serial dilutions method on Bengal Rose LAB-AGAR (BIOCORP,
104 Poland). Sequences of all strains were deposited in the National Centre for Biotechnology
105 Information (NCBI; http://www.ncbi.nlm.nih.gov) (Woodsmall & Benson, 1993) under the
106 following accession numbers: KX639331, KX639334, KX639335, KX639336, and KX639337,
107 respectively.
108 The compost consisted of the following organic substances: sewage sludge from wastewater
109 treatment, sawdust, biodegradable garden and park waste, soil, mouldings of medicinal plants
110 obtained by solvent extraction, lime sludge. The concentrations of the principal components of the
111 compost, i.e. total carbon, nitrogen and phosphorus were respectively 17.9%, 2.3%, and 0.75%,
112 respectively and pH was 5.3.
113

114 ### 2.2 Fungal DNA extraction

115

116 The analysed strains were cultured on 90 mm Petri dishes with Potato Dextrose Agar medium
117 (Oxoid Ltd, England) at 30°C for 14 days. 200 mg of fungal mycelium was taken from each of the
118 five strains and sterilely transferred into 2 ml tubes containing 250 mg of glass beads of 1.45 mm
119 diameter and 500 mg of glass beads of 3.15 mm diameter and they were homogenised with a
120 FastPrep-24 homogeniser (MPBio, USA) at 4 m/s for 20 seconds. The DNA was extracted in
121 accordance to the EURx GeneMATRIX Plant & Fungi DNA Purification Kit (EURx, Poland)
122 protocol. The quantity and purity of extracted DNA were evaluated with a NanoDrop-2000
123 Spectrophotometer (Thermo Scientific, USA).

124

125 2.3 D2 LSU rRNA sequencing

126

127 The sequencing of the D2 LSU region was performed with the use of universal primers (Table
128 1). The primary amplification of the target sequence was performed in a final volume of 20 µl in
129 a Veriti Fast thermal cycler (Applied Biosystem, USA). Each reaction contained 10 µl of 2X PCR
130 Reaction Master Mix (EURx, Poland), 1 µl of DNA template, 1 µl of 10 µM D2LSU2_F primer,
131 and 1 µl of 10 µM D2LSU2_R primer. The reactions were set up as follows: 95°C for 600 s
132 followed by 35 cycles of 95°C for 15 s, 53°C for 20 s, and 72°C for 20 s, and followed by a final
133 step at 72°C for 300 s. At the end of this reaction, 5 µl of products were purified with exonuclease
134 I – bacterial alkaline phosphatase, by mixing with 2 µl of Exo-BAP Mix (EURx, Poland). The
135 samples were then incubated at 37°C for 15 minutes and next at 80°C for another 15 minutes. In
136 the following step, the samples were diluted 1:10 with sterile water. The sequencing reactions were
137 performed in a final volume of 10 µl containing 0.5 µl of BigDye® Terminator v1.1 Reaction Mix

(Thermo Fisher Scientific, USA), 2 µl of sequencing buffer (400 mM Tris, 10 mM $MgCl_2$, pH 9.0), 1 µl of 3.33 µM D2LSU2_F or D2LSU2_R primer, and 1 µl of diluted PCR product. The reactions were performed using the specified conditions: 96°C for 60 s followed by 45 cycles of 96°C for 10 s, 50°C for 5 s, 60°C for 120 s. Subsequently, all samples were purified with Performa® DTR cartridges (Egde BioSystem, USA). The purified products were mixed with 10 µl of HiDi formamide (Applied Biosystems, USA) and incubated at 95°C for 180 s followed by 4°C for 180 s; next, they were loaded into the Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, USA) with a 50 cm capillary array filled with NanoPOP-7 Polymer (McLAB, USA).

2.4 AFLP analysis

The AFLP reactions were performed with the use of *Pst*I and *Mse*I restriction enzymes. The results of the analysis were visualised by capillary electrophoresis with the Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, USA). The sequences of the adapters and primers used in this study are shown in Table 2.

The AFLP reactions were performed in three biological replications for each isolate. The double-stranded *Pst*I and *Mse*I oligonucleotide adapters were formed in a final volume of 2 µl by incubating 0.5 µl of 10 µM *Pst*I_AF, 0.5 µl of 10 µM *Pst*I_AR, 0.5 µl of 100 µM *Mse*I_AF, and 0.5 µl of 100 µM *Mse*I_AR adapters at 95°C for 5 minutes followed by 15 minutes at room temperature. Next, the restriction-ligation (RL) reaction was performed. The genomic DNA (500 ng) was digested with 5 U of the *Pst*I restriction enzyme (EURx, Poland) and 5 U of the *Mse*I restriction enzyme (New England Biolabs, USA). The RL solution was composed of 1 U of T4 DNA Ligase (EURx, Poland), 2 µl of double-stranded adapters, 50 mM Tris-HCl, 10 mM $MgCl_2$,

10 mM DTT, 1 mM ATP, and 25 µg/ml of BSA in a final volume of 20 µl. The RL reaction was incubated for 1 hour at 37°C. At the end of this reaction, each RL reaction was diluted by addition of 80 µl of sterile water and 1 µl of this solution was used as a template in the selective amplification reaction. The selective PCR amplification reaction was performed in a final volume of 5 µl, which consisted of 2.5 µl of 2X Taq PCR Reaction Master Mix (EURx, Poland), 1 µl of diluted RL solution, 0.25 µl of 10 µM 6-FAM-*Pst*I+ACA primer (Genomed, Poland), and 0.25 µL of 10 µM *Mse*I+CA primer (Genomed, Poland). The reaction was performed in a Veriti Fast thermal cycler (Applied Biosystems, USA) in the conditions: 72°C for 120 s followed by 7 cycles of 94°C for 15 s, 63°C with a touchdown of -1°C per cycle for 30 s, 72°C for 45 s followed by 33 cycles of 94°C for 45 s, 56°C for 30 s, 72°C for 45 s, and followed by a final step at 72°C for 60 s. At the end of this step, purification of exonuclease I – bacterial alkaline phosphatase was performed by addition of 2 µl of Exo-BAP Mix (EURx, Poland) to each reaction tube. The samples were incubated at 37°C for 15 minutes and then at 80°C for another 15 minutes. In the next step, 28 µl of sterile water was added into each PCR-product and 0.5 µl of this solution was combined with 0.25 µl of GS-600 LIZ Standard (Applied Biosystems, USA) and 9.25 µl of HiDi formamide (Applied Biosystems, USA). This mixture was incubated for 150 s at 95°C and cooled down on ice for 5 minutes. The amplicons were separated by capillary electrophoresis with the Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, USA) in a 50 cm capillary array filled with NanoPOP-7 Polymer (McLAB, USA). The fragments were compared to the standard and visualized as an electropherogram with GeneMapper® version 4.0 software (Applied Biosystems, USA).

2.5 Fungal isolate phenotype profiles (FIPPs)

The phenotype profiles of *Petriella setifera* isolates, regarding their catabolic potential, were generated basing on the organism growth intensity on 95 substrates located on BIOLOG FF plates (Biolog Inc., USA) at low molecular weight carbon sources.

The inoculation procedure was based on the original FF microplate (BIOLOG™) method according to manufacturer's protocol modified by Frąc (2012). To prepare the inoculum, mycelia of each isolate were obtained by cultivation on Potato Dextrose Agar medium (Oxoid Ltd, England) in the dark at 30°C for 10 days. The transmittance of the mycelium homogenised suspension in inoculating fluid (FF-IF, BIOLOG™) was adjusted to 75% using a turbidimeter (BIOLOG™). Then, 100 µl of the mycelium suspension was added to each well and the inoculated microplates were incubated at 26°C for 10 days. The experiment was carried out in two biological replications. The optical density at 750 nm was determined in triplicates using a microplate reader (BIOLOG™) every day. Functional diversity was determined by the number of different substrates utilised by the individual isolates and expressed as the substrate richness (R) and Average Well Density Development (AWDD) indices. The AWDD index was determined through the optical density of each well corrected by the subtraction of the black (water) divide the number of the total wells (95-wells).

2.6 Statistical analysis

The sequences, which they were obtained from the Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, USA) through the D2 LSU analysis, were analysed through the Sequence Analysis program (Applied Biosystem, USA) and through the Mega version 6.0 software we obtained a dendrogram. Moreover, in the dendrogram we have added further published sequences

of fungal species belonging or not to the same *Petriella setifera* family. This process has been done to have a certain identification of the five fungal strains isolated from industrial compost and both to compare the *P. setifera* strains with others published fungal genome.

To illustrate the BIOLOG results, the similarity of the carbon utilization patterns between the strains, was presented by heatmaps graph and the percent of total carbon source utilization. For the substrate richness (R) and AWDD indices were assessed, by two-way ANOVA analysis, the effect of the incubation hours and the strain on them. Successively, the significant differences were calculated by a post hoc analysis using the Tukey test. In function of the carbon utilization, we drew a cluster analysis using a dendrogram calculated with the Ward method and Sneath's dissimilarity criterion which was calculated in function of the dissimilarity of fungal groups on the basis of their response to standard tests (Sneath & Sokal, 1973).

On the other hand, for the AFLP results, we have considered only the peaks of amplified fragments are longer than 200 bp. The results obtained were shown using dendrograms calculated with the Ward method and cluster analysis with Sneath's dissimilarity criterion (Sneath & Sokal, 1973).

All the statistical analyses, which are described above, were performed with the use of STATISTICA 10.0 software (StatSoft, Inc., USA).

3. Results

3.1 Fungal D2 LSU rRNA analysis

All the tested strains were identified as *Petriella setifera* and they were separated by another known species at the genus level (Figure 1), as shown with the analysis of their D2 LSU rRNA. In

fact, the phylogenetic analysis showed a clear separation of all isolates into two clusters. The first group included the species *Aspergillus versicolor* strain G30, whereas the other comprised the species belonging to the Microascaceae family (i.e. *Petriella* sp., *Trichurus spiralis* strain CBS 635.78, *Doratomyces purpureofuscus* strain CBS 523.63, *Scedosporium prolificans* strain CBS 114.90, and *Pseudallescheria africana* strain CBS 311.72). Furthermore, the analysis revealed the existence of subgroups within the Microascaceae group with a clear separation between *T. spiralis* strain CBS 635.78 plus *Doratomyces purpureofuscus* strain CBS 523.63 and species belonging to the *Scedosporium*, *Pseudallescheria* and *Petriella* genera. In the latter subgroup described above, the sequencing of the D2 LSU region did not lead to clear separation of the strains of *Petriella setifera* and *P. guttulata*.

3.2 AFLP fingerprinting analysis

The selective primers used in this analysis produced representative electropherograms. In this way, fluorescent AFLP banding between *Petriella setifera* isolates were revealed (Figure 2). The findings exhibited the presence of 28 polymorphic peaks in total with a minimum size of 205 bp and a maximum size of 484 bp, including 4 monomorphic peaks (14.29%), and only 12 of a total of 46 peaks (42.86%) were in common between all the five analysed isolates (Figure 3).

The genetic relationship between the isolates was presented by the dendrogram (Figure 4). The subdivision of all isolates is in accordance with the less restrictive Sneath criterion (66%). The isolates exhibited the following percentage of similarity: isolates G11/16 and G16/16 51% DNA profile similarity; isolates G17/16, G14/16, and G18/16 62% DNA profile similarity. In turn, at 33% of Sneath`s restrictive criterion, we noted separation between all the tested isolates.

255 Moreover, through this analysis, we saw that four monomorphic peaks were present only in one
256 strains.
257
258 3.3 Functional diversity using the BIOLOG system
259
260 The utilization profiles of carbon sources by these isolates revealed a broad intraspecific
261 variability (Figure 5). Significant differences (approximately up to 6 times) were demonstrated in
262 the substrate richness (R) index and especially, we saw that the strains G16/16, G11/16 and G17/16
263 presented a significant different substrate richness between them and between the two remaining
264 strains (G14/16 and G18/16) (Figure 6). These findings were supported by the ANOVA analysis
265 and the post hoc Tukey test. Through the ANOVA analysis, we found that the strain, the incubation
266 time and the interaction between these two factors had significant effect ($p < 0.05$) on the substrate
267 richness (Table 3). All the five strains used an average of 92% of the 95 available carbon substrates;
268 especially, they used more carbohydrate sources (average of 95.45% of the total 44 analysed
269 substrates). In total, each strain utilised more amino acid, carbohydrate and polymer; but for the
270 total utilization of carboxylic acid and miscellaneous, we saw a different utilization between the
271 strains (Figure 7).
272 We found that all the *P. setifera* strains were extensively capable of metabolising the carbon
273 substrates at relatively high levels, especially carbohydrates (i.e. N-Acetyl-D-Glucosamine, D-
274 Fructose, D-Galactose, D-Mannose, β-Methyl-D-Glucoside, D-Sorbitol, Sucrose and D-Xylose),
275 one polymer (i.e. Glycogen), one carboxylic acid (i.e. Quinic Acid), and two amino acids (i.e. L-
276 Alanine and L-Asparagine) (Figure 5). Furthermore, we found that a few substrates were not used
277 by the analysed strains. For example, *P. setifera* G18/16 had not metabolised N-Acetyl-D-
278 Galactosamine, N-Acetyl-D-Mannosamine, α-Cyclodextrin, L-Fucose, D-Galacturonic Acid,

Glucose-l-Phosphate, Glucuronamide, D-Glucuronic Acid, D-Melibiose, D-Raffinose, D-Ribose, L-Pyroglutamic Acid, L-Threonine, Putrescine and Uridine, but it metabolised two substrates (D-Saccharic Acid and Adenosine-5'-Monophosphate), which isolates G11/16, G17/16, G16/16 and G14/16 had not utilised (Figure 5).

The dendrogram showed that the strains were separated into three clusters, in accordance with Sneath's dissimilarity criterion (66%) (Figure 8). The first group included isolate G18/16 with metabolic profile similarity of 0%, the second one consisted of isolates G16/16 and G11/16, and the third included G17/16 and G14/16 isolates with metabolic profile similarity of 42% and 54%, respectively.

The fungal activity (Figure 9), presented by AWDD (Average Well Density Development), increased during the incubation time as a function of the intensity of the carbon substrates utilization. When we analysed this data through the two-way ANOVA, we found that strains, incubation time and the interaction between these two factors affected significantly the AWDD index (Table 4). The AWDD index provided us with a further information about the analysed strains. In the Fig 5 it was possible to observe how the five strains had an approximately same fungal activity until the 120-incubation time (h). After this time, we saw an increase of the fungal activity only for the G16/16 until the finish of the experiment.

4. Discussion

All the analysed strains can be regarded as *Petriella setifera* (Figure 1), as revealed in the phylogenetic tree and especially it confirmed that the five fungal isolates were not know and there is published partial genome for these strains. This analysis explained the good separation between

the other genera belonging to the Microascaceae family, but this approach did not show any significant differences within the *Petriella* sp.. The lack of the intraspecific variability may be related to the use of sequencing of the LSU region and not of the ITS region. In fact, to reveal the separation of strains at the family level in the fungal domain, the sequencing of LSU region should be carried out. Christ et al. (2011) revealed that to view the differences within a family, the best attempt is to sequence the ITS region because of its high variability and resolution at the species level. This was also confirmed by the phylogenetic study on the Microascaceae family performed by Lackner et al. (2014).

To the best of our knowledge, there are no reports describing the genetic and functional diversities of *Petriella setifera* through AFLP fingerprinting and BIOLOG FF Plates™. For the last 15 years many researchers used the AFLP analysis to identify the intraspecific variability within a fungal species (Bakkeren, Kronstad & Lévesque, 2000; Tooley et al., 2000; Abdel-Satar et al., 2003; Radišek et al., 2003; Schmidt, Niessen & Vogel, 2004; Perrone et al., 2006a,b, Pawlik et al., 2015a,b; Rola et al., 2015), and 7 years ago they began to use the methodology BIOLOG to estimate the functional diversity (Feng et al., 2009; Singh, 2009; Albrecht et al., 2010; Shengnan et al., 2011; Lucas et al., 2013; Janusz et al., 2015; Pawlik et al., 2015a,b; Rola et al., 2015; Panek, Frąc & Bilińska-Wielgus, 2016). All these investigations explain the validity and suitability of using these methodologies to discover the intraspecific differences between fungal species at the genetic and functional level.

The analysis of the metabolic potential has revealed the presence of intraspecific variability within the *P. setifera* strains and the differences were found in the affinity and modality to use these carbon substrates. When we analysed the dendrogram of the patterns of carbon sources utilization (Figure 8), we noted that the subdivision into the three clusters was a function of the

utilization of these substrates. Strains G16/16, G11/16 and G17/16 metabolised more substrates than the others, and this was confirmed by the high substrate richness index (R index, Figure 6). Another aspect that distinguishes the *P. setifera* strains in the functional diversity was the different pattern of substrates utilization between the isolates. Figure 7 showed clearly that cluster G11/16 and G16/16 used the five principal carbon source groups in the same way, which was completely different from cluster G14/16 and G17/16; in fact, these clusters exhibited metabolic profile similarity of 42% and 54%, respectively (Figure 8). We observed that strain G18/16 utilized these carbon substances in a different way than the other two groups, especially we saw this different utilization for carboxylic acid and miscellaneous (Figure 7). Moreover, Figure 7 demonstrated that all the strains were characterized by a different C-substrate utilization ratio, especially for carboxylic acids, polymers, and miscellaneous substrates, whereas the patterns for the other three groups (i.e. amines/amides, amino acids, and carbohydrates) were the same for all the strains. The results of the BIOLOG FF Plates ™ analysis indicated intraspecific differences in the phenotypic profiles. This means that these isolates have different metabolic abilities to degrade the analysed carbon sources. These findings were confirmed by the analysis of the density of each isolate. The AWDD showed that this measure for all the analysed isolates increased after the 24 incubation hours and it remained higher throughout the time of incubation. At the beginning of the experiment (until the 24 incubation hours) all the five strains had the same lower fungal activity and after this point, we saw a bigger increase (an exponential phase) of the activity for all strains from 48 to 72 incubation hours. From 72 to 120 incubation hours, the analysed strains had an equal activity (similar a plateau situation). After this moment, only for 144 and 192 hours of the incubation, we saw that four analysed strains had a same activity and only the G16/16 strain presented an increment of the activity until the end of the experiment. These modifications on the fungal activity

348 mean that in the moment when the strains come into contact with the carbon substrates, they
349 present a lower fungal activity followed by an exponential phase. In this last phase, it could be the
350 phase in which the substrates were more degraded. In the last 120 incubation hours, we saw a
351 plateau phase due to the possible limitation of the substrate amount or the excessive presence of
352 the inhibitor products. The significant different behaviour in the fungal activity between the five
353 strains, were seen at 144 and 192 incubation hours (Figure 9).

354 The *Petriella setifera*, which can be found in decaying wood, belongs to soft rot fungi that
355 degrade cellulose and hemicellulose. We found that all the isolates degraded at high level the
356 substances that can be produced during hemicellulose degradation (i.e. D-Arabinose, L-Arabinose,
357 D-Glucuronic Acid, Xylitol, γ-Amino-Butyric Acid, D-Mannose, D-Xylose and L-Rhamnose) or
358 during cellulose degradation (i.e. α-D-Glucose and D-Cellobiose). These results were associated
359 with the proprieties of soft rot fungi (Martínez et al., 2005; Schwarze, 2007; Mathieu et al., 2013).
360 Furthermore, we noted that all the analysed isolates degraded Quinic Acid at high level, which is
361 involved in the synthesis of the S- and G-type of lignin (Albrecht et al., 2010; Hatakka & Hammel,
362 2010). This could suggest possible involvement of *P. setifera* in partial degradation of lignin,
363 which is in accordance with findings reported by other researchers (Hammel, 1997; Schwarze,
364 2007; Janusz et al., 2013; Mathieu et al., 2013). In conclusion, the results of the BIOLOG FF
365 Plates™ analysis have demonstrated a great intraspecific variability of the analysed *P. setifera*
366 strains.

367 The findings obtained with the use of the AFLP fingerprinting analysis confirmed the presence of
368 genetic variability within the isolates of *Petriella setifera*. It is evident in Figure 4 that the
369 dendrogram based on cluster analysis divides the analysed strains into two groups (in accordance
370 with Sneath's dissimilarity criteria of 66%). However, at a 33% dissimilarity coefficient, the

analysed strains are not related to each other. This differentiation was made in function of the number of detected polymorphisms. The cluster with G11/16 and G16/16 had a 52% of AFLP profile similarity, since these two isolates exhibited in total an average of 24 common peaks of a total 27 polymorphic peaks and five polymorphic peaks were not observed in the other strains. The cluster with G14/16, G18/16, and G17/16 had a 62% of AFLP profile similarity with an average of 17 common peaks of a total 19 polymorphic peaks, there was only one common peak, which was not detected in the previous cluster. This means that more polymorphism peaks were detected in the cluster with G11/16 and G16/16 than in the other strains. The results of the AFLP analysis confirm that this new protocol has successfully differentiated the isolated *P. setifera* strains.

In general, the results of grouping obtained in the BIOLOG FF Plates™ and AFLP analyses revealed differences in the graphs (Figures 4 and 8). To evaluate intraspecific variability among isolates, the BIOLOG and AFLP are proper tools, as proved in our experiments, which is also consistent with other authors' findings (Bakkeren, Kronstad & Lévesque, 2000; Tooley et al., 2000; Abdel-Satar et al., 2003; Radišek et al., 2003; Schmidt, Niessen & Vogel, 2004; Stefanowicz, 2006; Perrone et al., 2006a,b; Singh, 2009; Janusz et al., 2015; Pawlik et al., 2015a,b; Rola et al., 2015). When we analysed the two dendrograms obtained in the BIOLOG and AFLP analyses, we found a cluster of isolates G16/16 and G11/16, which explained the differences between them. Therefore, isolates G16/16 and G11/16 show more variability in the genetic and metabolic patterns because of the lower similarities in the DNA and metabolic profiles. Isolate G18/16 presented metabolic profile similarity of 0% (Figure 8) and this resulted in initial separation of this strain from the other four isolates, due to the lower utilization of carbon substrates (80/95, 84.21%) and the lower substrate richness values (R index; Fig 6). For the DNA profile (Figure 4), strain G18/16 had profile similarity of 66% (it was clustered with strain G14/16),

as suggested by the detection of only 16 polymorphism peaks for this strain (16/28, 57.14%). For this reason, strain G18/16 displays lower variability in the genetic and metabolic profiles. Finally, isolate G17/16 had metabolic profile similarity of 54%, which was similar to strain G14/16, given their similar pattern of carbon substances utilization (Figure 8). Regarding the DNA profile, G17/16 exhibited similarity of 62%, which separated it from the cluster of isolates G14/16 and G18/16. We found that this separation between G17/16 and the latter cluster was revealed by the number of polymorphic peaks in common (14 out of a total of 19); additionally, a peak that was not present in the others two strains (G14/16 and G18/16) was detected for isolate G17/16.

5. Conclusions

This is the first report on the genetic and metabolic diversity of *Petriella setifera* strains isolated from the industrial compost and the first description of a protocol for the AFLP fingerprinting analysis optimised for these fungal species. Using these two methodologies we have found the existence of intraspecific variability within the *Petriella setifera* strains at functional and genetic levels and these findings confirm that the two methodologies descript in this study allows us to identify and elucidate the intraspecific diversity in DNA and metabolic profiles of unknown species until now. The results indicated that *P. setifera* strains were able to degrade substrates produced in degradation of hemicellulose (D-Arabinose, L- Arabinose, D-Glucuronic Acid, Xylitol, γ-Amino-Butyric Acid, D-Mannose, D-Xylose and L-Rhamnose), cellulose (α-D-Glucose and D-Cellobiose) and the synthesis of lignin (Quinic Acid) at a high level. Nevertheless, further studies are required, especially focused on the genetic and metabolic aspect of this species, since there are insufficient data on the utilization of the carbon sources from different organic wastes

417 containing e.g. cellulose, hemicellulose, and lignin. This analysis could lead light on the
418 degradation pathway of cellulose and hemicellulose by *P. setifera*. The results can help to
419 recognise whether these species are able to degrade lignin similar to soft rot fungi, which carry out
420 partial degradation of this substance, and to clarify whether this fungal can be included in the group
421 of brown rot fungi or only in the soft rot fungi.
422
423 **6. Acknowledgments**
424
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426 Development of Eastern Poland 2007–2013.
427
428 **7. References**
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592

Figure 1(on next page)

Fig 1 - Phylogenetic tree based on the D2 region of LSU rRNA sequences of *Petriella setifera* strains

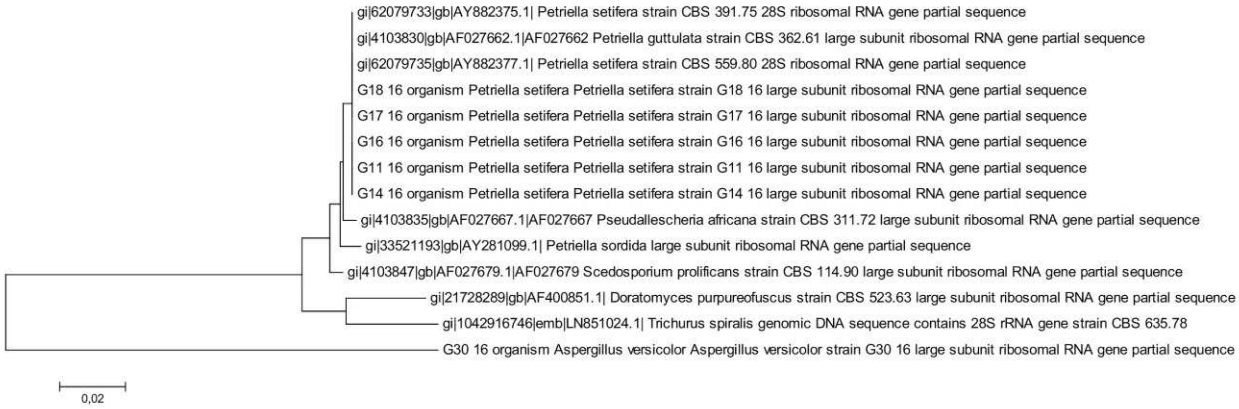
gi|62079733|gb|AY882375.1| Petriella setifera strain CBS 391.75 28S ribosomal RNA gene partial sequence
gi|4103830|gb|AF027662.1|AF027662 Petriella guttulata strain CBS 362.61 large subunit ribosomal RNA gene partial sequence
gi|62079735|gb|AY882377.1| Petriella setifera strain CBS 559.80 28S ribosomal RNA gene partial sequence
G18 16 organism Petriella setifera Petriella setifera strain G18 16 large subunit ribosomal RNA gene partial sequence
G17 16 organism Petriella setifera Petriella setifera strain G17 16 large subunit ribosomal RNA gene partial sequence
G16 16 organism Petriella setifera Petriella setifera strain G16 16 large subunit ribosomal RNA gene partial sequence
G11 16 organism Petriella setifera Petriella setifera strain G11 16 large subunit ribosomal RNA gene partial sequence
G14 16 organism Petriella setifera Petriella setifera strain G14 16 large subunit ribosomal RNA gene partial sequence
gi|4103835|gb|AF027667.1|AF027667 Pseudallescheria africana strain CBS 311.72 large subunit ribosomal RNA gene partial sequence
gi|33521193|gb|AY281099.1| Petriella sordida large subunit ribosomal RNA gene partial sequence
gi|4103847|gb|AF027679.1|AF027679 Scedosporium prolificans strain CBS 114.90 large subunit ribosomal RNA gene partial sequence
gi|21728289|gb|AF400851.1| Doratomyces purpureofuscus strain CBS 523.63 large subunit ribosomal RNA gene partial sequence
gi|1042916746|emb|LN851024.1| Trichurus spiralis genomic DNA sequence contains 28S rRNA gene strain CBS 635.78
G30 16 organism Aspergillus versicolor Aspergillus versicolor strain G30 16 large subunit ribosomal RNA gene partial sequence
0,02

Figure 2(on next page)

Fig 2 - DNA fingerprinting *Petriella setifera* strains based on Amplified Fragment Length Polymorphism

Explanation: (A) *P. setifera* G11/16; (B) *P. setifera* G14/16; (C) *P. setifera* G16/16; (D) *P. setifera* G17/18; (E) *P. setifera* G18/16; the x-axis -the size of amplified fragments; the y-axis- the relative intensity of the signal at the electropherogram.

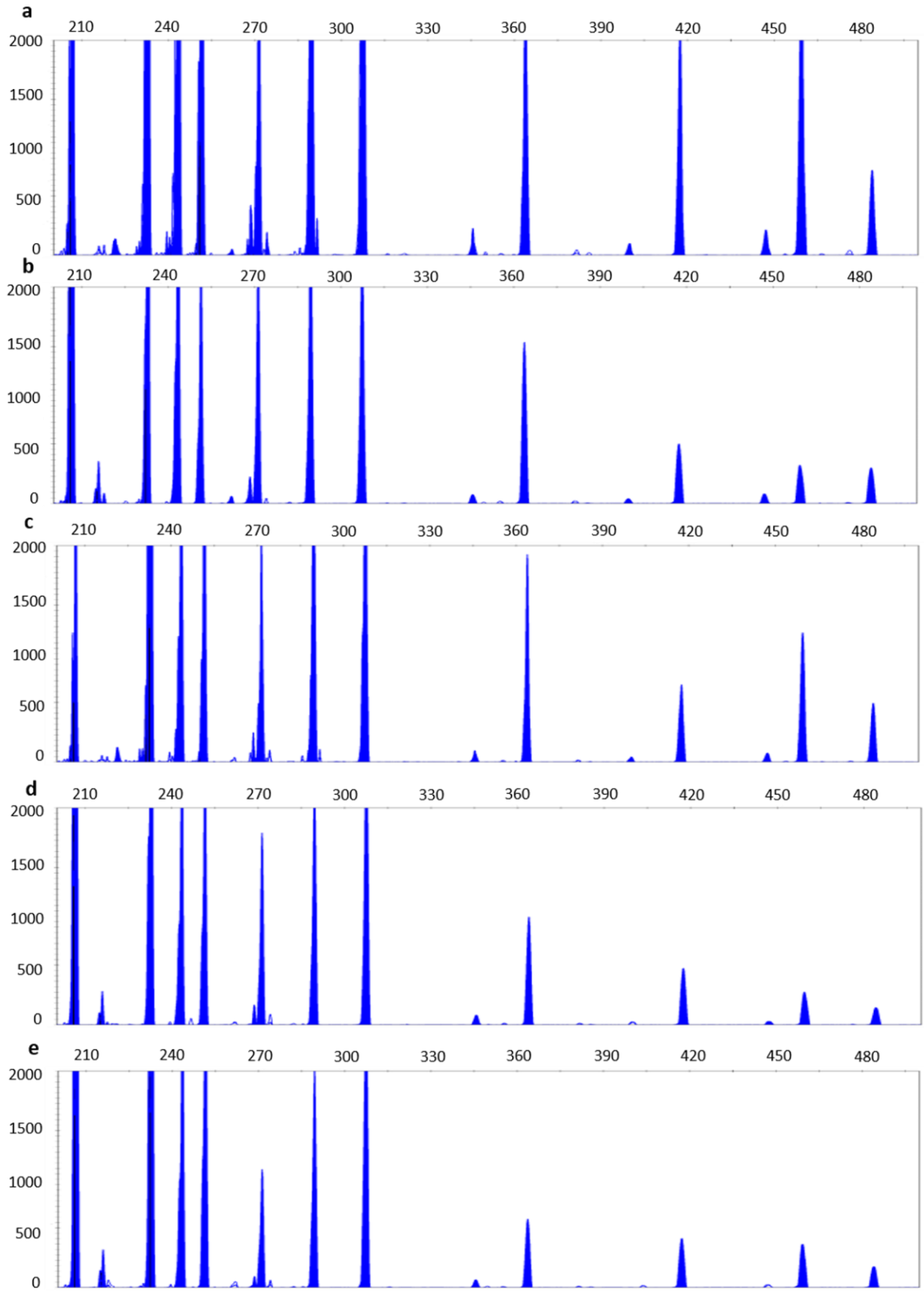

Figure 3(on next page)

Fig 3 - Genotype profile of *Petriella setifera* strains)

The colour scale at the heatmap indicates the presence (red) or absence (green) of the polymorphic peaks in each analysed strain.

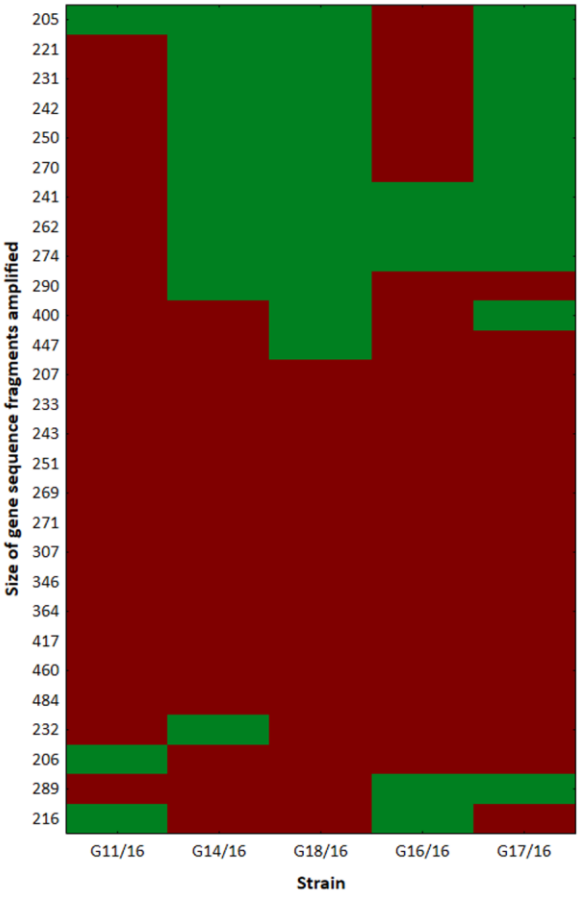

Figure 4(on next page)

Fig 4 - The dendrogram of *Petriella setifera* strains

This analysis depending on the presence or absence of the polymorphic peaks analysed through the AFLP analysis.

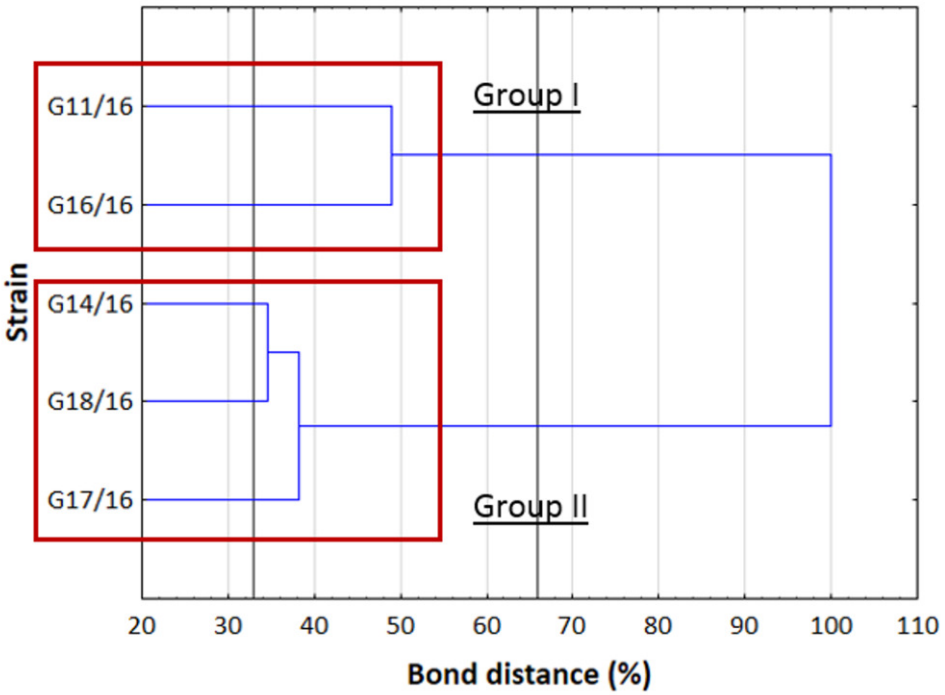

Figure 5(on next page)

Fig 5 - Phenotype profile of *Petriella setifera* strains

Colour scale at the heatmap indicates the growth of the organism (mycelial density measured at $A_{750\ nm}$) in carbons substrate for each analysed strain during the experiment.

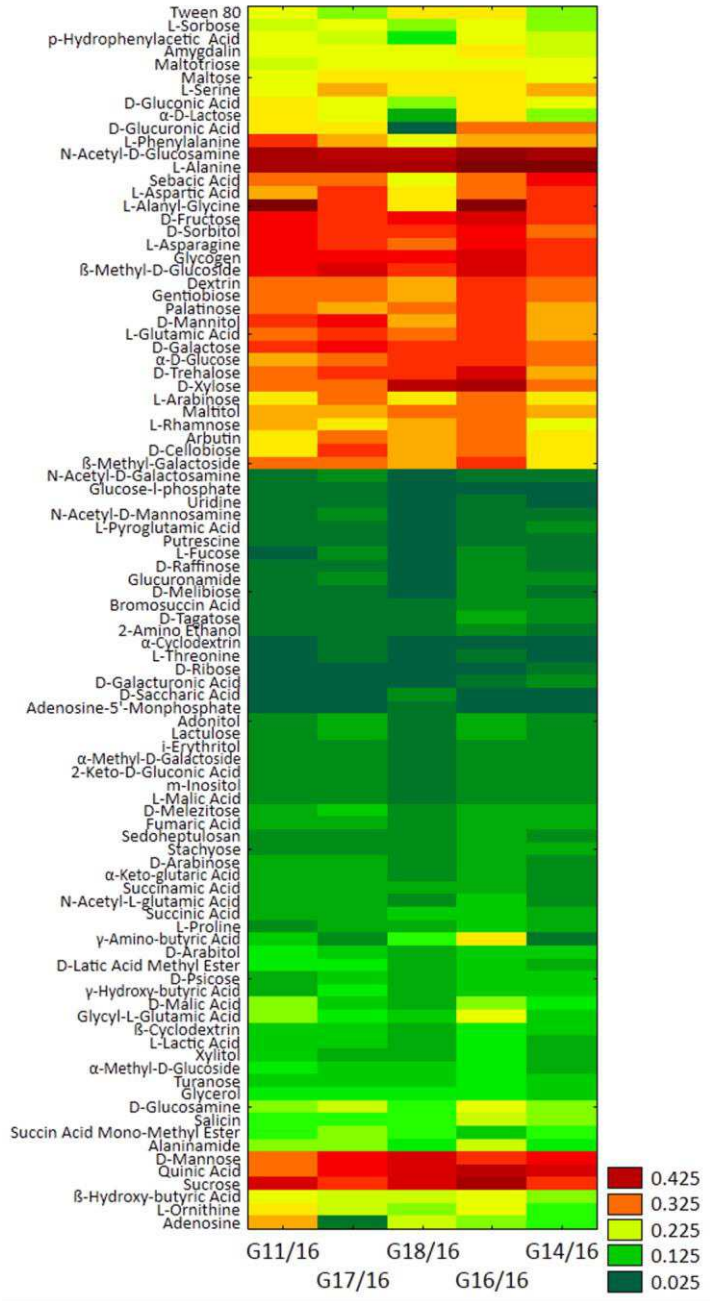

Figure 6(on next page)

Fig 6 - Functional diversity of *Petriella setifera* strains explained by substrate richness (R) index

The vertical bars indicate the confidence intervals at 0.95 and the lowercase letters indicate the significant difference (p 0.05) between each strain calculated through the post hoc Tukey test.

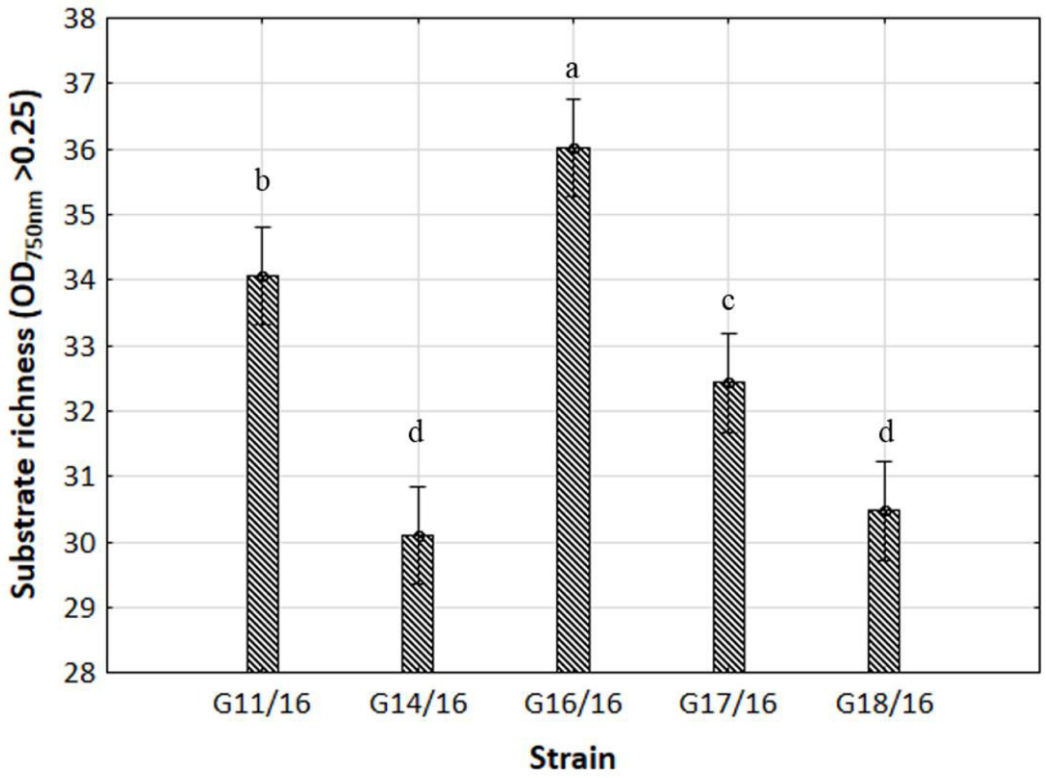

Figure 7 (on next page)

Fig 7 - Percent of total carbon source utilization for *Petriella setifera* strains

The carbon source utilization was drawn in function of the principal five carbon sources groups (AMINES/AMIDES, AMINO ACIDS, CARBOXYDRATES, CARBOXYLIC ACIDS, POLYMERS and MISCELLANEOUS). The vertical bars represent the deviation standard.

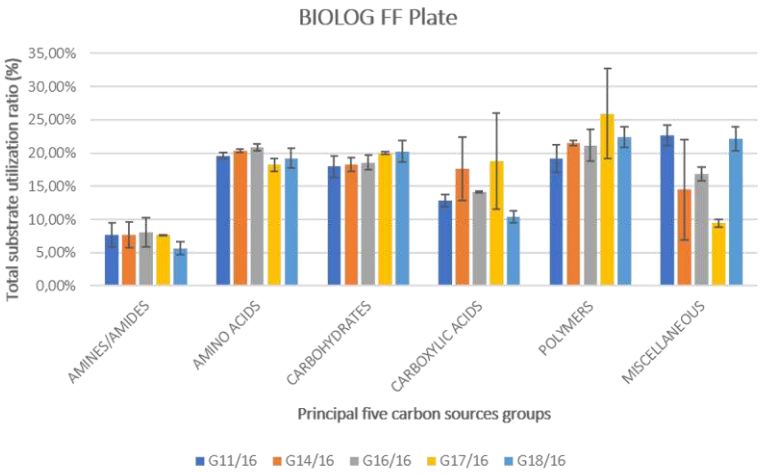
BIOLOG FF Plate
Total substrate utilization ratio (%)
35,00%
30,00%
25,00%
20,00%
15,00%
10,00%
5,00%
0,00%
AMINES/AMIDES
AMINO ACIDS
CARBOHYDRATES
CARBOXYLIC ACIDS
POLYMERS
MISCELLANEOUS
Principal five carbon sources groups
G11/16
G14/16
G16/16
G17/16
G18/16

Figure 8(on next page)

Fig 8 - Cluster analysis between *Petriella setifera* strains

The cluster analysis depending on the carbon sources utilization located inside BIOLOG FF Plates™.

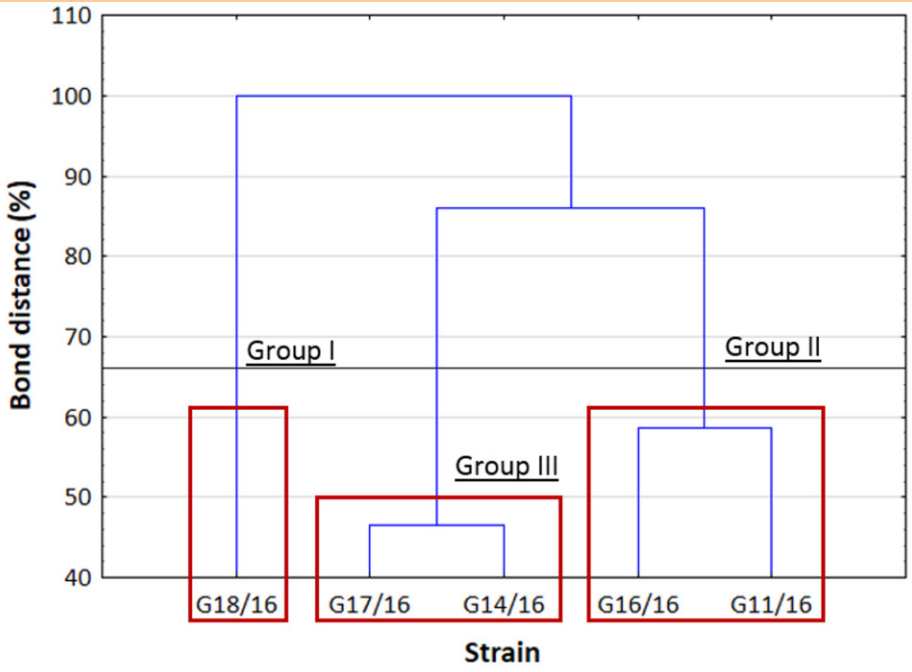

Figure 9(on next page)

Fig 9 - The growth of the analysed strains on the different carbon substrates during 192 hours of incubation

The growth of these fungal strains was explained by Average Well Density Development (AWDD) index. The vertical bars indicate the confidence intervals at 0.95. Each incubation hour was analysed by the two-way ANOVA and the post hoc Tukey test. The lower-case letters above each column describe the statistical difference between the treatments.

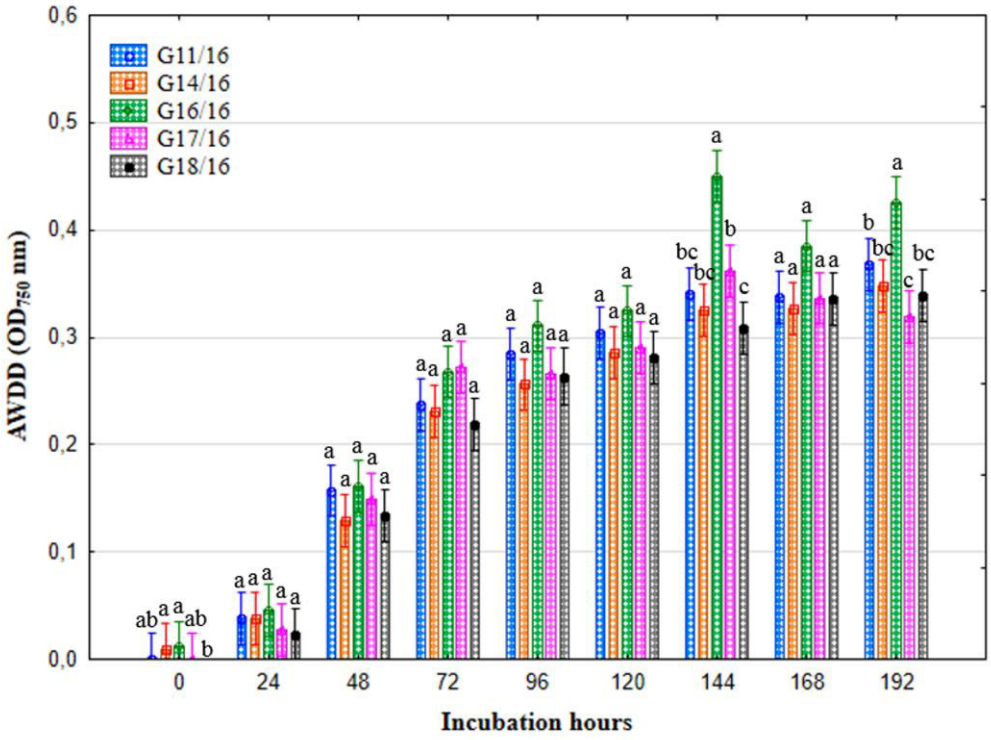

Table 1(on next page)

Table 1 - The list of oligonucleotide primers used in sequencing of D2 region of LSU rRNA

1

Primer name	Primer sequence 5'-3'
D2LSU2_F	AGA CCG ATA GCG AAC AAG
D2LSU2_R	CTT GGT CCG TGT TTC AAG

2

Table 2(on next page)

Table 2 - The list of oligonucleotide primers and adapters used in AFLP analysis

1

Adaptor name	**Adaptor sequence 5'-3'**
***Mse*I_AF**	GAC GAT GAG TCC TGA G
***Mse*I_AR**	TAC TCA GGA CTC AT
***Pst*I_AF**	CTC GTA GAC TGC GTA CAT GCA
***Pst*I_AR**	TGT ACG CAG TCT AC
Primer name	**Primer sequence 5'-3'**
6-FAM-*Pst*I+ACA	*FAM- GAC TGC GTA CAT GCA GAC A
***Mse*I+CA**	GAT GAG TCC TGA GTA ACA

2

Table 3(on next page)

Table 3 - *Petriella setifera* strains responses to substrates richness index (R)

The incubation time and strain effects on the substrate richness index (R) were determined by two-way ANOVA.

1

Effect	df	Average square sum	F	p
Incubation time (h)	8	11610.3	1495.92	0.000000
Strain	4	331.3	42.69	0.000000
Incubation time * strain	32	35.4	4.56	0.000000
Residual	1	285909.3	36837.76	0.000000

2

Table 4(on next page)

Table 4 - *Petriella setifera* strains responses to Average Well Density Development index (AWDD)

The incubation time and strain effects on the Average Well Density Development index (AWDD) were determined by two-way ANOVA.

1

Effect	df	Average square sum	F	p
Incubation time (h)	8	0.56126	623.74	0.000000
Strain	4	0.02395	26.62	0.000000
Incubation time * strain	32	0.00221	2.46	0.000070
Residual	1	14.11733	15688.91	0.000000

2