Journal of Basic & Applied Mycology 2 (2011): 83-90
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Aspergillus assiutensis, a new species from the air of grapevine plantation, Egypt

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Received 20/5/2010, Accepted 15/11/2010

Abstract: *Aspergillus assiutensis*, a new species isolated from the air of a grapevine plantation at Sahel-Saleem, Assiut, is described and illustrated. It is characterized by fast-growing whitish colonies, uniseriate conidiogenous cells, globose to radiate heads, thick-walled stipes and vesicles, pyriform to elongate conidia when young and globose to subglobose at maturity. ITS sequence of the isolate was determined and strongly supported the morphological differences. A new section (section *Assiuti*) of subgenus *Circumdati* is proposed for the species since it does not fit typically with any of the known sections of that subgenus.
Key words: *Aspergillus assiutensis*, Assiut, Egypt, air, grapevine plantation, ITS.

Introduction

During the course of a survey of mycobiota of grapevine plantations in Assiut Governorate, Egypt in 2008/2009, an interesting *Aspergillus* species was isolated from the air by settle-plate method (Hoekstra *et al.* 2004) on dichloran rose Bengal chloramphenicol agar plate (King *et al.* 1979) exposed for 5 minutes at a height level of 60 cm from the ground. The macro- and micro-morphological characteristics of the isolate proved to be sufficiently different from all described species of *Aspergillus* to warrant its description as a new species herein (Raper and Fennell 1965, Samson 1979, Samson and Gams 1985, Kozakiewicz 1989, Pitt and Samson 1993, 2007, Samson and Varga 2007).

Various molecular methods have been used for phenotypic and genotypic studies of aspergilli (Rinyu *et al*. 2000, Varga *et al.* 2000). The internal transcribed spacer (ITS) region, located between the 18S and 28S rRNA genes, is an area of particular importance in discriminating between closely related species or at intraspecific level, because it has areas both of high conservation and high variability. ITS has been used to identify *Aspergillus species* (Accensi *et al.* 1999, Henry *et al.* 2000).

Living culture of the new species as well as dried materials are deposited at the culture collection of the Assiut University Mycological Centre (AUMC 5748), Assiut, Egypt.

Materials and Methods

Strain examined: the strain examined was isolated from the air of a grapevine plantation in El-Khawaled village, Sahel-Saleem city at approximately 25 Km south-east of Assiut town, Egypt (about 6 km to the east border of the river Nile). The strain was isolated on 18 August 2008, on dichloran chloramphenicol agar plate, by Zeinab Soliman in a laboratory of Assiut University Mycological Centre (AUMC), Assiut, Egypt.

The new taxon is registered with a GenBank accession number JN393254 (http: //www.ncbi.nlm.nih.gov.).

Morphology: For macromorphological observations, the strain was grown in the dark on the following growth media: Czapek agar (Cz), Czapek yeast autolysate agar (CYA), yeast extract sucrose agar (YES), Blakeslee malt extract agar (MEA), Oatmeal agar (OA) at 25ºC for 7 days. To test temperature response, the strain was grown on Cz, CYA and MEA at 5ºC, 37ºC and 45ºC for 7 days (Tab. 1 & 2). (for medium formulations see Samson *et al.* 2004)

The strain was also grown on glycerol nitrate agar (G25N, Pitt 1973), Czapek agar with 20% sucrose (Cz20S), malt yeast 40% sucrose (M40Y) and Czapek agar with 70% sucrose (Cz70S) (Raper and Fennell 1965) at 25°C for 1 week.

Colony diameters were obtained from one-point inoculation of at least five replicate plates (90 mm diam) for each medium type. Colony colors were identified according to Kornerup and Wanscher (1978).

For micromorphological observations, microscopic mounts were made in lactophenol from CYA colonis after 7-10 days old.

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Growth of the fungus and DNA extraction and sequencing

The fungus was grown on CYA plates and incubated at 25° C for 7 days. A small amount of fungal mycelia was scraped and suspended in 100 µl of distilled water and boiled at 100° C for 15 minutes and stored at -70° C.

Samples were sent to SolGent Company (Daejeon, South Korea) to carry out the whole procedure from DNA extraction till the final step of DNA sequencing. Fungal DNA was extracted and isolated using SolGent purification bead. Internal transcribed spacer (ITS) sequences of nuclear ribosomal DNA were amplified using universal primers: ITS 1 (5' - TCC GTA GGT GAA CCT GCG G - 3'), and ITS 4 (5'- TCC TCC GCT TAT TGA TAT GC -3'). Then ampilification was performed 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) 0.25µl, template1.0 µl, DW to 25 µl. Then the amplification was carried out using the following PCR reaction conditions: One round of amplification was performed 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.

Then the PCR products were purified with the SolGent PCR Purification Kit-Ultra (SolGent, Daejeon, South Korea) prior to sequencing. Then the purified PCR products were reconfirmed (using size marker) by electrophoreses of the PCR products on 1% agarose gel. Then these bands were eluted and sequenced. Each sample was sequenced in the sense and antisense direction.

Initially the sequence alignments were performed using Clustal analysis and manual adjustments for improvement were made by eye. The Phylogenetic analysis was prepared using the MEGA 2.1 computer program (Kumar *et al.* 2001). The sequences of other *Aspergillus* species used for comparison were retrieved from GenBank database (http: //www.ncbi.nlm.nih.gov.).

Although this species is hitherto only represented by a single isolate, the combinations of macro- and micromorphological characters and ITS sequence makes it unique and indicates that *A. assiutensis* should be considered as the first representative of a new species in a new proposed section of subgenus *Circumdati*.

Aspergillus assiutensis Moubasher et Zeinab Soliman

Coloniae in 7 diebus et 25ºC expansae in agaro CYA 90 mm, in MEA 90 mm, in YES 90 mm, in Cz 56-68 mm, in OA 70-75 mm, in Cz20S 40-74 mm, in M40Y 90 mm, in G25N 24-30 mm, in Cz70S 34 mm, velutinae, conidiogenesis perplurima; in 7 diebus et 37ºC in agaro CYA 2-5 mm, in Cz 5-6 mm, in MEA 2-3 mm.

Conidia in agaro CYA in massa albae vel in margine flavo-albae (Methuen 4A1-2), albae vel aurantio-album ad centrum (M 5-6A1-2) (Cornerup and Wanscher 1978), reverso albae vel flavo-albae (M 2-3A1-2), capitula conidica globosa vel radiata. Conidiophora ex mycelio basali, uniseriata; stipites hyalini, 437-893 x 7.2-20.4 µm, interdum sinuosi, leves, parietibus 1.2-4.8 µm crassis, non constricti; vesiculae globosae vel subglobosae vel spathulatae, 22-60 µm diametro x 27-70.8 µm long, fertilis in tota superficie, phialides 3.6-9.6 x 3.0-4.8 µm. Conidia primum ellipsoidea vel pyriformia 2.5-3.5 x 6-8.4 µm, deinde globosa 3.6-4.8 µm vel subglobosa 3-4.8 x 4-6 µm, hyalina, levia vel scabrella. Sclerotia globosa vel subglobosa 450-700 µm.

Typus: AUMC 5748, isolatus ex aere campi *Vitidis vinifera*e, Assiut, Aegypto, 18 VIII. 2008, a Zeinab Soliman isolatus et in collection (AUMC 5748) conservatus.

Etymology: Latinized from the name Assiut, referring to the city of the type locality.

Colonies in 7 days at 25ºC on CYA, MEA, YES and M40Y fill the whole plate of 90 mm plates; Cz: 56-68; OA: 70-75; Cz20S: 40-74; Cz70S 34 mm; G25N: 24-30 mm diameters (Tab. 2).

Colony color at 25°C after 7d on CYA and Cz white to yellowish white at the edge (M 4A1-2), white to orange white towards the centre (M 5-6A1-2); on OA, G25N, M40Y and Cz70S pure white; on Cz20S white floccose mycelia at the colony periphery (as a circle of 15 mm diam) (Fig. 1& 2).

Sectors of slightly floccose mycelia of pure white color (not arising or originating from the colony, mostly with no or very few conidial structures near the colony edges centre), 1-2 sectors per colony on OA (Fig. 1), or only one on one Cz replicate-plate. Also, sectors (1 or 3 sectors have been seen on only 2 replicate-YES plates, however with yellowish white color (M 1-2A2).

Sporulation very heavy and abundant on all growth media used. On YES agar only, white mycelia felt with fewer numbers of yellowish white

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to pale yellow conidiophores (M 2A2-3) are produced.

Exudate absent on all growth media.

Reverse on CYA and Cz white to yellowish white (M 2-3A1-2), brownish orange (M 7C3) in the colony centre on Cz; on OA, Cz20S and G25N yellowish white (M 4A2); on MEA, YES and M40Y yellowish white to light yellow (M 4A2-3) (Fig. 2).

Sclerotia not observed after 7 days on all growth media incubated at different temperatures, however seen only on slants after prolonged incubation for up to several weeks, first white becoming creamish with age, globose to subglobose, measuring 450-700 µm.

Teleomorph not observed on all growth media incubated for up to 9 months in slant cultures.

Conidial heads (on CYA) globose at first and later radiate splitting into several, 2-5 (-6) conidial columns as they age, 133-292 µm diam; stipes 437-893 x 7.2-20.4 µm, or longer, sometimes sinuous, walls thick 1.2-4.8 µm, smooth, hyaline, not septate, not constricted below the vesicle; stipes longer on YES, up to 2100 µm or more; vesicles globose, subglobose to spathulate, hyaline, 22-60 µm diam x 27-70.8 µm long, thick-walled, 3.6-4.8 µm; uniseriate; phialides flask-shaped, covering the entire surface of the vesicle suggesting for the first impression section circumdati, measuring 3.6-9.6 long x 3.0-4.8 µm wide; conidia pyriform to elongate when young, 2.5-3.5 x 6-8.4 µm and globose 3.6-4.8 µm to subglobose 3-4.8 x 4-6 µm at maturity, smooth to finely rough (Fig. 3 & 4).

Foot cells in most cases 34.8x8.7 µm with a swollen part 23.2x 20.3 µm from which a conidiophore arises. Thick-walled hyphal cells (singly or in chains) extending from both sides of foot cell, somewhat globose 16.8x14.4-15.6 µm, or elongate 26.4-68.4x8.4-20.4; wall 4.8 µm thick.

No growth or germination is observed at 5ºC and 45ºC on CYA, CZ and MEA (Tab. 1).

Growth is restricted at 37ºC (2-6 mm) after 7 d on CYA, Cz and MEA (Tab. 1).

Strain examined: AUMC 5748 = type culture, isolated from the air of a grapevine plantation in El-Khawaled village, Sahel-Saleem at approximately 25 km south-east of Assiut town, Egypt on 18 August 2008, by Zeinab Soliman and deposited in the culture collection of Assiut University Mycological Centre (AUMC 5748), Assiut, Egypt, and the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands = CBS XXXX (awaiting accession number).

Taxonomic position

This species differs from the uniseriate species of subgenus *Aspergillus* (section *Aspergillus* and section *Restricti*) whose vesicles are fertile in the upper half and their conidial masses mostly in shades of green, but light brown in one species of section *Aspergillus*. Also, the uniseriate species of subgenera *Fumigati* and *Ornati* differ from *A. assiutensis* by their flask-shaped or clavate vesicles, the radiate to columnar conidial heads and the pale grey-green to dark blue-green (section *Fumigati*), pinkish fawn shades (section *Cervini*) of conidial masses, and the grayish or yellowish green to olive brown shades of conidial heads in Subgenus *Ornati*. The long clavate vesicle and the blue-green shades of conidial masses differentiate species of section *Clavati* from *A. assiutensis*. Therefore, the new species does not fit in any of the known uniseriate subgenera or their sections.

The new species differs also from the uniseriate *Aspergillus* species of Section *Nigri* by its weak growth at 37°C (2-6 mm; 15-40 mm in the uniseriate species of section *Nigri*), its larger conidial size (up to 6 µm or more; in section *Nigri* never exceed 5 µm) and smooth to very finely rough wall (echinulate to spinulose), its whitish colony color (black in section *Nigri*) and the wider stipes of *A. assiutensis* which may reach 20 µm (however only *A. aculeatus* of the four species known within the uniseriate species of section *Nigri*, has stipes width up to 30 µm, but it could be easily separated from this species by the conidia shape, size, wall and color).

Vesicles of the new species are entirely covered by phialides suggesting for the first impression a species of sections *Circumdati* or *Candidi*. This species differs from all species within these sections by the strictly uniseriate conidiogenus cells. Since this species has characters in common with those of subgenous *Circumdati*, especially those of vesicles which are perfectly globose-shaped, and fertile over their entire surface and the typically radiate conidial heads suggesting that it accommodates within subgenus *Circumdati*. However, it could not typically fit well in any of its sections because of colony colour. Within subgenus *Circumdati*, species of sections *Wentii* (of yellow-brown to dull buff conidial masses), *Flavi* (yellow-green to deep olive brown), *Nigri* (shades of black conidial masses), *Circumdati* (yellow, buff or ochraceous shades conidial masses), *Cremi* (buff brown, pale yellow-green shades, in addition to the constricted stipes below vesicles and ellipsoidal conidia), and *Sparsi* (light grey through shades of green to olive-buff).

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Aspergillus assiutensis resembles somewhat in colony color the species of Section *Candidi*, however species of this section have restricted growth rate on all media; biseriate large and uniseriate diminutive small heads, and small, mostly globose to subglobose conidia not exceed 5 µm diam. Also, species of whitish-colored colonies of Section *Fumigati* have columnar conidial heads in addition to their teleomorphs (*Neosartorya*).

Table 1: Growth rates (in mm) of *Aspergillus assiutensis* on three growth media after 7 days of incubation at various temperatures

Medium	Temperature ºC		
	5	37	45
CYA	NG	2-5 (3.8±1.03)	NG
Cz	NG	5-6 (5.4±0.52)	NG
MEA	NG	2-3 (2.6±0.52)	NG

- NG: no growth (no germination).

Table 2: Growth rates (in mm) of *Aspergillus assiutensis* on different growth media after 7 days of incubation at 25ºC

Growth rate	CYA	Cz	MEA	OA	YES	G25N	Cz20S	Cz70S	M40Y
Mean±SD	90±0	63.2±4.71	90±0	71.8±2.1	90±0	27.2±2.3	60±12.1	34±0	90±0
Range		56-68		70-75		24-30	40-74		

- CYA: Czapek yeast autolysate agar; Cz: Czapek agar; MEA: malt extract agar; OA: Oatmeal agar; YES: yeast extract sucrose agar; G25N: glycerol nitrate agar; Cz20S: Czapek agar with 20% sucrose; Cz70S: Czapek agar with 70% sucrose; M40Y: malt yeast 40% sucrose.
- Data based on average growth (mean) of 5 replicate cultures in 90 mm Petri dishes for each set of culture conditions.

Table 3: Comparison of diagnostic characteristics of sections with some similarities in common with *Aspergillus assiutensis*.

Character	*A. assiutensis*	*Candidi*	*Circumdati*	Uni *Nigri*	*Fumigati*
Conidial heads	whitish-orange white	whitish or sulphur yellow	yellow, buff or ochre	black	Shades of green
Conidiogenous cells	Uniseriate	Biseriate	Biseriate	Uniseriate	Uniseriate
Diminutive heads	No	Common, uniseriat	Common, biseriate	No	In some species
Vesicles (µm)	22-60x27-70.8 µm, globose	Not exceed 46 µm, globose or elongate	5-80 µm or more, globose to elongate	20-80 µm or more, globose	4-52 µm, flask-shaped
Conidia	Globose-subglobose-pyriform, up to 6 µm or more	Globose, smooth, never exceed 5µm	Globose, ovate or ellipsoidal, smooth to rough	Globose to ellipsoidal, not exceed 5 µm	Globose, subglobose-ellipsoidal, smooth–spinulose, (1.8) 2.5-3.5 (6)
Sclerotia	After long incubation, creamish	± purple to black or dark brown	± varying in color	±, white to cream or dark brown to black	-ve
Teleomorph	Unknown	Unknown	*Petromyces*	Unknown	*Neosartorya*
Growth at 25ºC	Fast	Restricted	Restricted	Fast	Fast
Growth at 37ºC	Weak (2-6 mm)	±	±	15-40 mm	13-85 mm

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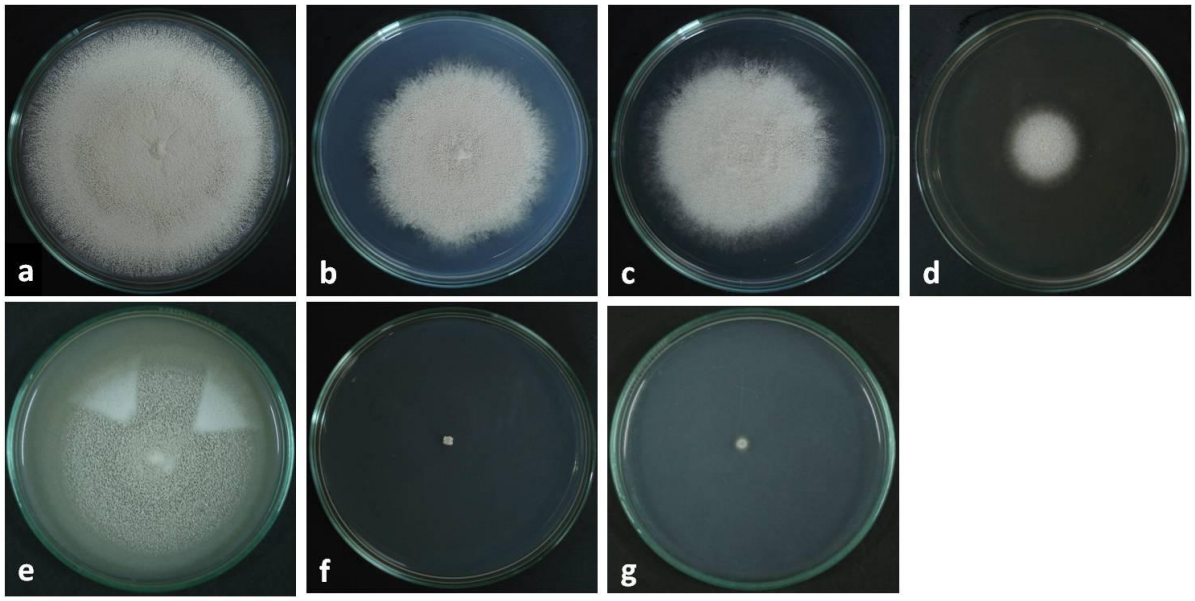

Figure 1: 7-day-old cultures of *Aspergillus assiutensis* AUMC 5748 on: a- CYA, b- Cz, c- Cz20S, d- G25N, e- OA (at 25°C) & f- CYA, g- Cz (at 37°C).

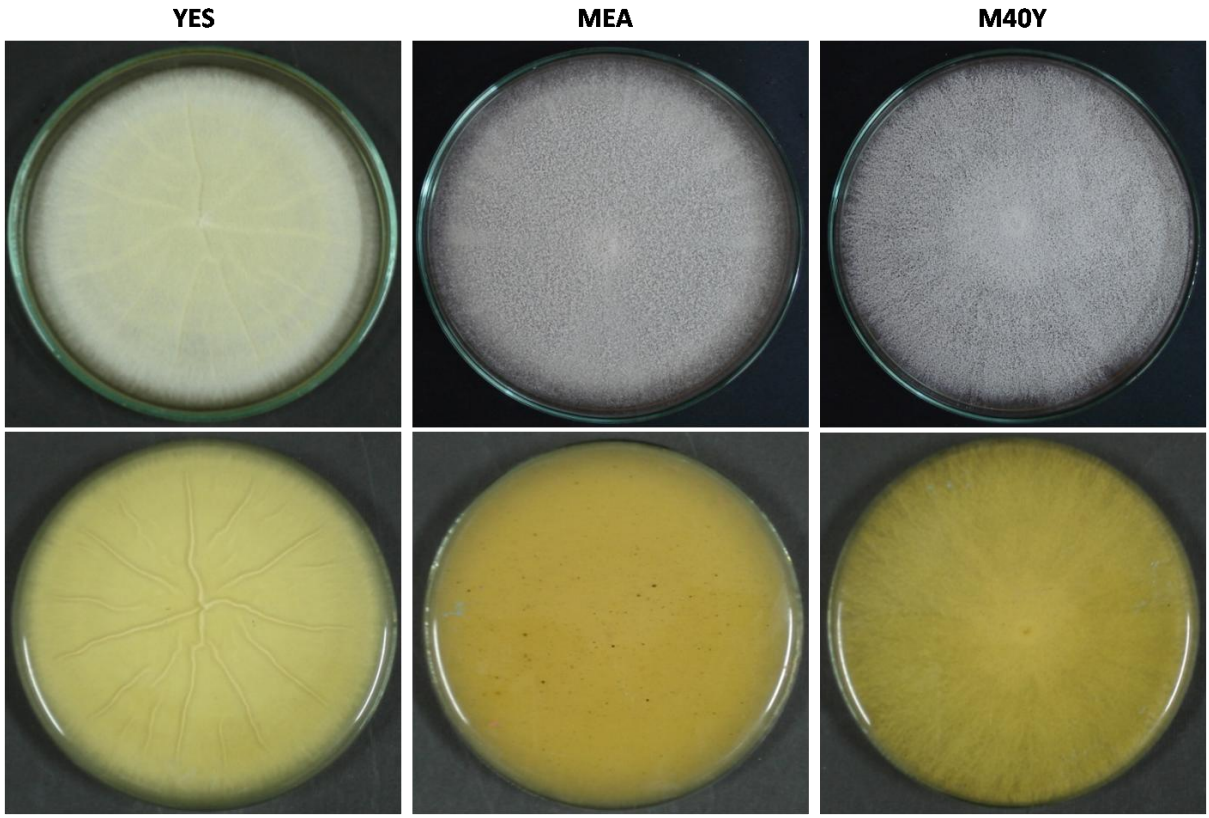

Figure 2: 7-day-old cultures of *Aspergillus assiutensis* AUMC 5748 at 25°C on YES, MEA and M40Y (Top obverse and bottom reverse).

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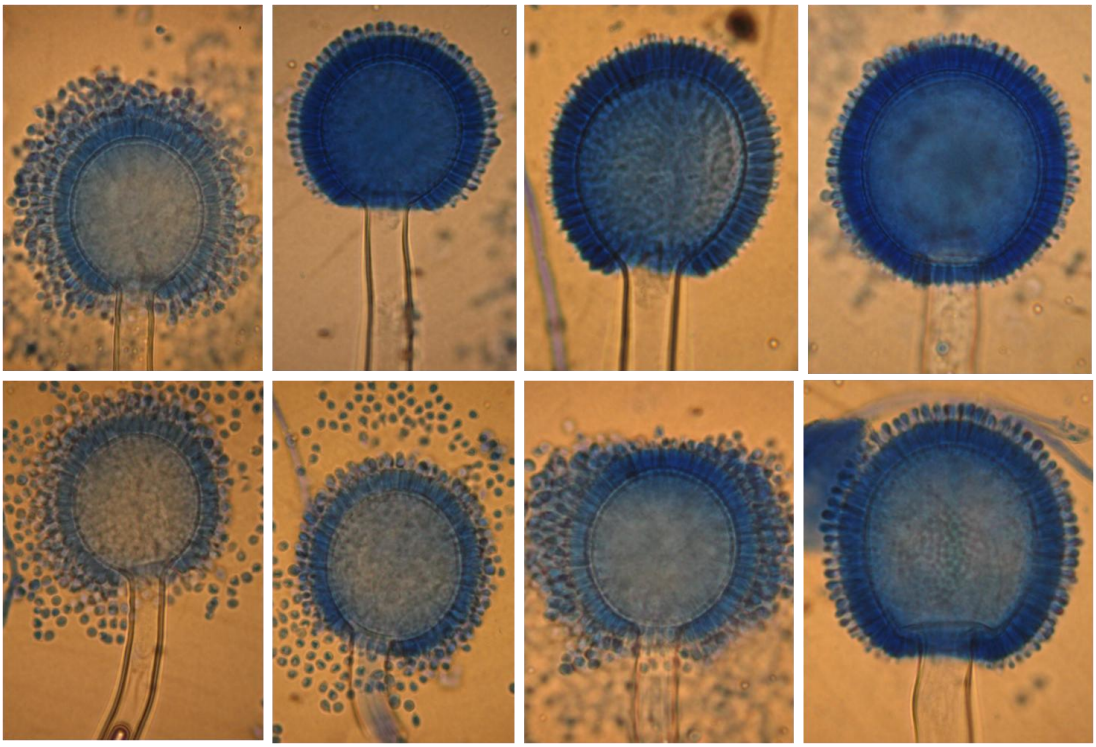

Figure 3: Conidial heads of *Aspergillus assiutensis* showing stipe, vesicle, uniseriate phialtdes and conidia .

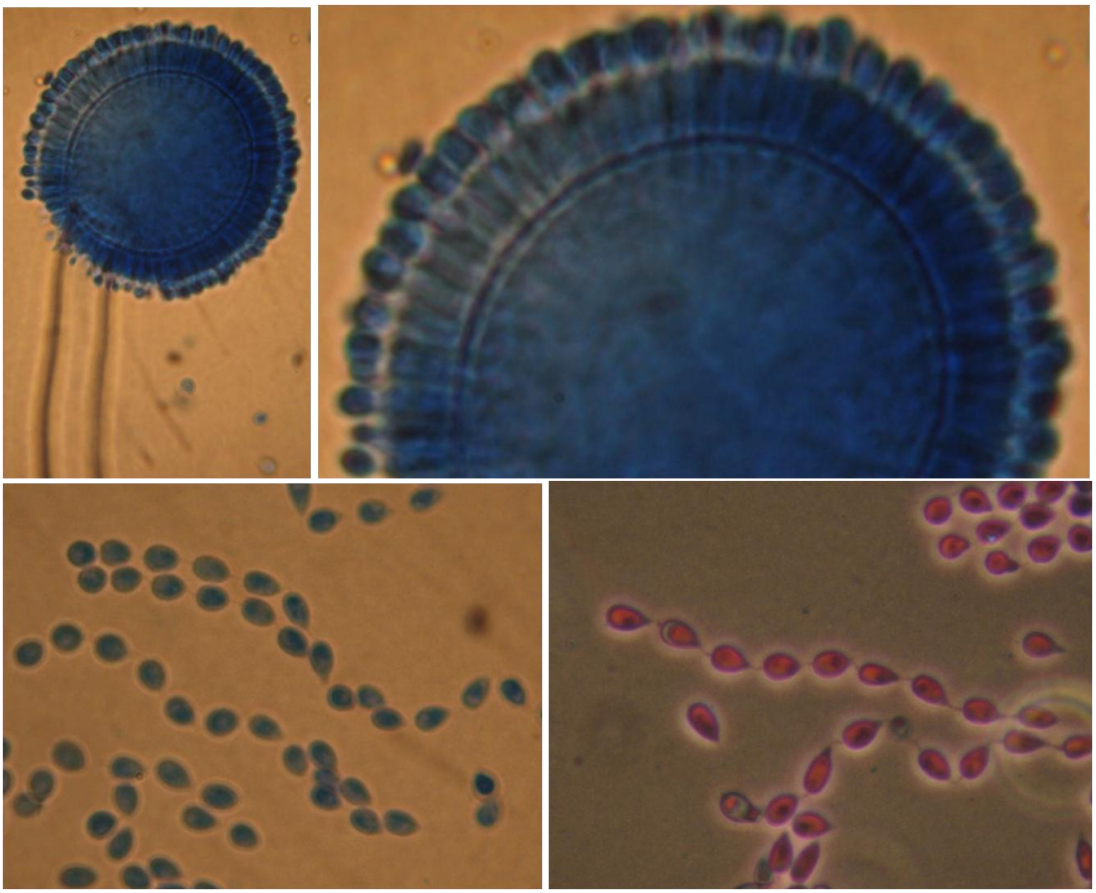

Figure 4: Conidial heads with uniseriate phialides and conidia of *Aspergillus assiutensis*.

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Phylogenetic analysis

The interspecific sequence divergences in the ITS region between *A. assiutensis* isolate and other uniseriate species ranged between 107.0 in *A. uvarum* and 120.1 in *A. fumigatus* (Fig 5). However, small values in divergence or even identical ITS sequences have been been obtained previously for many species of *Aspergillus* (Samson *et al.* 2007). In this respect, Varga *et al.* (2007) found that several species in *Aspergillus* section *Nigri* have identical ITS sequences (e. g. *A. niger* and *A. lacticofeatus*; *A. tubingensis*, *A. foetidus*, *A. vadensis* and *A. piperis*; *A. carbonarius* and *sclerotioniger*; *A. japonicus*, *A. aculeatus* and *A. uvarum*). Also, Zalar *et al.* (2008) found that *Emericella filifera* and *E. stella-maris* isolates respectively had identical ITS sequences to *E. variecolor* and *E. astellata*. In such cases, calmodulin and/or β- tabulin sequence data are needed for more confirmation (Varga *et al.* (2007, Zalar *et al.* 2008). The phylogenetic tree (Fig. 6) reveals that *A. assiutensis* forms a distinct clade.

Characters of *A. assiutensis* make it unique among all known *Aspergillus* species. Therefore we suggest a new section within subgenus *Circumdati* for this new species.

Aspergillus section *Assiuti* Moubasher and Zeinab Soliman, sect. nov.

Sectio Assiuti in subgenere *Circumdati* cum speciebus. Conidia in massa albae vel aurantio-album; stipitibus levibus, hyalinus; conidiophora uniseriata et 45^{0}C non crescents.

Section *Assiuti* in subgenus *Circumdati* containing species of fast growing, whitish-creamish colonies; not able to grow at 45^{0}C; with uniseriate, globose to radiate conidial heads; smooth stipes, hyaline and thick-walled vesicles and conidiophore stipes; and pyriform to elongate when young to globose or subglobose conidia at maturity.

Type species: *A. assiutensis* Moubasher & Zeinab Soliman.

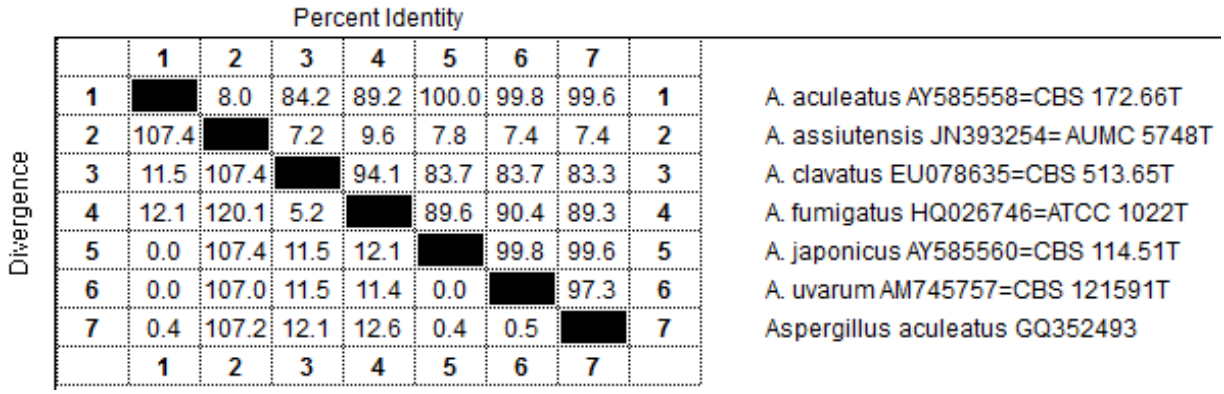

Figure 5: Sequence pair distance of *A. assiutensis* and the closely matched strains as determined by the Clustal W program (Meg Align Package).

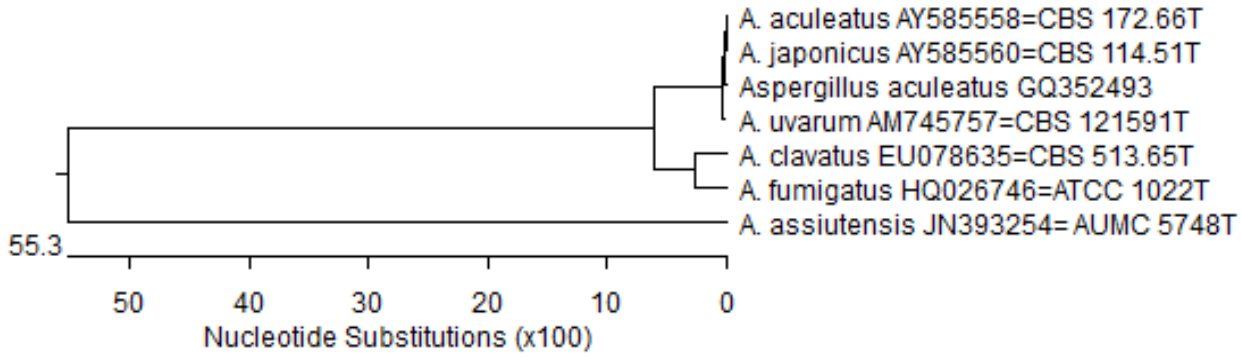

Figure 6: The phylogenetic tree of *A. assiutensis* based on partial nucleotide sequences (bp) of the ribosomal DNA internal transcribed spacer regions. The scale indicates the number of nucleotide substitutions per site.

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Acknowledgement

The authors would like to express their sincere gratitude for Dr. David Minter and to Dr. Paul Kirk, CABI, Egham, Surrey, UK, for critical revising the latin diagnosis. Sincere thanks are also due to Prof. A. M. Moharram for valuable discussion and for Prof. M. A. Ismail, for critical reading of the manuscript, the two from the Department of Botany, Faculty of Science, Assiut University,

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