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First report of entomopatogenic fungus *Akanthomyces muscarius* on stored apples in Romania

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Abstract Fungal identification using molecular tools become nowadays the general practice in laboratories that focus on identification of new natural plant protection products, as, for example, entomopathogenic fungi. This is justified both by the diversity of fungi, the lack of taxonomists, but also the endless taxonomical debates that brings continuous modifications in fungi nomenclature. In newly initiated laboratories, molecular methods are the researchers` lifesaver. During an identification study inside a project aiming at early detection of fungal pathogens of apples, some fungi colonies were isolated from 5 months stored apples. The fungus colonies on PDA had no similar characteristics with other pathogenic fungi, as *Neofabraea* sp., *Monilinia* sp., *Botrytis* sp., *Fusarium* sp, *Penicilium* sp. Morphologically, the colonies were fluffy, circular, white with pale yellow reverse, with septate, hyaline and smooth hyphae. The conidiophores were verticillium-like branched, septate and hyaline, with numerous solitary one-celled conidia. Further identification using molecular identification tools using conventional PCR and DNA barcoding was performed. According to existing data in NCBI, BOLDsystems and Q-bank, the new detected species was identified as *Akanthomyces muscarius* (*Cordycipitaceae*, *Hypocreales*). Our study is the first reports of *A. muscarius* on stored apples in Romania.

Key words

Akanthomyces muscarius, BOLD, DNA barcoding, entomopathogenic fungus

Akanthomyces belongs to the family Cordycipitaceae (Index Fungorum, Vinit at al., 2018), Hypocreales order. Cordycipitaceae species may occur on arthropods, plants, other fungi and in soil. Other well-known entomopathogenic species in this family belongs to Beauveria and Lecanicillium genera, but they are phylogenetically distinct. *Akanthomyces novoguineensis*, another pathogenic fungus, is mentioned as parasite for spiders.

When morphological identification is difficult, molecular methods can help identify unknown specimens. This is especially crucial when uncertainties appear in areas where the pest was not previously mentioned or is listed as quarantine pest. Since 2003, when the biological identification of species through DNA barcodes was proposed by Paul Hebert and his team, millions of barcodes have been generated for hundreds of thousands of living species and the process is continuing until all eukaryotic species are sequenced. All data (genetic and metadata) is stored and can be accessed through the Barcode of Life Data Systems (BOLD; www.boldsystems.org, available for comparisons and quicker identification of future analysis.

As taxonomists are becoming an endangered species themselves, the limitations of morphology-based insect identification become obvious, especially when plant health and quarantine staff must quickly identify specimens in different development stages. Molecular tests offer a sustainable alternative to these limitations. The EPPO standard PM 7/129 (1) describes the use of DNA barcoding protocols to identify regulated pests and invasive plant species of importance to the EPPO area (Europe and the Mediterranean Region) and details all the steps required for molecular and analytical processing in order to arrive to a species name.

Our study aimed at characterizing a newly identified fungus on stored apples, which had no similar characteristics with the known postharvest mycoflora in Romania.

Materials and Methods

Fungal strain and culture conditions. Our isolate was recovered from apple fruits. The apples were collected in October 2018 and cold stored until March 2019.
Pure cultures were maintained on potato dextrose agar (PDA) for further studies.

Molecular analysis

The first DNA barcoding steps were performed at the center for Studies of food quality and agricultural products and followed the standard protocols with molecular processing in 2 ml Eppendorf tubes.

For tissue subsampling, the white hyphae of the fungus was carefully removed from the PDA and the protocol of Qiagen Plant minikit was followed.

The 658-bp barcode region of the ITS gene (the standard barcode marker in fungi; (EPPO) was amplified using a primer cocktail ITS2 ITS4

The amplification was performed in an Eppendorf Mastercycler Nexus Gradient machine, with the following thermocycling program: initial denaturation at 94°C for 1 min, 5 cycles of 94°C for 40 s, 45°C for 40 s and 72°C for 1 min, followed by 35 cycles of 94°C for 40 s, 51°C for 40 s and 72°C for 1 min, and 1 cycle at 72°C for 5 min.

Confirmation of PCR amplification was first tried at one Nanodrop 1000, but was unsuccessful.

A conventional PCR reaction was performed at Universite d'Angers - IRHS, France. DNA was extracted from pure culture (mycelium collected by scrapping the surface of Petri plate culture). Nucleic acids were isolated according to the microwave miniprep procedure (Goodwin and Lee, 1993). The final DNA pellet was suspended into 100 µl TE buffer (10 mM Tris- HCI pH 8.0, 0.1 mM EDTA) and stored at -20^{0}C until used.

The universal primer pair ITS1 - ITS4 (TCCGTAGGTGAACCTGCGG/TCCTCCGCTTATT GATATGC) was used. The PCR reaction was performed for 35 cycles with an initial 3 min. at 95^{0}C for denaturation and a final 10 min at 72^{0}C for extension. Each cycle consisted of 30s at 95^{0}C, followed by 50s at 55^{0}C for annealing and 1 min at 72^{0}C for extension. The amplification results were visualized after electrophoresis on a 1.2% agarose gel. The resulting PCR products obtained were sequenced by Eurofins Genomics. The obtained sequence (528 nucleotides) was analysed by BLAST (Basic Local Alignment Search Tool).

The aligned sequences were translated into aminoacids to check for stop codons (an indication of potential pseudogene amplification) in MEGA7.0. Three molecular databases were used for sequence comparison, namely: BOLD (largest database dedicated to DNA barcodes; www.boldsystems.org, 37), GenBank (largest molecular database; https://www.ncbi.nlm.nih.gov/genbank/ ref number -> https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4702903/) and Q-bank (database focused on quarantine plant pests and diseases of importance to the EPPO region, the result of the Quarantine Organisms Barcode of Life; http://www.q-bank.eu, ref -> https://onlinelibrary.wiley.com/doi/full/10.1111/j.1365-2338.2009.02350.x).

Results and Discussions

Morphological observations

Morphological characteristics as colony color (above and below), shape, surface were observed. Colonies on PDA were fluffy, circular, white above (fig.1A) with pale yellow reverse (fig. 1B). The fungus mycelium presented septate, hyaline and smooth hyphae. Conidiophores are verticilium-like branched (fig.2A), sometimes unbranched, erect, flexuous, septate. Conidia were numerous (fig.2B), one-celled, solitary, variable in size, slimy, ellipsoidal, subcylindrical to cylindrical, smooth, hyaline to subhyaline. Our observation is similar with that of Vinit et al, 2018, although it was observed on different host plant (nipa palm).

Molecular analyses

All three public databases, BOLDsystems, GenBank and Q-Bank returned *Akanthomyces muscarius* with 98% similarity as match, upon using the BOLD ID Engine, Blast or Q-Bank identification tool. Based on ITS region amplification (ITS1/ITS4) our isolate is reported as a new record for Romania.

The obtained sequence had 528 nucleotides:

CCTTATGTGATACCTACTGTTGCTTCGGCGGAC
TCGCCCCGGCGTCCGGACGGCCTCGCGCCGCC
CGGGCCCGGACCCAGGCGGCCGCCGGAGACCT
CCAAACTCTGTATTATCAGCATTTTCTGAATCC
GCCGCAAGGCAAAACAAATGAATCAAAACTTT
CAACAACGGATCTCTTGGTTCTGGCATCGATG
AAGAACGCAGCGAAATGCGATAAGTAATGTG
AATTGCAGAATTCAGTGAATCATCGAATCTTT
GAACGCACATTGCGCCCGCCAGCATTCTGGCG
GGCATGCCTGTTCGAGCGTCATTTCAACCCTCG
ACTTCCCTTTGGGTCGGCGTTGGGGAACGGCA
GCATACCGCCGGCCCCGAAATGGAGTGGCGGC
CCGTCCGCGGCGACCCTGCGTAGTAATCCAAC
TCGCACCGGAACCCCGACGTGGCCACGCCGTA
AAACACCCAACTTCTGAACGTTGACCTCGGAT
CAGGTAGGAATACCCGCTGAACTTAAGCATAT
CAATAAGCGGAGGA

Akanthomyces species are most commonly known for their potential in biological control of plant pests and, recently, as fungal plant endophytes, in leaves or fruits (Sarma et al., 2018; Vinit et al., 2018).

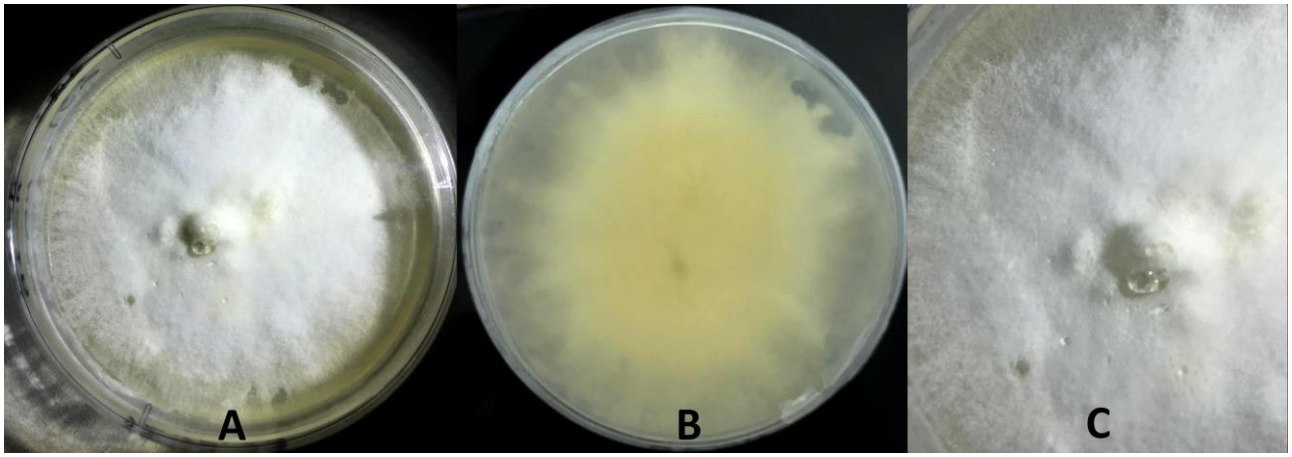

Fig. 1. *Akanthomyces muscarius* colony culture characteristics on PDA.
A. white above B. reverse pale-yellow C. colony growing detail

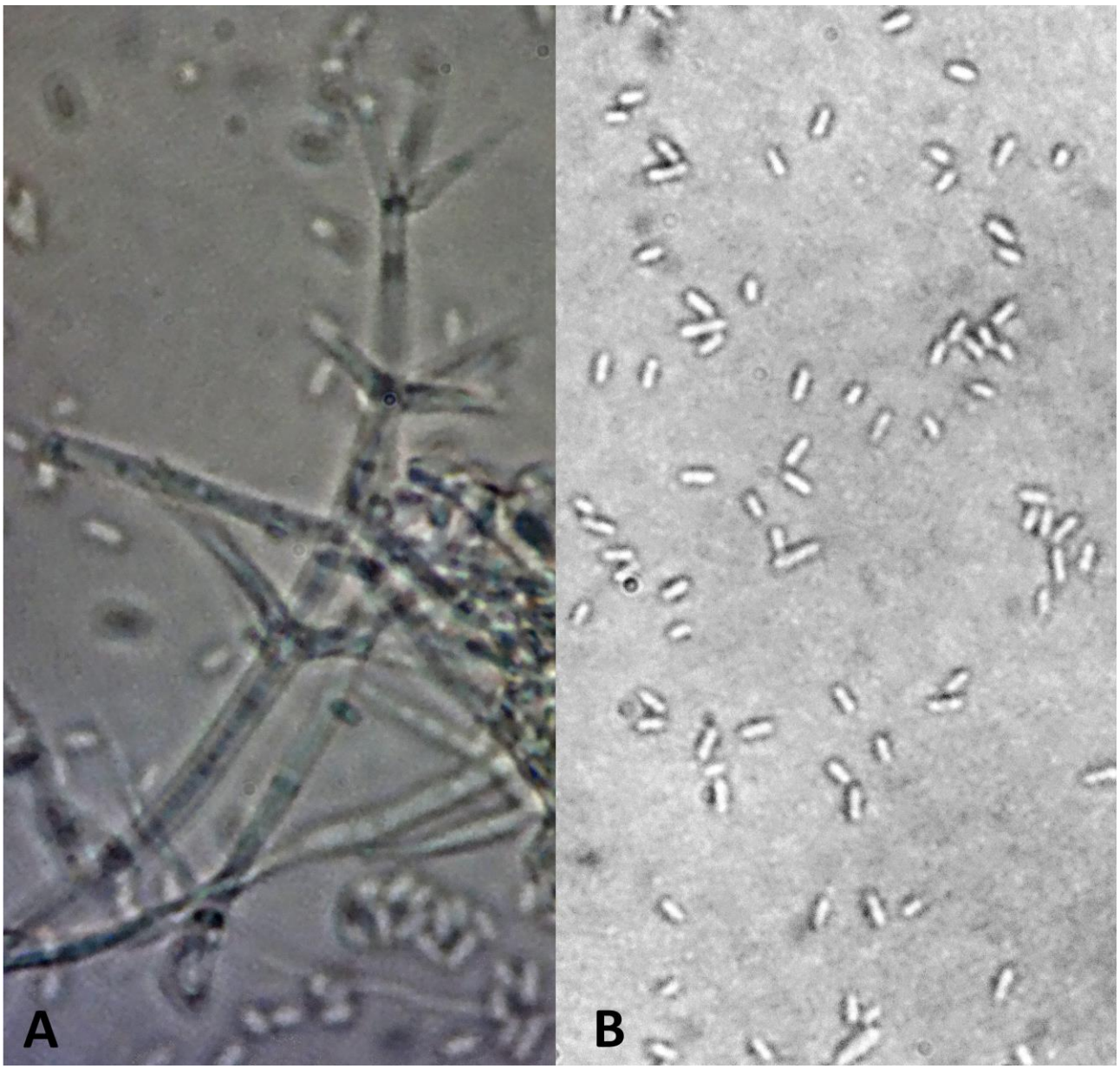

Fig. 2. *Akanthomyces muscarius*. A. verticilium-like branched conidiophores. B. Conidia

Conclusions

The present study reports the presence of *Akanthomyces muscarius* as a new record in Romania by morphological identification and DNA barcoding.

Descriptions of the isolate match well with previously published data and our phylogeny supports the species identification. This is the first attempt in identification of a new entompathognic fungus in Romania by barcoding.

Further studies are needed to characterize the newly found species from phylogenetic point of view and to determine its pathogenic potential against pest.

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