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

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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, anti-diabetic, 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 (Kubiçek 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...
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-Skłodowska University, Lublin, Poland. Pure cultures of *C. comatus* 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 Erlenmeyer flask by pouring out ca. 5 mm² of the slants with a sterilized cutter. The seeds were cultivated for 14 days at 24°C. Next, broth cultures were harvested by centrifugation (10000 × 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 twice with 1 volume of phenol. Subsequently, the aqueous phase was extracted with 1 volume of chloroform–isoamyl alcohol (24:1) and centrifuged (10000 × 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 (10000 × 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). Data-base 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 1000 random resamplings. Phylogenetic tree visualization was performed using the TreeView applet (Page, 1996).

**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 amplification separation, a Microchip Electrophoresis System for DNA/RNA analysis MCF9.202 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., 2004). On the basis of the DNA band patterns Dice’s similarity was determined as in Nei

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

<table>
<thead>
<tr>
<th>Strain number</th>
<th>Geographical origin</th>
<th>GenBank Accession</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. CCM1</td>
<td>Poznań (Poland)</td>
<td>JQ901432</td>
</tr>
<tr>
<td>2. CCM2</td>
<td>Poznań (Poland)</td>
<td>JQ901433</td>
</tr>
<tr>
<td>3. CCM3</td>
<td>Wojnowice (Poland)</td>
<td>JQ901434</td>
</tr>
<tr>
<td>4. CCM4</td>
<td>Przyprostynia (Poland)</td>
<td>JQ901435</td>
</tr>
<tr>
<td>5. CCM5</td>
<td>Wójnowo (Poland)</td>
<td>JQ901436</td>
</tr>
<tr>
<td>6. CCM6</td>
<td>Wilkanowo (Poland)</td>
<td>JQ901437</td>
</tr>
<tr>
<td>7. CCM7</td>
<td>Jarnatów (Poland)</td>
<td>JQ901438</td>
</tr>
<tr>
<td>8. CCM8</td>
<td>Borne Sulinowo (Poland)</td>
<td>JQ901439</td>
</tr>
<tr>
<td>9. CCM9</td>
<td>Swochowo (Poland)</td>
<td>JQ901440</td>
</tr>
<tr>
<td>10. CCM10</td>
<td>Smolędzin (Poland)</td>
<td>JQ901441</td>
</tr>
<tr>
<td>11. CCM11</td>
<td>Skierniewice (Poland)</td>
<td>JQ901442</td>
</tr>
<tr>
<td>12. CCM12</td>
<td>Kobiór (Poland)</td>
<td>JQ901443</td>
</tr>
<tr>
<td>13. CCM13</td>
<td>Tychy (Poland)</td>
<td>JQ901444</td>
</tr>
<tr>
<td>14. CCM14</td>
<td>Dębska Kujnia (Poland)</td>
<td>JQ901445</td>
</tr>
</tbody>
</table>

*FCL, Fungal Collection of Lublin, Department of Biochemistry, Maria Curie-Skłodowska University, Lublin, Poland; ULSP, Department of Vegetable Crops, University of Life Sciences, Poznan, Poland*
Table 2. List of oligonucleotide primers and adapters

<table>
<thead>
<tr>
<th>Primer/adapter name</th>
<th>Sequence 5’-3’</th>
<th>Melting temperature [°C]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. ITS1</td>
<td>TCCGTAGTGAACTCGGG</td>
<td>56.4</td>
</tr>
<tr>
<td>2. ITS4</td>
<td>TCCTCGCTTATGATGCG</td>
<td>49.7</td>
</tr>
<tr>
<td>3. PstI_AF</td>
<td>TCGTGACTGCATCAGGCA</td>
<td>51.0</td>
</tr>
<tr>
<td>4. PstI_AR</td>
<td>TGTACGCAGTCTAC</td>
<td>42.0</td>
</tr>
<tr>
<td>5. PstI_G</td>
<td>GACTGCGTACATGAGGG</td>
<td>49.5</td>
</tr>
<tr>
<td>6. PstI_GC</td>
<td>GACTGCGTACATGAGGCG</td>
<td>52.6</td>
</tr>
<tr>
<td>7. PstI_AAC</td>
<td>GACTGCGTACATGACAGAC</td>
<td>51.1</td>
</tr>
</tbody>
</table>

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 1000 replicates.

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).
AFLP analysis

The rare cutting restriction endonuclease PstI 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 UPGMA clustering using the Nei and Li’s genetic distance.

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

Figure 3. Phenotype profiles of Coprinus comatus generated from FF Micro-Plate using Biolog System; color scale in the heat maps indicates the growth of the organism (mycelial density A750nm) in a particular substrate during 168 hours

<table>
<thead>
<tr>
<th>0.1</th>
<th>0.3</th>
<th>0.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.7</td>
<td>0.9</td>
<td></td>
</tr>
</tbody>
</table>
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* 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 se-dheptulosan 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%.

**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
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). Favorable 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

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Accession numbers

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

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