Original paper

Aspergillus sakultaensis, a new species in section *Flavipedes* isolated from Sohag Governorate, Egypt

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ABSTRACT

During the routine sampling from Atawayel canal at Atawayel village, Sakulta city, Sohag Governorate, Egypt and isolation of fungi from these water samples, an interesting isolate of *Aspergillus* was recovered. The phenotypic characteristics of the isolate revealed its strong relationship to *Aspergillus* section *Flavipedes*. Sequencing of the Internal Transcribed Spacer (ITS) gene demonstrated that the isolate is a new species belonging to section *Flavipedes*. The new taxon morphological characters exhibited a huge differentiation between other species in brightly yellow mycelium color on MEA and CYA at 25 °C and on MEA, CYA, Cz and CREA at 30 °C, conidiophore length (up to 3 mm), accessory conidia absence and hull cells shape. The new strain was described and illustrated as *A. sakultaensis*. A pure culture of the new taxon was deposited in the culture collection of the Assiut University Mycological Centre as AUMC 13885, uploaded in MycoBank as MB 831480 and the ITS sequence was uploaded to GenBank as MK391495.

KEYWORDS

Aspergillus,
Flavipedes,
New taxon,
Phylogeny,
ITS.

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INTRODUCTION

The genus *Aspergillus* is one of the best-known and most studied fungi that occupying various habitats worldwide since the description of the genus Aspergillus from almost 300 years ago by Micheli (Dyer & O'Gorman 2012). They found in terrestrial habitats and are commonly isolated from soil particularly in tropical and subtropical environments (Carroll & Wicklow 1992; Klich 2002; Domsch *et al*. 2007). They also isolated from foods, indoor environments and as endophytes as well as from several cases of human and animal infections including osteomyelitis, cutaneous aspergillosis, otomycosis, onychomycosis and diskospondylitis (Stuart & Blank 1955; Roselle & Baird 1979; Barson & Ruymann 1986; Schultz *et al*. 2008; Gehlot *et al*. 2011).

Aspergillus species have been described morphologically into 18 groups (Raper & Fennell 1965) namely: *clavatus*, *gluacus*, *ornatus*, *cervineus*, *restrictus*, *fumigatus*, *ochraceus*, *niger*, *candidus*, *flavus*, *wentii*, *cremeus*, *sparsus*, *versicolor*, *nidulans*, *ustus*, *flavipes*, and *terreus*. Depending on biochemical and molecular analysis, *Aspergillus* species were distinguished into eight groups namely: *Eurotium*, *Fumigati*, *Ornati*, *Clavati*, *Nidulantes*, *Circumdati*, *Stilbothamnium* and *Ochraceoroseus* (Samson 1992; Verga *et al*. 2000; Klich 2006). Traditionally, filamentous fungi have been defined by morphology, wherein a species is recognized as

a group of isolates that share a set of morphological characteristics distinguishing it from other isolates. Thus, identification of aspergilli is based upon a cumulative evaluation of their colony characteristics and microscopic morphology: color of the aerial parts of the Aspergillus colony, pigmentation of the basal mycelium or the underlying exudates, the rate of colony growth, the texture of the surface growth of the colony, growth zones and colony topography (Balajee *et al*. 2006). However, the morphological identification of *Aspergillus* suffers from several limitations and recognizing these limitations as early as 1965, Raper and Fennell noted that in studying aspergilli, it is important to realize that strains may vary within a species, that species may vary within a group and that groups overlap within the genus (Raper & Fennell 1965). Several recent studies have brought into focus the difficulties of phenotypic methods of identification in aspergilla (Alsohaili & Bani-Hasan 2018).

Molecular identification of the filamentous fungi was conducted based on sequence of mitochondrial cytochrome b gene (Wang *et al*. 2001), mycotoxin regulatory genes, alkaloids (Chang *et al*. 1995), DNA topisomerase gene (Kanbe *et al*. 2002), and ribosomal RNA identity (Hinrikson *et al*. 2005). However, ribosomal RNA regions as 18S rRNA and internal transcribed spacers (ITS 1 and ITS 2), between the small and large subunit, is a universal fungal probe as species-species identifiers for saprophytic filamentous fungi (White *et al*. 1990; Henry *et al*. 2000; Iwen *et al*. 2002; De-Aguirre *et al*. 2004). Furthermore, the 5'-end of the large rRNA subunit (D1 and D2 domain) have been studied to emphasize the molecular approaches for identification of fungi (Scorzettiet *et al*. 2002; Hall *et al*. 2004; Peterson 2008). Schoch *et al*. (2012) proposed the use of nuclear ITS sequences for BarCode identification of fungal species. Others have included additional loci such at β-tubulin or calmodulin to confidently identify isolates (Peterson 2012). While DNA sequence analysis is perhaps the most accurate method of species identification, it is still to provide descriptions that allow for recognition of the species with morphological criteria. The *Aspergillus flavipes* group (Thom & Church 1926) originally contained only *A. flavipes* while Raper & Fennell (1965) placed *A. flavipes*, *A. carneus* and *A. niveus* in the group. Gams *et al*. (1985) erected *Aspergillus* section *Flavipedes* to contain species of the informal *A. flavipes* group.

The addition of new species of fungi isolated from different environments will remain one of the most important goals of scientific research to identify the treasures contained in those environments that may have a great impact in the areas of discovering and producing new useful materials. In this study, a new interesting species of fungi belonging to *Aspergillus* section *Flavipedes* was isolated during the routine sampling from Atawayel canal at Atawayel village, Sakulta city, Sohag Governorate, Egypt and identified based on their phenotypic and genetic characters.

MATERIALS & METHODS

Sampling site

Sohag Governorate is located in the southern part of Egypt (Upper Egypt), and covers a stretch of the Nile Valley. It lies between 26.56°N and 31.7°E with total area of 11022 km^2 and extends 125 km long and 16-25 km wide. The governorate consists of 12 centers, comprising 12 cities, 3 districts and 51 major villages. The inhabited area is about 1593.92 km^2, about 14.5% of the total area of the Governorate. Using sterile 1L bottles, water samples were collected from Atawayel canal at Atawayel village, Sakulta city, Sohag Governorate.

Strain isolation

The fungus was isolated from a water sample using pour plate technique (Sanders 2012) on potato dextrose agar of the following composition (g/L): potato infusion from 200 g, glucose, 20 and agar, 20. Rose Bengal (0.05 g/L) and chloramphenicol (0.25 g/L) were added to the medium to prevent the bacterial growth. The medium pH was adjusted at 7.3. After autoclaving,

aliquots of 20 ml medium were poured into sterile Petri plates each containing 1ml of water sample. The Cultures were then incubated for 7-15 days at 25°C. The developing fungal colonies were isolated as pure cultures and maintained in slants of MEA at 4°C. One of the fungal isolates expected to be a new species was sent to the Assiut University Mycological Centre for reidentification and preservation in the culture collection.

Phenotypic studies

Morphological characteristics of the fungal isolate were studied by culturing on different media such as Yeast Extract Sucrose agar (YES, Frisvad 1981), Malt Extract Agar (MEA, Samson *et al*. 2010), Czapek Yeast Autolysate agar (CYA, Pitt 1979), Czapek's agar (Cz, Raper & Thom 1949) and Creatine sucrose agar (CREA, Frisvad 1981). Plates were inoculated in three-point pattern using a micropipette and inoculum size of 1 µl per spot. Unwrapped plates were incubated in the dark reverse side up at 25 °C. Microscopic features of the fungal culture grown on MEA were examined in lacto-phenol cotton blue mounts.

Molecular characterization of the fungal isolate

DNA extraction

The protocol employed in Moubasher *et al*. (2019) was followed, in which 0.2 g of 7-day-old fungal mycelia of *A. sakultaensis* grown on MEA, were grounded using liquid nitrogen and transferred to 1.5 ml microfuge tubes. 800 µl CTAB buffer composed of 3 % CTAB, 1.4 M NaCl, 0.2 % Mercaptoethanol, 20 mM EDTA, 100 mM TRIS-HCl pH 8.0 and 1 % PVP-40, were added to each tube. After incubation at 65 °C for 30 min, 800 µl of CI Mix with the composition of 24 ml chloroform and 1 ml isoamyl alcohol, were gently added and mixed with the tube contents. A clear supernatant was obtained by centrifugation at 10000 xg for 10 min. For DNA precipitation 2/3 volume of isopropanol (precooled at -20 °C) was added and mixed gently. The samples were incubated at 4 °C overnight, thereafter centrifugation at 13000 xg for 10 min. The supernatant was discarded, and the pellet was pooled and washed with 200 µl washing buffer composed of 76 % ethanol and 10 mM ammonium acetate. The washing buffer was carefully decanted, and the pellet was suspended in 200 µl TE buffer supplemented with 10 mg/ml RNase. After incubation at 37 °C for 30 min, 100 µl of 7.5 M ammonium acetate and 750 µl ethanol were added and mixed gently. Samples were centrifuged at 13000 xg for 10 min at room temperature. The supernatant was completely discarded, and the pellet was suspended in 100 µl sterile distilled water.

PCR amplification and sequencing of rDNA using ITS1 and ITS4 primers

The fungal DNA was sent to SolGent Company, South Korea for polymerase chain reaction (PCR) and sequencing of rRNA gene. PCR was performed using SolGent EF-Taq and the universal primers ITS1 and ITS4 (White *et al*. 1990). In the PCR tubes 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 ddH_2O, 10 pmol of ITS1 (5´ TCC GTA GGT GAA CCT TGC GG 3´) and ITS4 (5´ TCC TCC GCT TAT TGA TAT GC 3´) were added. Then the PCR amplification was carried out using the following sequence: 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) prior to sequencing. The purified PCR products were confirmed on 1% agarose gel by electrophoresis using 100 bpDNA ladder as a size marker. The bands were eluted and sequenced in the forward and reverse directions using the same pimers and incorporation of ddNTPs in the reaction mixture. The obtained sequences were analyzed using Basic Local Alignment Search Tool (BLAST) from the National Center of Biotechnology Information (NCBI) website.

Phylogenetic analyses

The phylogenetic analysis was inferred by the Maximum Likelihood method based on the General Time Reversible model (Nei & Kumar 2000). The bootstrap consensus tree inferred from 100 replicates (Felsenstein 1985) is taken to represent the evolutionary history of the taxa analyzed (Felsenstein 1985). Branches corresponding to partitions reproduced in less than 50 % bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches (Felsenstein 1985). Initial tree(s) for the heuristic search were obtained automatically by applying the Maximum Parsimony method. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.1000). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 48.95 % sites). The analysis involved 15 nucleotide sequences. All positions with less than 95 % site coverage were eliminated. That is, fewer than 5 % alignment gaps, missing data, and ambiguous bases were allowed at any position. There were 522 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*. 2016).

RESULTS

Phylogenetic analysis

The phylogenetic analysis was inferred using the Maximum Likelihood method based on the General Time Reversible model. The tree with the highest log likelihood (-794.17) is shown in Figure 1. The ITS data set composed of 15 sequences including the ITS sequence of *A. sakultaensis*. The new species was consistently grouped with *A. flavipes*, *A. templicola*, and *A. mangaliensis* forming a highly supported clade.

Sources, localities and percentages coverage and similarity of *Aspergillus* strains aligned with *A. sakultaensis*.

Data in table 1 summarise some basic information on 16 *Aspergillus* strains closely related to the strain isolated in the current study. Although *A. sakultaensis* showed 100% similarity with some strains of *A. flavipes* and *A. mangalensis* (from Turkey, China, Egypt and Czech Republic), the percentages of sequence coverage were generally low (91% - 95%). This means that *A. sakultaensis* is still geneitically different from these strains. Another important criterion is the low percentages of identity and coverage of *A. sakultaensis* sequences (98.04% and 94% respectively) when aligned with those of the type material of *A. flavipes* NRRL302T (NR_135397). Similar observations of low identity and coverage can be observed with sequences of the type strains of *A. neoflavipes*, *A. templicola*, *A. frequens* and *A. lizukae* as shown in Table 1.

Taxonomy

Aspergillus sakultaensis OA Al-Bedak, AA Zohri & MM Abel-Kareem sp. nov. Fig. (2)
GenBank: ITS=MK391495
MycoBank: MB 831480

Typification: EGYPT. Sohag Governorate: Sakulta city, Atawayel village, from water sample of Atawayel canal, 10Aug 2018, Marwa Abdel-Kareem, MAK-2008 (Holotype AUMC13885).

Etymology: Named after Sakulta city in Sohag Governorate (Egypt) that is located close to the place of isolation.

Cultural characteristics

Colonies on MEA reaching a diameter of 26-30 mm in 7 days at 25 °C, velutinous to floccose with granular surface, plane, zonate, with moderately raised central part of colonies, brownish-grey to greyish brown (5C2-5D3) in the center, margin entire, paler with yellow to vivid yellow mycelia (2A7-2A8) forming complete zone around the colony center, reverse raw umber to brownish-grey (5F8-6F8), On CYA after 7 days at 25 °C, colonies attaining a diameter of 28-30 mm, brownish orange to greyish brown (5C3-5D3) in the center, velutinous to floccose with granular surface, zonate, radially wrinkled with raised colony center, yellow mycelia (2A7-3A7) form a complete zone around the center, 2-3 mm from the

margin, margin paler, entire, reverse light brown to yellowish brown (5D6-8 – 5E6-8). On Cz after 7 days at 25 °C colonies attaining 16-18 mm diameter, brownish grey to greyish brown (5C2-5D3) in the center, floccose with granular surface, zonate, plane, margin entire, paler than the center, reverse greyish orange (5B4-5) to brownish orange (5C4). On CREA colonies reaching 20-23 mm diameter after 7 days at 25 °C, velutinous to floccose, plane, slightly sulcate, brownish grey to greyish brown(5C2-5D3) in the center, margin entire, white, reverse reddish brown (8D-F7), no acid production. Colonies in 7 days at 30 °C attaining a diameter of 32-35 mm on MEA, 29-30 mm on CYA and Cz, and 27-30 mm on CREA, yellow mycelia dominant on all media.

Colonies on CYA at 37 °C after 7 days were similar to those on CYA at 30 °C attaining a diameter of 28-30 mm. and dominant yellow mycelia (Figure 2).

Conidial heads brownish-grey to greyish brown (5C2-5D3), loosely columnar to radiate, conidiophores biseriate, stipes hyaline to light brown, smooth-walled, up to 3 mm long× 5-8µm width (n=50), diminutive conidiophores not observed. Vesicles variable in shape, globose, subglobose or spathulate, most commonly, (10 -) 12-20 µm diameter (n=50), metulae 4–6 µm long (n=50), phialides 6.0-7.5 µm long (n=50), Conidia globose, subglobose, polygonal, mostly 2-3 µm in diameter (n=50). Accessory conidia not observed. Hülle cells formed as yellow mycelial clumps, variable in shape and dimensions, globose, elliptical, ossiform, lobate, irregularly elongated and narrow, exudate colourless. No ascomata or ascospores observed (Figure 3).

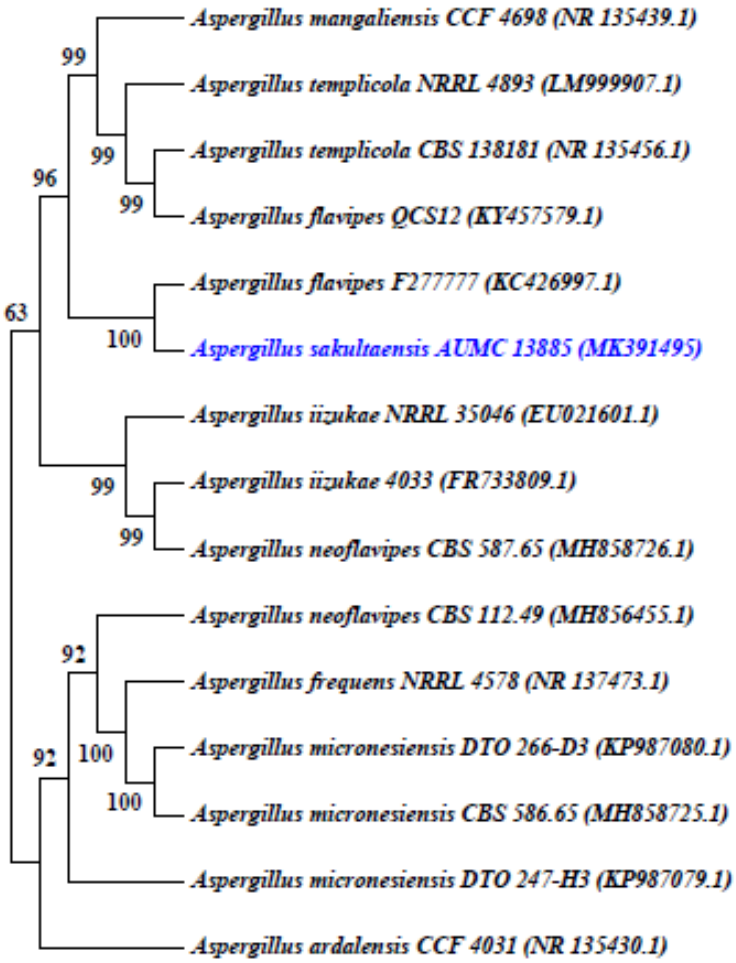

Figure (1) Maximum Likelihood phylogenetic tree of ***Aspergillus sakultaensis*** **AUMC13885** with other related *Aspergillus* species in section *Flavipedes* based on their nucleotide sequences of the ITS region. The new species in blue color.

Table (1) Sources, localities and percentages coverage and similarity of *Aspergillus* strains aligned with *A. sakultaensis*

***Aspergillus* strain of the current study**	**Closely related strains of *Aspergillus* accessed from the GenBank (T=Type material)**						
Aspergillus sakultaensis AUMC 13885 (MK391495) from a water sample, Sohag/Egypt	Fungal species	Strain No.	Accession No.	Source	Country	Coverage	Similarity
	A. flavipes	QCS12	KY457579	Soil	China	98%	99.66%
	A. flavipes	63	KF671231	Leaves of *Stevia rebaudiana*	India	98%	99.14%
	A. flavipes	F277777	KC426997	Soil	Spain	96%	99.82%
	A. flavipes	08CK020	KF938457	marine sediment	Turkey	94%	!00%
	A. flavipes	M1	HM595494	endophytes of *Abies beshanzuensis*	China	95%	100%
	A. flavipes	AUMC1201	MN428799	Soil	Egypt	95%	100%
	A. flavipes	DTO303-14	KP987083	Unknown	Iran	99%	97.97%
	A. flavipes	NRRL 302**T**	NR_135397	Unknown	USA	94%	98.04%
	A. flavipes	E14	GU566238	Rhizosphere of *Phalaris arundinacea*	Czech Republic	99%	97.98%
	A. neoflavipes	NRRL5504**T**	NR_137475	Unknown	USA	94%	98.21%
	A. mangaliensis	CCF4698	NR_135439	European caves	Czech Republic	91%	100%
	A. templicola	CBS138181**T**	NR_135456	indoor house dust	Moxico	91%	99.81%
	A. templicola	DTO 267-H4	KP987081	Unknown	Iran	99%.	99.48%
	A. frequens	NRRL4578**T**	NR_137473	Unknown	USA	94%	98.04%
	A. ardalensis	CCF4031T	NR_135430	Soil	Czech Republic	94%	98.40%
	A. lizukae	NRRL3750**T**	NR_135398	Unknown	USA	94%	98.05%

Distinguishing features

Long conidiophore (up to 3 mm), brightly yellow mycelia on MEA and CYA at 25 °C and on MEA, CYA, Cz and CREA at 30 °C, as well as absence of accessory conidia are the distinguishable morphological characters that differentiate the new taxon from other species in section Flavipedes.

DISCUSSION

The novel species in the present study was isolated from a freshwater sample collected from Atawayel canal, Sakulta city, Sohag Governorate, Egypt. The morphological examination of the new taxon in the current study showed typical phenotypic characteristics of *Aspergillus* section *Flavipedes*. The sequencing of the ITS gene revealed the strong relationship of the new taxon with members of section *Flavipedes*. Phylogenetic tree showed that the new species *A. sakultaensis* was consistently grouped in subclade with *A. flavipes*, *A. templicola* and *A. mangaliensis*.

In section *Flavipedes*, the cultural morphology is very useful in the differentiation between species, since brightly yellow mycelia are present in A. sakultaensis colonies on MEA and CYA at 25 °C and on MEA, CYA, Cz and CREA at 30 °C, in *A. ardalensis* on MEA, and in both *A. neoflavipes* and *A. luppii* on all media at 25 °C, while colonies of *A. movilensis* are whitish and those of the remaining species are mostly in shades of brown at 25 °C. In addition, the red or brownish soluble pigment is present in *A. iizukae* and *A. frequens* while it is absent in *A. sakultaensis*.

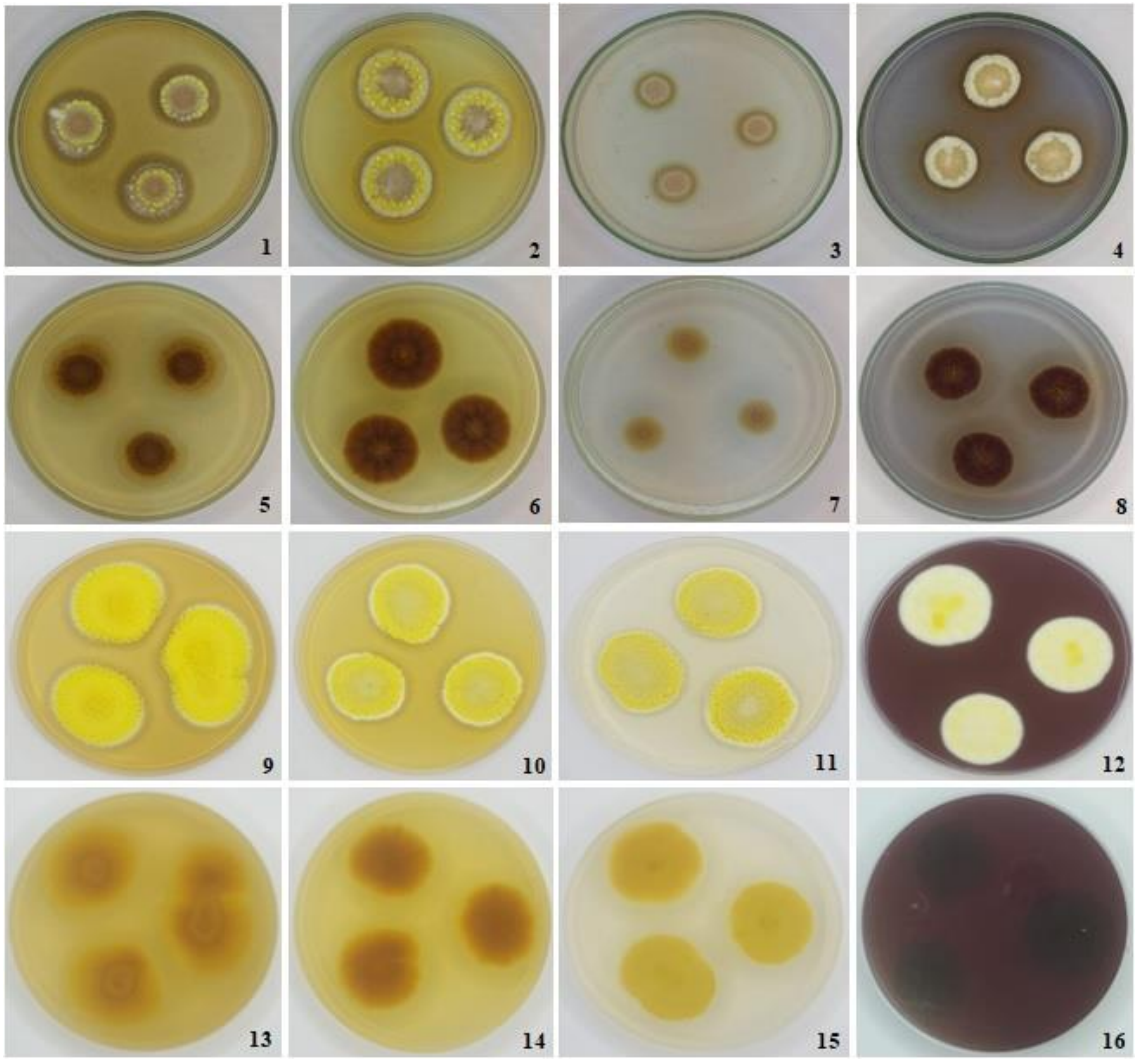

Figure (2) ***Aspergillus sakultaensis*** AUMC13885. Colonies after 7 days at 25 °C, **1-4**: on MEA, CYA, CZ and CREA at 25 °C; **5-8**: reverse on MEA, CYA, CZ and CREA at 25 °C; **9-12**: on MEA, CYA, Cz and CREA at 30 °C; **13-16**: reverse on MEA, CYA, Cz and CREA at 30 °C.

The vesicle shape and diameter are also very important features in species differentiation as the vesicles of *A. flavipes* are predominantly spathulate and their diameter not exceeding 11 µm and mostly with a constriction at the base, those of *A. iizukae* commonly exceed 20 µm diam in contrast to other species while *A. sakultaensis* has globose, subglobose or spathulate vesicles, that most commonly measure 12-20 µm diameter and this character can distinguish the new species from *A. flavipes* and *A. iizukae*.

Conidiophore is another important morphological character that helps in species differentiation in this section. The conidiophore of *A. sakultaensis* is a distinctive morphological character among other members of the section. It measures up to 3 mm long being the longest conidiophore recorded in all species in section *Flavipedes*, while the conidiophores of *A. ardalensis*, *A. mangaliensis*, *A. flavipes*, *A. frequens*, *A. iizukae*, *A. movilensis*, *A. neoflavipes*, *A. templicola*, *A. micronesiensis* and *A. capensis* may exceed 1 mm long but they are shorter than that of *A. sakultaensis*. In addition to the previous morphological features, Hülle cells are considered as important microscopic criteria that help in the differentiation between these species. In *A. sakultaensis*, Hülle cells are formed as masses of yellow clumps at the colony margin, Hülle cells are variable in shape and dimensions, globose, elliptical, ossiform, lobate, irregularly elongated and narrow. They resemble those of *A. frequens*, *A. iizukae*, *A. mangaliensis* and *A. movilensis* while A. *flavipes*, *A. frequens* and *A. polyporicola*

lack Hülle cells in their cultures. Also, the accessory conidia are missing in the new taxon; which considered, with the long conidiophore, the most distinguishable morphological characters that can differentiate *A. sakultaensis* from all other species in section *Flavipedes*.

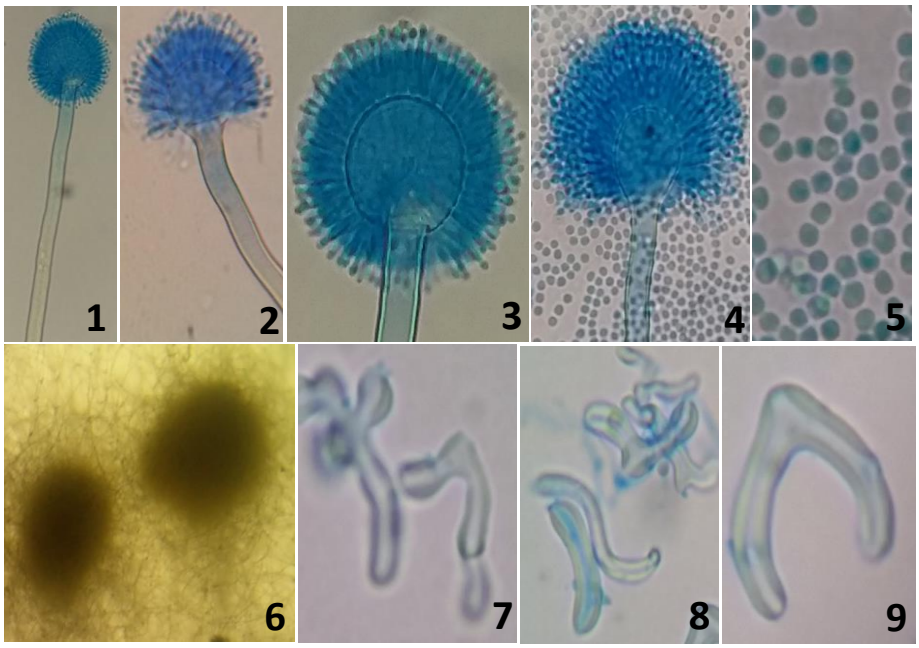

Figure (3) *Aspergillus sakultaensis* AUMC13885. **1-4**: Conidial heads; **5**: conidia; **6**: clumps of Hülle cells; **7-9**: Hülle cells with different shapes.

CONCLUSION

In this research, a new interesting species of fungi named Aspergillus sakultaensis belonging to Aspergillus section Flavipedes was isolated, described, identified and added to the science. The studies are still ongoing on this fungal species in our laboratory by the same researcher group to identify its physiological characteristics and the possibility of using it in producing compounds of economic value.

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