



***Aspergillus curvatus*, a new species in section *Circumdati* isolated from an alkaline water of Lake Khadra in Wadi-El-Natron, Egypt**

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Abstract

In this study, a novel *Aspergillus* belonging to the Ochraceous group was isolated from a sample of alkaline water taken from the Khadra lake in Wadi-El-Natron Depression, Egypt, and is identified in the current study as *Aspergillus curvatus*. The novel species was introduced based on phenotypic characters and molecular evidence. *Aspergillus curvatus* is characterized by its strongly-curved conidiophores; this species is distinguishable from other species in section *Circumdati* by its morphological characteristics. The pure culture of *Aspergillus curvatus* is deposited at the Assiut University Mycological Centre cultural collection (AUMC 11038) and the Egyptian Microbial Culture Collection Network (EMCCN 2213). ITS gene sequence was deposited in the GenBank (MN006961). The novel species was registered at the MycoBank (MB831990) with its description.

Key words – ITS – lakes – new taxon – phylogeny – saline

Introduction

Aspergillus species of section *Circumdati* are important producers of several mycotoxins such as ochratoxin A (Van der Merwe et al. 1965, Ciegler 1972, Hesseltine et al. 1972), penicillic acid (Ciegler 1972), xanthomegnin, viomellein and vioxanthin (Robbers et al. 1978, Stack & Mislivec 1978). Some members also used in biotransformation's industry (Singh et al. 1968, Miski & Davis 1988). The *Aspergillus ochraceous* group has been taxonomically revised by Christensen (1982). This section has subsequently introduced in a molecular study on genetic variation, and many new species were introduced during the last few decades (Tuthill & Christensen 1986, Udagawa et al. 1994, Varga et al. 1998, Frisvad & Samson 2000, Zotti & Montemartini Corte 2002, Frisvad et al. 2004, Davolos et al. 2012, Gil-Serna et al. 2015, Al-Bedak & Moubasher 2020). The current study, therefore, offers insights into the morphological and phylogenetic evaluation of the new species within the *Aspergillus* genus that is isolated from an extreme Wadi-El-Natron environment in Egypt.

Materials and methods

Sampling site

Wadi-El-Natron is located at 30°17' and 30°38"N, 30°2' and 30°30' E in a Western desert

parallel to Egypt's Nile Delta. It lies 23 meters downstream and 38 meters below the Nile Rosetta branch (Abd-el-Malek & Rizk 1963). Khadra lake is approximately 0.77 km² and has a greenish appearance. In summer, it dries up (Fig. 1).



Fig. 1 – Lake Khadra in Wadi-El-Natron region where the new species was isolated.

Strain isolation

The *Aspergillus* species has been isolated from alkaline water collected in February 2012 at Lake Khadra, in depression at Wadi-El-Natron (Ismail et al. 2017). The isolation medium composed of (g/l): glucose, 10; Na₂NO₃, 2; K₂HPO₄, 1; KCl, 0.5; MgSO₄.7H₂O, 0.5; FeSO₄, 0.01; ZnSO₄, 0.01; CuSO₄, 0.005, agar, 15; Rose Bengal, 0.05 and chloramphenicol, 0.25 and the pH was adjusted to 7.3. Sterile dishes with 1 ml of each sample and 15 ml of medium were incubated at 25°C for 15 days. The colonies were purified on the same medium and preserved for further research at 4°C on slopes of Malt extract agar as pure cultures.

Morphological studies

Morphological characteristics in the culture and growth rates of the new species were studied on yeast extract sucrose agar (YES) (Frisvad 1981), malt extract agar (MEA) (Samson 2010), Blakeslee's malt extract agar (MEAbI) (Blakeslee 1915), malt extract (20%) with sucrose agar (M₂₀S) (Samson 2010), M₄₀S, Czapek's yeast Autolysate agar (CYA) (Pitt 1979), CYA with 20% sucrose agar (CY₂₀S) (Klich 2002), Czapek's agar (CZ) (Raper & Fennell 1965), Creatine sucrose agar (CREA) (Frisvad 1981), urease enzyme (Paterson & Bridge 1994), ammonium salt agar (mannitol agar) (Brayford & Bridge 1989) and tannin sucrose agar (TAN) (Thrane 1986). Inoculations were made from spore suspensions prepared in a 0.2% agar and 0.05% Tween 80 solution (Samson et al. 2014). Plates were inoculated in a three-point pattern using a micropipette and inoculum size of 1 µl per spot. Unwrapped cultures were incubated in the dark, reverse side up at 25°C with additional CYA plates incubated at 37°C. Microscopic features on MEA were examined in lactophenol cotton blue.

Molecular studies

DNA extraction

Before DNA extraction, a small piece of fungal mycelia of a 7-day-old culture of *A. curvatus* AUMC 11038 grown on MEA was collected and transferred to 2 ml-Eppendorf tubes. DNA extraction was carried out according to the method of Moubasher et al. (2019).

PCR for rDNA and sequencing using ITS1 and ITS4 primers

The PCR reaction was performed using SolGent EF-Taq. The universal primers ITS1 and ITS4 (White et al. 1990) were used to amplify ITS region. The PCR mixture composed of 1 µl of DNA template, 1 µl 2.5 mM dNTP mix, 0.2 unit of Taq polymerase, 5 µl of 10x complete buffer and 40 µl of sterile ddH₂O, 10 pmol of ITS1 (5' TCC GTA GGT GAA CCT TGC GG 3') and ITS4 (5' TCC TCC GCT TAT TGA TAT GC 3'). Then the PCR amplification was carried out using the following program: one round of amplification consisting of denaturation at 95°C for 15 min followed by 30 cycles of denaturation at 95°C for 20 sec, annealing at 50°C for 40 sec and extension at 72°C for 1 min, with a final extension step of 72°C for 5 min. The PCR products were then purified with the SolGent PCR Purification Kit-Ultra (SolGent, Daejeon, South Korea) before sequencing. The purified PCR products were confirmed on 1% agarose gel by electrophoresis using a size marker. The bands were eluted and sequenced in the forward and reverse directions.

Phylogenetic analysis

The ITS dataset included 28 sequences, 26 of which were sequences for the nearest strains, including the available type species in section *Circumdati* and a sequence for *A. flavus* as the outgroup, in addition to the new species sequence obtained in the current study. DNA sequences of *A. curvatus* AUMC 11038 were edited with the DNASTAR computer package (DNA star version 5.05). Assembled sequences of *A. curvatus* was aligned with those downloaded from GenBank using MAFFT (Kato & Standley 2013). Alignment gaps and parsimony uninformative characters were treated by BMGE (Criscuolo & Gribaldo 2010). Maximum-likelihood (ML) and Maximum parsimony (MP) phylogenetic analyses were performed using PhyML 3.0 (Guindon et al. 2010). The robustness of the most parsimonious trees was evaluated by 100 bootstrap replications (Felsenstein 1985). The best optimal model of nucleotide substitution for the ML analyses was determined using akaike information criterion (AIC) as implemented in Modeltest 3.7 (Posada & Crandall 1998). The phylogenetic tree was visualized using BioNJ (Gascuel 1997) and edited using FigTree version 1.4.3 (Rambaut & Drummond 2012).

Results

Aspergillus curvatus Al-Bedak OA & Moubasher AH, sp. nov.

Figs 2–3

Index Fungorum number: IF557788; Facesoffungi number: FoF08713

Mycobank number: MB831990

Etymology – Referring to the strongly-curved conidiophores.

Colonies on MEA attaining a diameter of 42–55 mm after 7 days at 25°C, floccose, raised in the center, orange grey to dark blond to yellowish-brown (5B2–3/5D–E4). Margin entire, pale yellowish-brown. Sporulation moderate to heavy. Reverse mustard-brown to snuff brown in the center (5E6/5F6), Pompeian yellow to honey yellow at the margin (5C–D6). Colonies on CYA attaining a diameter of 40–46 mm after 7 days at 25°C, floccose, centrally raised, sulcate, hair brown, bronze to mustard brown at the center (5E4–6). Margin regular, pale yellowish-brown. Sporulation heavy, compact in the center. Reverse vandyke brown, burnt umber to dark brown in the center (6F6–7), greyish-orange to topaz at the margin (5B–C5). Soluble pigments absent. Exudates present (Fig. 2). Conidial heads loosely columnar to radiate, splitting into columns by age, mostly (45–) 60–80 (–90) µm. Conidiophores brown, thick-walled, rough, sinuous, or even strongly-curved, commonly 200–300 (–500) µm × 5–10 µm. Vesicles globose to subglobose,

mostly (10–) 20–25 (–30) μm . Metulae 10–17 μm . Phialides 5–8 μm . Conidia globose 2–3 μm (Fig. 3). Sclerotia, cleistothecia and Hülle cells not observed. Colony diameters of *A. curvatus* on different media are summarized in Table 1.

Teleomorph – Not observed.

Known distribution – an alkaline water of Lake Khadra in Wadi-El-Natron.

Material examined – Egypt, Behira Governorate, Wadi-El-Natron, Lake Khadra, alkaline water sample, 3 Feb 2012, Osama A. Al-Bedak, Osa-643 (holotype AUMC 11038).

GenBank number – ITS: MN006961

Distinguishing features: The strongly-curved conidiophores make this unique species.

Table 1 The colony diameter of *A. curvatus* on different media

Medium	Diameter (mm)	Medium	Diameter (mm)
Cz	3.6–4.5	MEAb1	3.5–5.0
Yes	3.8–5.8	CREA (no acid)	2.8–3.2
CY ₂₀ S	4.2–6.0	Urea (negative)	2.6–3.0
CYA37	1.2–1.5	Mannitol (negative)	4.2–5.2
M ₂₀ S	4.0–7.0	Tannic acid	1.3–1.4
M ₄₀ S	4.2–6.5		

Phylogenetic analyses

The entire ITS dataset comprised 28 sequences. The maximum parsimony dataset consisted of 579 characters with 384 constant characters (no gaps, no N), 103 variable characters which were parsimony-uninformative (20.8% of constant characters), and 42 characters were counted as parsimony informative (10.9% of constant). The GTR was the best fit model for nucleotides substitution. Maximum Parsimony analyses resulted in 4 most parsimonious trees with a tree length of 124 steps. Maximum likelihood analysis yielded one tree (Log-likelihood = –2191.30657) with a tree size of 0.56522 (Fig. 4). The closest matches in the GenBank nucleotide database based on a mega blast search using the ITS sequence of the new species was *A. ochraceopetaliformis* CAF 073 (= ATCC 12066 = CBS 123.55 = IMI 211804 = NRRL 4752) [(GenBank KU 821470; Identities 515/558 (92.29%), 15 gaps (2%) with 95.0% coverage of the ITS gene]. The novel species was placed in the phylogenetic tree in the same subclade along with *A. ochraceopetaliformis* CAF 073 endorsing a strong bootstrap value (100% ML/100% MP) (Fig. 4).

Molecular study

Sequences of the ITS region confirmed the closest relationship of the new species with the members of *Aspergillus* section *Circumdati*. The type strain was deposited in the culture collection of Assiut University Mycological Centre as AUMC 11038 and Egyptian Microbial Culture Collection Network as EMCCN 2213. The ITS gene sequence was deposited in the GenBank with the accession number MN006961 and the name is registered in Mycobank (MB831990) with the description of the new species. The percentage of sequence similarity to the closest matching *Aspergillus* species in section *Circumdati* did not exceed 93% revealing that the current species is a new taxon (Fig. 4).

The morphological differences between the new species, *A. curvatus* and the closely similar species in section *Circumdati* are summarized in Table 2.

Discussion

The morphological features of the new species *A. curvatus* displayed the features of the *Circumdati* group, such as yellowish-brown color, biseriate and radiate conidial heads in addition to rough-walled conidiophores and small conidia. Sequences of the ITS region confirmed the closest relationship of the new species with the members of *Aspergillus* section *Circumdati*. The internal transcribed spacer (ITS) region, located between the 18S and 28S rRNA genes, is a field of

special interest to differentiate between closely related or intraspecific species as it has areas of high survival and high variability and was used to classify *Aspergillus* species (Accensi et al. 1999, Henry et al. 2000, Al-Bedak & Moubasher 2020).

The new fungus located in a single branch within the same subclade including *A. ochraceopetaliformis* CAF 073 making this unique species. The interspecific differences in the ITS sequences varied between *A. curvatus* and other species in section *Circumdati* from 65 nucleotides in case of *A. ochraceopetaliformis* CAF 073, to 189 nucleotides in *A. insulicola* NRRL 6138, 204 nucleotides in *A. pulvericola* CBS 137327, 205 nucleotides in *A. ochraceopetaliformis* CBS 123.55, and 218 nucleotides in *A. flocculosus* CBS 112785.



Fig. 2 – Cultural features of *Aspergillus curvatus* AUMC 11038. 1-5 colonies on YES, MEA, MEAbI, M20S, M40S. 5-10 reverse on YES, MEA, MEAbI, M20S, M40S. 11-15 colonies on CYA, CY20S, Cz, CYA37, CREA. 16-20 reverse on CYA, CY20S, Cz, CYA37, CREA. 21-23 colonies on urea, mannitol, TAN. 24-26 reverse on urea, mannitol, TAN.

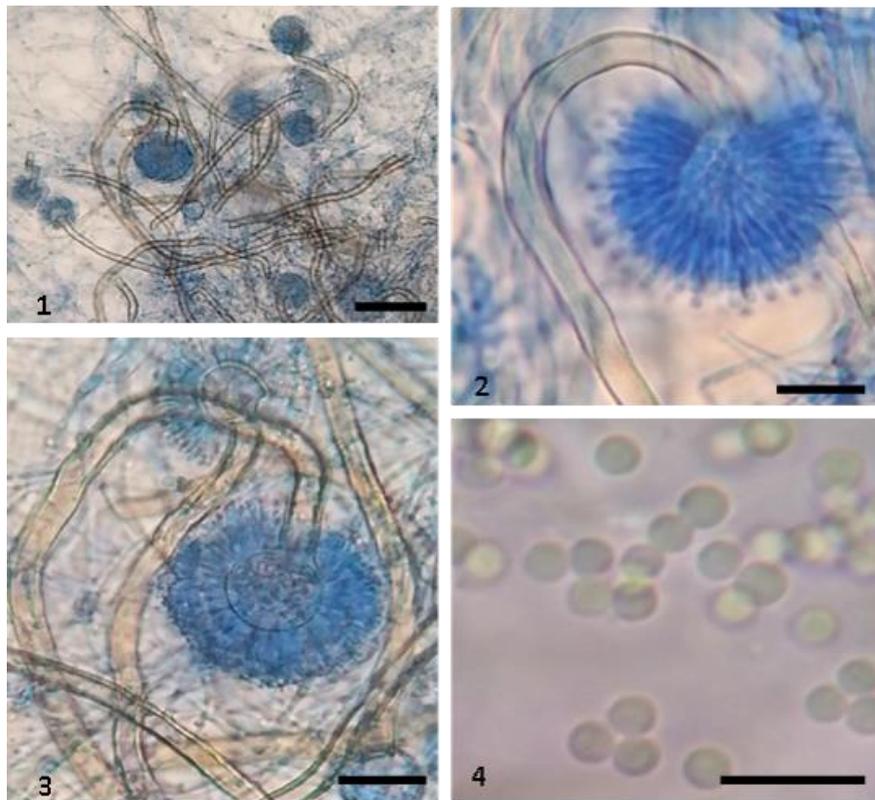


Fig. 3 – Microscopic characteristics of *A. curvatus* AUMC 11038. 1-3 strongly-curved conidiophores. 4 globose to subglobose conidia. Scale bars: 1 = 50 μm , 2–3 = 20 μm , 4 = 10 μm .

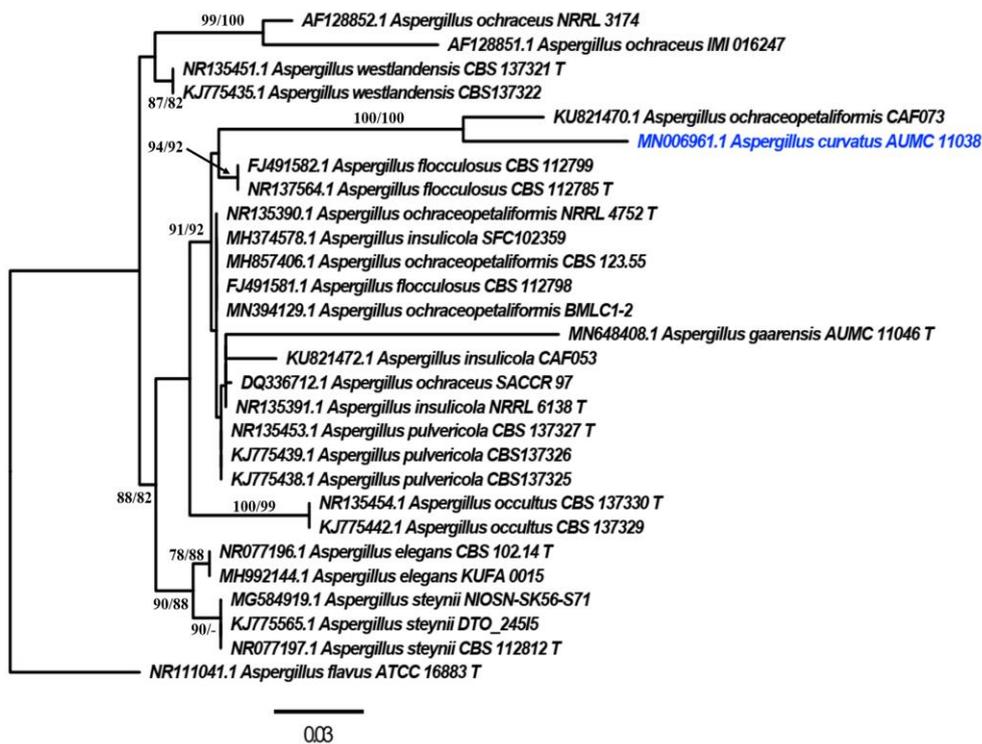


Fig. 4 – Maximum Likelihood (ML/MP combination) phylogenetic tree of *A. curvatus* sp. nov. strain AUMC 11038 aligned with other related *Aspergillus* taxa in section *Circumdati* based on the ITS gene sequences. Sequence of the new species is in blue color. The numbers near the branches are the bootstrap values (100 replications). Values <70 % are not shown. The tree is rooted to *A. flavus* ATCC 16883. T = type strain.

Table 2 Morphological characteristics of *A. curvatus* AUMC 11038 compared with some other species in section *Circumdati* (involved in the phylogenetic analysis) on MEA after 7 days at 25°C.

Morphological features	Sporulation	Sclerotia	Conidiophores	Vesicles	Metulae	Conidia
<i>A. curvatus</i> This study	Orange grey, dark blond to yellowish brown	Absent	Brown, rough-walled, sinuous, strongly-curved, 200-300 (-500) $\mu\text{m} \times 5-10 \mu\text{m}$	Globose to subglobose, (10-) 20-25 (-30) μm	10-17 μm	Globose to subglobose, smooth, 2-3 $\mu\text{m} \times 2-3$
<i>A. ochraceopetaliformis</i> (Visagie et al. 2014)	Dull yellow to olive brown to brown	Sclerotia-like structures sparsely produced in some isolates, reddish brown, 350–650 μm	Hyaline to yellow to brown, rough walled, 260–1300 $\times 8-10 \mu\text{m}$	Globose to pyriform, 25–45 μm	10–20 (-28) μm	Globose, smooth, 2–3 $\times 2-3 \mu\text{m}$
<i>A. pulvericola</i> (Visagie et al. 2014)	Yellowish white	Sometimes present, white to cream, 250–510 μm .	Hyaline to brown, rough, 190–1000 $\times 5-9 \mu\text{m}$	Globose, 15–53 μm	5.5–16.5 μm	Globose, smooth, 2–3 $\times 2-3 \mu\text{m}$
<i>A. insulicola</i> (Christensen 1982) (Visagie et al. 2014)	Light orange to greyish orange	Absent	Hyaline to yellow to brown, rough walled, 250–600 $\times 6-8.5 \mu\text{m}$	Globose, 15–30 μm	7–12 μm	Globose to subglobose, smooth, 2–3 $\times 2-3 \mu\text{m}$
<i>A. flocculosus</i> (Frisvad et al. 2004)	Dull yellow to greyish yellow	(360-)400–590(-650) μm	Light brown, rough, 1000–1500 μm	Globose to pyriform, (16-) 20–44 (-46) μm	(7-)8–26(-28) μm	Globose, (1.9-)2–2.5(-2.7) μm
<i>A. ochraceus</i> (Christensen 1982)	Warm buff to cinnamon buff	In some strains, 1000-2000 μm	700-1500 $\times 10-14 \mu\text{m}$	Globose to somewhat 35-50 μm	15-20 μm	Globose to subglobose, finely roughened, 2-3.5 μm
<i>A. westerdijkiae</i> (Frisvad et al. 2004)	Velvety, pale to light or dull yellow	Sparsely formed, (460-)480–760(-840) \times (430-)480–660 (-720) μm on CYA and (440-)450–720(-750) \times (430-)430–650(-700) μm on OA	Up to 1800 μm	Globose to spathulate, (16-)20–35(-42) \times (3-)3.5–5.7(-7.1) μm	(10.5-)11 \times 19(-23) μm	Globose, finely roughened, (2.3-)2.5–3(-3.1) \times (2.2-)2.3–2.8(-3.1) μm
<i>A. sclerotiorum</i> (Christensen 1982)	Pale yellow	In some strains, 1000-1500 μm	Up to 800-1200 μm	Less than 40 μm	6.5-12 μm	Globose, smooth or delicately roughened, 2-3 μm
<i>A. auricomus</i> (Christensen 1982) (Visagie et al. 2014)	Golden yellow to orange yellow	Abundant on Czapek's agar	Hyaline to dark brown, rough, 190–1360 $\times 5-11 \mu\text{m}$	13-53 μm	6.5–14.5 $\times 4-6 \mu\text{m}$	Smooth, mostly 3-4 $\times 2.5-3 \mu\text{m}$

The new species is characterized by strongly curved-conidiophores, a specific morphological character that has never been defined in other *Circumdati* species. The new taxon can also be differentiated from similar species in the section by the size of its vesicle measuring 20–25 µm; somewhat resembling that of *A. insulicola* (15–30 µm), but smaller than the *A. ochraceopetaliformis* (25–45 µm), *A. pulvericola* (15–53 µm) or *A. flocculosus* (20–44 µm). Moreover, the length of the conidiophore may be regarded as a distinctive morphological character that separates the new species from the other related species as it commonly measures 200–300 µm relative to the longer conidiophores of *A. ochraceopetaliformis* (260–1300 µm), *A. pulvericola* (190–1000 µm), *A. insulicola* (260–600 µm) or *A. flocculosus* (1000–1500 µm).

Accessibility of data

A pure culture of the type material of the novel species is deposited in the culture collection of Assiut University Mycological Centre as AUMC 11038 and in Egyptian Microbial Culture Collection Network as EMCCN 2213. Sequence alignments have been submitted for all data sets to TreeBASE <http://purl.org/phylo/treebase/phyloids/study/TB2:S26506> (study no. 26506).

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