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First report of *Aspergillus nomius* isolation from Hungarian apple: An emerging mycotoxigenic threat in Central Europe

E. Leandro Rodrigues[1*], Zs. Zalán[1] and I. Bata-Vidács[2]

[1] Department of Bioengineering and Fermentation Technology, Hungarian University of Agriculture and Life Sciences, Budapest, Hungary

[2] Centre for Research and Development, Eszterházy Károly Catholic University, Eger, Hungary

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ABSTRACT

Anthropogenic climate change is reshaping fungal dynamics in European agro-ecosystems, favoring thermotolerant and xerotolerant species once restricted to tropical and subtropical zones. Among these, *Aspergillus* section *Flavi* poses a major concern due to its production of carcinogenic aflatoxins, particularly aflatoxin B1 (AFB1). In this context, *Aspergillus nomius*, a known producer of both B- and G-series aflatoxins, has gained attention for its growing presence in non-tropical regions. We report the first confirmed isolation of *A. nomius* from a Hungarian crop, apple, marking its entry into the national food supply. Morphological characteristics aligned with species descriptions, and molecular identification was confirmed *via* ITS and β-tubulin gene sequencing. The isolate exhibited AFB1 production, highlighting its potential role in mycotoxin contamination in temperate environments.

This discovery expands the known ecological range of *A. nomius* and supports evidence that aflatoxigenic fungi are shifting their distribution due to rising temperatures and altered environmental conditions. The detection of this high-risk species in a Central European crop underscores the urgent need to revise monitoring protocols and develop climate-adaptive mycotoxin management strategies. As fungal biodiversity rapidly adjusts to climatic pressures, integrated surveillance using morphological, molecular, and toxin-based approaches will be critical to ensuring food safety in increasingly vulnerable agro-ecosystems.

KEYWORDS

Aspergillus nomius, climate change, Hungarian crops, aflatoxin

* Corresponding author. E-mail: emelin.l.rodrigues@gmail.com

1. INTRODUCTION

Anthropogenic climate change is reforming biological groups from microbes to megafauna, and filamentous fungi respond especially quickly to the new selective landscape. Warmer mean temperatures, longer heat-wave episodes, shifting rainfall regimes, and extended crop-growing seasons are altering the competitive balance among fungal taxa, favoring thermotolerant and xerotolerant species that were historically restricted to lower latitudes (Intergovernmental Panel on Climate Change (IPCC), 2022).

Within European agro-ecosystems this ecological drift is already evident: members of *Aspergillus* section *Flavi*, long regarded as tropical or subtropical, are now encountered with increasing frequency in Southern and Central Europe (Miraglia et al., 2009). The phenomenon is more than an academic curiosity. Many *Flavi* species synthesise highly toxic secondary metabolites, and shifting fungal community structure, therefore, has direct repercussions for food-chain safety. Because mycotoxins survive most processing and thermal treatments, even low-level contamination of staple commodities can translate into chronic dietary exposure for humans and livestock.

Aflatoxin B1 (AFB1) has surfaced as the most pressing toxin in this context. Classified by the International Agency for Research on Cancer as a Group 1 carcinogen, AFB1 is genotoxic, hepatocarcinogenic, immunomodulatory, and strictly regulated worldwide. Europa's first large-scale aflatoxin episode occurred during the record heatwave of 2003, when *Aspergillus flavus* colonised Italian maize and drove AFB1 concentrations well above the European Union's legal limit of $5\ \mu g\ kg^{-1}$ in raw grain (Battilani et al., 2016). A second, continent-wide outbreak followed the unusually hot and dry summer of 2012. Both incidents aligned closely with meteorological conditions known to favour *Flavi* growth and toxigenesis, providing empirical validation for modelling studies that couple regional climate scenarios with fungal-crop interaction kinetics. In a pan-European simulation, Battilani and colleagues demonstrated that a modest $+2\ ^{\circ}C$ rise in mean temperature is sufficient to push vast swaths of maize acreage beyond the threshold of regulatory concern, with contamination risks escalating further under a $+5\ ^{\circ}C$ trajectory (Battilani et al., 2016).

Approximately 30 years ago, Richard et al. (1992) tested the mycotoxin producing abilities of 22 isolates collected from various sources in Hungary, and none of the isolates were found to produce aflatoxins. However, Borbély et al. (2010) in 2010 examined mycotoxin levels in cereal samples and mixed feed samples collected in eastern Hungary, and detected aflatoxin B1 levels above the EU limit in 4.8% of the samples. Dobolyi et al. (2013) were the first to identify aflatoxin producing *A. flavus* isolates in several maize fields in Hungary in 2012–2013.

These projections underscore a critical food-safety dilemma: the suite of fungal pathogens confronting European agriculture is in flux, yet surveillance and mitigation frameworks remain calibrated to historical baselines. Continuous monitoring of fungal communities, through molecular diagnostics, ecological modelling, and toxin analytics, has therefore become an indispensable component of climate adaptation in the food sector (Miraglia et al., 2009; Medina et al., 2017).

Against this backdrop, *Aspergillus nomius* has attracted growing attention. First described by Kurtzman et al. (1987), *A. nomius* is phylogenetically allied to *A. flavus* and *Aspergillus parasiticus* but differs in its toxin profile: it routinely produces both B- and G-series aflatoxins, whereas *A. flavus* generally synthesises only B-types. Morphologically, colonies on Czapek yeast-extract agar are fast-growing, yellow-to olive-green, with rough-walled conidiophores 400–800 μm long that terminate in globose vesicles 20–45 μm in diameter; conidia are typically

3–6 μm, smooth to finely roughened. Optimal growth and aflatoxin production occur between 33 °C and 37 °C, and the species is tolerant of low water activity, a physiological suite that confers competitive advantage under the hotter, drier field conditions projected for many European summers (Olsen et al., 2008).

Historically, *A. nomius* was isolated from tropical substrates such as Brazilian nuts, peanuts, and maize, and from warm agricultural soils across the Americas and Southeast Asia (Kumeda et al., 2003; Reis et al., 2014; Moore et al., 2015). Over the past decade, however, its geographic footprint has expanded. Isolates capable of high-level AFB1 and AFG1 production have been recovered from maize in Serbia, Croatia, and most recently Central Italy, challenging long-held assumptions about the bioclimatic limits of aflatoxigenic fungi (Olsen et al., 2008; Battilani et al., 2016). The arrival of species into temperate agroecosystems is therefore a good example of the kind of pathogen redistribution that climate change is expected to accelerate.

The present study adds new data to this relating narrative by documenting the first confirmed isolation of *A. nomius* in a Hungarian crop. By reporting the appearance of this high-risk species in a region once considered climatically secure from aflatoxins, we underscore the urgency of adaptive surveillance and risk-management strategies capable of keeping pace with a rapidly changing mycobiota.

2. MATERIALS AND METHODS

2.1. Apple samples

In September 2022, healthy apple fruits (*Malus domestica*) were collected from a commercial orchard in Újfehértó (47°47′55.64″N, 21°40′59.88″E), Hungary, equipped with an anti-hail ice net system. Fruits were placed in sterile bags, transported under refrigeration, and processed within 24 h.

2.2. Fungal isolation

Fungal isolation was performed using Malt Extract Agar (MEA; VWR). An apple was placed into sterile Stomacher® bags containing 90 mL of sterile 0.1% peptone saline solution. After vigorous shaking for 2 min, a tenfold serial dilution was prepared and 100 μL aliquots were spread onto MEA plates. Plates were incubated at 37 °C for 7 days to favour the growth of *Aspergillus* species and hinder *Penicillium* species that are expected in higher numbers on apples. Colonies showing *Aspergillus*-like morphology were subcultured on fresh MEA to obtain pure isolates and stored in glycerol-Malt Extract Broth (MEB) at −20 °C. This procedure was done in triplicate.

2.3. Morphological and cultural characterisation

The isolate identified as *A. nomius* was cultivated on three different media: MEA (VWR), Potato Dextrose Agar (PDA; VWR), and Czapek-Dox Agar (CDA; VWR) to assess colony morphology. Plates were incubated at 37 °C for 7 days. Colony characteristics were recorded.

2.4. PCR amplification

PCR amplification targeted the β-tubulin and internal transcribed spacer (ITS) regions using the ITS1 and ITS4 primers, which amplify the ITS1-5.8S-ITS2 region of the rDNA. The reaction

mixture (50 µL) consisted of 35 ng of DNA template, 5 µL of 10X DreamTaq Green Buffer (Thermo Fisher Scientific, USA), 0.2 µM of forward primer, 0.2 µM of reverse primer (Integrated DNA Technologies), 0.1 µM of dNTP, and 0.5 mL of 1U of Taq DNA polymerase (Thermo Fisher Scientific). The final volume was adjusted to 50 µL with nuclease-free water.

PCR conditions followed (Nasri et al., 2015), with an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 45 s, and 72 °C for 1 min, concluding with a final extension at 72 °C for 5 min. The primer sequences can be found in Table 1.

2.5. Sanger sequencing

The Sanger sequencing reactions were prepared in a final volume of 5 µL, containing 0.75 µL of Big Dye Terminator v1.1, v3.1 5X Sequencing buffer (Thermo Fisher Scientific, USA), 0.5 µL of Big Dye™ Terminator v3.1 enzyme, 0.5 µM of forward primer (Bt2a or ITS1), 3 µL of Milli-Q water, and 0.5 µL of PCR template at a concentration of at least 35 ng μL^{-1}. The thermal cycling conditions included 28 cycles of 96 °C for 10 s for denaturation, 50 °C for 5 s for primer annealing, and 60 °C for 4 min for elongation.

Following the sequencing reaction, a precipitation step was performed to remove excess reagents and buffer components. The purification mixture consisted of 3 µL of 3M sodium acetate, 14.5 µL of Milli-Q water, and 62.5 µL of ethanol. A volume of 80 µL of this mixture was added to each sample, followed by incubation for 10 min at 25 °C. The samples were then centrifuged at 43,800×*g* for 20 min at 4 °C. The supernatant was carefully removed, and 180 µL of 70% ethanol was added. The samples were centrifuged again under the same conditions. After removing the supernatant, the tubes were inverted and centrifuged at 180×*g* for 1 min to allow complete drying of the pellet.

Subsequently, the purified PCR product was resuspended in 20 µL of Hi-Di™ (Thermo Fisher Scientific, USA) Formamide, a reagent that ensures proper DNA denaturation and stability before sequencing. The samples were then subjected to sequencing using the Hitachi 3500 Genetic Analyzer (Applied Biosystems, USA) machine. The resulting sequences were analysed using MEGA X software and compared against the National Center for Biotechnology Information (NCBI) database for identification and verification.

2.6. Aflatoxin detection by TLC

The *A. nomius* isolate was cultivated in 20 mL of Malt Extract Broth (MEB) at 35 °C for 10 days. After incubation, 4 mL of the suspension was transferred into a 15 mL Falcon tube, and 2 mL of dichloromethane (DCM) was added. The mixture was shaken vigorously and let stand in the dark for 20 min. One millilitre of the lower DCM phase was transferred to an Eppendorf tube and centrifuged at 14,000 r.p.m. for 1 min. A 0.5 mL aliquot of the supernatant was transferred to a clean tube and evaporated at 40 °C in a thermoshaker (BIOSAN TS-100) under a fume hood. The

Table 1. Primer sequences used in the study

	Sequence
Forward β-tubulin (Bt2a)	5′-GGT AAC CAA ATC GGT GCT GCT TTC-3′
Reverse β-tubulin (Bt2b)	5′-ACC CTC AGT GTA GTG ACC CTT GGC-3′
Forward ITS1	5′-TCC GTA GGT GAA CCT GCG G-3′
Reverse ITS4	5′-TCC TCC GCT TAT TGA TAT GC-3′

residue was dissolved in 100 µL of 10% acetonitrile. A 10 µL aliquot was applied to TLC Silica Gel 60 plates (20 × 20 cm; Merck). The mobile phase was toluene:ethyl acetate:formic acid (6:3:1, v/v/v). Plates were developed, dried under a fume hood, and examined under UV light (365 nm). Aflatoxins were identified based on fluorescence and retention factors in comparison with aflatoxin B_1 and G_1 mixture standard (LGC, Toronto Research Chemicals, Canada).

3. RESULTS AND DISCUSSION

3.1. Fungal isolation

From the three healthy apple samples collected in the Hungarian orchard, a total of 6 fungal isolates exhibiting *Aspergillus*-like morphology were recovered on Malt Extract Agar after incubation at 37 °C for 7 days. Among these, one isolate was selected for further characterisation based on morphological traits.

3.2. Molecular identification

Genomic DNA was successfully extracted from the isolate, and PCR amplification of ITS and partial β-tubulin (*BenA*) regions produced clear amplicons of expected sizes. Sequence analysis using BLASTn revealed ≥99% identity and over 95% query coverage with reference *A. nomius* sequences available in the NCBI GenBank. Phylogenetic analysis based on the Maximum Likelihood method placed the isolate firmly within the *A. nomius* clade, clearly distinct from closely related *A. flavus* and *A. parasiticus* strains, confirming its molecular identification (Fig. 1).

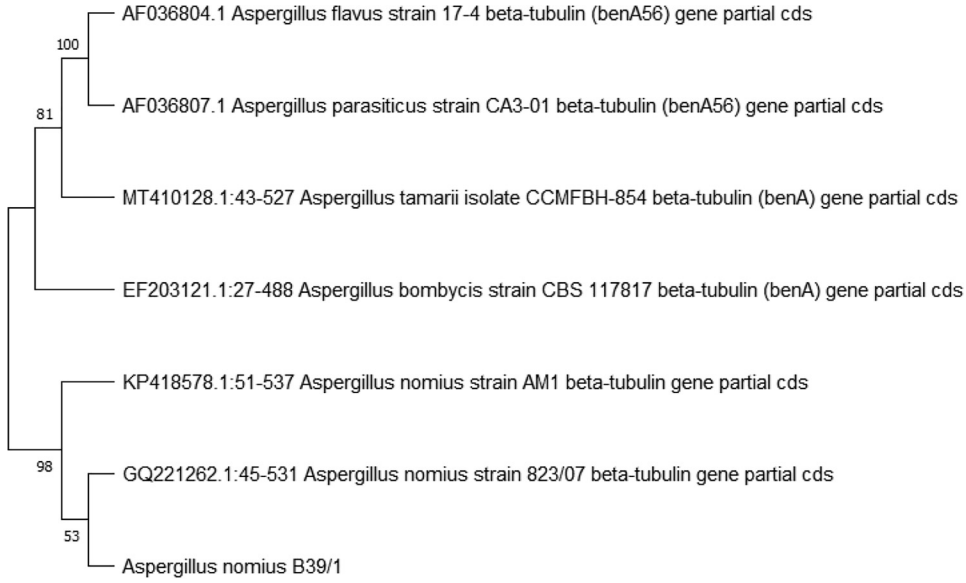

Fig. 1. Phylogenetic tree based on partial β-tubulin (*BenA*) gene sequences showing the relationship of the Hungarian isolate *Aspergillus nomius* B39/1 to reference strains of *Aspergillus* section *Flavi*

Our study presents the first known isolation of *A. nomius* from a crop source in the country, specifically from an apple. To the best of our knowledge, this represents the first report of *A. nomius* associated with a fruit crop in Hungary.

3.3. Morphological characteristics of *A. nomius* B39/1

Microscopic examination revealed rough-walled conidiophores terminating in globose vesicles, and conidia were spherical to sub-spherical, smooth to finely roughened (Fig. 2). These characteristics align with those described for *A. nomius* in previous studies (Kurtzman et al., 1987; Olsen et al., 2008).

The macromorphological characteristics of *A. nomius* B39/1 were evaluated on three different culture media: Malt Extract Agar (MEA), Potato Dextrose Agar (PDA), and Czapek Agar (CZA), after incubation at 25 °C for 7 days (Fig. 3).

On MEA, colonies exhibited a dense, velvety to floccose texture with a dominant white mycelial growth and yellow pigmentation concentrated in the center and periphery. Radial furrows were evident, while sporulation appeared limited. The colony reverse showed an intense yellowish-brown coloration. On PDA, colonies were fast-growing and showed abundant sporulation, resulting in a powdery to granular olive-green surface. The colony margin was regular and circular, with slight central elevation and concentric zonation. In contrast, growth on CZA was more compact and restricted, displaying a fine granular texture with yellow to dull green pigmentation, and moderate conidiation.

These morphological differences across media highlight the adaptability of the fungus under varying nutritional conditions.

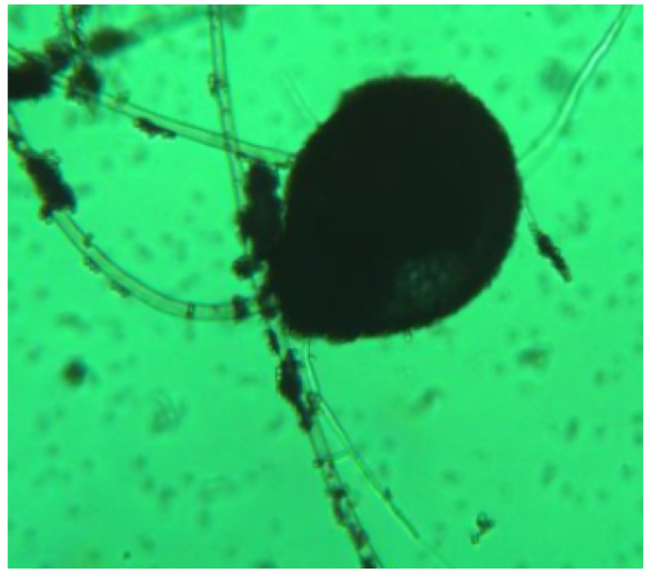

Fig. 2. Microscopic morphology of *Aspergillus nomius* isolate B39/1 under 40 × objective lens

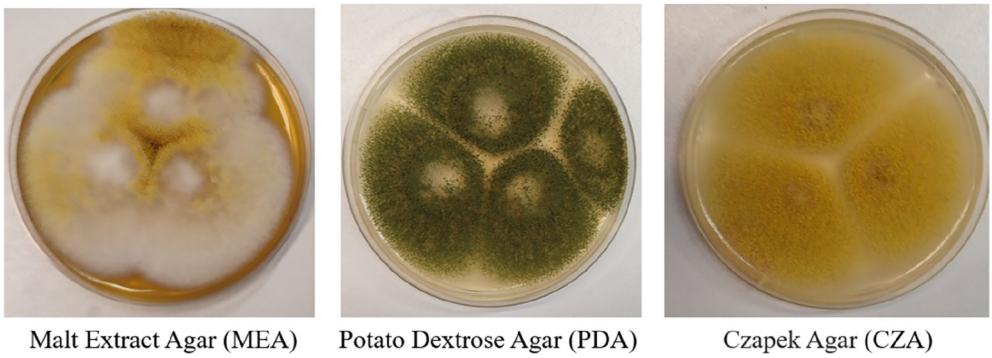

Fig. 3. Colony morphology of *Aspergillus nomius* B39/1 on different culture media after incubation at 25 °C for 7 days

3.4. Aflatoxin detection by TLC

Following the macroscopic and microscopic characterisation, the potential for aflatoxin production was further investigated to assess its toxigenic profile. Thin-layer chromatography analysis of culture extracts demonstrated that the *A. nomius* B39/1 isolate produces only B type aflatoxin (Fig. 4).

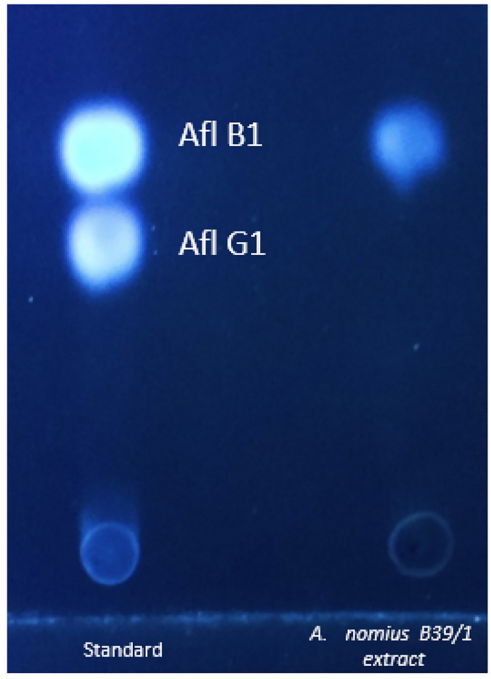

Fig. 4. Thin layer chromatography for aflatoxin production of *A. nomius* B39/1

Fluorescent spots matching the retention factors of aflatoxin B_1 standards were detected under UV light (365 nm), confirming the isolate's aflatoxigenic potential, in contrast to the findings of Baranyi et al. (2015), who reported *A. nomius* isolated from cheese samples in Hungary with the ability to produce both B- and G-type aflatoxins. This phenotypic divergence suggests potential strain-level variation in toxigenic profiles, and highlights the need for further mycotoxicological surveillance of *A. nomius* in diverse agroecological niches.

4. CONCLUSIONS

This study presents the first report of *A. nomius,* named B39/1, isolated from apple in Hungary, representing a new occurrence of this aflatoxigenic species in a Hungarian crop. Identification was confirmed through detailed morphological analysis and molecular characterisation using ITS and β-tubulin gene sequencing. The isolate demonstrated the ability to produce aflatoxin B1, underscoring its potential risk to food safety.

The emergence of *A. nomius* in Hungary, in a previously unreported source and geographic location, highlights a growing concern regarding the shifting distribution of toxigenic fungi. In the context of global climate change, rising temperatures and changing environmental conditions may favour the proliferation of aflatoxin-producing species in temperate regions. This finding emphasises the need for enhanced monitoring and mycological surveillance in agricultural systems, as well as proactive risk assessment strategies to address the potential impact of climate-driven shifts in fungal biodiversity on crop contamination and public health.

Conflict of interest: I. Bata-Vidács is a member of the Editorial Board of the journal, therefore she did not take part in the review process in any capacity and the submission was handled by a different member of the editorial board. The submission was subject to the same process as any other manuscript and editorial board membership had no influence on editorial consideration and the final decision.

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