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Full Length Research Paper

Morphological and molecular characterization of L-methioninase producing *Aspergillus* species

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Six species of L-methioninase producing *Aspergillus* species, isolated from Egyptian soil, were selected for comprehensive morphotypic and molecular characterization. Based on morphological and physiological features, these isolates were identified as *Aspergillus flavipes*, *Aspergillus carneus*, *Aspergillus flavus*, *Aspergillus tamari*, *Aspergillus oryzae*, and *Aspergillus parasiticus*. Regarding to the maximum enzyme productivity by *A. flavipes,* it was selected as authentic strain for ribosomal ribonucleic acid (rRNA) primer design. Using primer combinations for 18S rRNA and internal transcribed spacers (ITS)1 amplification, these isolates gave the same polymerase chain reaction (PCR) amplicon size, revealing the relative molecular identity. Moreover, using ITS2 primers, among the six isolates, *Aspergillus flavipes* EK and *A. carneus* displayed PCR products on agarose gel, approving the actual morphological and biochemical similarities of these two isolates, *A. flavipes* group. By sequencing of ITS1-5.8S-ITS2 region, blasting and alignment from the data base, *A. flavipes* EK showed a typical identity to gene bank deposited *A. flavipes* isolates. The rRNA sequence of *A. flavipes* EK was deposited to genbank under accession number JF831014.

Key words: *Aspergillus*, morphological descriptions, 18 S rRNA, internal transcribed spacers (ITS) regions.

INTRODUCTION

L-Methioninase is a pyridoxal phosphate dependent enzyme catalyzing α and γ-elimination of L-methionine to α-ketobutyrate, methanethiol and ammonia (Tanaka et al., 1977). It was comprehensively characterized as a potent therapeutic agent towards various types of tumor cell lines (Hoffman, 1984; Tan et al., 1998). From the screening profile of L-methioninase producing fungi, isolated from Egyptian soils, twenty one fungal isolates were recovered (Khalaf and El-Sayed, 2009), belonging to eight genera namely; *Aspergillus*, *Cladosporium*, *Fusarium*, *Humicola*, *Mucor*, *Penicillium*, *Scopulariopsis* and *Trichoderma* according to the universal morphological accepted keys (Raper and Fennell, 1965; Booth, 1971; Ellis, 1971). Among these fungal isolates, *Aspergillus* were the most frequent group (43%) represented by nine species namely; *Aspergillus flavipes*, *Aspergillus carneus, Aspergillus flavus*, *Aspergillus tamarii, Aspergillus oryzae*, *Aspergillus parasiticus*, *Aspergillus subolivaceus, Aspergillus niger* and *Aspergillus ochraceus,* as identified morphologically. Thus, during this study, we focused on the molecular description of *Aspergillus* species. Apparently, a few publications about L-methioninase producing fungi were documented, otherwise, this is the first report focused on characterization of L-methioninase producing filamentous fungi from Egyptian soil.

Aspergillus species are the most abundant sporulating filamentous fungal genera in soil and decaying vegetations (Garrett, 1951; Raper and Fennell, 1965; Domsch et al., 1980), particularly in Egyptian soils (El-Abyad and Abu-Taleb, 1993; Moubasher, 1993; Mouchacca, 2005). *Aspergillus* species are used in commercial enzyme production generating more than US$5 billion (Lubertozzi and Keasling, 2009), more than 80 recombinant enzymes of fungal origins are universally used, and 55 of these proteins were produced from *Aspergillus* species (Yoder and Lehmbeck, 2004). Therapeutically, *Aspergillus* species have a great

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contribution on production of various antitumor enzymes as L-glutaminase, L-asparaginase (Klimberg and McClellan, 1996; Agrawal et al., 2003) and L-methioninase (Ruiz-Herrera and Starkey, 1969; El-Sayed, 2009, 2010).

Aspergillus species have been characterized morphologically into 18 groups (Raper and Fennell, 1965) namely; *clavatus*, *gluacus*, *ornatus*, *cervineus*, *restrictus*, f*umigatus*, *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). Identification of *Aspergillus* species was traditionally relied on macro and micro-morphological features of the colonies on different growth media. However, the development of specific phenotypic criteria (anastomosis) as fruiting bodies, mycelial aggregation and conidial heads, conidial ornamentations, may require several days, with specific medium constitution. Recently, 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 (Peterson, 2000; Scorzettiet et al., 2002; Hall et al., 2004). Therefore, the objective of this study was to assess the utility of molecular approaches as 18S rRNA, ITS1, ITS2, and 28S rRNA parallel to morphological characters for identification of L-methioninase producing *Aspergillus* species. Owing to the potentiality of *Aspergillus flavipes* as L-methioninase producer, it was used as reference fungus for primer design of 18S rRNA and ITS regions. The obtained sequences of rRNA were blasted and aligned towards their closely related fungal isolates, for phylogenetic analysis.

MATERIALS AND METHODS

Fungal Isolates

Six species of *Aspergillus* (*A. flavipes*, *A. carneus, A. flavus*, *A. oryzae*, *A. tamarii* and *A. parasiticus*) as a potent L-methioninase producers (Khalaf and El-Sayed, 2009), were selected for further morphological and molecular characterization. These isolates were identified phenotypically according to the universal key of *Aspergillus* identification (Raper and Fennell, 1965; Klich, 2002; Geiser et al., 2007). The fungal isolates were grown on Czapek's agar for 10 days at 28 ± 1°C, then for 2 days at 37°C to confirm their species purity. The purified fungal isolates were used for further identification.

Morphotypic identification

Fungal inocula were pointedly inoculated to Czapek's and malt extract agar as prepared by (Raper and Fennell, 1965) then incubated for 15 days at 28°C. The macroscopic features as colony diameter, conidium color, extracellular exudates, pigmentation and reverse mycelium color were examined daily. Microscopical properties as conidial heads, fruiting bodies, degree of sporulation and identity of conidiogenous cells were examined by light microscope after staining by lactophenol solution according to the instructions of (Raper and Fennell, 1965). The macromorphological features of the recovered colonies were photographed using digital camera (Panasonic, DMC-FS7 (LUMIX), Japan).

Scanning electron micrography (SEM)

The conidial suspension of the fungal isolate was prepared from their slope cultures after 8 days of incubation at 28°C. The slants were scratched with 10 ml of sterile saline then washed two times by the same solution. After collection of conidia by centrifugation, it was fixed by 2.5% glutraldehyde at 4°C for 2 h in 0.1 M sodium cacodylate buffer, and then washed twice by the same buffer for 15 min. The samples were post-fixed by 1% osmium tetraoxide for 2 h at 4°C, followed by washing by the same buffer. The fixed samples were dehydrated by ascending gradient of ethanol (Tsai et al., 1998). After drying, the samples were coated by gold, and then examined by scanning electron microscope JXA-8230 (Electron Probe Microanalyser-JEOL, Japan). Spores ornamentations were photographed.

Molecular identification

Preparation of the fungal cultures

After incubation of Czapek's slants of the tested fungal isolates at 28 ± 1°C for 4 days, the spores were collected by vigorously pipetting 5 ml of sterile saline to each slant. Then the spore suspension was inoculated to 100 ml of Czapek's in 250 ml Erlenmeyer flask. After incubation for 4 days, the cultures were filtered; the mats were collected and washed by distilled water prior to genomic DNA extraction.

Genomic DNA extraction

Mechanical freeze fracturing using liquid nitrogen was first applied to fungal mycelium. Fungal genomic DNA was extracted according to the protocol of Sharma et al. (2007) with slight modifications as follow; 50 to 100 mg fungal mycelia/spores were homogenized with pestles. Five hundred microliter (500 µl) of DNA extraction buffer (200 mM Tris-HCl pH 8, 240 mM NaCl; 25 mM EDTA, and 1% SDS) were then added to the homogenized fungal materials. One (1) volume of phenol/$CHCl_3$, in the ratio of 1:1 (v/v), was added and mixed gently for 10 min on a shaker followed by centrifugation at 15000 x g for 10 min. The upper phase was transferred to a new tube and 0.1 vol of 3 M Na-acetate (pH 5.2) and 2 volume of ethanol (96%) were added and mixed well, incubated for 30 min at -20°C followed by centrifugation (15000 x g/4°C/20 min). The resulting pellet was washed with 700 µl of 70% ethanol, air dried

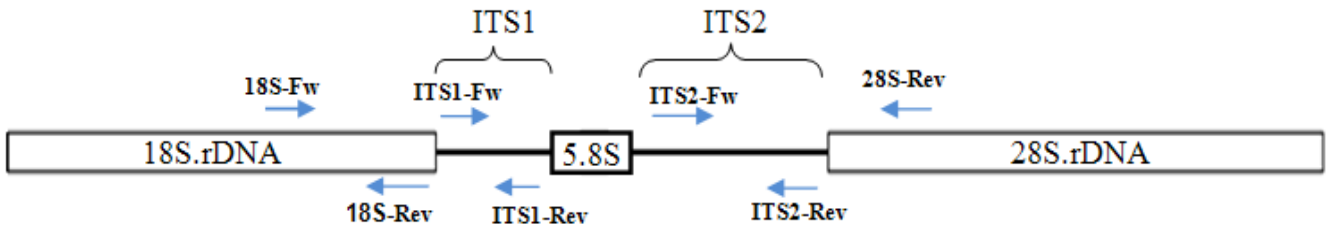

Figure 1. Sketch representing the 18S rDNA-ITS1-5.8S-ITS2-28S rDNA sequence domain with the used primer position.

Table 1. List of the oligonucleotides used in this study.

Primer	Sequence 5′- 3′
18S-Fw	TCG GCA CCT TAC GAG AAA TC
18S-Rev	GCT ATT TAA GGG CCG AGG TC
ITS1-Fw	CGA GTG AGG GTC CTC GTG
ITS1-Rev	GGC TTT CAG AAA CAG TGT TCG
ITS2-Fw	GGC TTG TAT TGG GTC CTC GT
ITS2-Rev	AAG GAT GAT GTG CGT GGG CT
28S Rev	CAC GTG CTG TTT AAC TCT CT

and re-suspended in 100 µl of sterile bi-distilled water

PCR amplification

Fungal isolates were molecularly identified on the basis of their 18S rRNA gene, internal transcribed spacer regions (ITS1-5.8S-ITS2), and 28S rRNA sequences in a similar manner to the studies performed by De Santi et al. (2010) as shown in Figure 1. PCR reactions contains 2 to 10 ng of DNA, 5 µl of 10x reaction buffer, 1.25 U of Taq DNA polymerase, 200 µM of the four deoxynucleotides (Fermentas, Germany) and 0.2 mM of each oligonucleotide in a total volume 50 µl. The PCR protocols was adjusted as follow; denaturation at 95°C for 30 s, annealing at 56°C for 30 s, elongation at 72°C for 60 s, for 35 cycles. The amplified products were resolved using 2% agarose gel containing ethidium bromide, then visualized by gel documentation system (Biorad, USA). The PCR products were purified with the PCR purification kit (Fermentas, Germany). The primers for PCR are listed in Table 1.

Sequencing of 18S-28S rDNA amplicons and phylogenetic analysis

The amplified PCR products were sequenced using an ABI 377 DNA auto sequencer (PerkinElmer, Applied Biosystems Div., Waltham, USA) using the same primers (Table 1). After construction of the retrieved sequence of 18S-28S rDNA, the whole sequence was used for searching of compatible sequences from database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogenetic profile of our samples was constructed using multiple sequence alignment software (http://www.genome.jp/tools/clustalw/), declaring the molecular identities with the closely related isolates in the database (Thompson et al., 1994).

Nucleotide sequence accession numbers

The 18S rRNA partial-ITS1-5.8S-ITS2-28S rRNA partial gene sequence of *A. flavipes*, isolated from Egyptian soil was deposited in database (http://www.ncbi.nlm.nih.gov/nuccore/JF831014) under accession number JF831014.

RESULTS AND DISCUSSION

L-Methioninase producing fungi

Using L-methionine as sol carbon and nitrogen sources, twenty one fungal isolates belonging to nine genera were recovered as L-methioninase producers from Egyptian soil samples. Among L-methioninase producing genera, the frequency of genus *Aspergillus* was about 43%, represented by nine species (Khalaf and El-Sayed, 2009). Among the nine species of *Aspergillus*, the maximum enzyme productivity was reported for *Aspergillus flavipes,* followed by *A. carneus, A. flavus, A. tamarii* and *A. oryzae*, depending on our preliminary screening studies (Khalaf and El-Sayed, 2009). *Aspergillus flavipes*, as potent L-methioninase producers was selected by our research group (El-Sayed, 2009, 2010, 2011; El-Sayed and Shindia, 2011) for production and characterization of this enzyme, for the maximum exploitation in various biotechnological aspects. These fungal isolates were identified morphologically according to Raper and Fennell (1965) and Domsch et al. (1980); however, the morphotypic description of these *Aspergillus* species was not cited during our screening studies (Khalaf and El-Sayed, 2009). Consequently, identification of *Aspergillus* species producing L-methioninase was extensively studied relying on morphological and molecular approaches in the following sections.

Morphological features of L-methioninase producing *Aspergillus* species

The macro and microscopical features of the *Aspergillus* species were observed on Czapek's and malt extract agar media (Figures 2 to 8). *Aspergillus* sp1 appeared as irregular furrowed buff-yellowish colonies on Czapek'[s media with off-white spores. A yellow to orange mass of hyphae with red-brown reverse, no obverse exudates was observed (Figure 2). Conidial heads are loosely columnar to radiate with subglobose vesicles.

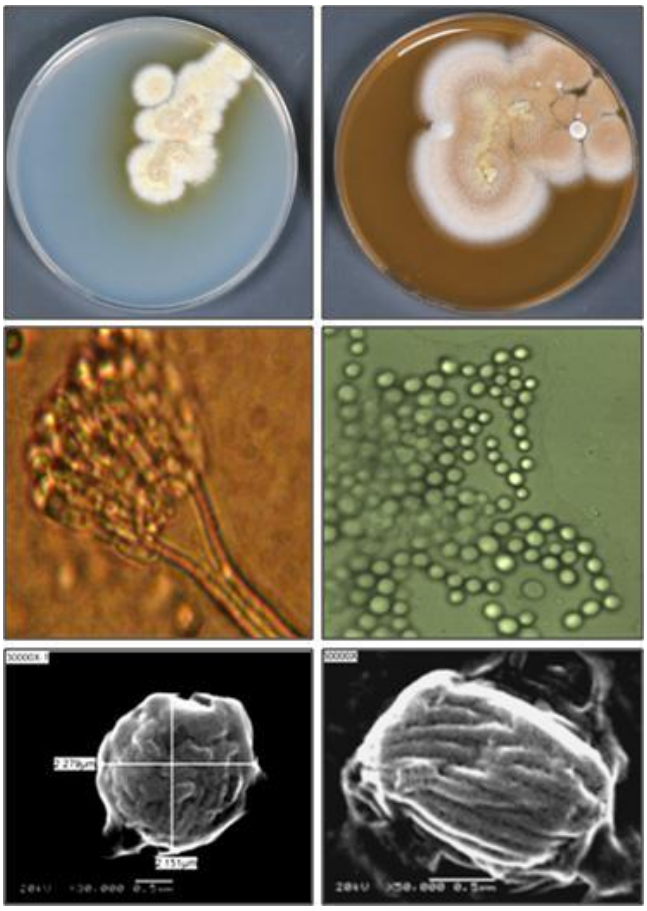

Figure 2. Macro and Microscopical features of *Aspergillus flavipes.* Colonies after 4 days at 28°C on Czapek's medium (A) and malt extract agar (B), conidial head and conidiophores (C, D), conidia by light microscope 1000X (E). Conidial surface ornamentation by SEM at 30000X (F) and 50000X (F).

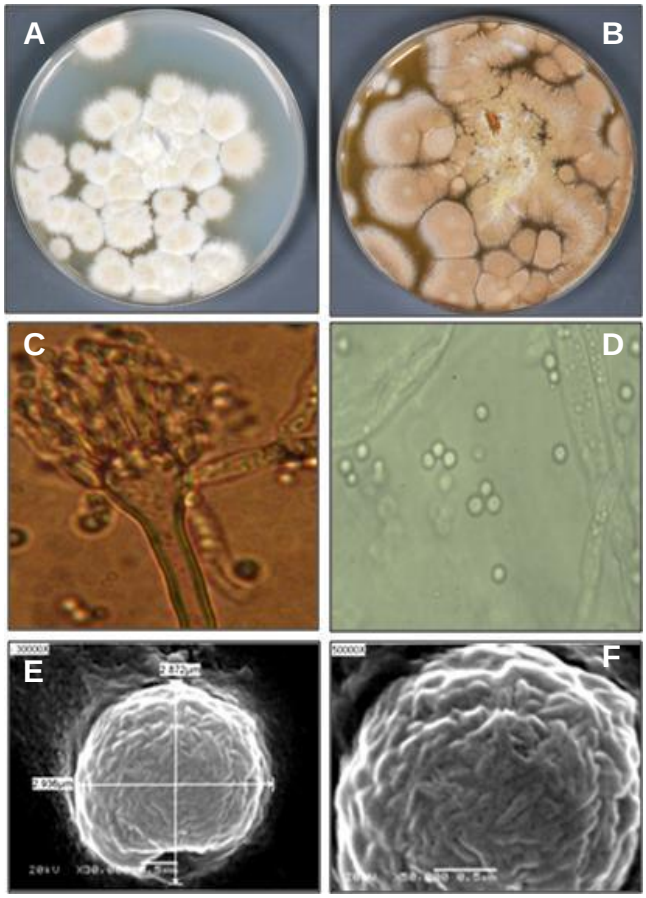

Figure 3. Macro and Microscopical features of *Aspergillus carneus.* Colonies after 4 days at 28°C on Czapek's medium. (A), malt extract agar (B), conidial head and conidiophores (C, D), conidia by light microscope 1000X (E). Conidial surface ornamentation by SEM at 30000X (F) and 50000X (F).

Conidiogenous cells are strict biseriate, haploplastic conidia, covering the upper third of the vesicle surface. Hulle cells are distributed. Conidia are globose to subglobose with dimensions 2.15 to 2.27 µm. The conidial surface was smooth as observed by 50000X SEM. The slightly ornamentation of the conidial surface may attributed to the mechanical stress during process of fixation or dehydration prior to scanning micro-graphing. Colonies on malt extract agar were growing rapidly, with dark shades close to pinkish buff, abundant sporulation without surface exudates and brown reverse. The macro and micro-morphological description of this isolate typically follows *Aspergillus flavipes* (Bainier and Sartory), Thom and Church according to Raper and Fennell (1965), Domsch et al. (1980), Hoog et al. (2000) and Klich (2006). Consistently, *A. flavipes* was frequently identified from Egyptian soils (Sabet, 1939; El-Abyad and Abu-Taleb, 1993; Moubasher, 1993, 2005; Valera et al., 2005).

Aspergillus sp2, pale yellow-tan colonies of thin margin and relative fructification, grow rapidly on Czapek's agar (Figure 3). Heavy sporing mycelium with fructification, white at first, then turned to vinaceous fawn persistence. Reverse with shades of yellow to red-brown with no obverse exudates. Conidial heads are loosely columnar with hemispherical vesicle. Biseriate Strigma covers the upper third to half of the vesicle. Conidia are globose with dimension 2.88 to 2.93 µm, with slightly wrinkled surface as observed by SEM. Colonies are growing rapidly on malt extract agar, more sporulation, with deep vinaceous fawn color. This isolate typically follows the morphological descriptions of *Aspergillus carneus* (Van Tieghem) Blochwitz as adopted by Raper and Fennell (1965), Klich and Pitt (1992), Hoog et al. (2000) and Domsch et al. (2007).

Aspergillus sp3, yellow green to deep grape green colonies in old cultures (Figure 4). Conidial heads radiate to columnar with spherical vesicles. Conidiogenous cells are uniseriate in young cultures to dominant biseriate with age. Reverse was red brown without surface exudates. Sclerotia are frequently present on plates. Conidia are globose to sub-globose with dimension 2.4

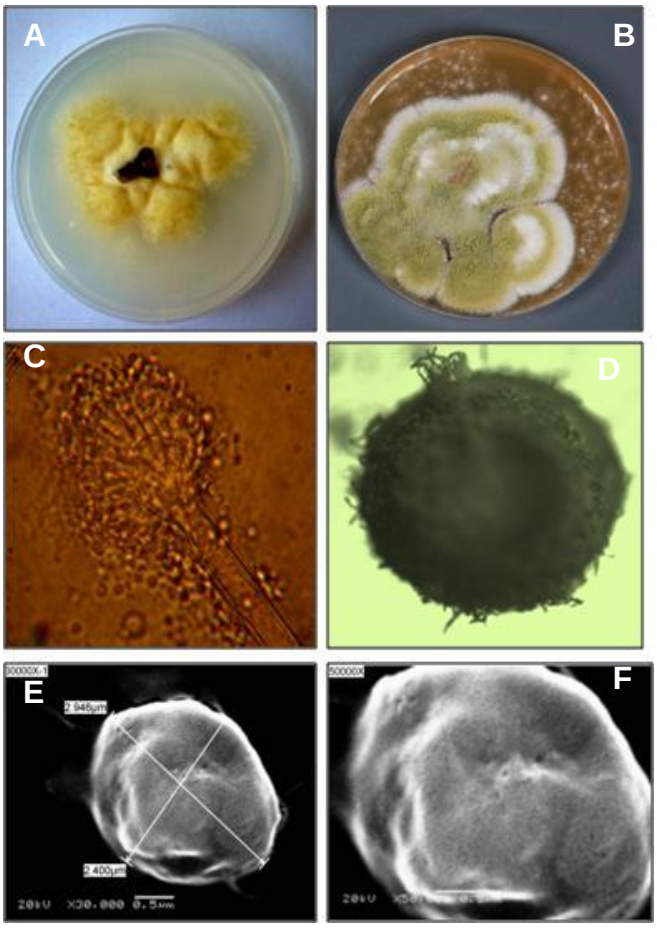

Figure 4. Macro and Microscopical features of *Aspergillus flavus.* Colonies after 4 days at 28°C on Czapek's medium (A), malt extract medium (B), conidial head and conidiophores (C). Sclerotia on Czapek's after 7 days (D). Conidial ornamentation by SEM at 30000X (E) and 50000X (F).

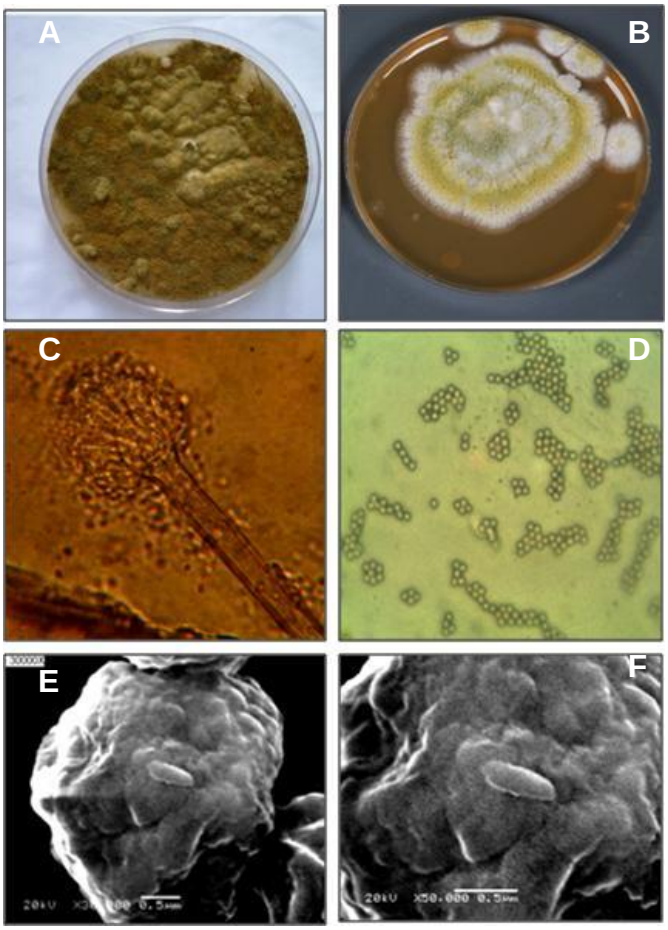

Figure 5. Macro and Microscopical features of *Aspergillus tamarii.* Colonies after 4 days at 28°C on Czapek's medium (A), malt extract agar (B), conidial head and conidiophores (C & D). Conidial ornamentation by Scanning Electron Microscope at 30000X (E) and 500000X (F).

to 2.95 µm. conidial surface are conspicuously echinulate as appeared by SEM micro-graphing. On malt extract agar, the colonies were rapidly grown bearing numerous sclerotia with yellowish-green shades and grayish reverse. Depending on morphological features, this isolate was identical to *A. flavus* Link as described by Ainsworth and Austwick (1959), Lancaster et al. (1961) and Raper and Fennell (1965). *A. flavus* Link was frequently isolated from Egyptian soils (El-Khadem et al., 1976; Moubasher et al., 1979; Mohamed et al., 2002).

Aspergillus sp4, yellow green colonies at the first 3 days of growth on Czapek's, shifted to brown or brownish green after 7 days without exudates, pinkish reverse (Figure 5). Conidial heads loosely radiates with sub-globose vesicles. Strigma are uniseriate to biseriate at maturity covering the entire surface of the vesicles. Conidia are subglobose to cylindrical with average dimensions 3.21 to 3.32 µm. Under 5000X magnification, the conidial surface appears conspicuously roughening. Colonies on malt extract were yellow-green to olive-brown, with gray reverse. This isolate follow the morphological and microscopical features of *A. tamarii* Kita (Raper and Fennell, 1965; Klich and Pitt, 1992; Domsch et al., 2007).

Aspergillus sp5, a yellow green colonies that slightly shitted olive-yellow on Czapek's with aging, uncolored reverse. Conidial heads radiate with uniseriate to biseriate prominent at maturity. Stigmata cover the whole surface of vesicles. Conidia were globose with average dimension 3.68 to 4.40 µm, conidial surfaces were slightly ornamented, echinulate, under high magnification (50000X). Colonies on malt extract were deep yellow-green, rapidly grown with abundant sporulation (Figure 6). This description follows that of *A. oryzae* (Ahlburg) Cohn as previously reported (Raper and Fennel, 1965; Domsch et al., 1980; Klich and Pitt, 1992; Hoog et al., 2000; Domsch et al., 2007). As well as, *A. oryzae* (Ahlb) Cohn was identified by (Naguib and Yassa, 1973; Moubasher, 1993).

Aspergillus sp6, the colonies were of yellow-green

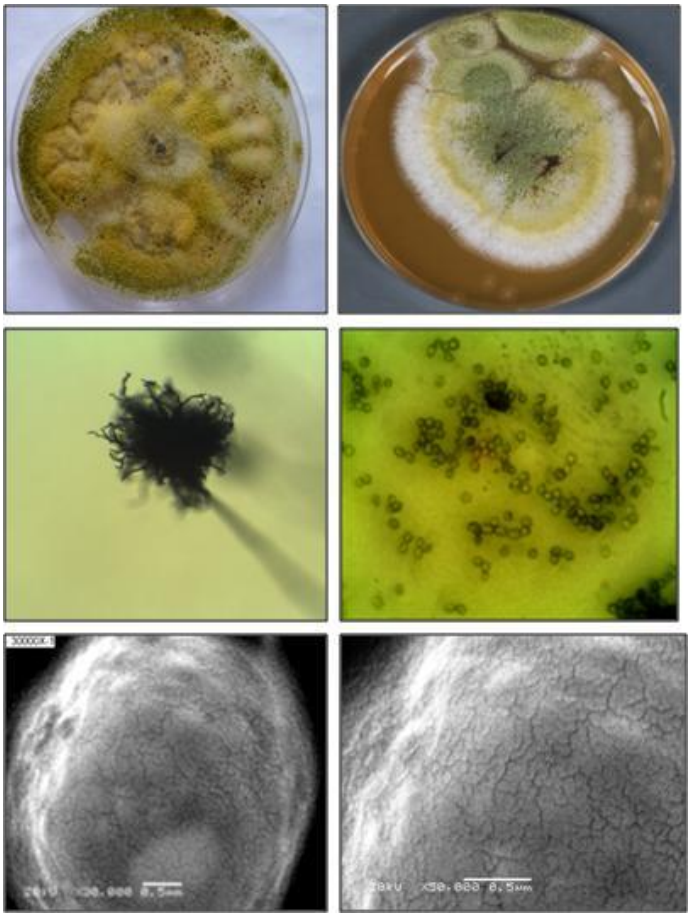

Figure 6. Macro and Microscopical features of *Aspergillus oryzae.* Colonies after 4 days at 28°C on Czapek's medium (A), on malt extract agar (B), conidial head and conidiophores (C). Conidia by light microscope 1000X (D). Conidial ornamentation by Scanning Electron Microscope at 30000X (E) and 50000X (F).

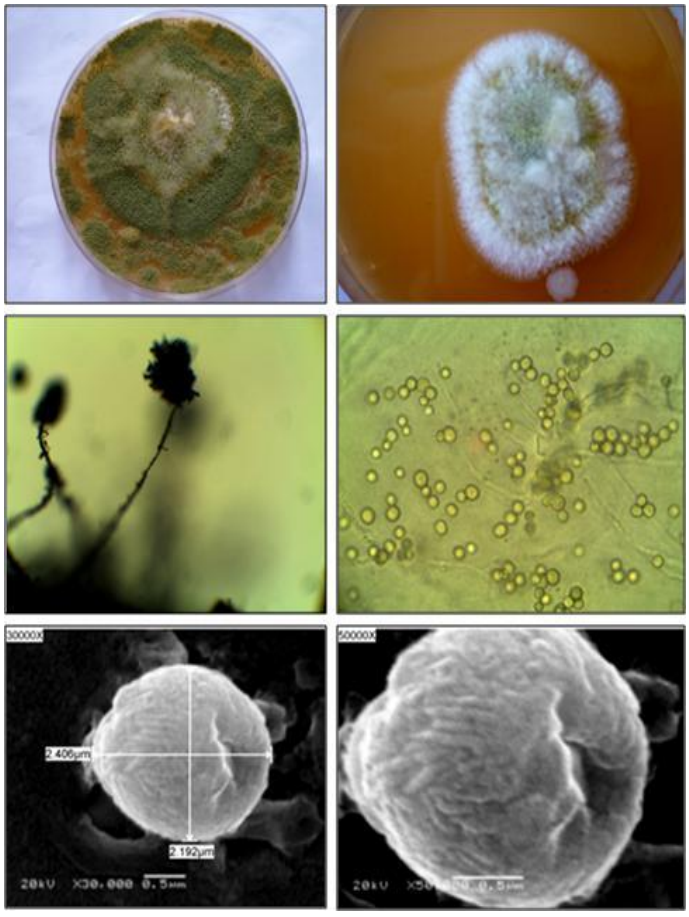

Figure 7. Macro and Microscopical features of *Aspergillus parasiticus.* Colonies after 4 days at 28°C on Czapek's medium (A), on malt extract agar (B), conidial head and conidiophores (C). Conidia by light microscope 1000X (D). Conidial ornamentation by Scanning Electron Microscope at 30000X (E) and 50000X (F).

color turned to deep grape green with slightly creamy reverse. Conidial heads loosely radiate, with strict uniseriate stigmata covering the entire surface of vesicle. Conidia are globose with coarsely echinulate with average dimension of 2.19 to 2.40 µm by 30000X SEM. On malt extract agar, colonies are widely spread, heavily sporulation with uncolored reverse (Figure 7). The morphological description is identical to *A. parasitica* Speare by Thom and Raper (1945) and Raper and Fennell (1965).

Practically, among nine *Aspergillus* species producing L-methioninase, depending on our previous screening studies (Khalaf and El-Sayed, 2009), six isolates (*A. flavipes*, *A. carneus*, *A. flavus*, *A. tamarii*, *A. oryzae* and *A. parasiticus*); belonging to *A. flavipes* and *A. flavus* groups were extensively characterized morphologically according to Raper and Fennell (1965). However, regarding the relative accuracy of macro and micro-morphological identification, due to some limitations as development of specific phenotypic structure and physiological criteria that requires specific type of media. Consequently, combination of molecular approaches with morphological descriptions could achieve a plausible accuracy for fungal identification. Also, to fulfill our previous studies concerning L-methioninase enzyme production (El-Sayed, 2009, 2010, 2011; El-Sayed and Shindia, 2011); these isolates were further characterized via molecular approaches.

Molecular studies of L-Methioninase producing *Aspergillus* species

For the maximum enzyme productivity and based on morphological identification, *Aspergillus flavipes* (Bain. and Sart.) was used as authentic strain for primer design of 18S rRNA, ITS regions, and 28S rRNA sequences. After extraction of genomic DNA from the six fungal isolates (*A. flavipes*, *A. carneus*, *A. flavus*, *A. tamarii, A. oryzae*, and *A. parasiticus*), their DNA purity and

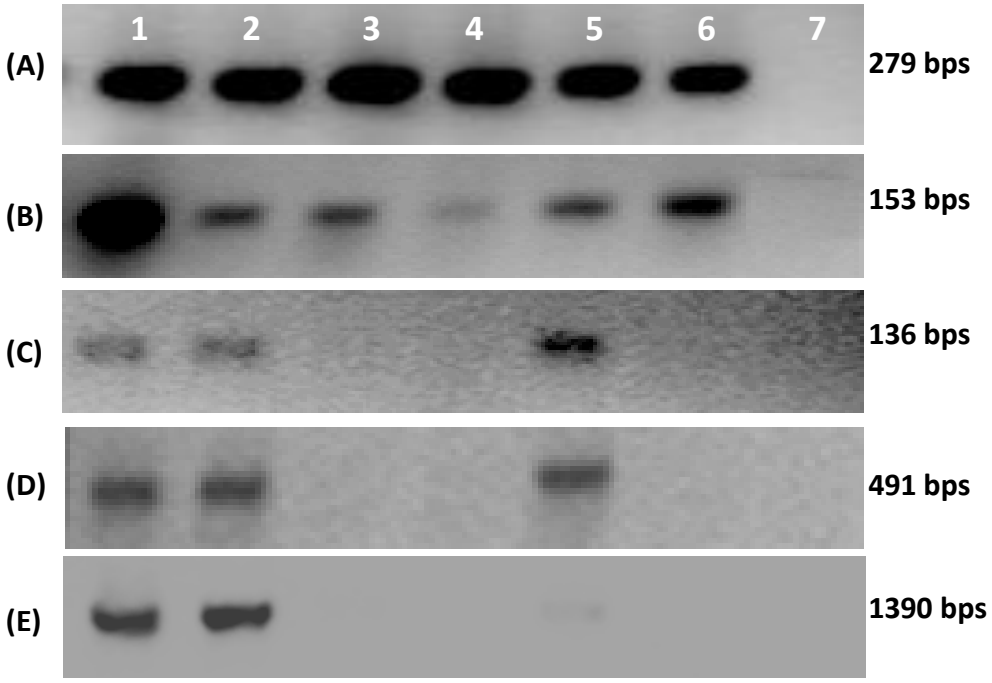

Figure 8. Results of PCR analysis of *A. flavipes* EK and other six L-methioninase producing *Aspergillus* species (lanes 1-6), lane 7: negative control. PCR amplification products were visualized on 2% agarose gel. PCR amplicons of 18S rDNA (A), ITS1 (B), ITS2 (C), ITS1-ITS2 (D) and 18S-28S rDNA (E). Based on morphological description, lane 1:*A. flavipes*, lane 2: *A. carneus*, lane 3: *A. flavus*, lane 4: *A. tamarii*, lane 5: *A. oryzae*, and lane 6: *A. parasiticus.* DNA markers were reported on the corresponding agarose gel.

homogeneity were checked on 0.8% agarose gel. Using 18S-Fw and 18S-Rev primers (Table 1) for 18S rRNA amplification, the PCR amplicon for all samples were about 279 bps (Figure 8A). Apparently, the similarity on the sizes of the 18S rRNA amplicon among the tested fungal isolates not only reveal the utility of the design primer as universal one at least for *A. flavipes* and *A. flavus* group, but also ensure the genetic identity of this domain among these isolates.

Also, using the ITS1 specific primers, the PCR product of ITS1 PCR amplicon was about 153 bps for the six tested L-methioninase producing *Aspergillus* species, ensuring the molecular and morphological similarity among these two groups (Figure 8B). In consistent, the size of ITS1 amplicon of *A. flavipes* ATCC 1030 (Haugland et al., 2004), *A. flavipes* E14 (Bukovska et al., 2010) and *A. flavipes* NRRL 295 (Peterson, 2008) is 156 bps. Similarly, the size of ITS1 region of these isolates was in the range of those deposited in the database for *A. flavus* MUM: 10.206 (HG: 340106.1), *A. flavus* NRRL: 6412 (HG340109.1), *A. oryzae* NJP18 (HQ710546.1), *A. oryzae* NJP15 (HQ710543.1), *A. parasiticus* NRRL: 3386 (HQ340110.1), *A. tamarii* NRRL SRRC1088 (AY373870.1).

Furthermore, the amplicon of ITS2 and ITS1-5.8S-ITS2 were resolved only for the three isolates of *A. flavipes* group by about 136 and 491 bp, respectively assuming the relative identity of their genetic loci, other than the remaining isolates (Figure 8C, D). Unlike the genetic proximity of ITS1 size, among the six fungal samples, a great genetic variability of ITS2 was observed that was consistent with that deposited in the database. Interestingly, the identity of ITS2 amplicon directly anticipates with the morphological and biochemical relatedness of the fungal isolates, as ensured by (Marcellino et al., 2001). From the profile of ITS2 (Figure 8C), similarity of size, at least, for ITS2 amplicon of *A. flavipes*, *A. carneus, and A. oryzae* are ensuring the morphological and biochemical proximity of these isolates as adopted by Raper and Fennell (1965).

Similarly, the ITS2 identity usually correlates with the similarity of phenotypic features as observed for *Penicillium requeforti* and *Geotrichum candidum* (Larsen et al., 1998; Berger et al., 1999; Florez et al., 2007). However, unequivocal identification of L-methioninase producing *Aspergillus* species relying on only ITS2 amplicon size as appeared on agarose gel was absolutely not reliable. De-Aguirre et al. (2004) has reported similar result for various species of *Aspergillus*. For more accuracy, the 18S rRNA forward and 28S rRNA reverse primers were used in PCR under standard conditions for the complete amplification of ITS1-5.8S-ITS2 region. The PCR amplicon of 18S rRNA- ITS1-5.8S-ITS2- 28S rRNA sequence was 1390 bp (Figure

Table 2. Proximity of *A. flavipes* EK ITS1-ITS2 sequence with database *Aspergillus* species.

Number	Isolate	Strain no.	Accession no.	18S-28S Size (bp)	Maximum Identity of ITS1-ITS2 (%)	Aligned score of 18S-28S region (%)
1	*A. flavipes* EK	This study	JF831014	1385	-	-
2	*A. flavipes*	NRRL302	EF669591.1	1137	99	29.55
3	*A. iizukae*	NRRL3750	EF669597.1	1138	99	29.34
4	*A. flavipes*	NRRL4578	EF669602.1	1134	98	29.01
5	*A. flavipes*	NRRL295	EF669588.1	1134	98	29.01
6	*A. flavipes*	NRRL4263	EF669600.1	1135	98	29.07
7	*A. flavipes*	ATCC1030	AY373849.1	613	97	34.47
8	*A. carneus*	NRRL527	EF669611.1	1148	94	24.56
9	*A. carneus*	NRRL298	EF669590.1	1148	94	24.56
10	*A. carneus*	NRRL1928	EF669581.1	1149	94	24.80
11	*F. nivea*	NRRL6134	EF669616.1	1149	94	24.54
12	*F. nivea*	SRRC333	AY373853.1	626	92	28.43
13	*A. flavus*	NRRL4998	EF661566.1	1152	92	23.09
15	*A. oryzae*	NJP18	HQ710546.1	589	89	25.29
16	*A. oryzae*	NJP10	HQ710539.1	595	88	23.02
17	*A. tamarii*	NRRL427	HQ340111.1	914	90	23.74
18	*A. candidus*	NRRL313	EF669594.1	1138	93	26.01
19	*A. parasiticus*	NRRL6433	EF661568.1	1152	92	22.91
20	*A. pulvinus*	NRRL5078	EF652159.1	1140	92	22.98
21	*A. niger*	NRRL1956	EF661191.1	1144	92	25.26
22	*A. terreus*	NRRL1923	EF669580.1	1152	94	25

8E) for lane 1 (*A. flavipes*), lane 2 (*A. carneus*) as appeared on agarose gel, approving their morphological and genetic relatedness. Thus, sequencing of this domain (18S rRNA-ITS1-5.8S-ITS2-28S rRNA) and alignment with similar DNA sequences of closely related species deposited on genbank may significantly figure out the genetic identity of these isolates.

Sequencing of ITS1-5.8S-ITS2 amplicon and phylogenetic analysis

Depending on the PCR profile (Figure 8E), *A. flavipes* (lane 1) 18S rRNA partial -ITS1-5.8S-ITS2-28S rRNA partial sequence amplicon was sequenced using the previously reported primer sets (Table 1). The sequence of 18S-28S rRNA region includes the complete sequence of ITS1-5.8S-ITS2 amplicon revealing the actual identity of rRNA. Owing to the uniqueness of *A. flavipes* for L-methioninase production in our studies (Khalaf and El-Sayed, 2009; El-Sayed, 2009, 2011; El-Sayed and Shindia, 2011), its 18S-28S rRNA was selected for further sequencing and alignment with closely related *Aspergillus* species deposited in the database. The obtained sequence of *A. flavipes* EK 18S-28S rRNA was deposited in genbank with accession number JF831014 (http://www.ncbi.nlm.nih.gov/nuccore/JF831014).

From the alignment profile of the obtained sequence using multiple alignment software (http://blast.ncbi.nlm.nih.gov/Blast.cgi), *A. flavipes* EK 18S-28S domain displays a great similarity with *A. flavipes* NRRL302 (EF669591.1), *A. iizukae* NRRL3750 (EF669597.1), *A. flavipes* NRRL4578 (EF669602.1), *A. flavipes* NRRL295 (EF669588.1), *A. flavipes* NRRL4263 (EF669600.1) by about 99% (Table 2). However, *A. flavipes* EK exhibit a relative identity with *A. carneus* (NRRL527, NRRL298 and NRRL1928) by about 94% with alignment score of 24.2%. The higher divergence was relatively appeared with *A. oryzae* (NJP10) with 88% maximum identity. However, by alignment of *A. flavipes* 18S-28S amplicon sequence using Clustal W (http://www.genome.jp/tools/clustalw) (Thompson et al. 1997), the matched scores were maximally calculated with *A. flavipes* ATCC1030 (34.5%), *A. flavipes* NRRL302 (29.5%), *A. iizukae* NRRL3750 (29.34%), *A. flavipes* NRRL4578, NRRL295, NRRL4263 (29.01-29.07%) followed by *F. nivea* SRRC333 (28.43%) and *A. carneus* NRRL527, NRRL298 and NRRL1928 (24.5 to 24.8%) (Table 2).

From the phylogenetic analysis of the whole sequence of *A. flavipes* EK 18S-28S rRNA (Figure 9) with the closely related strains from the database, it appears a distinct identity with *A. flavipes* and *A. iizukae* isolates followed by *A. carneus.* Morphologically, *A. flavipes* and *A. carneus* belonging to the same group (*A. flavipes* group) according to Raper and Fennell (1965), approve

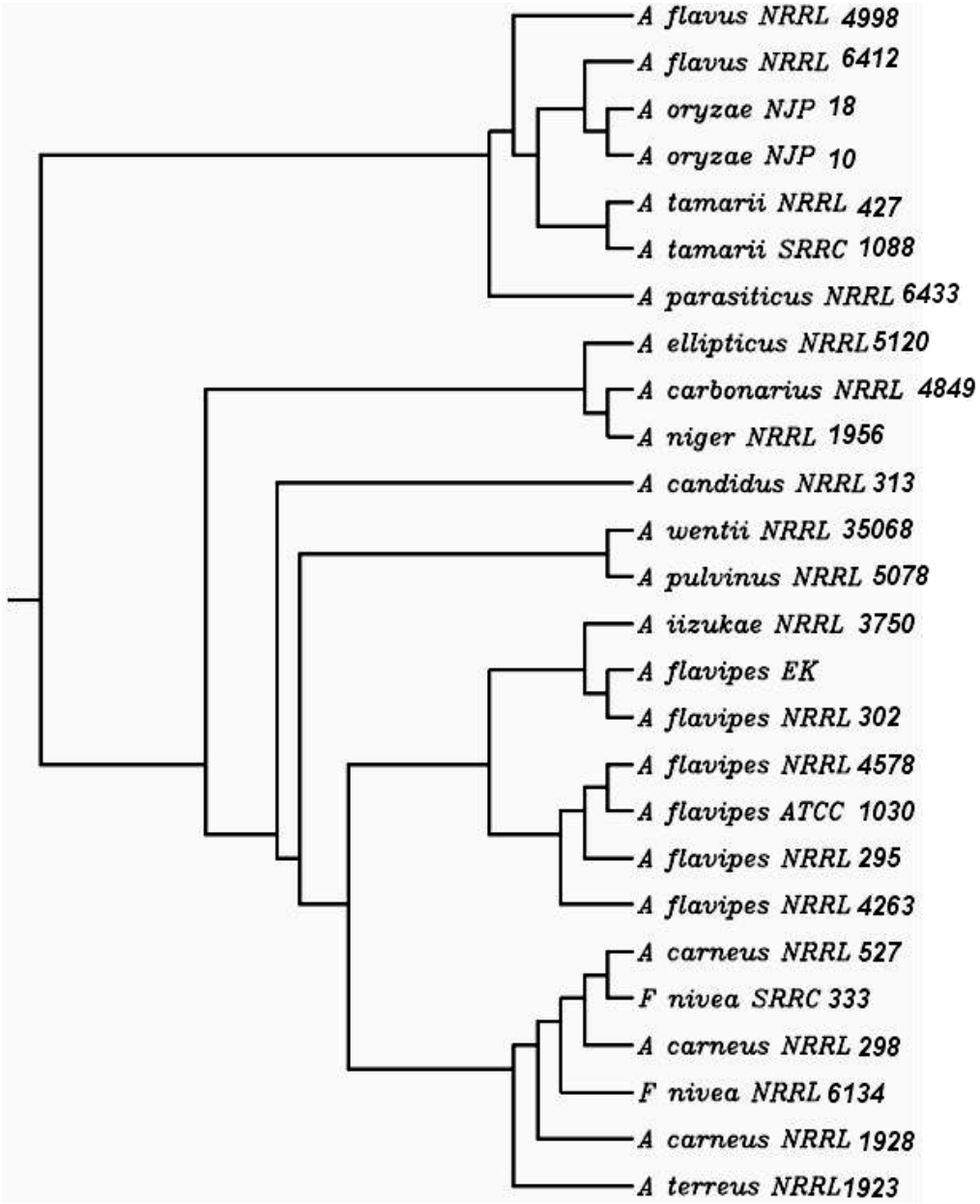

Figure 9. Phylogenetic analysis of *A. flavipes* EK 18S-28S rRNA.

the molecular inspections of these results. The phylogenetic analysis reveals the molecular identity of *A. flavipes* EK isolate with closely related strains of *Aspergillus* spp. that directly correlated with the morphotypic promiscuity of *Aspergillus* species (Thom and Raper, 1945; Raper and Fennell, 1965; Domsch et al., 1980). The sequence of ITS1-5.8S-ITS2 region appears the reliable molecular approach for fungal identification (Dupont et al., 1999; Boysen et al., 2000; Florez et al., 2007). Consistently, ITS2 was frequently used for discrimination of medically related *Aspergillus* species (Hinrikson et al., 2005; Embong et al., 2008). Practically, ITS regions of rRNA are the most reliable molecular approach for fungal discrimination (Henry et al., 2000; Chen et al., 2002; De-Aguirre et al., 2004). Approving to our results, the 18S-28S region flanking ITS1, 5.8S and ITS2, was the probe for intra-species of *Aspergillus* species (Yamakami et al., 1996).

Unlike the broader classification of *Aspergillus* spp depending on the sequence analysis of more conserved rDNA region (18S rRNA), ITS1-ITS2 regions shown a plausible accuracy on molecular identification as ensured by Henry et al. (2000) and De-Aguirre et al. (2004). Interestingly, morphological and physiological identification of *Aspergillus* species (Thom and Raper, 1945; Raper and Fennell, 1965) was plausibly ensured via

development of different molecular aspects, along the last 60 years, up to date (Geiser et al., 2007; Lubertozzi and Keasling, 2009). Thus, molecular identification is a relevant supplementary tool approving the traditional systematic fungal classification. To the best of our knowledge, this is the first context dealing with extensive morphological and molecular characterization of L-methioninase producing *Aspergillus* species.

Conclusion

L-Methioninase producing *Aspergillus* species isolated from Egyptian soil were comprehensively characterized relying on various morphological and molecular approaches. Depending on macro and micro-morphological features, the isolates were identified as *A. flavipes*, *A. carneus, A. flavus*, *A. tamarii*, *A. oryzae* and *A. parasiticus*. *A. flavipes* was used as reference fungal isolate for molecular analysis. *A. flavipes* EK 18S-28S rRNA, flanking ITS1, 5.8S, ITS2 regions, was amplified, sequenced and aligned with the closely related fungal isolates. From phylogenetic analysis, the isolate was determined to be *A. flavipes*, according to the genetic loci of *Aspergillus* species isolates deposited on database. Interestingly, molecular aspects of rRNA studies strongly approve the corresponding morphological identification among related species, ensuring the divergence among dissimilar morphotypic isolates. The sequence of *A. flavipes* EK 18S-28S rRNA was deposited in Gene bank with accession No. JF831014. To the best of our knowledge, this is the first study for extensive morphological and molecular characterization of L-methioninase producing *Aspergillus* species.

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