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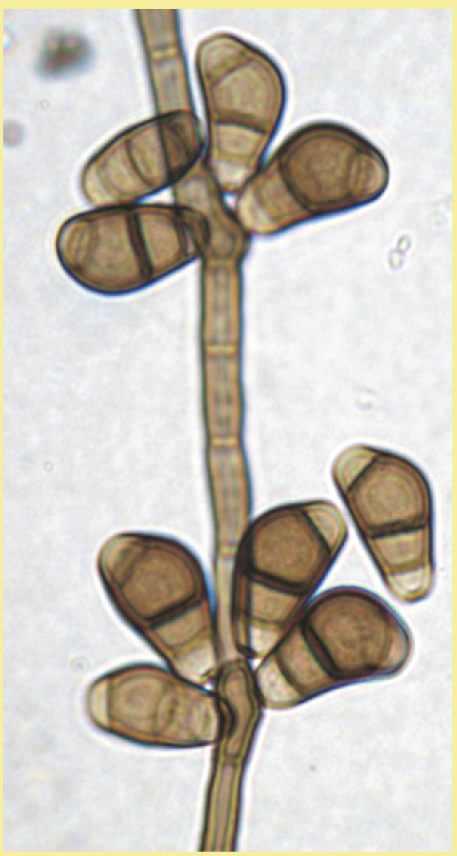

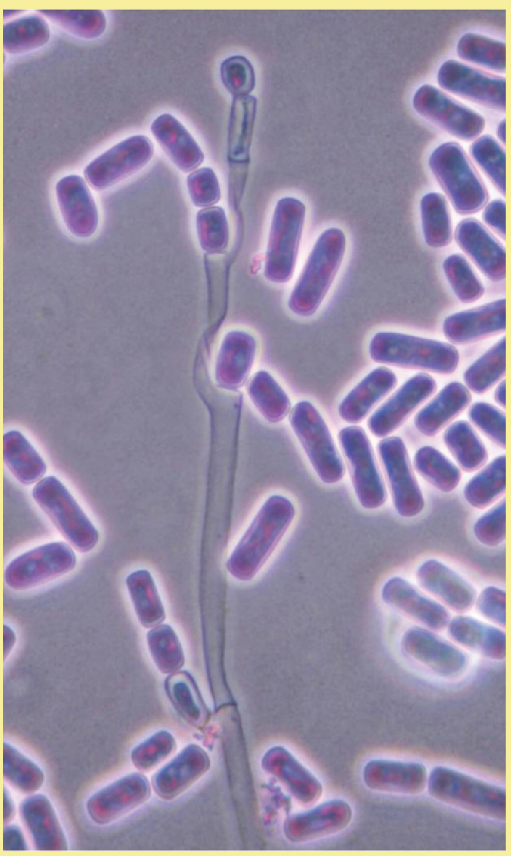

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Aspergillus calidoustus Varga, Houbraken and Samson a new record of section Usti from the air of Assiut, Egypt

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Abstract: *Aspergillus calidoustus* is a human pathogen, causing an invasive infection to the immunocompromised patients. The occurrence of this fungus in the air had a serious effect on human health.A strain of *Aspergillus* related to section *Usti* was isolated from air at Assiut area, Egypt in 2003. The strain was grown on different media for morphological description, as well as, molecularly identified based on their ITS sequence.From the morphological description and molecular analysis, this strain has been confirmed as *A. calidoustus*, the well-known human pathogen. The strain was able to grow well at 37°C. To the best of our knowledge, this isthe first record of this species in Egypt.The strain was deposited in the culture collection of Assiut University Moubasher Mycological Centre as AUMC 2007 (=CCF 5184 in culture collection of fungi at the Department of Botany, Prague)and the ITS gene sequence was deposited at the National Centre of Biotechnology Information (NCBI) with GenBank accession number: MK729534.A brief description of the fungus was presented.

Key words: Human pathogen, *Aspergillus calidoustus*, Phenotypiccriteria, Genotypic characterization**.**

INTRODUCTION

Aspergillus ustus (together with *A. conjunctus*, *A. deflectus*, *A. panamensi s*and *A. puniceus*) was classified in the *Aspergillus ustus* group according to the manual of Raper& Fennell (1965). The group was then named section *Usti*(Gamset al.1985). Later, Kozakiewicz (1989) revised the group, and included *A. ustus*, *A. conjunctus*, *A. panamensis*, *A.puniceus*, *A. pseudodeflectus* and *A. granulosus* in the *A. ustus* group, and established the*A. deflectus* group to include *A. deflectus*, *A. pulvinus* and*A. Silvaticus* based on morphological studies. Klich (1993) treated *A. granulosus* as a member of section *Versicolores* and found that *A. pseudodeflectus* is weakly related to this section based on morphological treatment of section *Versicolores*. Peterson (2000) transferred *A. conjunctus*, *A. funiculosus*, *A. panamensis A. silvaticus* and *A. anthodesmis* to section *Sparsi*. More recently, Peterson (2008) examined the relationships of the *Aspergillus* genus using phylogenetic analysis of sequences of four loci andassigned 15 species to this section. In 2011, Samson et al.based on phylogenetic analysis of sequence data, described 5 species in *Usti* section (*A. carlsbadensis*, *A. californicus*, *A. germanicus*, *A. pseudoustus*, *A. turkensis*) and proposed a new combination *A. monodii* for *Fennellia monodii* and included 21 species in section *Usti*, two of which are able to reproduce sexually: *A. heterothallicus* (≡*Emericella heterothallica*) and *A. monodii* (≡*Fennellia monodii*). On 2012, Novakova *et al*. described 2 more species namely *A. baeticus* and *A. thesauricus* in section *Usti* from Spanish caves. On 2014, Visagie *et al.* added another novel species, *A. porphyreostipitatus*, on 2016, Jurjevic & Peterson described 2 new species *A. asper* and *A. collinsii*, and finally Romero *et al.* (2018) isolated *A. fuscicans* from Argentinean semi-arid soil as a new species in the section to reach a total of 27 species.Out of these species, 9 were recorded in Egypt, from different sources and localities (Table 1). Also, it was stated that, from 27 identified species within section Usti, *A. calidoustus* is the most relevant human pathogen (Glampedakis *et al.* 2020). A notable feature of this section is that about one third of the described species are known

only from the type isolate or specimen(Jurjevic and Peterson 2016), so addition of new strains to such species has significant importance.

The holotype of *Aspergillus calidoustus* was firstly identified by Varga *et al.* (2008) from bronchoalveolar lavage fluid, proven invasive aspergillosis, Nijmegen, The Netherlands.In Tertiary Care Hospital in Kuwait, it was isolated from outdoor and indoor air samples, as well as, from the bronchoalveolar lavage fluid of a patient with pneumonia (Khan *et al.* 2014). In 2020, it was recovered in Switzerland from woman underwent allogeneic hematopoietic stem cell transplantation for acute myeloid leukemia (Glampedakis *et al.* 2020). Also, it was observed in female with primary myelofibrosis underwent allogeneic-matched unrelated donor HCT, USA (Mendoza *et al.* 2020).

Species of *Aspergillus* section *Usti* are common in foods, stored maize, soil, dung and indoor air environments (Moubasher 1993, Samson *et al.* 2004, Houbraken *et al.* 2007, Samson *et al.* 2011). *A. ustus* was reported as a relatively human pathogen that can cause invasive infection in immunocompromised hosts (Weiss & Thiemke 1983, Iwen *et al.* 1998, Verweij *et al.* 1999, Pavie *et al.* 2005, Panackal *et al.* 2006, Yildiran *et al.* 2006, Krishnan-Natesan *et al.* 2008, Florescu *et al.* 2008, Vagefi *et al.* 2008). However, recent studies clarified that infections attributed to *A. ustus* are caused in most cases by another species, *A. calidoustus* (Houbraken *et al.* 2007, Varga *et al.* 2008, Balajee *et al.* 2009, Peláez *et al.* 2010), *A. granulosus* can cause disseminated infection in a cardiac transplant patient (Fakih*et al.* 1995), and *A. deflectus* can cause disseminated mycosis in dogs (Jang *et al.* 1986, Robinson *et al.* 2011 and Schultz *et al.* 2008). Several phenotypic and genotypic studies of aspergilli have been carried out (Rinyu*et al.* 2000, Varga *et al.* 2000 a,b). The internal transcribed spacers (ITS) regions, flanking the 5.8S region, located between the 18S and 28S rRNA genes, is the area of particular importance in discriminating the closely related species or at intraspecific level, due to their highly conserved and highly variable regions. ITS region has been frequently used to identify *Aspergillus* species (Accensi *et al.* 1999, Henry *et al.* 2000).

MATERIALS AND METHODS

Strain examined: the strain examined was isolated on dichloran Rose-Bengal chloramphenicol agar plates (King *et al.* 1979) from the air in October 2003 (AUMC 2007), in the city of Assiut. It was isolated by OA Al-Bedak in the Mycology laboratory of Assiut University Mycological Centre (AUMC), Assiut, Egypt. It was designated as *A. deflectus* of section Usti. Later it was re-examined, macro- and micro-morphological characteristics and proved to be related to section *Usti*, and DNA sequencing revealed its identity as *A. calidoustus*.

M**acro-morphological observations**, the strain was grown in the dark on the following standard media: Czapek´s yeast autolysate agar (CYA, Samson & Pitt 1985); Czapek´s agar (Cz, 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); mannitol agar (MA, Brayford & Bridge 1989); tannin sucrose agar (TAN, Thrane 1986); creatine sucrose agar (CREA, Frisvad 1985) and Christensen's urea agar (UA, Christensen 1946).Three replicate-plates of 3-point inoculations of all media were incubated at 25ºC except for CYA plates were incubated at 5, 25, 37 and 45ºC for 7 days. Growth rates were recorded on CYA, Cz and MEA and on media with reduced water activity Cz20S, G25N and M40Y) after 7 days of incubation.

Table 1.*Aspergillus* species of Section *Usti* recorded from Egypt

Species	Strain No.	Source	References
A. calidoustus	AUMC 2007 (= CCF 5184)	Air, Assiut	The current study
A. carlsbadensis	AUMC 6717	phyllosphere and nonrhizo-sphere soil of the orange & grapevine plantations, Assiut	(Moubasher *et al.* 2018)
A. aegyptiacus	AUMC 3603, AUMC 9488, AUMC 6120, AUMC 7335, AUMC 7614 and many others	Dung, Assiut, wood from monumental places, Cairo, textile, Assiut, temples soil, El-Minya, air	AUMC catalogue (2010)
	CBS 656.73^{T} = NRRL 5920	Sandy soil, under *Olea europaea*, Ras-El-Hikma	(Moubasher & Moustafa 1972, Samson *et al.* 2011)
	CBS 991.72C, CBS 991.72A, CBS 991.72B, CBS 991.72F.	Bare ferruginous soil, Dahkla Oasis, Western desert	(Samson *et al.* 2011)
A. heterothallicus	AUMC 6715	Citrus soil, Assiut	(Abdel-Sater *et al.* 2016)
A. lucknowensis	----------	indoors in the urban and rural homes	(Awad *et al.* 2013)
A. porphyreostipitatus	AUMC 6930	phyllosphere and nonrhizo-sphere soil of the orange plantation, Assiut	(Moubasher *et al.* 2018)
A. pseudodeflectus	AUMC 6744, AUMC 6926, AUMC 6944	Citrus soil, citrus soil, grapevine soil, Assiut	(Abdel-Sater *et al.* 2016)
	CBS 756.74^{T}	Desert soil, Western Desert	(Samson *et al.* 2011
A. puniceus	AUMC 6006, AUMC 6927, AUMC 6943	Soil, citrus soil, grapevine soil, Assiut	(Abdel-Sater *et al.* 2016), AUMC Catalogue (2010)
A. ustus	AUMC 2935, AUMC 4942, AUMC 6136, AUMC 6931, AUMC 7096, AUMC 7392	Acidic solution, moldy book, textile, citrus and grapevine soil, , waste water, air, phylloplane of onion, Assiut	(Abdel-Sater *et al.* 2016,Abdel-Gawad *et al.* 2017) AUMC catalogue (2010)

Turning the colour to pink on UA medium referred as urease positive. Results of 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. Colony colour was identified according to Kornerup and Wanscher (1978).

Micromorphological observations, microscopic mounts were made in lactophenol from CYA colonies after 7-10 days old.

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 fungalgrowth was scraped and immersed in 100 µl of distilled water and boiled at 100ºC for 15 minutes and sent to SolGent Company, Daejeon, South Korea for DNA extraction and sequencing. The fungal genomic DNA was extracted using SolGent purification bead in SolGent Company, Daejeon, South Korea. Internal transcribed spacer (ITS) sequences of nuclear rDNA were amplified using primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC). Then amplification was performed using the polymerase chain reaction (PCR) (The GeneAmp® PCR System 9700 thermal cycler, Applied Biosystems, Foster City, California, USA). 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 (2.5 U) 0.25 µl, template 1.0 µl, DW up to 25 µl. The amplification program was: initial denaturation at 95 °C for 15 min, followed by 30 cycles of denaturation at 95 °C for 20 s, annealing at 50 °C for 40 s and extension at 72 °C for 1 min., with a final extension step of 72 °C for 5 min. The PCR products were 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 electrophoreses on 1% agarose gel. These bands were then eluted and sequenced. Each sample was sequenced in sense and antisense direction. Contigs were created from the sequence data using the CLC Genomics Workbench Version 12. The sequence obtained from each isolate was analyzed using BLAST of the National Center of Biotechnology Information (NCBI) website. Sequences obtained together with those retrieved from the GenBank database were subjected to the Clustal W analysis using MegAlign software version 5.05 (DNASTAR Inc., Madison, Wisconsin, USA) for the phylogenetic analysis (Thompson *et al.* 1994). The sequences of other *Aspergillus* species used for comparison were retrieved from GenBank database ((http: //www.ncbi.nlm.nih.gov).

Results and Discussion

***Aspergillus calidoustus* Varga, Houbraken and Samson 2008**

Brief description

The representative strain was isolatedfrom the air in Assiut city, Egypt in 2003, at 25°C. The macro- and micro-morphological characteristics of the isolate proved to be related to section *Usti*, and DNA sequencing revealed its identity as *A. calidoustus*, recalling its significance reports as human pathogen. It is recorded here for the first time from Egypt. The strain is deposited at the culture collection of Assiut University Moubasher Mycological Centre (AUMC), Assiut, and assigned to AUMC 2007. The stain was also registered with a GenBank accession number MK729534(Table 4)(http: //www.ncbi.nlm.nih.gov.).

Colony diameters (range and mean ± SD) after 7 days at 25°C on CYA, CZ, MEA, CREA, UREA, MAN, TAN and the low water activity media Cz20S, M40Y and G25N; and at 5°, 37° and 45°C on CYA are shown in Table 2. Abundant growth was detected on UREA agar at 25°C and on CYA at both 25° and

37°C. No growth was detected on CYA at 5°C and at 45°C and faint growth on G25N at 25°C. Urease was produced, however no acid was detected on CREA or Mannitol agar at 25°C (Figure 1).

Colony colour: at 25°C on CYA whitish in the center and edge, brownish grey to brownin the middle (7E2-4), Cz whitish, MEA grey to brownish grey (5D-F1-2, with some white sectors), Cz20S whitish at the edge, grey to brownish center (5D-E1-2) and MAN grey to dull green (27D-E1-3), M40Y greenish grey to dull green(26D-E2-3) and on CYA at 37°C whitish with greenish brown to yellowish brown sectors (5F3-4).

Reverse: at 25° on CYA pale yellow to dull yellow (3A-B3-4), and Cz pale yellow to greyish yellow (4A3-4B3-5), MEA dull blond to yellowish brown(5D-E4-5), Cz20S centerbrownish grey (5D-F2) withfaint edge, MAN yellowish white (2A2). No reverse on M40Y at 25°C and CYA at 37°C.

Colony texture: floccose on CYA and Cz at 25°C.

Conidial heads loosely columnar; stipes 25-150 µm long x (2.5) 6–7 (9) µm width, smooth, brown;some showing curvature along its main axis and bent below the vesicles, vesicles 10-12 µm wide, pyriform to broadly spathulate, biseriate; metulae 4-6x 2µm, covering the upper part 50- 75% of the vesicle; phialides 6-7 x 2-3 µm; conidia globose 3-4 (4.5) µm (Table 3), with very prominent echinulations (Figure 2); Hull cells were sparsely produced, irregularly elongated (Varga *et al.* 2008).

Diagnostic features: good growth at 37°C and coarsely roughened to echinulate conidia.

This species was first described in 2008 (Varga *et al.* 2008) from bronchoalveolar lavage fluid, proven invasive aspergillosis, and many other clinical isolates, Nijmegen, The Netherlands; Postcataract surgery endophthalmitis, Turkey, indoor air, Germany; Wooden construction material, Finland; Pasteur Institute, Paris, France; Oslo, Norway; Osteorickets; as a culture contaminant, Peoria, USA (Houbraken *et al.* 2007, Varga *et al.* 2008, Samson *et al.* 2011); Miami, Florida, USA (Mendoza *et al.* 2020).

Features presented in Varga *et al.* (2008) and Samson *et al.* (2011) revealed significant morphological differences of the closely related species *A. calidoustus* and *A. pseudoustus*. The width of the stipes is larger in *A. calidoustus* (4-7 µm) than in *A. pseudoustus* (3.5-5 µm) and *A. ustus* (3-6µm). Moreover, the growth of *A. calidoustus* at 37°C distinguishes it from *A. ustus* and *A. pseudoustus*. The conidia ornamentation and sizes can be used as a reliable feature for distinguishing the two related species, *Aspergillus calidoustus* (coarsely rough to echinulate, 2.7-3.5 µm) from *A. pseudoustus* (smooth to echinulate, 2.5-3.0 µm).Thevesicle shapes and diameter of both *A. calidoustus* (pyriform to broadly spathulate, 7-20 µm) and*A. pseudoustus* (globose,10-14 µm) show also further important differences. Hull cellswere not observed in *A. pseudoustus* in contrast with *A. calidoustus*, which irregularly elongated and produced in scattered groups.

A. calidoustus has been described as an emerging cause of invasive aspergillosis (IA) and pulmonary infection, particularly in hematopoietic stem cell and solid organ transplant recipients (Peláez *et al.* 2013), from the bronchoalveolar lavage fluid of a patient with pneumonia (Khan *et al.* 2014). All *A. calidoustus* isolates tested by Khan *et al.* (2014), were susceptible to caspofungin and micafungin. However, they showed low susceptibilities to itraconazole, posaconazole, voriconazole, amphotericin B and several other antifungal drugs (Varga *et al.* 2008). Seroy *et al.* (2017) described four cases and reviewed 8 additional cases of *A. calidoustus* from immunocompromised persons. These cases occurred in patients with hematologic malignancy, receipt of hematopoietic cell transplantation, and/or solid organ transplant recipients.

Table 2: Growth measurements (range and mean ± SD of 9 readings) of *A. calidoustus* AUMC 2007.

	Growth measurements (in mm)												
	25°										**37°**	**5°**	**45°**
	CYA	**Cz**	**MEA**	**CREA**	**UREA**	**MAN**	**TAN**	**Cz20S**	**M40Y**	**G25N**	**CYA**	**CYA**	**CYA**
range	38-46	33-40	30-40	22-35	34-52	15-25	13-30	31-40	25-32	2-7	23-60	0.0	0.0
mean	40.56±2.19	35.78±2.4	33.11±3.0	29.33±3.7	43.17±5.0	19.67±3.0	20.56±6.5	35.61±2.4	28.61±1.7	4.5±1.7	40.67±14.3		

Table 3: Microscopic measurements (means of at least 10 readings, in µm) of *A.calidoustus* AUMC 2007.

Species	**Stipe length**	**Stipe width**	**Vesicle diam**	**Conidia size**	**Acid from creatine**	**Urease**
Aspergillus calidoustus	25-150	(2.5) 6–7 (9)	10-12	3-4 (4.5)	-	+

Table 4: Genetic similarities of *A. calidoustus* isolate no. AUMC 2007 (accession number: MK729534) isolated from air with the closest match in the GenBank database and sequence similarity in percent to the match as inferred from Blastn searches of ITS sequences.

Closest GenBank match # ITS	**Culture collection code**	**Sequencing similarity**	**Length**	**Species name**	**Isolation source**	**Country**	**Year of isolation**	**References**
MH863122	CBS 121601^{T}	366/374 (97.86%)	582	*A. calidoustus*	Man bronchoalveolar lavage (BAL)	Netherlands	1992	Varga et al. 2008
NR_135372	NRRL 6135^{T}	366/374 (97.86%)	591	*A. pseudodeflectus*	Desert	Egypt	2008	Peterson 2008
FJ531146	CBS 123887^{T} = DTO 27D9	348/355 (98.03%)	495	*A. germanicus*	Indoor air	Germany	2011	Samson et al. 2011
NR_131292	CBS 107.25^{T} = NRRL 279^{T}	366/374 (97.86%)	591	*A. insuetus*	Unknown	Cape town, South Africa	2008	Peterson 2008
NR_135432	CCF 4166^{T}	366/375 (97.60%)	590	*A. thesauricus*	Spanish caves	Spain	2012	Novakova et al. 2012
NR_135446	NRRL58999^{T}	353/376 (93.88%)	594	*A. subversicolor*	coffee berries,	Karnataka, India	2012	Jurjevic et al. 2012
NR_131284.1	CBS 261.67^{T}	306/343 (89.2%)		*A. ustus*	Culture contaminant	USA	2008	Varga et al. 2008
FJ531147.1	IBT 28161	280/318 (88.1%)	464	*A. pseudoustus*	Stored maize	South Africa	2011	Samson et al. 2011

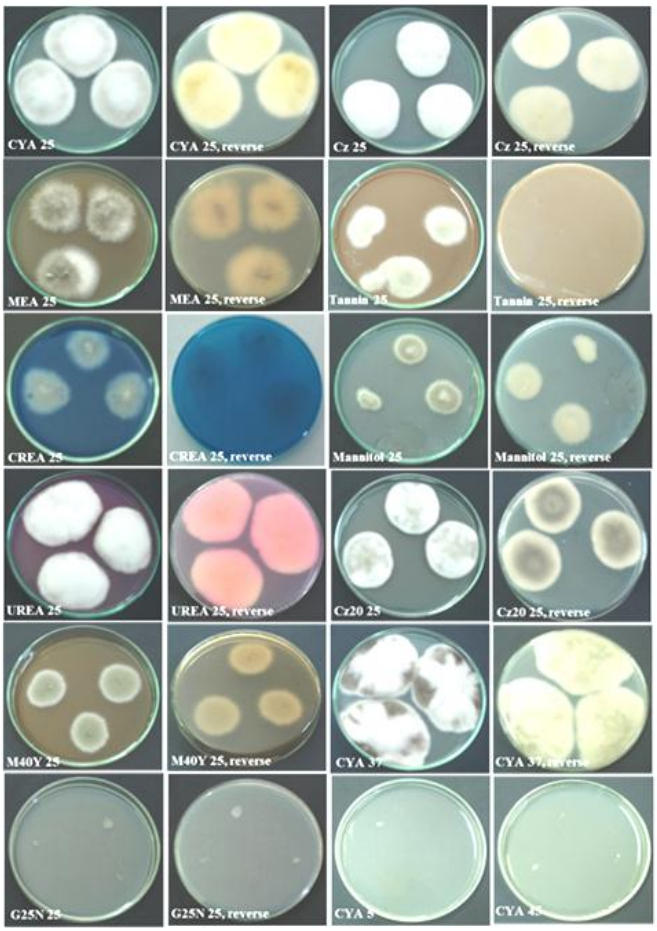

Fig. 1. *Aspergillus calidoustus* stain AUMC 2007 grown at 25°C on CYA, Cz, MEA, Tannin, Creatine, Mannitol, Urea, Cz20%S, M40Y and G25N and at 5°, 37° and 45°C on CYA.

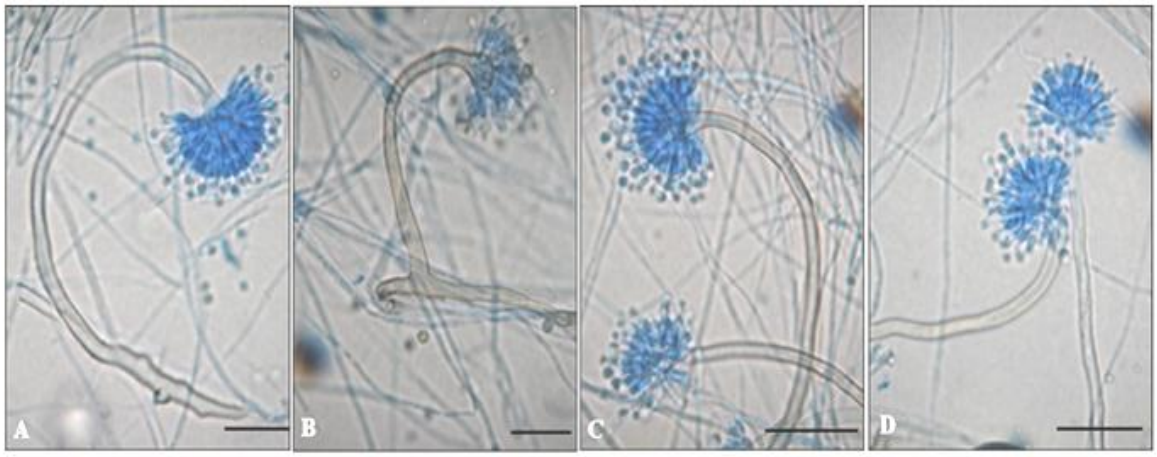

Fig. 2. Microscopic structures of *Aspergillus calidoustus* stain AUMC 2007; A-D, Conidial heads; bar 30 µm.

Also, it was observed that, *A. calidoustus* colonizes the water systems and this may be considered a potential health risk for human because the water systems play a potential role as a source of nosocomial fungal infections (Hageskal *et al.* 2011).

A. calidoustus has been demonstrated as a potential source of many pharmaceutical products such as drimanes, and ophiobolins G (Cutler *et al.* 1984), and austins (Chexal *et al.* 1976). Ophiobolins have bioactivity towards leukemia cells with induction of apoptosis at nanomolar concentrations and exhibit inhibitory activity against cancer cell lines including lung cancer, colon cancer, melanoma, leukemia and ovarian cancer (Bladt *et al.* 2013).

Molecular identification: The strain was molecularly identified according to ITS gene sequence as *Aspergillus calidoustus* and deposited in Gene Bank with accession number, MK729534 (Table 4, Figure 3).

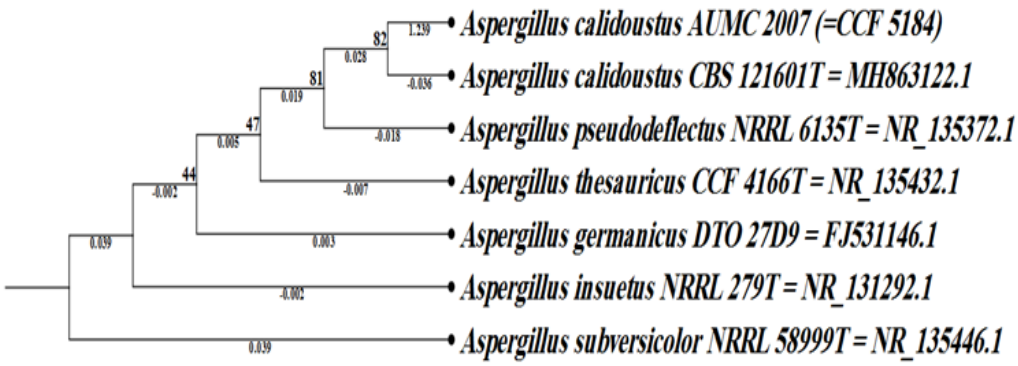

Fig. 3. Phylogenetic tree of *Aspergillus calidoustus* stain AUMC 2007 (accession GeneBank number: MK729534) with closely related species from GeneBank database.

Dichotomous key for identification of species of section Ustithat wererecorded from Egypt

(based on phenotypic characteristics)

1. Ascospores produced (heterothallic species) ……..…….……..… ……... ***A. heterothallicus***
1. Ascospores not produced ……...…….……..……...… **2**

2. Colony diam on CYA at 25ºC in 7 days attaining 20 mm or less, with sulfur bright yellow colour, becoming reddish in age ……..……..……. ***A. lucknowensis***
2. Colony diam on CYA at 25ºC in 7 days exceeding 25 mm or less, not sulfur yellow ……..……..... **3**

3. Growth on CYA at 37ºC …….....……..…….……..…….. **4**
3. No growth on CYA at 37ºC ……..…….……..……. **9**

4. Weak growth on CYA at 37ºC (5–10 mm diam), stipe 185–410µm ……..... ***A. heterothallicus***
4. Good growth on CYA at 37ºC (up to 45 mm diam), stipes not exceeding 300 µm long ……...…..…. **5**

5. Conidia up to 5.5 µm diam, Hull cells absent.……..…….... ***A. pseudodeflectus***
5. Conidia less than 5 µm diam, Hull cells present ……..……..……..……..…….. **6**

6. Vesicle pyriform to broadly spathulate,9-15 µm in diameter ……..... ***A. calidoustus***
6. Vesicle globose to dome-shaped, 6–15 µm in diameter ……..…..…….. **7**

7. Stipe short, 20-35 µm long, conidia globose to almost cylindrical, smooth, 3.5-5 in diameter, Hull cells 15-20 µm diam .....…… ***A. egyptiacus***

7. Stipe long, (20)50–245 µm long, conidia globose to subglobose, or ellipsoidal, spiny to tuberculate,

3–4 µm diam, Hull cells 15-92 long ..…….....……..……..……..….. **8**

8. Stipe long, 105–245 µm long, Hull cells 15–30 µm long, globose to broadly ellipsoidal, colony diam on Cz at 25ºC 12–22 ……... ***A. carlsbadensis***

8. Stipe short, (20)50–125 µm long, Hull cells 26–92 µm long, predominantly elongate, twisted, colony

diam on Cz at 25ºC 24–35 ……..…….. ***A. porphyreostipitatus***

9. Conidia ellipsoidal 3.5–4.0 µm diam, Hull cells globose to broadly ellipsoidal, 15-30 µm Long ……..……...……. ***A. carlsbadensis***

9. Conidia globose, 2.5-3.5 µm diam, Hull cells not as above……..……..……..…… **10**

10. Hull cells crescent shape or twisted, aggregated in yellow masses, vesicle subglobose (8–16 µmdiam) to elliptical (15–18), colonies on Cz 40-50 mm diam, exudate wine red ……..…….....… ***A. puniceus*** ……..……..…

10. Hull cells ovate, elongate, helicoid or twisted, vesicle hemispherical to subglobose, 7–15 µmdiam,

colonies on Cz 45-60 mm diam exudate colourless or yellow to brown…….....…….…..***A. ustus***

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