ACTA ABP BIOCHIMICA POLONICA

Vol. 62, No 4/2015
683–689
http://dx.doi.org/10.18388/abp.2015_1102

Regular paper

Determination of biodiversity of *Coprinus comatus* using genotyping and metabolic profiling tools*

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Coprinus comatus **strains (CCMs) originating from Poland were identified using ITS region sequencing. Based on the sequences obtained, the genetic relationship between the CCM strains was determined and a clear separation of all strains into two main clusters was obtained. The *Coprinus* strains were also genetically characterized for the first time by the AFLP technique. The analysis showed that the CCMs separated into four main clusters and a high complication of a UPGMA-based dendrogram was achieved. *C. comatus* strains included in the analysis displayed an AFLP profile similarity level in the range from 44 to 66%. The highest similarity coefficient, 0.490, was found between CCM12 and CCM13, and the lowest (0.202) between the CCM2 and CCM5 isolates. Biolog FF MicroPlates were applied to obtain data on utilization of 95 carbon sources and mycelial growth. The analysis allowed comparison of the functional diversity of the CCM strains and revealed a broad variability within the analyzed *Coprinus* species based on substrate utilization profiles. Significant differences (2–48) have been shown in the substrate richness values. The Biolog experiments proved to be a good profiling technology for studying the diversity in shaggy manes due to metabolic differences and demonstrated that all the strains might be considered individually. It is evident that the strain metabolic grouping does not correlate with the grouping based on the ITS sequences and AFLP profiles, however, some similarities may be observed.**

Key words: AFLP, ITS, Biolog, fungal diversity, *Coprinus comatus,* shaggy mane mushroom

Received: 15 July, 2015; revised: 17 September, 2015; accepted: 02 October, 2015; available on-line: 26 October, 2015

INTRODUCTION

Coprinus comatus, the shaggy mane mushroom, is commonly seen on newly disturbed grounds, grassy places, and road sides. Moreover, it has been cultivated as a delicious and highly nutritious edible species in recent years in China (Sabo *et al.*, 2010; Stojkovic *et al.*, 2013). Beside its culinary value, *C. comatus* is regarded as a medicinal mushroom and in recent years numerous publications have been produced indicating that it may possess antioxidant, antitumor, antidiabetic, immuno-modulating, hypolipidemic, and antibacterial properties (Han *et al.*, 2006; Li, Lu, *et al.*, 2010; Sabo *et al.*, 2010; Ren *et al.*, 2012; Zhao *et al.*, 2014). In addition, its ability to kill nematodes is intensively studied (Luo *et al.,* 2004; Luo *et al.*, 2007).

Up to date, only a few of laboratory-based techniques have been used to study the genetic diversity in *Coprinus*, such as random amplified polymorphism DNA (RAPD) (Muraguchi *et al.*, 2003; Jang *et al.*, 2009), internal transcribed spacers (ITS) 25S ribosomal DNA sequencing technique (Ko *et al.*, 2001; Keirle *et al.*, 2004), large subunit rDNA sequencing technique (Hopple & Vilgalys, 1999), RFLP markers (Muraguchi *et al.*, 2003), sequence related amplified polymorphism (SRAP) (Cai *et al.*, 2010), and functional characterization of specific gene families (Agger *et al.*, 2009).

Although the *Agaricales* include many edible and medicinal species (Stamets, 2000), these mushrooms are often poorly characterized or intractable to genetic analysis (Muraguchi *et al.*, 2003), and there are many gaps to be filled in the current knowledge on their taxonomy and biology. The traditional generic concept for *Coprinus* Pers. has existed for over 200 years. Members of the genus were recognized by a suite of morphological characters. Phylogenetic analyses of the DNA sequences are beginning to demonstrate relationships among fungi that have not been obtainable previously through morphological characterization alone (Keirle *et al.*, 2004). The development of tools aimed at clear-cut and safe identification and assessment of the genetic variability of fungal strains is thus a fundamental goal of molecular genetics research (Urbanelli *et al.*, 2007). However, the use of genetic techniques alone in fungal diversity studies has sometimes failed (Hoyos-Carvajal *et al.*, 2009). Additionally, phylogenies based only on a selected molecular method do not necessarily have the same topology as trees made from morphological or biochemical data (Kubicek *et al.*, 2003; Tripathi *et al.*, 2011). Recently, metabolic profiling technologies have been applied to investigate the taxonomy and metabolic relationships within fungi (Tripathi *et al.*, 2011; Janusz *et al.*, 2015; Pawlik *et al.*, 2015). Bearing this in mind, it seems highly reasonable and fully justified to use a comprehensive approach, taking into account molecular, morphological, physiological, and metabolic data in the research concerning identification and differentiation of fungal species.

Therefore, the aim of the present work was to determine the intraspecific diversity of *Coprinus comatus* based

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*The results concerning *Coprinus comatus* genetic identification (ITS sequencing) were presented in the form of a poster at the 47th National Scientific Conference "Microorganisms-plants the environment under changing climate conditions" (2013 Puławy, Poland). The results on *C. comatus* Biolog metabolic profiling were presented in the form of a poster at the 6th International Weigl Conference on Microbiology, Gdańsk, Poland (8–10 July, 2015).

Abbreviations: CCMs,*Coprinus comatus* strains

on a complex approach using genetic and biochemical profiling tools. In addition, we investigated the usefulness of these methods for identification and establishing the genomic and metabolic relationships between *C. comatus* strains.

MATERIALS AND METHODS

Fungal strains and cultivation. *Coprinus comatus* strains (CCMs, Table 1) were obtained from the Department of Vegetable Crops, University of Life Sciences, Poznan, Poland (ULSP) and deposited at the Fungal Culture Collection (FCL) of the Department of Biochemistry, Maria Curie-Sklodowska University, Lublin, Poland. Pure cultures of *C. camatus* isolates were obtained by excising pieces of trama from carpophores and transferring them onto Malt Extract Agar medium (Difco, BD, USA). Next, the incubation was carried out for 14 days at 25°C and the mycelium was transferred onto PDA medium (HiMedia, India). The stock culture of the fungal strains was maintained on Malt Extract Agar slants. The slants were inoculated with mycelia and incubated at 24°C for 8 days, and then used for seed culture inoculation. The mycelia of C. *comatus* strains were transferred into a 40 ml liquid Lindeberg-Holm (LH) medium (1952) in 100 ml Erlenmayer flask by punching out ca. 5 mm^2 of the slants with a sterilized cutter. The seeds were cultivated for 14 days at 24°C. Next, broth cultures were harvested by centrifugation (10 000 × *g*, 10 min) and used for DNA isolation.

DNA extraction. The mycelia from liquid cultures were used for DNA extraction according to the modified protocol developed by Borges *et al.* (1990). To extract DNA, 20 mg of fresh mycelium was transferred to a 1.2 ml Lysing Matrix A tube (MP Biomedicals, USA) supplemented with 1 ml of 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) and homogenized 3 × 40 seconds using a benchtop homogenizer FastPrep-24 Instrument (MP Biomedicals, USA). The mixture was then centrifuged, transferred (0.6 ml) to sterile 1.5 ml Eppendorf tubes, and immediately extracted two times with 1 volume of phenol. Subsequently, the aqueous phase was extracted with 1 volume of chloroform–isoamyl alcohol (24:1) and centrifuged (10 000 × *g*, 10 min, 4°C). Next, a 0.1 volume of 3 M sodium acetate (pH 5.5) was added to the aqueous phase. DNA was then precipitated by the addition of 2 volumes of ice-cold 96% ethanol and recovered by centrifugation (10 000 × *g*, 4°C for 10 min). DNA was dried in a vacuum centrifuge (JWE, Poland) and re-dissolved in 50 µl of sterile MilliQ water. The purity and quantity of the DNA samples were evaluated using an ND-1000 spectrophotometer (Thermo Scientific, USA).

PCR amplification and sequencing of the ITS region. PCRs were performed using DreamTaq Green PCR Master Mix (Thermo Scientific, USA) in a MyCycler Thermal Cycler (Bio-Rad, USA). To confirm the taxonomic identity of the fungus, the ITS region in the nuclear ribosomal repeat unit was determined by direct sequencing of the PCR products amplified with ITS1-ITS4 primers (Table 2) as described previously (White *et al.*, 1990; Gardes & Bruns, 1993). Automatic sequencing was performed using a BigDye™ Terminator Cycle Sequencing Kit and an ABI PRISM 310/3730 XL sequencer (Applied Biosystem).

Data from ITS sequencing were analyzed with Lasergene v.11.0 software (DNASTAR, Inc, USA). Database searches were performed with the BLAST program at the National Centre for Biotechnology Information (Bethesda, MD, USA). The multiple DNA sequence alignments were performed with the Clustal-W algorithm (Thompson *et al.*, 1994). The neighbour-joining (NJ) algorithm was employed to construct phylogenetic tree for *C. comatus* strains as implemented in MEGA v.6.0 software. Fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. The topology of the tree was evaluated by bootstrap analysis of the sequence data based on 1 000 random resamplings. Phylogenetic tree visualization was performed using the TreeView applet (Page, 1996).

Table 1. List of *Coprinus comatus* strains (CCMs) used in this study

	Strain number[a,b]	Geographical origin	GenBank Accession
1.	CCM1	Poznań (Poland)	JQ901432
2.	CCM2	Poznań (Poland)	JQ901433
3.	CCM3	Wojnowice (Poland)	JQ901434
4.	CCM4	Przyprostynia (Poland)	JQ901435
5.	CCM5	Wojnowo (Poland)	JQ901436
6.	CCM6	Wilkanowo (Poland)	JQ901437
7.	CCM7	Jarnatów (Poland)	JQ901438
8.	CCM8	Borne Sulinowo (Poland)	JQ901439
9.	CCM9	Swochowo (Poland)	JQ901440
10.	CCM10	Smołdzino (Poland)	JQ901441
11.	CCM11	Skierniewice (Poland)	JQ901442
12.	CCM12	Kobiór (Poland)	JQ901443
13.	CCM13	Tychy (Poland)	JQ901444
14.	CCM14	Dębska Kuźnia (Poland)	JQ901445

[a]FCL, Fungal Collection of Lublin, Department of Biochemistry, Maria Curie-Sklodowska University, Lublin, Poland; [b]ULSP, Department of Vegetable Crops, University of Life Sciences, Poznan, Poland

AFLP fingerprinting. The AFLP (Amplified Fragment Length Polymorphism) analysis was performed as described by Vos *et al.* (1995) with some modifications (Pawlik *et al.*, 2012). Adapters and primers were synthesized by GensetOligos, France, and IBB PAN, Poland. All reagents and chemicals were of molecular biology grade. Amplification reactions were performed as described elsewhere (Pawlik *et al.*, 2012). The adapters and primers employed for AFLP are shown in Table 2.

For amplicon separation, a Microchip Electrophoresis System for DNA/RNA analysis MCE®-202 MultiNA (Shimadzu, Japan) and a DNA-2500 reagent kit were applied. A 5 µl aliquot of the PCR reaction mixture was combined with 1 µl of separation buffer and fluorescent dye SYBR® Gold in a 96-well plate. The PCRs were run at 1.5 kV using a WE-C microchip according to the manufacturer's protocol.

Gel images/pherograms were visualized and analyzed using MultiNA Control & Viewer Software (Shimadzu, Japan). Presence or absence of the band between 100 and 2500 bp was regarded as a single trait and values 1 or 0 were assigned respectively. This binary information was used to calculate Jaccard's pairwise similarity coefficients as implemented in the program FreeTree v.0.9.1.50 (Hampl *et al.*, 2001). On the basis of the DNA band patterns Dice's similarity was determined as in Nei

Table 2. List of oligonucleotide primers and adapters

	Primer/adapter name	Sequence 5′-3′	Melting temperature [°C]
1.	ITS1	TCCGTAGGTGAACCTGCGG	56.4
2.	ITS4	TCCTCCGCTTATTTGATATGC	49.7
3.	*Pst*I_AF	CTCGTAGACTGCGTACATGCA	51.0
4.	*Pst*I_AR	TGTACGCAGTCTAC	42.0
5.	*Pst*I_G	GACTGCGTACATGCAGG	49.5
6.	*Pst*I_GC	GACTGCGTACATGCAGGC	52.6
7.	*Pst*I_GAG	GACTGCGTACATGCAGGAG	53.3
8.	*Pst*I_AAC	GACTGCGTACATGCAGAAC	51.1

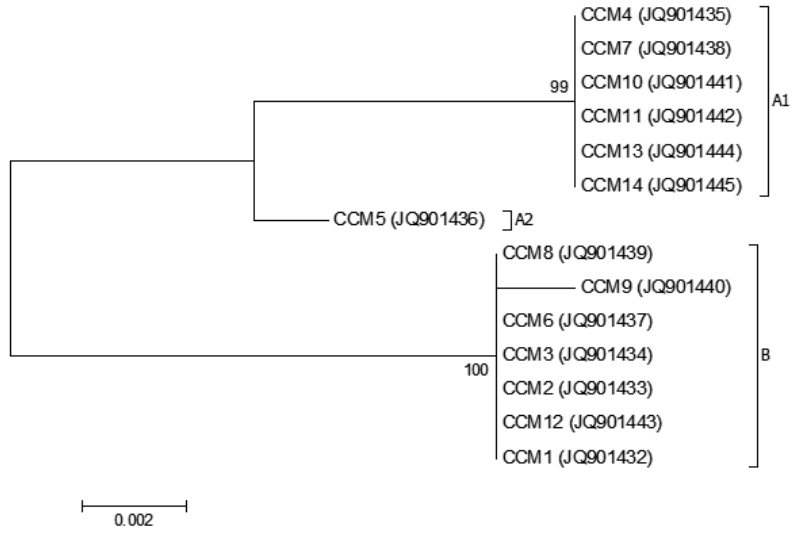

Figure 1. Phylogenetic tree constructed with the NJ method based on ITS region sequences for the 14 *Coprinus comatus* strains; the numbers in parentheses are accession numbers of ITS sequences; scale bar indicates base substitutions per 100 bases; bootstrap values at nodes are percentages of 1 000 replicates

& Li (1979) and cluster analysis was performed. The UPGMA (unweighted pair-group method with arithmetic averages) method was used for clustering, employing NTSYSpc software v.2.01. (Exeter Software Co., USA).

Biolog FF MicroPlate analysis. The global phenotypes and utilization of particular nutrients by each of the *C. comatus* strains based on 95 low molecular weight carbon sources were evaluated using the Biolog FF MicroPlate (Biolog, Inc., USA). The inoculation procedure was based on the original FF MicroPlate (Biolog Inc., USA) technique (manufacturer's supplied protocol) and the protocol developed by Janusz *et al.* (Janusz *et al.*, 2015). For inoculum preparation, mycelia of each strain were obtained by cultivation on 2% Malt Extract Agar plates (Difco, BD, USA) in the dark at 27°C for 14 days. The mycelia were thoroughly macerated using a spatula to fragment the mycelia. The suspension of the mycelia in the inoculating fluid (FF-IF, Biolog) was adjusted to 75% of transmittance as measured by a turbidimeter (Biolog). 100 µl of the above-mentioned mycelial suspension was added to each well and the inoculated microplates were incubated at 27°C in the OmniLog ID System (Biolog, Inc., Hayward, CA). The optical density was determined in triplicates using a Biolog microplate reader for each plate at 24-h intervals over a period of 192 h at 750 nm (mycelial growth). The most consistent readings came from the 7-day old Biolog plates and these were used in the analyses.

Data from all the experiments were combined in a single matrix and analyzed with the STATISTICA 10.0 (StatSoft, Inc., Tulsa, OK) software package. All data were subjected to descriptive statistical evaluations (mean, minimum, maximum, and standard deviation values) and checked for outliers. 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 168 h of incubation. Functional diversity was measured as substrate richness. The number of different substrates utilized by the 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 was determined by the greatest distance between any two objects in the different clusters. One-way or main-effect analyses of variance ANOVAs (confidence interval 0.95) were performed to compare the growth of the selected strains on the individual carbon sources. ANOVA was followed by a post-hoc analysis using the Tukey's HSD (Honestly Significant Difference) test. The summed data matrixes were also evaluated following multidimensional scaling to detect additional relationships between the variables.

RESULTS

ITS region sequencing

One product was obtained from the amplification reaction with ITS1-ITS4 primers and followed by direct sequencing. The complete sequences of these products (669 ± 1 bp) revealed over 99% identity to *Coprinus comatus*, as shown in the NCBI-BLAST search system. The GenBank accession numbers assigned to the nucleotide sequences determined in this study are presented in Table 1.

The alignments of the obtained *C. comatus* ITS sequences indicated similarities between the strains ranging from 97.9 to 100% identity (not shown). Phylogenetic analysis of these DNA sequences showed a clear separation of all strains into two main clusters (group A and B). In general, group B included strains with a lower sequence variation (Fig. 1).

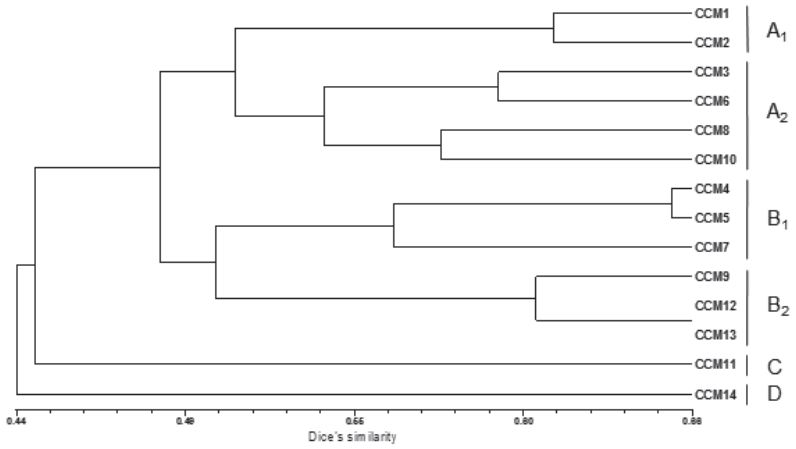

Figure 2. AFLP-based dendrogram of the 14 *Coprinus comatus* generated by UPGMA clustering using the Nei and Li's genetic distance

AFLP analysis

The rare cutting restriction endonuclease *Pst*I and 4 primers listed in Table 2 were used separately in selective DNA amplification of 14 *C. comatus* strains in the AFLP fingerprinting analysis.

Each of the four primers generated a fingerprint pattern markedly distinct from those of the other primers, even when the primers differed in only one selective nucleotide in the extension. The AFLP method applied has provided characteristic genomic markers to differentiate among the *C. comatus* strains. Although a variable number of amplified bands was obtained in the PCR reaction with each primer, all of them generated polymorphic and unambiguously scored fragments. High resolution and high reproducibility of the biological data obtained was achieved by application of an automated electrophoresis system (Shimadzu, Japan). The primers differed in their ability to detect polymorphism between the strains. Selective primers generated a total of 600 fragments, including 42 monomorphic (7.0%) and 558 polymorphic ones (93.0%) across all fourteen isolates, which demonstrates that the AFLP analysis is a robust and efficient method for detecting genomic differences between the analyzed strains. The number of scorable amplicons produced high variation and ranged from 1 to 16 fragments with an average of 150 per primer combination.

A binary matrix was used to compute similarities between the *Coprinus* strains (not shown). The average Jaccard's similarity coefficient (Jaccard, 1912) among the studied strains was low, i.e. 0.326. The highest similarity coefficient, 0.490, was found between CCM12 and CCM13, and the lowest (0.202) between the CCM2 and CCM5 isolates.

The results of the AFLP analysis are presented on the dendrogram constructed with the UPG-

Figure 3. Phenotype profiles of *Coprinus comatus* generated from FF MicroPlate using Biolog System; color scale in the heat maps indicates the growth of the organism (mycelial density A_{750nm}) in a particular substrate during 168 hours

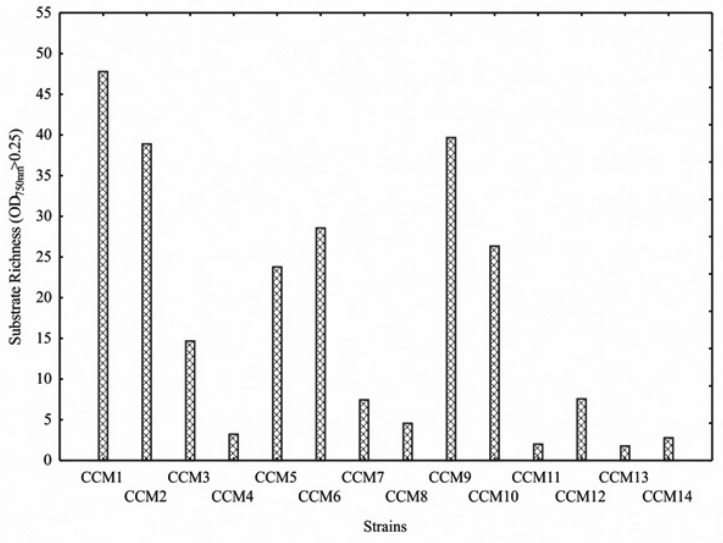

Figure 4. Functional diversity of the analyzed *Coprinus comatus* strains (substrate richness) based on the capability of C-sources from FF MicroPlate utilization

MA clustering (Fig. 2). The analysis showed that the *C. comatus* strains separated into four main clusters. Out of the 14 fungi analyzed in this study, 6 were classified as group A and 6 as group B at a DNA profile similarity of 48%. *Coprinus* CCM11 and CCM14 were clustered in separate groups (C and D) at the AFLP profile similarity level about 44%. The analysis revealed existence of subgroups within group A and B. The highest genetic similarity, 0.66, was exhibited by the CCM12 and CCM13 strains within cluster B_2.

C. comatus metabolic profiling using the Biolog FF MicroPlate system

The application of the FF MicroPlates allowed comparison of the functional diversity of the 14 *Coprinus comatus* strains. The substrate utilization profiles for the isolates tested revealed a broad variability (Fig. 3). Significant differences (up to 20 times) were demonstrated in the substrate richness values (Fig. 4). CCM1, CCM9, and CCM2 strains showed the highest catabolic activities, which were reflected by their capabilities to decompose 48/95 (50.53%), 41/95 (43.15%), and 39/95 (41.05%) of the total number of the substrates tested, respectively. In turn, CCM11, CCM13, CCM14, CCM4, and CCM8 strains were able to assimilate only 2 to 5 C-sources, i.e. 3.0% of the substrates on average. Biolog data allowed detection of the strain-specific properties of *Coprinus*. Although there is no clear correlation in the metabolic preferences of the analysed CCM strains for a particular group of substrates, most fungal strains were easily capable of utilization of carbohydrates and amino acids (Fig. 3). Only one carbon source, adenosine, was used most universally. Merely two strains (CCM8 and CCM11) were unable to utilize this compound. N-acetyl-D-glucosamine was utilized only by *Coprinus* CCM2, whereas sedoheptulosan only by the CCM9 strain. All the CCM isolates were grouped into two major clusters (A and B) at a 100% similarity level, which were then arranged in subclusters (Fig. 5). In general, the strains from group A comprise all slowly metabolizing strains that used fewer substrates (2–7), than the isolates from group B. It is worth noticing that the rapidly metabolizing *Coprinus* CCM1 and CCM9 were clustered together in a subgroup at a bond distance of 18%.

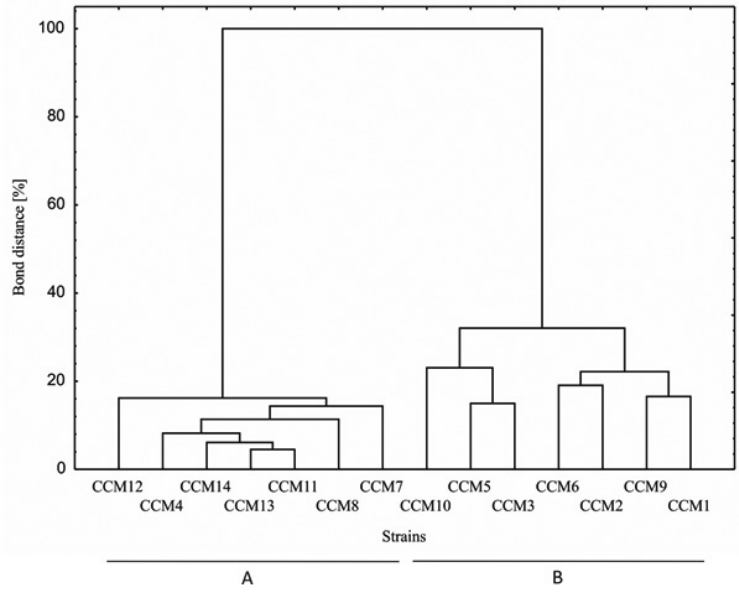

Figure 5. Cluster analysis-based dendrogram showing correlation between the *Coprinus comatus* strains in relation to utilization of C-sources from the FF MicroPlate; strains grouping into two main clusters (A and B) have been indicated

DISCUSSION

Coprinus has often been considered as one of the most recognizable genera of agarics and it was regarded by FAO and WHO as one of the 16 species of rare mushrooms because it is natural, nutritious, and healthy (Li, Yin, *et al.*, 2010). However, molecular studies made it clear that the genus includes several distinct phylogenetic groups and that the type species of the genus, *C. comatus* is more allied to lepiotaceous and *Agaricus* fungi (Redhead *et al.*, 2001).

Sequence data from ribosomal genes have been an important source of phylogenetic information for fungal systematics and taxonomy (Hopple & Vilgalys, 1999). Recently, the ITS region has been indicated as a standard barcode marker for fungi (Schoch *et al.*, 2012). In the present work, sequencing of the ITS region, including the intervening 5.8S gene, from the total DNA using primers ITS1 and ITS4 successfully allowed identification of the *Coprinus comatus* strains from Poland (Table 1). The sequenced ITS region varied slightly in length (669 ±1 bp). Alignment of ITS sequences performed here resulted in construction of a phylogenetic tree covering all CCM strains (Fig. 1). The genetic identification of *C. comatus* based on ITS

sequencing has also been studied previously (Ko *et al.*, 2001; Keirle *et al.*, 2004).

To our knowledge there is no report describing the diversity of *Coprinus comatus* by AFLP, which until now has been successfully used for identification and differentiation of microorganisms at the intraspecies level, as well as for determining their genomic relationships (Mueller & Wolfenbarger, 1999; Urbanelli *et al.*, 2007; Pawlik *et al.*, 2015). Moreover, due to the quantity of information generated, reproducibility, resolution, ease of use and cost efficiency of AFLP markers, AFLPs are compared to RAPDs and SSRs or even RFLPs (Mueller & Wolfenbarger, 1999). The use of a simplified AFLP protocol as described previously (Pawlik *et al.*, 2012) and an automated microchip electrophoresis system made it possible to produce more reliable and reproducible DNA bands in gel pherograms. It was possible to obtain a unique genetic fingerprint of the whole microorganism in the case of all the 14 *C. comatus* strains. Based on the AFLP profiles and UPGMA clustering, the isolates were grouped into 4 main clusters (Fig. 2).

In general, the results of grouping obtained in the AFLP and ITS analysis gave differences in the topology of the trees (Fig. 1 and 2). An extremely high complication (the number of clusters and subclusters) was achieved in the dendrogram constructed based on AFLPs and the UPGMA method (Fig. 2). The source of the existing differences can be assigned to the fact that the ITS method is based on a about 650 bp conserved DNA fragment, while the AFLP technique amplifies randomly the whole genome (White *et al.*, 1990; Gardes & Bruns, 1993; Vos *et al.*, 1995). Nevertheless, CCM11 and CCM14 strains which belonged to separate groups (C and D) in the AFLP dendrogram, were clustered together (A_1) in the ITS-based phylogram.

Metabolic features are becoming increasingly important in fungal taxonomic studies (Kubicek *et al.*, 2003; Druzhinina *et al.*, 2010; Janusz *et al.*, 2015) and biochemical properties of strains should be treated as an important part of the fungal identification and distinguishing process. On the other hand, it was observed that the enzyme synthesis by fungal isolates belonging to the same species may exhibit a significant/drastic variation and is affected by culture conditions, fungal age, and mycelium physiological state (Cilerdzic *et al.*, 2014; Janusz *et al.*, 2015). In this study, the Biolog FF MicroPlates analysis was performed to assess the ability of *C. comatus* to decompose various substrates. Using this method, the metabolic diversity of several fungal species has already been determined (Hoyos-Carvajal *et al.*, 2009; Janusz *et al.*, 2015; Pawlik *et al.*, 2015). To our knowledge, this is one of the first such complex surveys on the metabolic diversity of shaggy mane mushrooms. Until recently, the metabolic diversity of *C. comatus* has only been investigated by functional characterization of specific gene families (Agger *et al.*, 2009). The grouping analysis showed a clear separation of the strains into two main clusters: A — slowly metabolizing strains and B — rapidly metabolizing strains (Fig. 5). In general, there was a compliance of the overall tree topology and a considerable consistency between the results of grouping obtained in the ITS analysis and Biolog experiments (Fig. 1 and 5), despite the fact that the discrepancies observed between the trees result from the different features analyzed. A closer examination of the Biolog data revealed interesting differences in the metabolic properties of the analyzed CCM strains (substrate utilization profiles), which may result from strain-specific specialization. The results of phenotype grouping (Fig. 5) placed the slowly metabolizing CCM4, CCM14, CCM13, CCM11, and CCM8 strains (2-4 C-sources) together (group A). In turn, CCM3, CCM6, CCM2, CCM9, and CCM1, which were able to assimilate 14-48 carbon sources and were characterized by the highest catabolic activities, occupied the same subgroup in cluster B, as in the case of the phylogenetic tree based on ITS sequences. Only one carbon source, adenosine, was used most universally. This is not surprising since adenosine is considered as a universal building block of biochemical energy transfer (ATP, ADP) and signal transduction molecules (cAMP). Preferable utilization of carbohydrates, likewise for other saprophytic strains (Kubicek *et al.*, 2003; Druzhinina *et al.*, 2010), was also observed. The Biolog experiments have demonstrated a great variability within the analyzed *C. comatus* strains and have proved to be a good profiling technology for studying the diversity in shaggy manes. Moreover, the Biolog FF MicroPlate analysis proved that all the strains might be considered as individuals due to metabolic differences (Fig. 3).

In summary, this is the first report on the genetic and metabolic diversity of *Coprinus comatus* strains. It is evident that the Biolog groupings do not correlate with the grouping based on the ITS sequences and AFLP profiles; however, some similarities may be observed. As shown, the fungal variability is a complex issue and a cautious approach is needed. Further large-scale NGS studies are required, especially considering that metabolic differences among strains are much greater than may be expected based on a classical genome research.

Acknowledgements

This work was financially supported by BS/Bioch/UMCS grant. The Biolog analyses were performed using equipment bought with European Union funds – The Eastern Poland Development Programme 2007–2013 – Regional Laboratory of Renewable Energy, IA PAS.

Accession numbers

JQ901432; JQ901433; JQ901434; JQ901435; JQ901436; JQ901437; JQ901438; JQ901439; JQ901440; JQ901441; JQ901442; JQ901443; JQ901444; JQ901445

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