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Aspergillus homomorphus, a first global record from millet grains

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Abstract

During surveys of mycobiota associated with millet (*Pennisetum glaucum* L.) grains from Taiz Governorate, Yemen, an interesting isolate of *Aspergillus* section *Nigri* was isolated. It was recorded once on Czapek´s agar at 28ºC. The isolate was examined and identified macroscopically, microscopically and genotypically as *Aspergillus homomorphus*. It is recorded for the first time from millet grains and for only the third time since its original description in 1994. The strain was deposited at Assiut University Mycological Centre (AUMC), and the ITS sequence was deposited in the National Centre of Biotechnology Information (GenBank).

Key words – Assiut University Mycological Centre – Czapek´s agar – DNA sequencing – Section *Nigri* – Yemen.

Introduction

Members of *Aspergillus* section *Nigri* (black aspergilli) are important in biotechnological processes as well as in biodeterioration. Some strains of black aspergilli are often misidentified as *A. niger* due to the difficulties of identifying the species in this group (Samson et al. 2007). Recent surveys on the mycobiota of distinct commodities revealed new species of section *Nigri* unknown to science (Perrone et al. 2007). For many years black aspergilli, and indeed all *Aspergillus* spp. were identified to species by morphological criteria (Raper & Fennell 1965, Pitt & Hocking 2009, Oliveri & Catara 2011). such as colour, density and colony appearance and microscopic observations (conidial head, conidiophore and conidial characters). The taxonomy of the section was revised by Abarca et al. (2004). and 15 taxa were accepted in the last critical revisions: *A. aculeatus, A. brasiliensis, A. carbonarius, A. costaricaensis, A. ellipticus, A. japonicus, A. foetidus, A. heteromorphus, A. homomorphus, A. lacticoffeatus, A. niger, A. piperis, A. sclerotioniger, A. tubingensis* and *A. vadensis* (Samson et al. 2004, 2007). Subsequently, many species of this section were described based mainly on their genotypic characteristics (Serra et al. 2006, Noonim et al. 2008, Perrone et al. 2008, Sørensen et al. 2011, Varga et al. 2011, Jurjević et al. 2012). *A. homomorphus* was not only separated from related species in the section by morphological distinction but also by molecular analyses (Kallow et al. 2006). Among morphological differences, this species has conidia that are 5–7 µm in diameter, which separates it from *A. carbonarius* (7–9

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µm diam.) and *A. niger* aggregate (3–5 µm diam.) (Samson & Varga 2007). Perrone et al. (2007) reported that nine different black *Aspergillus* species are often difficult to identify with classical methods, but can be distinguished by molecular techniques.

During mycobiotic surveys carried out continuously in the Mycological Laboratory at the Department of Botany and Microbiology, Faculty of Science, Assiut University, an unusual black strain of *Aspergillus* was isolated from millet grains collected from Taiz City, Yemen and placed on Czapek´s agar medium at 28ºC. It was identified based on macro – and micromorphology and genotypically as belonging to *Aspergillus homomorphus*. This species was first isolated and described (invalidly) together with the closely related species (*A. pseudo-heteromorphus*), as new species from soil samples collected near the Dead Sea (Steiman et al. 1994). Samson et al. (2004). validated *A. homomorphus* and synonymized *A. pseudo–heteromorphus* with *A. homomorphus* since they were identical in both extrolites and DNA sequences. *A. homomorphus* was later recorded from a soil sample in Mexico (Borrego–Terrazas et al. 2014). and soil in the arid regions of Oman (Al-Sadi /ncbi website).

Materials & Methods

Source of the isolate

The isolate was obtained during surveys of grain-borne fungi of millet (*Pennisetum glaucum* L.) samples collected from Taiz Governorate, Yemen. For isolation of fungi the grains were placed on Czapek´s agar medium and incubated at 28ºC.

Media and growth conditions for identification

The isolate was cultivated and examined on the following standard media: Czapek´s yeast autolysate agar (Samson & Pitt 1985). Czapek´s agar (Raper & Thom 1949). Czapek´s with 20% sucrose ,(Cz20S), (Raper & Fennell 1965). malt extract agar, (MEA) (Blakeslee 1915). malt yeast with 40% sucrose agar, (M40Y), (Raper & Fennell 1965). glycerol 25% nitrate agar, (G25N), (Pitt 1973). creatine sucrose agar, (CREA), (Frisvad 1985). tannin sucrose agar, (TAN), (Thrane 1986). urea agar (UA), mannitol agar, (MA), (Brayford & Bridge 1989). and Czapek´s starch agar (CzSA) in which sucrose was replaced by soluble starch (Bridge 1985). Three replicates of 3-pointed inoculated plates of all media were incubated at 25ºC except for CYA, plates were incubated at 5º, 25º, 37º and 45ºC. Growth rates were recorded on CYA, Cz and MEA after 7 days of incubation. Assessment of growth on media with reduced water activity (Cz20S, G25N and M40Y). was also carried out after 7 days?. Turning the UA medium pink was referred as urease positive. A positive amylase result was indicated by the appearance of a clear zone around the colony after flooding with iodine solution (Cowan 1974).

Growth rates

Colony diameters were measured on all media and all tested temperatures. The results on MA were assessed by growth and acid production turning phenol red pH indicator from red to yellow. Growth and base production on CREA were also recorded by visible colour change of medium from purple to yellow.

Phenotypic characters

Assessment of colony characteristics (colony texture, colour, exudate, reverse and soluble pigment) was carried out. The Methuen Handbook of Colour (Kornerup & Wanscher 1989). was used for evaluation of colour. Microscopic measurements of conidial heads, stipes (conidiophores), vesicles, supporting cells (metulae), conidiogenous cells (phialides) and conidia were determined. Surface and colour of the stipe and conidia were also recorded.

Sample preparation for Scanning Electron topography Microscope (SEM)

Discs from culture grown on CYA at 25ºC for 5 days were soaked in a fixative solution at pH

7.2. The discs were then washed in cacodilate buffer, post-fixed in 1% osmium tetroxide solution and water for 1 hour, followed by dehydration in increasingly more concentrated acetone solutions. Afterwards, the samples were dried in a desiccator containing silica. The specimens were clustered on a film of aluminum foil, then coated with gold in a sputter and observed in a scanning electron microscope, SEM (JEOL, JSM–5400 LV unit, Assiut University, Assiut, Egypt) (Silva et al. 2011).

Photography

Growth on all agar media as well as microscopic structures were photographed. Moreover, a number of images from SEM were digitally produced and registered at variable magnifications.

Genetic identification

The isolate was grown on MEA plates and incubated at 28ºC for 4 days. Mycelial growth was scraped off and immersed in 100 µl of distilled water and boiled at 100ºC for 15 minutes. It was then sent to SolGent Company, Daejeon, South Korea for DNA extraction and sequencing. Internal transcribed spacer (ITS) sequences of nuclear rDNA were amplified using universal primers ITS1 (5' – TCC GTA GGT GAA CCT GCG G – 3') and ITS4 (5'– TCC TCC GCT TAT TGA TAT GC – 3'). Then amplification was carried out using the polymerase chain reaction, PCR (ABI, 9700). The PCR reaction mixtures were prepared using SolGent EF–Taq as follows: 10X EF–Taq buffer 2.5 µl, 10 mM dNTP (T) 0.5 µl, primer (F–10p) 1.0 µl, primer (R–10p) 1.0 µl, EF– Taq (2.5U) 0.25 µl, template 1.0 µl, DW to 25 µl. Then the amplification was conducted using the following PCR reaction conditions: one round of amplification consisting of denaturation at 95ºC for 15 min followed by 30 cycles of denaturation at 95ºC for 20 sec, annealing at 50ºC for 40 sec and extension at 72ºC for 1 min, with a final extension step of 72ºC for 5 min. The PCR products were then purified with the SolGent PCR purification Kit–Ultra (SolGent, Daejeon, South Korea) prior to sequencing. The purified PCR products were reconfirmed (using size marker) by electrophoresis of the PCR products on 1% agarose gel. The bands were eluted and sequenced. Each sample was sequenced in the forward and reverse direction. The sequence alignments were performed using Clustal W analysis and the phylogenetic analysis of ITS retrieved from GenBank database (Moubasher et al. 2016). Contigs were created from the sequence data using CLCBio Main Workbench program. The sequence obtained from the target isolate was further analyzed using BLAST from the National Center of Biotechnology Information (NCBI) website. The sequence was subjected to Clustal W analysis using MegAlign (DNAStar) software version 5.05 for the phylogenetic analysis. The ITS sequence was deposited in Genbank (Thompson et al. 1994).

Results

Colony characteristics of Aspergillus homomorphus Steiman, Guiraud, Sage & Seigle-Murandi ex Samson and Frisvad

On Cz at 25ºC after 7 days: Colonies 20–28 mm diam, floccose, margin white, conidiogenesis moderate to heavy; colour black (M. 6E–F1) to carbon black (Kornerup & Wanscher 1989). sclerotia absent, exudate, reverse and soluble pigment absent.

On CYA at 25ºC after 7 days: Colonies 45–64 mm diam, floccose, margin white, conidiogenesis as on Cz; colour carbon black; sclerotia absent; exudate and soluble pigment absent; reverse colour yellowish white, pale yellow to pastel yellow (M. 2A2–4).

On CYA at 5º and 45ºC after 7 days: No growth.

On CYA at 37ºC after 7 days: Colonies 30–50 mm diam, colony texture, colour, conidiogenesis and reverse as on CYA at 25ºC after 7 days.

On MEA at 25ºC after 7 days: Colonies 40–60 mm diam, floccose, margin white, conidiogenesis moderate; colour black; sclerotia; exudate, reverse and soluble pigment absent (Fig 1). Colony diameters of the strain on other media was presented in (Table 1).

Table 1 Growth rate of *Aspergillus homomorphus* on different media.

Medium	Colony diameter (in mm)
Malt yeast with 40% sucrose agar (M40Y)	40 – 55
Czapek´s with 20% sucrose (Cz20S)	32 – 38
Creatine sucrose agar (CREA)	21 – 32
Tannin sucrose agar (TAN)	24 – 35
Glycerol 25% nitrate agar (G25N)	2 – 10
Czapek´s starch agar (CzSA)	22-24
Urea agar (UA)	35 – 48
Mannitol agar (MA)	17 – 25

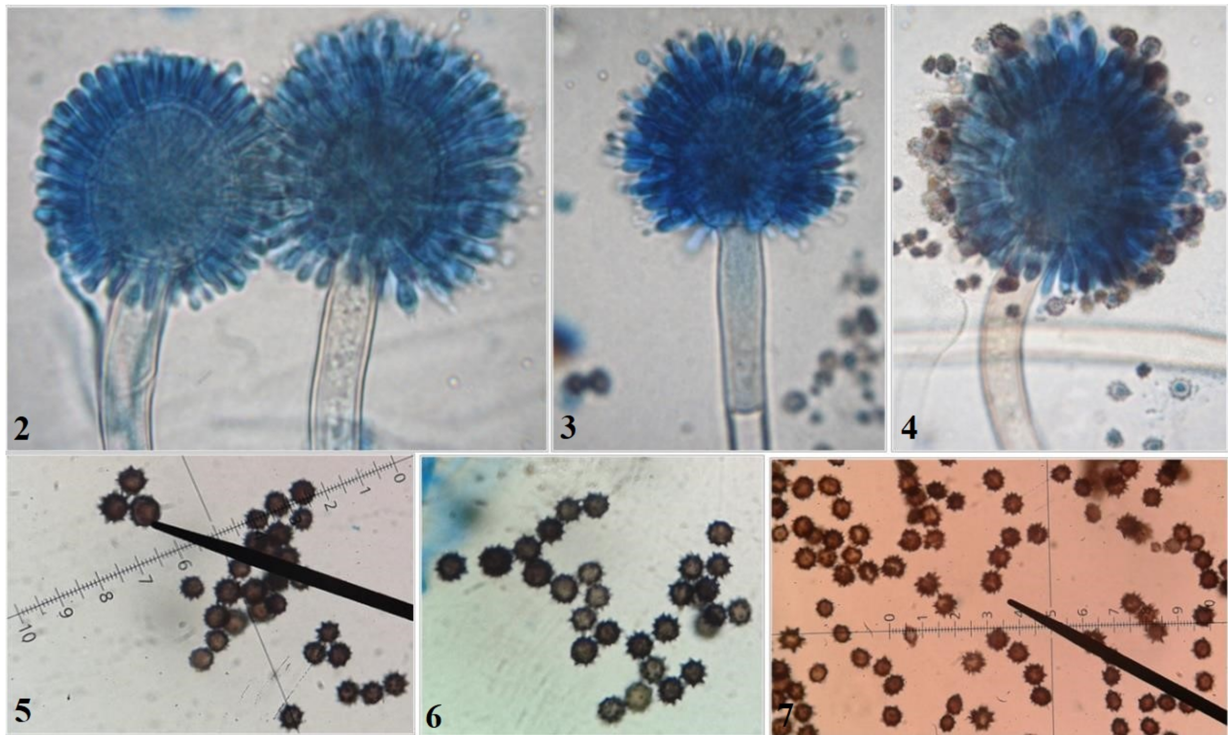
Note: The fungus showed base production on CREA, amylase production, but negative urease activity. In respect to MA, acid production was not observed.

Fig 1 – *Aspergillus homomorphus* AUMC 10521 grown on CYA at 5º, 25º, 37º and 45ºC; and MEA, Cz, Cz20%S, G25N, mannitol, creatine, tannin, M40Y and urea at 25ºC.

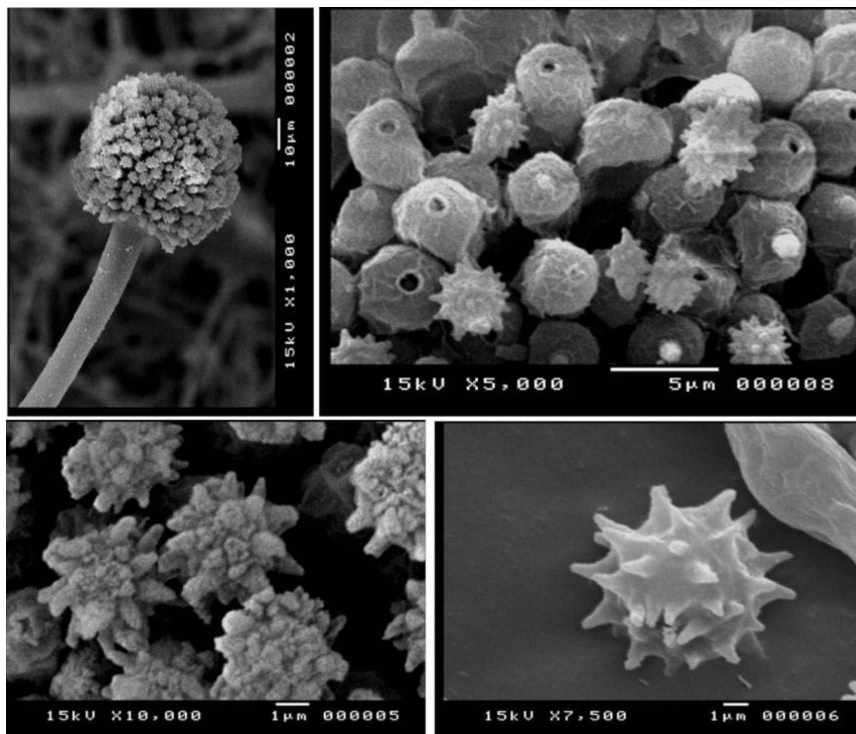
Microscopic features

On CYA, conidial heads biseriate, radiate to globose, 80–180 µm diam, splitting with age into defined columns, stipes smooth-walled, light yellowish brown colour below the vesicle, 400–875 x 9–15 µm, sometimes with septum below the vesicle, vesicle globose, subglobose to less

commonly spathulate, brown, 27–50 µm diam, but slightly smaller in uniseriate heads (20–45 µm diam), metulae present, 7–8 µm long; phialides 9–15 x 4–5 µm, conidia globose to subglobose, conspicuously spiny to echinate at maturity, mature conidia dark brown, 5–7 µm diam, but up to 8 µm diam on MEA, with spines up to 2 µm in length (Figs 2–11).



Figs 2–7 – Microscopic structure of *A. homomorphus* AUMC 10521. 2–4 Conidial heads. 5–7 Conidia.



Figs 8–11 – Scanning electron micrographs of *A. homomorphus* AUMC 10521. 8 Stipes and conidial head. 9 Phialides and conidia. 10–11 Conidia.

Molecular identification

ITS sequence of *A. homomorphus* AUMC 10521 showed high similarity (> 99%) with GenBank accession numbers NR-077189 = CBS 101889^T (type strain), EU821330 = CBS 101889^T, KU94906 = SQU-MA06 and KJ888832 = TAA50 and 94.2-94.7 with *A. aculeatinus* (NR_135417 CBS 121060^T)*, A. indologenus* (AJ28305 = CBS 114.80)*, A. uvarum* (NR_135330 = CBS 121591^T)*, A. brunneoviolaceus* (NR_138279 = NRRL4912^T)*, A. assiutensis* (JN393254 = AUMC 5748^T)*, A. japonicus* (NR_131268 = CBS 114.51^T) and *A. aculeatus* (NR_111412 = CBS 172.66^T) (Fig 12).

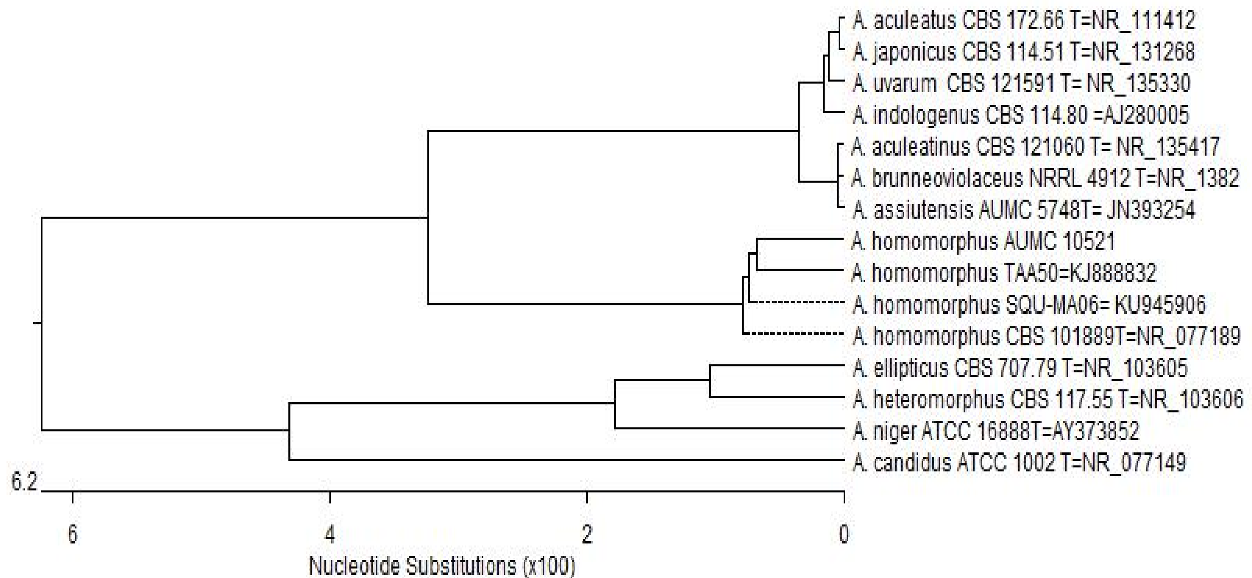


Fig 12 – Phylogenetic tree based on DNA sequence data of ITS region of *Aspergillus homomorphus* AUMC 10521, compared with 13 reference strains in GenBank of closely related species belonging to *Aspergillus* section *Nigri* and *A. candidus* as outgroup.

A. homomorphus is most similar phenotypically to *A. ibericus* and *A. carbonarius*; the three species have black biseriate aspergilli with long stipes and relatively larger conidia (> 5 µm) when compared to species of the *A. niger* aggregate (conidia 3–5 µm diam), and smaller when compared to *A. carbonarius* (7–10 µm) (Kallow et al. 2006, Perrone et al. 2007). Samson et al. (2007). studied morphological characteristics of the type strains of *Aspergillus* Section *Nigri* and reported that several species produce large conidia up to 7–9 µm including *A. carbonarius, A. ibericus, A. sclerotioniger, A. homomorphus* and *A. scleroticarbonarius*, but *A. homomorphus* and *A. ibericus* produce conidia with spiny appearance. Other characters may help in the distinction of these species such as the dark or olive green colony reverse on Cz of *A. carbonarius* (Raper & Fennell 1965), versus absence of colour in the reverse in *A. homomorphus* and *A. ibericus*. Furthermore, on MEA, the latter two species grow faster than *A. carbonarius* (Raper & Fennell 1965). *A. ibericus* produces sclerotia whereas *A. homomorphus* does not (Frisvad et al. 2014). In agreement with the current results, *A. homomorphus* could grow at 36°C, but not at 45ºC (Meijer et al. 2011.

CREA medium can be used as a semi-selective medium for classification of black aspergilli into groups (Samson et al. 2007). The present isolate grew and produced acid from CREA (turning the medium from purple to yellow). *A. homomorphus* showed colony diameter less than 40 mm at 37ºC, short metulae, echinate conidia (spines up to 1.5 µm).

Blast search revealed a similarity of the current strain of *A. homomorphus* with the uniseriate aspergilli; *A. aculeatinus, A. aculeatus*, *A. assiutensis, A. brunneoviolaceus, A. indologenus, A. japonicus* and *A. uvarum* ranging from 94.2–94.7%. In similar studies, *A. homomorphus* and uniseriate species were located in either one subclade or two neighboring clades (Samson et al. 2004, Silva et al. 2011).

AUMC 10521 strain exhibited slightly smaller vesicles (up to 50 µm) than its original description of Steiman et al. (1994) (up to 65 µm). The current strain of *A. homomorphus* was isolated from millet grain and thus, the species is not limited to soil (Steiman et al. 1994, Borrego–Terrazas et al. 2014, Al–Sadi /ncbi website)

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