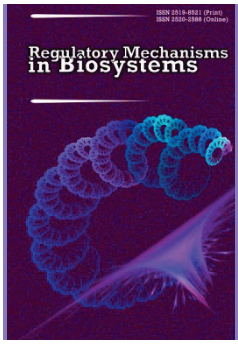

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Identification of *Albifimbria verrucaria* (*Myrothecium verrucaria*) using new polyphasic taxonomy approaches

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Filamentous fungus *Myrothecium verrucaria* UCM F-13522 (current name *Albifimbria verrucaria*) was isolated and identified by cultural and morphological characteristics, deposited in the Depositary of Microorganisms of the D.K. Zabolotny Institute of Microbiology and Virology of the NAS of Ukraine. *Albifimbria verrucaria* generally is a saprophytic fungus that belongs to the Stachybotryaceae family. A recent multigene analysis of the Stachybotriaceae family has allowed some species from the genus *Myrothecium* to be separated into a new introduced genus *Albifimbria*, and a species previously described as *M. verrucaria* has been named *A. verrucaria* as a typified representative. Accurate identification of *Albifimbria verrucaria* is of particular interest because of the wide range of its fungal activities. This species is ubiquitous plant-pathogenic fungus, cause of foliar spots in a large number of important crops and cultivated vegetables, and has exhibited bioherbicidal, nematicidal and antimalarial activities. Recently it was shown that *A. verrucaria* has become an opportunistic pathogen of humans. The aim of this work was to confirm the new taxonomic affiliation of the *A. verrucaria* strain UCM F-13522, for which we used a modern multilevel identification of the fungus with polyphasic taxonomy approaches. Cultural and morphological observations of the fungal mycelium confirm the previously described characteristics of *A. verrucaria*. The internal transcribed spacer (ITS) region of nuclear ribosomal DNA (rDNA) was used for DNA barcoding. Our research has shown that molecular identification with the ITS rDNA region is of sufficient variability to discriminate *A. verrucaria* from the species *A. lateralis* and *A. terrestris*. Comparison of ITS rDNA gene sequences revealed 3 different transition point mutations that distinguish *A. lateralis* and *A. terrestris* from *A. verrucaria*, at positions 51–52 and 54 nucleotides of the ITS2 region. The phylogenetic tree analysis based on ITS rDNA classified *Albifimria* spp. into a monophyletic clade, as in the multigene phylogeny. This indicates that it is possible to discriminate fungi of *Albifimbria* spp. using a single genetic marker. We studied the possibility of using the secondary structure model of the ITS rDNA region to identify *Albifimbria* spp. The set of the *A. verrucaria* verified culture collection strains and *A. terrestris*, *A. viridis*, *A. lateralis* type strains sequences were used fto construct a consensus model predicted minimum free energy secondary structures of the ITS2 region. An important characteristic in polyphasic taxonomy of fungi is the use of species-specific metabolites. Chemotaxonomic characters such as secondary metabolites and specific enzymes have not previously been used for taxonomic studies of *Albifimbria* genus. For chemotaxonomic identification, the bilirubin-oxidase (BOD) activity, unique to the Stachybotryaceae family, was used. Under submerged cultivation conditions *A. verrucaria* UCM F-13522 synthesized BOD with an activity of 3.6 units/mL. The enzymatic activity of BOD can be recommended as an important chemotaxonomic marker in *A. verrucaria* identification. Based on the culture, morphologic, molecular, and chemotaxonomic data obtained, the fungus *Myrothecium verrucaria* UCM F-13522 should be renamed *Albifimbria verrucaria* UCM F-13522. This study provides insight into the development of new species-specific markers for the discrimination of closely related to *Albifimbria* spp. by deploying polyphasic taxonomy approaches.

Keywords: *Albifimbria verrucaria*; *Myrothecium verrucaria*; polyphasic taxonomy; bilirubin-oxidase; ITS rDNA; ITS secondary structure.

Introduction

Albifimbria verrucaria (Alb. & Schwein.) L. Lombard & Crous, previously named *Myrothecium verrucaria* (Alb. & Schwein.) Ditmar, has a worldwide distribution as a plant pathogen, saprophytic and endophytic fungus that belongs to the Stachybotryaceae family (Hypocreales, Sordariomycetes). The need for re-identification of this fungal species is due to changes in the taxonomy of Stachybotryaceae. Lombard et al. (2016), based on phylogenetic analysis, revised the genus *Myrothecium* which resulted in the recognition of 13 new genera based on the polyphyletic origin of its species, and 4 species have been reported within the renamed genus *Albifimbria*.

Filamentous fungus *A. verrucaria* is of particular interest because it causes foliar spots in a large number of important crops and cultivated vegetables including soybean, cotton, tomato, rice, corn, potato, cucumber, spinach and other (Herman et al., 2020). At the same time, *A. verrucaria* exhibited bioherbicidal activity against several important weeds and it is a promising biological control agent (Li et al., 2020). The properties of this fungus are successfully used for weed control, so mycelial preparation of *A. verrucaria* along with glyphosate synergistically managed the hard-to-kill perennial weeds redvine (*Brunnichia ovata*) (Boyette et al., 2020), kudzu (*Pueraria lobata*) (Abbas et al., 2001), and trumpet creeper (*Campsis radicans*) (Anderson & Hallett, 2004; Weaver et al., 2021).

The fungus synthesizes a number of extracellular enzymes such as chitinases, proteinases, lipases, glucanases and laccases, which are actively used in medicine, agriculture and in the development of new energy technologies. Chitinase, proteinase and β-1,3-glucanase produced by *A. verrucaria* are effective against the root infecting fungal pathogen *Sclerotium rolfsii*, which causes disease in a wide variety of plants, including vegetables, fruits, and ornamental crops (Chavan et al., 2017). Hydrolytic enzymes of *A. verrucaria* were successfully used for the control of woolly aphid, *Ceratovacuna lanigera*, on sugarcane, and were effective against mealy bug in grapes (Ahmad & Rasool, 2021). A complex of *A. verrucaria* chitinase, lipase and proteinase, which act as insect cuticle-breaking enzymes, has been shown to have great potential in the control of *Aedes aegypti* mosquitoes (Mendosa et al., 1996) and exhibited nematicidal activities against root-knot nematode, *Meloidogyne incognita*, a parasite of sugar beet (*Beta vulgaris*) (Hagag, 2021).

In addition to the enzymes mentioned above, secondary metabolites produced by the fungus are also responsible for the nematicidal activity. *A. verrucaria* synthesizes a range of macrocyclic trichothecene mycotoxins such as verrucarins A, J and roridins A, E, H. The research of Nguyen et al. (2018) concludes that the verrucarin A and roridin A are major active metabolites with nematicidal activity against the root-knot nematode *Meloidogyne incognita*. Trichothecenes (roridin A and E, verrucarin A and J) isolated from *A. verrucaria* showed high antimalarial activity *in vitro* against *Plasmodium falciparum* K1 strain with EC_{50} values less than 1 ng/mL (Isaka et al., 1999).

It was recently shown that *A. verrucaria* has become an opportunistic infectant of humans. Liu et al. (2021) reported that *A. verrucaria* is the causative agent of keratitis in immunocompetent patients. Masetti et al. (2020) report for the first time the occurrence of a filamentous fungus, *A. verrucaria*, in the blood of a pediatric neuroblastoma patient.

In addition to secondary metabolites and hydrolytic enzymes, *A. verrucaria* synthesizes a number of other important ones. Cyanamide hydratase (EC 4.2.1.69) enzyme belongs to the family of the hydrolyases, which cleave carbon-oxygen bonds. An important feature of this enzyme is the ability to convert herbicide cyanamide (CN_2H_2) into urea. Transgenic tobacco and soybean have been made resistant to cyanamide by transformation with *A. verrucaria* gene that encodes cyanamide hydratase (cah). Moreover, the Cah gene also been used as selectable marker in gene cassettes constructed for genetic transformation to produced economically important transgenic plants (Zhang et al., 2005; van Driel et al., 2014).

Filamentous fungus *A. verrucaria* has been known as a producer of bilirubin oxidase (BOD, EC 1.3.3.5) (Murao & Tanaka 1981), which has various biotechnological applications such as manufacturing biobatteries and biosensors, bleaching of clothes, degradation of effluents, and dye decolorization. BOD belongs to the subgroup of multicopper oxidases, has attracted significant interest due to its ability to oxidize bilirubin to biliverdin, which is utilized in the determination of bilirubin levels in the blood. Because the accumulation of bilirubin in the blood is a common symptom of liver diseases, its measurement in plasma (serum) is an important diagnostic marker (Guo et al., 1991; Mano, 2012). Furthermore, BOD is an attractive candidate for enzyme cathodes in biofuel cells and biosensors because unlike laccase, it is stable under physiological conditions (Tsujimura, 2019; Suzuki et al., 2022).

Multicopper oxidases (MCOs) are ubiquitous enzymes that catalyse the oxidation of various substrates via the reduction of O_2 to H_2O. Members of the MCO family have been previously classified as true laccases (EC 1.10.3.2), BODs and some other enzymes. Laccases, representing the largest subgroup of MCOs, are enzymes with p-diphenol oxidase activity, which oxidize polyphenols using oxygen as final electron acceptor. In fungi, laccases have been studied extensively because they have a broad range of biotechnological applications including wood delignification for paper manufacturing, dye destaining in the textile industry, and detoxification of water pollutants. BOD, as well as laccases, has the ability to oxidize some phenolic compounds, as ABTS and Syringaldasine. However, unlike laccases, BOD catalyzes the oxidation of bilirubin to biliverdin (green tetrapyrrolic bile pigment) and then to a purple pigment (Janusz et al., 2020).

$$2\ \text{bilirubin} + O_2 \leftrightarrow 2\ \text{biliverdin} + 2\ H_2O.$$

Classical fungal oxidoreductases are multicopper oxidases, mainly laccases. Laccase-like oxidoreductases are quite widespread in fungi of various taxonomic groups, while BOD is found in a limited number of species in the basidiomycete *Trachyderma tsunodae* (family Ganodermataceae) and ascomycetes: rice blast fungus *Pyricularia oryzae* Cavara (Teleomorph: *Magnaporthe oryzae* B. C. Couch), a model aging fungus *Podospora anserina* (order Sordariales) and *Penicillium janthinellum* (Mano, 2012). Therefore, BOD can be considered as a species-specific enzyme that can serve as an important marker for *A. verrucaria* identification using polyphasic taxonomy approach.

The traditional mycological methods and DNA barcoding were synergistically used for taxonomy of genus *Myrothecium* fungi. The phylogenetic analyses (Lombard et al., 2016) which combined LSU rDNA, ITS rDNA, *tef1*, *rpb2*, *tub2* and *cmdA* genes showed that the ex-neotype strain of *Myrothecium verrucaria* (= *Peziza verrucaria*) CBS 328.52 = IMI 45541 (Tulloch, 1972). Fungal species such as *M. viridis*, *M. terrestris* and *M. lateralis* were clustered in a highly supported clade distant to the *Myrothecium* sensu stricto clade. This clade was introduced the new generic name, *Albifimbria*. Members of the *Albifimbria* genus are characterised by the formation of verrucose setae surrounding the sporodochia and conidia sometimes bearing a funnel-shaped mucoid appendage, not observed in *Myrothecium* s. str. Chemotaxonomic characters such as secondary metabolites and specific enzymes have not previously been used for taxonomic studies of this genus.

In this context, taking into account the changes in the taxonomy of the genus *Myrothecium*, the aim of our study was to identify the filamentous fungus *Myrothecium verrucaria* UCM F-13522 using polyphasic taxonomy approaches, which in this case are based on cultural and morphologic, molecular: determination and sequence analysis of the ITS rDNA region; and biochemical: the ability to synthesize BOD, characteristics.

Materials and methods

Myrothecium verrucaria UCM F-13522 (current name *Albifimbria verrucaria*) was isolated from the rhizosphere of corn (*Zea mays*) in Kyiv region of Ukraine. The fungus previously had been identified by cultural and morphological characteristics and deposited in the Depositary of Microorganisms of D. K. Zabolotny Institute of Microbiology and Virology of the NAS of Ukraine and stored in Ukrainian Collection of Microorganisms.

The strain was grown on potato-dextrose agar (PDA) for 10–14 days at 26 ± 2 ºC. A spore suspension (1 x 10^6 spore/mL) was cultivated in 750 mL Erlenmeyer flask with potato-dextrose medium under submerged conditions at 26 ± 2 ºC for 48 h.

To obtain the culture filtrate (CF), the filamentous fungus was cultivated under submerged conditions at 26 ± 2 ºC for 4 days, the amount of inoculum introduced into the medium was 5%. After cultivation, the mycelium of the fungi was separated by filtration through ashless paper filters (blue tape).

For the phenotypic identification, the fungus was cultivated on Petri dishes with potato dextrose agar (PDA), malt extract agar (MEA) and Czapek agar (CzA) at 26 ± 2 ºC for 14 days. During this time, the growth rate and morphology of the colonies were examined using the light microscope Leica DM500 at 400 magnification with Leica Imaging Software LAS EZ. The fungus was identified using the morphological characteristics according to Pidoplichko & Kirilenko (1969), Tulloch (1972) and Lombard et al., (2016).

Genomic DNA was isolated by the microwave-based method Orsini (2001) with some modifications (Yurieva et al., 2017) and calibration generated by the magnetron radiation at 2.45 MHz. Internal transcribed spacer (ITS) regions of nuclear ribosomal DNA (rDNA) were amplified using Taq PCR Core Kit (Qiagen NV, USA) with the primer pairs: forward ITS1 (GGAAGTAAAAGTCGTAACAAGG) and reverse ITS4 (TCCTCCGCTTATTGATATGC) (White et al., 1990). The amplification was performed as follows: 1 cycle of 94 ºC for 1 min; 30 s at 94 ºC, 30 s at 55 ºC and 30 s at 72 ºC for 26 cycles; followed by a final extension of 10 min at 72 ºC. Amplification products were purified with a PCR Purification Kit (Qiagen NV, USA) and Sanger sequenced on the Applied Biosystems 3730xl DNA Analyzer at the PrimBio Research Institute (Exton, PA, USA). The sequences were edited and analyzed with the Geneious software 8.1.8 (Biomatters, NZ). The nucleotide sequence of *A. verrucaria* ITS region rDNA data obtained was deposited in GenBank under accession number OQ608004.

Blastn of NCBI GenBank nucleotide database was used for searching for the best hit for query ITS sequence. Multiple sequence alignments were produced using the ClustalW algorithm. Phylogenetic analysis and construction of an unrooted tree were performed by the software MEGA-X with using Maximum Likelihood (ML) and Neighbour Joining (NJ) methods.

The ITS2 sequences from whole ITS region were extracted from the *Albifimbria* spp. alignment between the 5.8S and 28S gene using the data of Lombard et al. (2016). Prediction of the secondary structures of ITS2 rRNA was performed based on the minimum free energy calculation using RNAfold WebServer of ViennaRNA Web Services (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi). List of verified strains used for secondary structure prediction (strain/NCBI ID): *A. verrucaria* UCM F-13522, OQ608004.1; CBS328.52 (TYPE), NR_153550.1; CBS 208.72, KU845891.1; CBS 962.95, KU845895.1; CBS 207.30, KU845890.1; CBS 188.46, KU845888.1; CBS 231.56, KU845892.1; CBS 231.56, MH857598.1; CBS 176.27, KU845886.1; CBS 121142, KU845896.1; CBS 189.46, KU845889.1; IHEM:18499, OW985686.1; IHEM:03073, OW983028.1; CGMCC 3.2190, FJ235085.1; *A. lateralis* CBS 117712 (TYPE), NR_153548.1; *A. viridis* CBS 449.71 (TYPE), NR_153551.1; *A. terrestris* CBS 126186 (TYPE), NR_153549.1.

The method (Murao & Tanaka, 1981) was used to determine the activity of bilirubin oxidase (BOD). Bilirubin from the diagnostic kit Bilirubin-calibrator NK005.02 (NVP "Filisit-Diagnostics", Ukraine) was used as a substrate. The enzyme activity was assayed as 2.0 mL of bilirubin (40 µg/mL 0.004% (w/v) Bilirubin Solution) dissolved in Tris-HCl buffer with 1 mM EDTA, pH 8.0, 2.0 mL culture filtrate were incubated at 37 ºC for 5 min. The activity of BOD is followed by the oxidation of bilirubin, which leads to a decrease in the absorbance measured at 440 nm. One unit of BOD activity was defined as the amount of the enzyme that oxidized 1 µmol of substrate (bilirubin) per min under the assay conditions. Molar extinction coefficient of free bilirubin at 440 nm is 4.97×10^4 $M^{-1}cm^{-1}$.

Results

To identify the fungus, we used cultural and morphological, genetic, and biochemical methods of analysis. When the fungus was cultivated on Petri dishes with PDA medium, the size of colonies reached 5–6 cm diameter after 12 days of incubation at 26 ºC. The size of colonies when cultured on YEM medium under the same conditions was 6–7 cm diam (Fig. 1). Mycelium was snow-white to rosy buff, low-fluffy, dense. Sporodochia round, convex, conical, at first olivaceous, then almost black. Hyphae septate, colorless to white, smooth. Conidiophores colorless to white. Phialides columnar, 11.5–18.0 * 1.5–2.0 µm. Conidia (phialospores) lemon-shaped or spindle-shaped with rounded apex, some at base with cut papillae, 7.0–8.0 * 2.0–3.0 µm (Fig. 2).

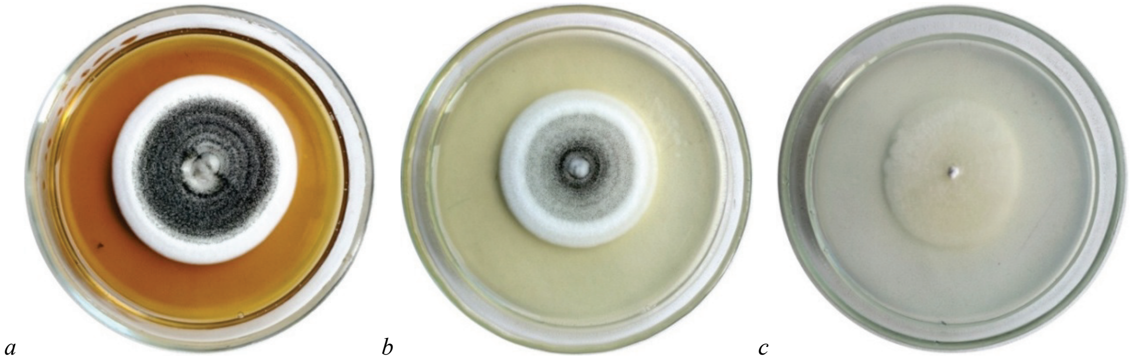

Fig. 1. Colony characteristics of *A. verrucaria* UCM F-13522 grown for 14 days at 26 ºC on: *a* – potato dextrose agar (PDA), *b* – malt extract agar (MEA) and *c* – Czapek's agar (CzA)

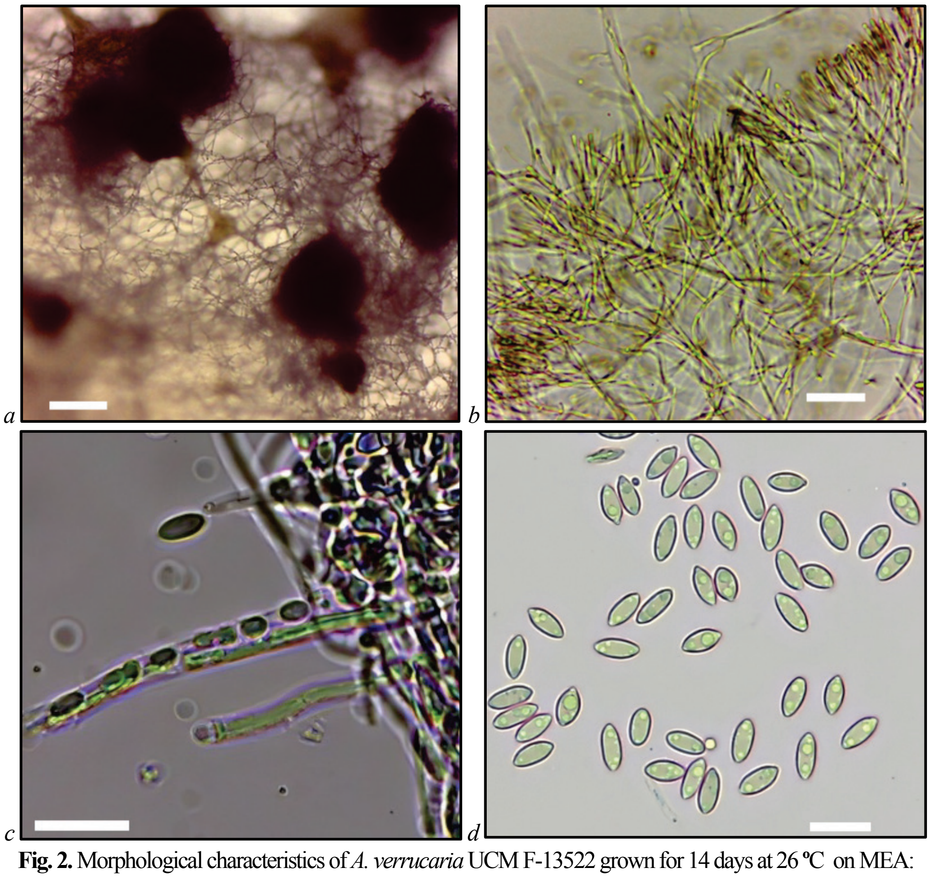

Fig. 2. Morphological characteristics of *A. verrucaria* UCM F-13522 grown for 14 days at 26 ºC on MEA: *a* – black sporodochium on colony surface; *b* – conidiophores; *c* – phialides; *d* – conidia; scale bars: *a* – 100 µm, *b* – 50 µm, *c* – 20 µm, *d* – 10 µm

PCR amplification of genomic DNA of *A. verrucaria* with universal primers ITS1 and ITS4 yielded a 515 bp product. Sequencing of this fragment and further blastn analysis established a high degree of relatedness to fungi of the genus *Albifimbria*. ClustalW multiple sequence alignments of the ITS region of the rDNA M. verrucaria UCM F-13522 showed complete identity with both the type culture of *A. verrucaria* CBS328.52 and a number of verified museum strains (see Materials and methods).

Sequence analysis of the ITS region coding rRNA showed that alignment of the ITS2 fragment sequences was suited to resolve closely related species of *Albifimbria* genus, but with some limitations. Thus, nucleotide comparison of ITS gene region revealed 3 bp difference between species *A. lateralis* and *A. terrestris* from *A. verrucaria* in positions 51–52 and 54 nucleotides. In both cases, C-T and T-C pyrimidine nucleotide substitutions in the ITS2 fragment sequence are observed, which correspond to transition type of mutations. At the same time the full sequences of ITS region rRNA of species *A. viridis* and *A. verrucaria* completely coincide.

The data of phylogenetic analysis based on the dataset of ITS sequence of *Albifimbria* spp. and selected Stachybotryaceae representatives recommended by Lombard et al. (2016) showed that phylogenetic trees constructed by ML and NJ methods indicate that *A. verrucaria* UCM F-13522 with other *Albifimbria* spp. form a distinct cluster within closely related representatives of genera *Myrothecium* and *Paramyrothecium* (Fig. 3).

Biochemical analysis of fungal culture filtrates for bilirubin oxidase activity showed that under submerged cultivation conditions *A. verrucaria* UCM F-13522 synthesized BOD with an activity of 3.6 units/mL.

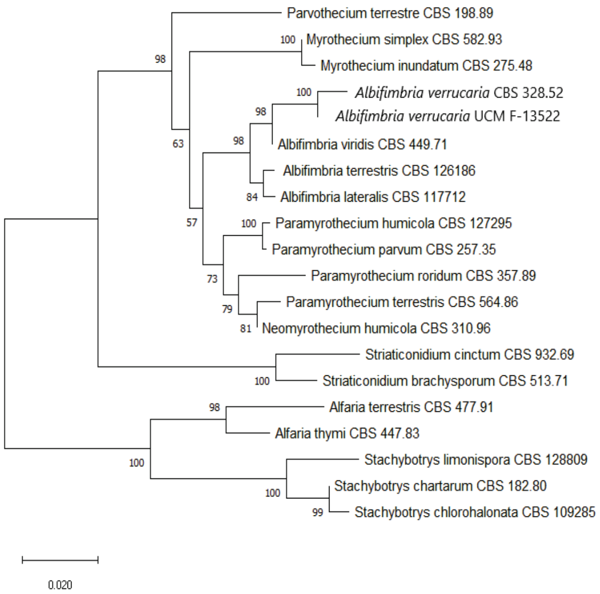

Fig. 3. Phylogenetic tree constructed by Maximum Likelihood method based on the dataset analyses of ITS sequence of selected Stachybotryaceae representatives recommended by Lombard of Stachybotryaceae genera showing the position of *A. verrucaria*

The secondary structure of ITS2 rDNA region with the minimum free energy (MFE) algorithm was predicted as single strained RNA. Folding pattern of 177 bp ITS2 region is characterized by the formation of a conserved core structure as a central unpaired bulge with three double-stranded helices radiating from it (Fig. 4). The conformational free energy of sequence ΔG required for the formation of secondary structure was −74.40 kcal/mol. All three mutation points are located in the terminal hairpin loop of Helix III ITS2 rDNA and do not affect the structure of the paired helixes (Fig. 5).

Discussion

Keys for the identification of fungal species of the genus *Myrothecium*, currently classified in the genus *Albifimbria,* in particular *A. verrucaria*, were proposed by Pidoplichko & Kirilenko (1968). Detailed morphological descriptions of fungal species of the genus *Albifimbria* are provided by Tulloch (1972). The studied morphological and cultural characteristics were in good agreement with the descriptions of *M. verrucaria* given by Pidoplichko and Tulloch. Because fungal species of the genus *Albifimbria* are difficult to discriminate morphologically, molecular identification and phylogenetic analysis were necessary to accurately determine the taxonomic affiliation of species. However, morphological observations continue to play an important role in fungal and in particular *Albifimbria* species identification (Senanayake et al., 2020).

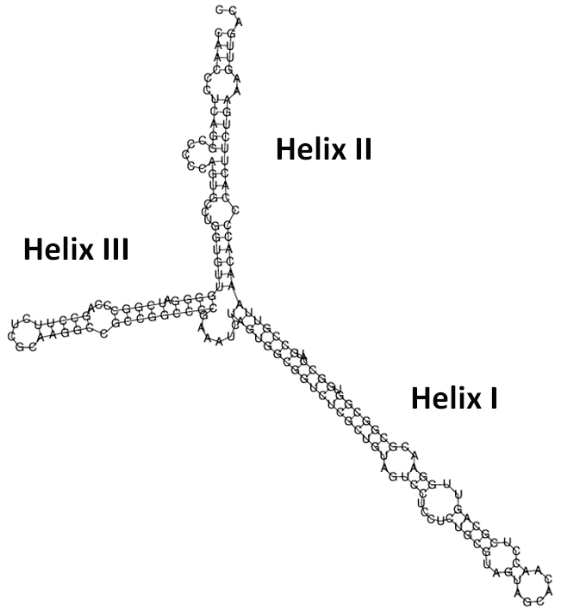

Fig. 4. Consensus predicted minimum free energy (MFE) secondary structures of ITS2 region from four *Albifimbria* spp.

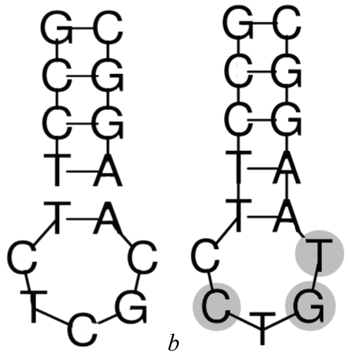

Fig. 5. Terminal hairpin loop of Helix III ITS2 rDNA MFE predicted secondary structure: *a* – *A. verrucaria*, *A. viridis*; *b* – *A. lateralis* and *A. terrestris*; mutation points are indicated by gray circles

Modern fungal taxonomy has moved forward using a combination of morphology with chemotaxonomy, phylogeny and molecular biology. Some modern methods of identification were not possible, so matrix-assisted laser desorption/ionization time of flight analysis (MALDI-TOF mass spectrometry) is ruled out as the spectrum of *A. verrucaria* was not present in the filamentous fungi reference database (Masetti et al., 2020). Therefore molecular markers have become the main criteria for the taxonomy of fungi. Nuclear ribosomal internal transcribed spacer – ITS region of rDNA region that includes ITS1, 5.8S and ITS2, is proposed as the primary fungal barcode marker (Schoch et al., 2012; Nilsson et al., 2014).

In addition to the ITS region of rDNA gene sequences of highly conserved proteins are now widely used, m- the RNA polymerase II second largest subunit (rpb2), calmodulin (cmdA), translation elongation factor 1-alpha (tef1) and β-tubulin (tub2), and, to a lesser extent, the large subunit of the nuclear ribosomal RNA (rRNA) gene (LSU) and the small subunit (SSU) (Lücking et al., 2021).

The use of different sets of markers for identification and comparison with unverified strains introduces some confusion and forces us to look for more unambiguous criteria for identification. However, many researchers believed that the use of a single marker does not provide a clear identification of the species. As a result, in most cases, different sets of protein-coding marker genes were used for high taxonomic resolution. The problem of studying the molecular taxonomy of the genus *Albifimbria*, as well as Stachibotryaceae in general, is the use of different sets of genetic markers and the lack of a generally accepted assessment of the suitability of a particular marker. Lombard et al. (2016) used *ITS*, *rpb2*, *cmdA*, *tub2* genes and did not include *tef1* and *LSU* when studying *Albifimbria* taxonomy. So Yin et al. (2022) besides ITS as the main marker used *cmdA* and *rpb2* genes, while Samarakoon et al. (2022) as additional ones used *rpb2* and *tub2*. Matić et al. (2019) used all markers to study *Paramyrothecium*-like strains, which included *A. verrucaria* sequences *ITS*, *tub2*, *cmdA*, *tef1*, *rbp1*, *LSU* as well as the rarely used marker ATP6. The most detailed study of this issue was conducted by Pinruan et al. (2022), whose research showed that the use of *LSU*, *rpb2*, *tef1*, *tub2* sequences separately did not allow adequate phylogenetic relationships to be obtained for representatives of the genus *Albifimbria*. Bao (2023) used the multi-gene dataset with *LSU*, *SSU*, *ITS*, *tef1*, *rpb2* and obtained conflicting data, where *A. viridis* is phylogenetically more closely related to *A. terrestris* than to *A. verrucaria*. However, Rehman et al. (2021) considered that the use of the ITS region alone was sufficient to identify the pathogenic isolate, which is in principle consistent with our results.

Thus, in most cases, use of an integrated set of markers allows one to obtain an adequate phylogenetic tree, but it should be concluded that if the main aim is to identify *A. verrucaria*, this approach does not have significant advantages, because, in fact, it is based on the selection of most appropriate markers for each specific case of identification. Comparative analysis of the obtained phylogenetic tree did not reveal any fundamental differences with the trees constructed on a phylogenetic analysis of combined *LSU* rDNA, *ITS*, *tef1*, *rpb2*, *tub2* and *cmdA* sequence data by Lombard et al. (2016) and Pinruan et al. (2022). Considering the fact that *A. viridis* compared to *A. verrucaria* is a fungal species that is rarely found in nature, and can also be discriminated on a cultural level it can be assumed that the ITS sequence is a sufficient molecular marker for the identification of the latter. *A. viridis* and *A. verrucaria* species can be separated on the basis of morphological characters since *A. viridis* forms exudates on the colony surface unlike *A. verrucaria* (Tulloch, 1972).

An important approach to polyphasic taxonomy of fungi is the use of species-specific secondary metabolites, but it is difficult or even impossible to use them for species identification, since the mycotoxins that are synthesized by *Albifimbria* spp. – roridins and verrucarins – are also synthesized by other closely related species of microscopic fungi (Sun et al., 2020). Zaichenko et al. (2008) suggested using the ratio of the amount of mycotoxins synthesized by fungi of the genus *Myrothecium* as one of the identification criteria. However, this assumption has not yet received its scientific confirmation.

We analyzed species-specific secondary metabolites of *A. verrucaria* and decided to explore the possibility of the fungus synthesizing BOD enzyme as a chemotaxonomic trait. We have previously shown that synthesis of extracellular polyphenol oxidases (laccases) is intrinsic to many species of filamentous fungi (Kurchenko et al., 2023). At the same time, only the studied fungus exhibited rare BOD activity. This fact indicates that the enzymatic activity of BOD is a unique characteristic for representatives of the family Stachybotryaceae. The feasibility of synthesizing such an enzyme with such specificity is not clear, perhaps it is a side effect of the evolution of laccase enzymes.

One of the promising directions of fungal taxonomy is to analyze the secondary structure of the *ITS* region rDNA. The secondary structural features of the *ITS1* and *ITS2* regions provided advantageous data for clades, species groups, and closely related species analysis (Schultz & Wolf, 2009, Zhang et al., 2020). Since the mutation points were detected in the *ITS2* rDNA sequence, secondary structure folding and analysis were performed with *ITS2* sequences of *Albifimbria* spp. We used the sequences of 14 *A. verrucaria* verified culture collection strains and *A. terrestris*, *A. viridis*, *A. lateralis* type strains to construct the consensus model predicted minimum free energy (MFE) secondary structures of the *ITS2* region. It should be noted that the transition type of mutations we have detected is in accordance with the main trend of evolutionary drift in Ascomycota (Shen et al., 2020; van Wyk et al., 2021).

Next-generation sequencing (NGS) techniques are being actively introduced in mycological research. Short-read sequencing technologies provide a potentially powerful tool in clinical diagnosis of mycosis and species identification. Compared to Sanger sequencing, NGS are more accurate and cost-effective. Taking into account that NGS produces short reads with length less than 300 bp, the use of the shorter *ITS2* fragment as a *A. verrucaria* barcoding marker compared to the entire ITS region may have advantages.

Conclusions

It was found that the cultural and morphological characteristics of the studied micromycete correspond to the recognized classical descriptions *A. verrucaria*. Despite the claims of some researchers, morphological observations of fungi continue to play an important role in the identification of *A. verrucaria*.

Sequence analysis of the ITS rDNA region of the *M. verrucaria* UCM F-13522 showed complete identity with both the type culture of *A. verrucaria* CBS 328.52 and a number of verified culture collection strains. For the taxonomy of *Albifimbria* spp., only type and verified collection species should be used. Among the ITS rDNA regions, *ITS2* sequence is the most informative for the identification of species of the genus *Albifimbria*. Analysis of the secondary structure features of the *ITS2* region allowed us to present a consensus structure of the region and spatial patterns of mutation points. The consensus model of the secondary structure of the *ITS2* region is a promising object of research into the possibility of using it as an additional barcode in polyphasic taxonomy of the *Albifimbria* spp.

The absence of closely related species capable of synthesizing BOD, a simple and cheap method of determining the enzyme activity using a standard clinical bilirubin assay kit, allows us to recommend BOD as one of the powerful markers for *A. verrucaria* identification using a polyphasic taxonomy approach.

Thus, morphological and genetic observations, production of BOD activity support the renaming of the fungus that was previously recognized as *Myrotheicum verrucaria* UCM F-13522 as *Albifimbria verrucaria* (current name: *Albifimbria verrucaria* (Alb. and Schwein.) L. Lombard and Crous).

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