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Aspergillus creber and *A. keveii*, two new records as endophytes from wild medicinal plants in Egypt

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Abstract. In the present research and during surveys of mycobiota inhabiting leaves and roots of some wild and medicinal plants in El-Kharga Oasis, New Valley Governorate, Egypt, two significant isolates were isolated for the first time in Egypt and for the second time worldwide after type species inhabiting leaves of *Convolvulus arvensis* and *Moringa oleifera*. Those isolates were phenotypically classified as *Aspergillus creber,* and *A. keveii* referred to the representative type species belonging to the genus *Aspergillus* section *Versicolores* and section *Usti*, respectively. Pure cultures of both species have been deposited in the Culture Collection of Assiut University Mycological Centre with *A. creber* AUMC 14298 and *A. keveii* AUMC 14299. Their identification was verified by molecular recognition based on the sequencing of the ITS gene for each. Sequences of the two fungi's ITS genes have been submitted to GenBank with respective accession numbers MN542346 and MN542353, respectively. These species are recorded here for the first time worldwide as endophytes. The current research provides brief descriptions and photographs of both species.

Keywords: A*spergillus*, *Convolvulus*, endophytes, medicinal plants, *Moringa*, New Valley

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1. Introduction

The present research is at the New Valley Governorate, Egypt, in the field of environmental mycology. It is an extension of the systematic surveys performed in various Egyptian habitats, which have been extended to other mycological fields for over 50 years. It is known that only a small proportion of total fungi have been recorded on Earth, but the actual number of fungal species on Earth is likely to be seven-digit and may even be of higher magnitude. In particular, mycologists are expected to concentrate on identifying new or established organisms in the diverse habitats around them [1].

Endophytic fungi are those that colonize plant tissues in which they spend part or all of their life cycle in their hosts without triggering any signs of disease [2]. Host tissues can include fungal endophytes in different bodies, including leaves, branches, bark, roots, fruits, flowers, bulbs, and seeds [3]. These are exploited by efforts to classify and identify endophytic fungi from medicinal plants. In the present study, therefore, two *Aspergillus* species were isolated for the first time in Egypt, after the type species, as endophytes from some wild medicinal plants collected from the New Valley Governorate, Egypt.

Aspergillus section *Versicolores* was initially established as the *Aspergillus versicolor* group by [4] and was subsequently updated [5] to include four species. Raper and Fennell [6] overhauled the genus *Aspergillus* and accepted 18 species in the *A. versicolor* group. Several updates were performed for the section *Versicolores* [7], culminating in recognizing four phylogenetically distinct species based on multilocus DNA sequence analysis and putting the remaining 14 species in separate *Aspergillus* clades.

Aspergillus ustus is widespread filamentous fungi found in the environments of food, soil, and indoor air [8]. These species are rare human pathogens in immunocompromised hosts that can cause invasive infection [9,10-13]. However, *Aspergillus*

granulosus was found to cause spread infection in patients with cardiac transplantation [14], while it was confirmed that *A. deflectus* caused disseminated mycosis in dogs [15-17].

2. Materials and methods

2.1. Samples collection

In one season during April 2018, leaves and roots of healthy and mature wild medicinal plants (*Convolvulus arvensis* and *Moringa oleifera*) have been obtained from El-Kharga Oasis, New Valley Governorate, Egypt. Ten replicates of the growing plant were gathered in sterile polyethylene bags and immediately brought to the laboratory for fungal analysis.

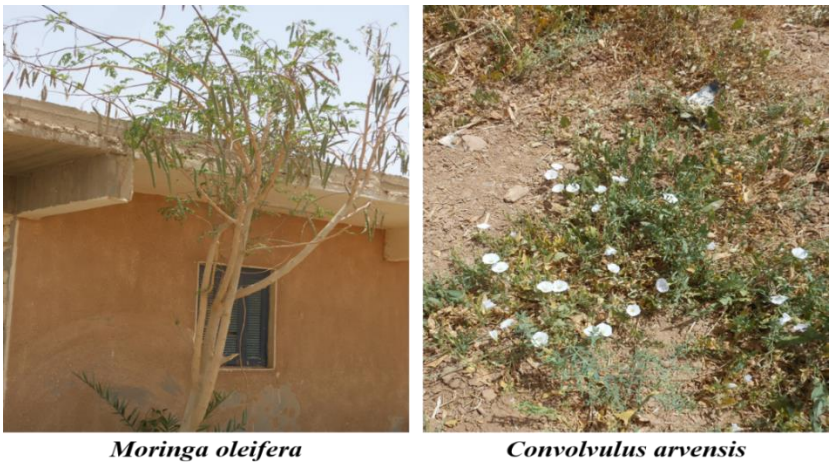

Figure 1. Locations of samples collected from El-Kharga Oasis, New Valley Governorate.

2.2. Sample preparation

Each collected sample's leaves and roots were vigorously washed with tap water to extract dust, then with sterile distilled water, and cut into segments of 5 cm.

2.3. Surface sterilization

Surface sterilization was achieved by immersing leaf and root segments in 5% sodium hypochlorite for 3 min, 70% ethanol for 1 min, and washing with sterile distilled water 3 times for 1 min. Both ends of each segment (1 cm) were cut off in aseptic conditions to create a 3-cm piece.

2.4. Isolation of endophytic fungi

Segments of each sample were plated on Petri dishes containing 1% glucose-Czapek's agar medium (Cz) with the following composition (g/L): Glucose, 10; Na_2NO_3, 2; K_2HPO_4, 1; KCl, 0.5; $MgSO_4.7H_2O$, 0.5; $FeSO_4$, 0.01; $ZnSO_4$, 0.01; $CuSO_4$, 0.005; Rose Bengal, 0.05; chloramphenicol, 0.25; agar, 15 and the final pH 7.3. The plates were incubated for 7-21 days at 25 °C. Counts of CFUs of each developed fungal isolate were calculated per 25 segments in every sample. Pure cultures of the developed fungal isolates were preserved on Cz slants at 4 °C for further investigations.

2.5. Morphological studies

Cultural morphological characteristics and growth rates of *A. creber* and *A. keveii* were studied on Czapek's yeast Autolysate agar (CYA) [18], Czapek's agar (CZ) [6], malt extract agar (MEA) [19] and Creatine sucrose agar (CREA) [20]. The plates were inoculated with a micropipette and an inoculum scale of 1 µL per position in a three-point pattern. Unwrapped cultures were

incubated in the dark reverse side up at 25 °C, with additional CYA plates incubated at 5 °C, 37 °C and 40 °C. Microscopic features on MEA were examined in lactophenol cotton blue.

2.6. Molecular identification of the fungal isolate

2.6.1. DNA extraction

Until DNA extraction, tiny fragments of fungal mycelia from the 7-day-old colony of *A. creber* AUMC 14298 and *A. keveii* AUMC 14299, grown on MEA, was individually collected and moved to the 2 mL-Eppendorf. The extraction of DNA was carried out using the process of Moubasher et al. [21].

2.6.2. PCR for rDNA and sequencing using ITS1 and ITS4 primers

SolGent EF-Taq was used to conduct the PCR reaction. The standardized primers ITS1 and ITS4 were used for DNA amplification. 1 µL of DNA base, 1 µL 2.5 mM dNTP combination, 0.2 units of Taq polymerase, 5 µL of 10 x buffer, 40 µL of sterile ddH2O, 10 pmol of ITS1 (5' TCC GTA GGT GGT GAA CCT TGC GG 3') and ITS4 (5' TCC TCC GCT TAT TGA TAT GC 3') was applied to the PCR tube. 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 secs, 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 processed before sequencing with the SolGent PCR Purification Kit-Ultra (SolGent, Daejeon, South Korea). Using the scale symbol, electrophoresis verified the distilled PCR products on 1% agarose gel. The bands were eluted in both forward and reverse directions and sequenced.

2.6.3. Alignments and phylogenetic analysis

DNA sequences *of A. creber* AUMC 14298 and *A. keveii* AUMC 14299 were edited with the DNASTAR computer package (DNA star version 5.05). Published sequences for the closest matching strains, including the available type materials, were downloaded from GenBank. Assembled sequences of *A. creber* and *A. keveii* were aligned using MAFFT [22]. Alignment gaps and parsimony uninformative characters were treated by BMGE [23]. Maximum-likelihood (ML) and Maximum parsimony (MP) phylogenetic analyses were performed using PhyML 3.0 [24]. The robustness of the most parsimonious trees was evaluated by 100 bootstrap replications [25]. The best optimal model of nucleotide substitution for the ML analyses was determined using the Akaike Information Criterion (AIC) as implemented in Modeltest 3.7 [26]. GTR was the best fit for the nucleotides substitution. The phylogenetic tree was visualized using BioNJ [27] and edited using FigTree version 1.4.3 [28].

3. Results and discussion

3.1. Phylogenetic analysis

The entire dataset consisted of 60 nucleotide sequences, two of which are obtained in this study; one for *A. creber* AUMC 14298, and the other for *A. keveii* AUMC 14299 and 58 sequences of the nearest related *Aspergillus* species downloaded from GenBank. The maximum parsimony dataset consisted of 535 characters with 397 characters as constant information (no gaps, no N), 66 characters as variable characters, which were parsimony-uninformative (16.6% of constant characters), and 39 characters were counted as parsimony-informative characters (9.8% of constant). Maximum Parsimony analyses resulted in 6 most parsimonious trees with a tree length of 94 steps. The maximum likelihood analysis yielded one tree with the highest log likelihood (Log-likelihood= -1559.7535) (Figure 2). Both strains *A. creber* and *A. keveii* were consistently located in the phylogenetic tree within their respective *Aspergillus* groups forming highly supported clades (94% ML/ 93% MP and 77% ML/79% MP), respectively. A megablast search with a sequence of the ITS of *A. creber* AUMC 14298 revealed that the most similar species in GenBank were *A. creber* DTO 245B2 [(GenBank MN413176; identities 551/551 (100%); no gaps with 100% coverage of the ITS gene)], *A. creber* STG-57 [(GenBank MN266993; identities 551/551 (100%); no gaps with 100% coverage of the ITS gene)], *A. versicolor* STG-25G [(GenBank MN260015; identities 551/551 (100%); no gaps with 100% coverage of the ITS gene)] and *A. versicolor* CBS 117.34 [(GenBank MN855466; identities 551/551 (100%); no gaps with 100% coverage of the ITS gene)]. The fungus was consistently located within the subtree, including *A. creber* DTO 245B2, NRRL 58592 (type species), and STG-57 (Figure 2). A megablast search with a sequence of the ITS of *A. keveii* AUMC 14299 revealed that the most similar species in GenBank were *A. ustus* 99-168 [(GenBank KT323974; identities 566/566 (100%); no gaps with 100% coverage of the ITS gene)], *A. ustus* NRRL 1974

[(GenBank AY373879; identities 566/566 (100%); no gaps with 100% coverage of the ITS gene)] and *A. keveii* CBS 209.92 [(GenBank MF004311; identities 554/555 (99.82%), no gaps with 98% coverage of the ITS gene)]. The fungus was consistently located within the subtree, including *A. keveii* CBS 209.92 (ex-type), *A. keveii* CML 2968, *A. keveii* ONI 75, and *A. keveii* F277880 (Figure 2).

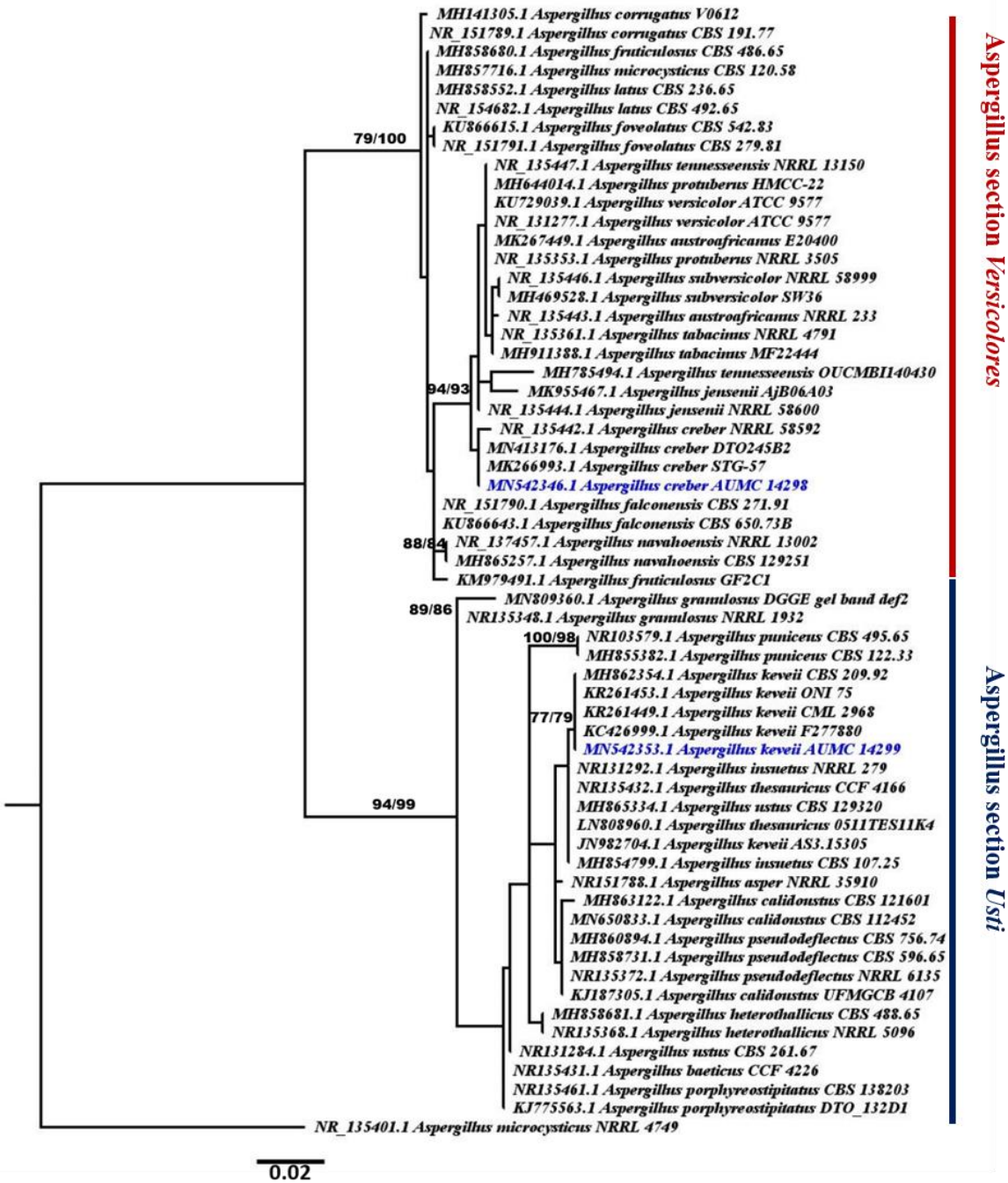

Figure 2. The maximum likelihood of phylogenetic tree of *A. creber* AUMC 14298 and *A. keveii* AUMC 14299 aligned with the closest matching sequences of other related taxa downloaded from GenBank on the ITS gene sequences. The sequence of the species in this study is in blue. The numbers near the branches are the bootstrap values (100 pseudoreplicates). Branches corresponding to partitions reproduced in less than 70% bootstrap replicates are not shown. The tree is rooted in *A. mictocysticus* NRRL 4749 as an outgroup.

3.2. Description of *Aspergillus creber* AUMC 14298 (GenBank MN542346.1)

Etymology*:* From the Latin word, Creber meaning numerous or frequent.

Colonies on CYA attaining a 26-30 mm diameter at 25 °C for 7 days, radially sulcate with good sporulation in the center. Conidial heads with some shades of green. Margin entire, white. Exudates few, yellowish to reddish. Soluble pigments absent. Reverse reddish-brown. No growth was recorded on CYA at 5 °C, 37 °C or 40 °C when incubated for 7 days. On Cz, MEA, and CREA at 25 °C after 7 days' colonies achieved 20-25 mm, 12-14 mm, and 12-13 mm in diameter, respectively; exudates and soluble pigments were absent. Conidiophores smooth-walled, commonly (60-) 200-300 (-400) µm × 3-6 µm. Vesicles are pyriform to spathulate and sometimes subglobose, 6-15 µm. Conidial heads are biseriate. Metulae 5-7 × 2-5 µm. Phialides 3-8 × 2-3 µm. Conidia are globose to subglobose to short-ellipsoidal to lemon-shaped, smooth-walled, 2-3 × 2-3 µm. Hülle cells present, globose to subglobose to pyriform, 10-20 × 7-18 µm and Ascomata not observed (Figure 3).

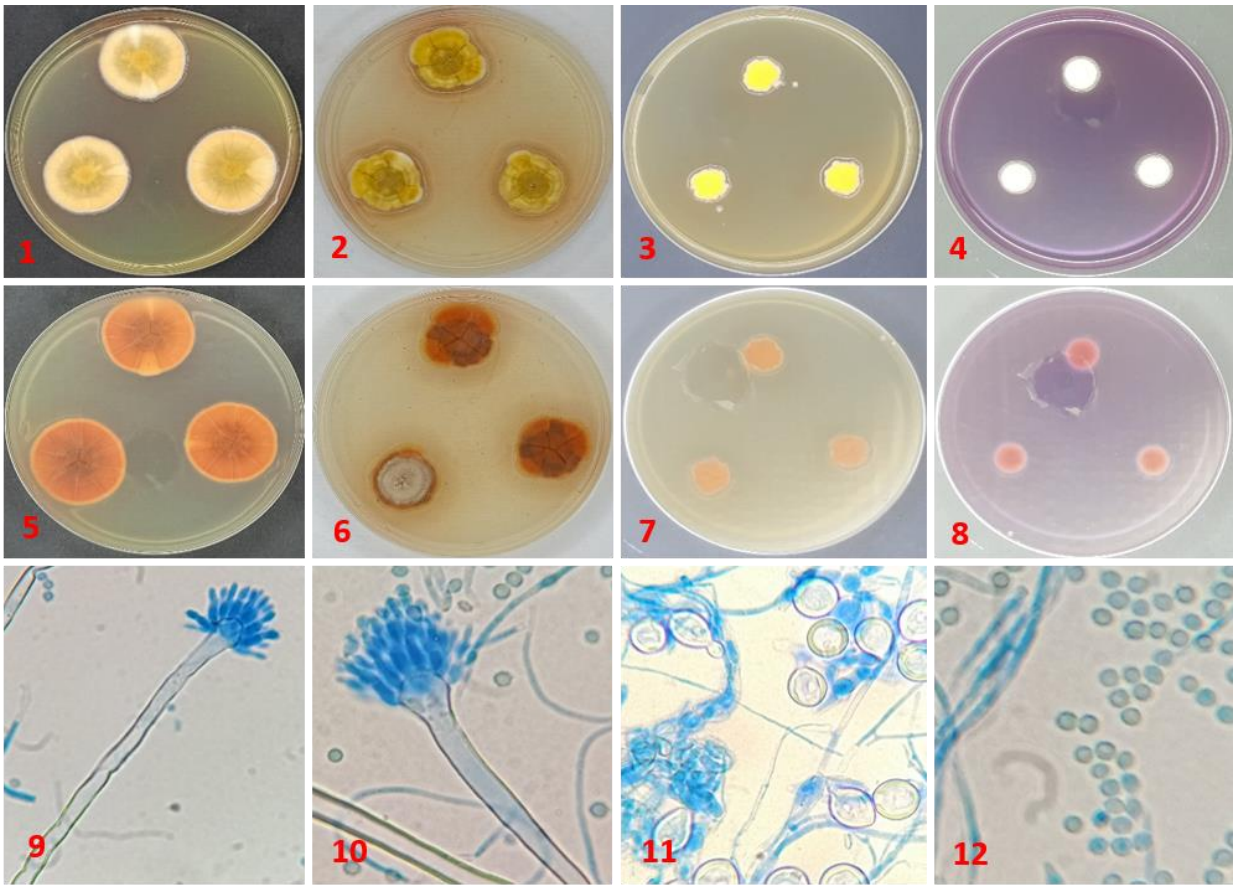

Figure 3. *Aspergillus creber* strain AUMC 14298: 1-4, colonies on CYA, Cz, MEA, and CREA after 7 d at 25 °C; 5-8, reverse on CYA, Cz, MEA and CREA; 9-10, conidial heads and conidiophores; 11, Hülle cells and 12, conidia.

3.3. Description of *Aspergillus keveii* AUMC 14299 (GenBank MN542353.1)

Etymology: Named after Prof. Ferenc Kevei, eminent mycologist devoting his life to *Aspergillus* research.

Colonies on CYA attaining 35-37 mm, after 7 d at 25 °C, no growth on CYA after 7 d at 5 °C and 40 °C and very restricted growth (5-6 mm) was obtained on CYA at 37 °C. Conidial heads are abundant in CYA, Cz, and MEA. Colony color on CYA brownish-grey to pinkish brown. Colony texture floccose. Reverse yellow olive-brown to dark brown. On Cz, MEA, and CREA, colonies attained 36-40 mm, 40-42 mm, and 28-30 mm in diameter at 25 °C after 7 days. Very restricted growth ranged from 5-6 mm was obtained on CYA at 37 °C when incubated for 7 days and no growth at 5 °C and 40 °C. Exudates and soluble pigments absent. Conidial heads are loosely columnar. Stipes 60-300 × 3-5 µm, smooth-walled, brown. Vesicles 6-10 µm wide, pyriform,

biseriate. Metulae covers the upper half to three-fourths of the vesicle, measuring 4-6 × 3-4 µm. Phialides 5-6 × 2-4 µm. Conidia globose 3-4 µm, coarsely roughened to echinulate. Hülle cells and ascomata are not seen (Figure 4).

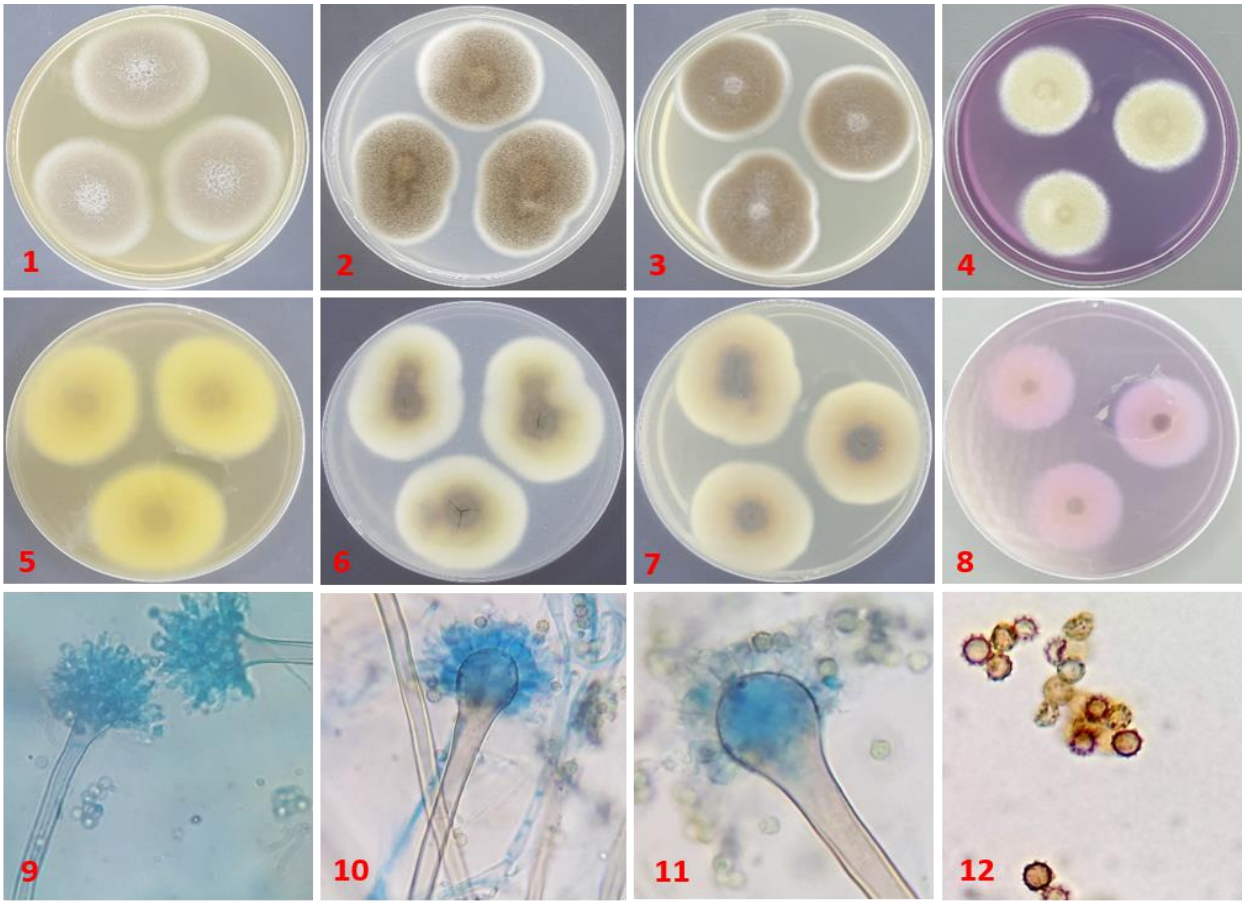

Figure 4. *Aspergillus keveii* strain AUMC 14299: 1-4, colonies on CYA, Cz, MEA, and CREA after 7 d at 25 °C; 5-8, reverse on CYA, Cz, MEA and CREA; 9-11, conidial heads and conidiophores; 12, conidia.

In this study, two new *Aspergillus* species, namely *A. creber* and *A. keveii* were obtained from *Convolvulus arvensis,* and *Moringa oleifera* leaves within tissues. In their representative sections, they were classified by comparing their phenotypic characteristics with the species *Aspergillus*. Both strains in this study have similar morphological characters as their representative type strains, and they differ in some other characters. A comparison between the morphological characteristics of *A. creber* and *A. keveii* in this study with those of the representative type strains is given in table (1).

Also, the sequencing of their ITS genes confirmed their identification as *A. creber* and *A. keveii*. In this regard, many studies have shown that molecular approaches based on DNA sequences combined with traditional methods based on phenotypic characteristics are the most accurate means of reliably characterizing the *Aspergillus* species. The internal transcribed spacer (ITS) region, located between the 18S and 28S rRNA genes, is a field of particular 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 [29-31]. National Center for Biotechnology Information (NCBI) data have shown that A. creber has been published from various sources worldwide in several countries. It was collected in Capetown, South Africa, from unknown sources, from floating tar balls in the Atlantic Ocean, and tea field soils in Ibaraki, Japan. The indoor air samples in Pennsylvania, California, New Jersey, Georgia, and Ohio, USA, have also been recorded [32]. It has also been recorded from clinical samples in Spain [33]; ancient painting in Moscow, Russia; house dust in the UK; the roots of Kalmia angustifolia and Gaultheria hispidula in Nava Scotia, Canada, as well as

freshwater fish commercial feeds and wastewater of the slaughterhouse in Mexico (unpublished work). On the other hand, *A. keveii* was isolated from soil in Las Palmas, Spain [34]. As a result, the two species were identified worldwide for the first time as endophytes in this study. Their presence in New Valley, Egypt, suggests that they are cosmopolitan fungi and can be found across the globe in diverse climates.

Table 1. Comparison of morphological characteristics of *A. creber* AUMC 14298 and *A. keveii* AUMC 14299 with indicative type strains

Morphological characters	***A. creber***		***A. keveii***	
	AUMC 14298	Type strain	AUMC 14299	Type strain
Colony color				
CYA	Shades of green	Pea green or Artemisia green	Brownish grey to pinkish brown	Brownish grey to pinkish brown
MEA	Yellow to vivid yellow	Yellow green	Brown	Not available
Colony diameter at 25 °C				
CYA	26-30	18-26	35-37	30-39
MEA	12-14	18-22	40-42	36-41
Exudates	Absent	Yellowish to reddish	Absent	Absent
Soluble pigments	Absent	Absent	Absent	Absent
Growth				
5 °C	No	No	No	No
37 °C	No	No	5-6	No
40 °C	No	No	No	No
Microscopic				
Conidiophores	60-400	70-450	60-300	150-300
Vesicles	6-15	7-17	6-10	9-13
Metulae	5-7	4-6	4-6	4.7-6.7
Phialides	3-8	5-8	5-6	5.7-7
Conidia	2-3	(2.5-) 3-4 (-9)	3-4	2.4-2.8
Hülle cells	10-20	Absent	Absent	10-65

4. Conclusion

In the current research, two new *Aspergillus* strains, namely *A. creber* and *A. keveii* are isolated in Egypt for the first time and are considered a new global record as desert plant endophytes. This means that these species are widespread and can be reported worldwide from various sources and localities.

Conflicts of interest. The authors declare that there is no conflict of interest.

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