INTRODUCTION

Poultry ration ingredients are rich in microbial growth enhancers i.e. cereals. The majority of fungi and fungi-producing mycotoxins present in cereals are preprocessing stable, thus contaminate the final feedstuffs into which they are incorporated. Among Aspergillus, section Nigri species (black Aspergilli) is considered the most frequent fungi causing feed and food spoilage (Mohamed et al. 2017; Schuster et al. 2002) as well as ochratoxin A producers (Samson et al. 2007). Based on phentotypical analysis alone, the classification of section Nigri fungi remains difficult and is hampered by minor morphological differences between the species (Abarca et al. 2004; Varga et al. 2011). Therefore, the current protocols for
section Nigri fungi classification include a combination of phenotypic, chemotaxonomic and molecular as well as chromatographic approaches (Storari and Broggini 2017). Using these aforementioned methods two species namely, *Aspergillus niger* (*A. niger*) and *Aspergillus tubingensis* (*A. tubingensis*) can be separated from the fungal complex (Abarca et al. 2003).

It is generally accepted that *A. niger* is one of the most commonly reported *Aspergillus* species. Furthermore, major ochratoxin A producers belong to the *A. niger species complex* *i.e.* *A. foetidus, A. carbonarius, A. japonicas* and *A. niger* according to previous reports (Abarca et al. 2003; Battilani et al. 2003; Magnoli et al. 2003; Teren et al. 1996). Ochratoxin A can be produced in feed and food due to improper storage and/or shipment (Pfohl-Leszkowicz et al. 2002). Further, ochratoxin A is a potent nephrotoxin in several animal species, and exerts immunotoxic and genotoxic properties (Kamp et al. 2005) as well as a neurotoxic potential (Razafimanjato et al. 2010). Generally, fungal contamination is associated with nutrient losses and animal health concerns, also for poultry farms (Pitt 2000). In this context, several reports have shown that exposure of poultry to low levels of ochratoxin (0.5 mg/kg feed) decreases feed consumption and lowers feed conversion capacity (Prior et al. 1980; Wang et al. 2009). In Egypt, particularly its southern regions, little is known about the mycotoxin contamination of feedstuffs. However, few articles have shown the levels of mycotoxins in this region compared with middle and northern parts (Badr et al. 2017; Sultan and Magan 2010). Therefore, the characterization and identification by PCR of food-spoilage mold species is becoming essential in order to control and prevent food contamination by these microorganisms and thus lower potential mycotoxin production (Mohamed et al. 2017; Parvathi et al. 2017). Therefore, we aim in the current study to identify and characterize *Aspergillus section Nigri* isolates, particularly potential ochratoxin A producing species, in poultry feed. We show that poultry feed is contaminated with *A. niger* and *A. tubingensis*. In addition, these fungi are able to produce ochratoxin A under optimal *in vitro* conditions.

**MATERIAL AND METHODS**

**Collection of samples:** Forty poultry ration samples were collected from different markets (Table 1) in Qena governorate (Egypt). All samples (rations and cereals) were collected in the winter season (2017).

| City          | Samples (n)                  | #Positive isolates (%) |
|---------------|------------------------------|------------------------|
| Nag-Hammady   | Commercial prepared feed (10)| 4 (40%)                |
|               | Mixed poultry feed (10)      | 4 (40%)                |
| Elhemadat     | Commercial prepared feed (10)| 2 (20%)                |
|               | Mixed poultry feed (10)      | 6 (60%)                |
| Total         | 40                           | 16 (40%)               |

**Table 1:** Incidence of positive isolates for *Aspergillus* at Qena district (Egypt) in poultry feed.

**Fungal isolation and identification:** The isolation technique was performed according to Van Pamel and co-workers (Van Pamel et al. 2009) with some modifications. Briefly, 1 g of
the sample was diluted in 9 ml distilled water, then 1 ml of the mixture was plated onto Petri dishes containing Dichloran Rose-Bengal Chloramphenicol agar medium (DRBC; (King et al. 1979) at 25°C. After 5-7 days, colonies exhibiting the characteristic morphological features of *Aspergillus section Nigri* were purified on Malt Extract Agar (MEA) using the spot inoculation method (Pitt 1973) at 25°C. After 5 days, both macroscopic and microscopic identifications were performed as previously described by Leong et al. (Leong et al. 2007).

**Extraction of genomic DNA:** Extraction of DNA was performed with slight modification according to a previous report (Moller et al. 1992). The collected isolates of *A. niger* (n=17 isolates) were cultured in Potato dextrose agar (PDA) medium. Five days post-incubation, mycelium was collected by sterilized spatula and ground to a fine powder with 0.7 ml 2x cetyl trimethyl ammonium bromide (CTAB) buffer and transferred to a 1.5 ml Eppendorf tube and mixed. The Eppendorf tubes were incubated at 65°C for 30 min, then 0.7 ml of chloroform was added and briefly mixed. After centrifugation at 15,000 rpm for 30 min, the supernatant was transferred into a new tube mixed with 0.6 ml of isopropanol chilled to 20°C, followed by centrifugation for 5 min at maximum speed. The supernatant was discarded, and the remaining pellet was washed twice with 1 ml of 70% ethanol, followed by drying under vacuum and, thereafter, dissolved in 1 ml of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5). DNA integrity and concentration was evaluated by agarose gel electrophoresis.

**PCR-based identification of Aspergillus section Nigri:** We investigated the nucleotide sequence variability in a region of the β-tubulin gene to design a primer to amplify target DNA from *A. niger*. Primers used to amplify a region of the β-tubulin gene (Glass and Donaldson 1995) were applied (Table 1). PCR was performed in a reaction volume of 25 μl according to Massi and co-workers (Massi et al. 2016), contained 1 μl of genomic DNA, 5 mM Tris-HCl (pH 8.3), 25 mM KCl, 2.0 mM MgCl₂, 0.1 mM of dNTP, 0.5 μl of each primer and 1 U of Taq DNA polymerase (Invitrogen, Darmstadt, Germany). Then, 1.5 μl for each primer, the volume was completed by PCR grade water. The reactions were done in a Thermo Cycler (BioRad, Munich, Germany) with denaturation at 94°C for 5 min, followed by 36 cycles at 94°C for 45 s, annealing temperature at 46°C for 45 s and extension at 72°C for 1 min, then final step as extension at 72°C for 7 min. PCR products were separated on a 1.5% agarose gel, and subsequently stained with ethidium bromide. A similar protocol was applied to identify the ochratoxin metabolic genes (Aopks, Table 1) according to Reddy and colleagues (Reddy et al. 2013).

**Sequence analysis:** PCR products were prepared for sequencing by adding 5 units of exonuclease I (Fermentas, USA) and 0.5 units of alkaline phosphatase (Fermentas, USA) to 8 μL of PCR product and incubating at 37°C for 30 min followed by 80°C for 15 min. Cleaned products were separated on an ABI 3770 sequencer at the Massey Genome Service (Palmerston North, New Zealand) following the manufacturer’s recommendation (Applied Biosystems, USA). The results of β-tubulin gene sequences were submitted to the Gene Bank [http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROG RAM=blastn&PAGE TYPE= Blast Search & LINK LOC= blasthome#](http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROG RAM=blastn&PAGE TYPE= Blast Search & LINK LOC= blasthome#).
DNA sequences of the gene coding for the DNA product were aligned by PAUP Version 4.0b10 for 32-bit Microsoft Windows.

**Phylogenetic analyses:** Maximum parsimony phylogenetic analyses were conducted using PAUP4.0b10 on the data set. Heuristic searches were conducted with 1000 random stepwise addition replicates, holding and saving 5 trees per replicate, with TBR branch swapping, and all characters treated as equally weighted and unordered. Bootstrap support analyses (Felsenstein 1985) were performed running 1000 bootstrap replicates (each with 1000 replicates of random sequence addition) equal weighting and TBR branch swapping, holding a maximum of 10 trees per replicate. Bayesian analyses were performed with MrBayes 3.1 according to (Ronquist and Huelsenbeck 2003). The best fit models were determined using the AIC criterion as implemented in MrModeltest version 2.3. Two independent runs were conducted in MrBayes on the β-tubulin dataset. For each analysis, four simultaneous runs with one cold and three heated chains each (using the default heating parameters) with random starting tree were run for $2 \times 10^6$ generations, with tree sampling every 1000 generations. This allowed the standard deviation of split frequencies to stabilize at levels lower than 0.01. The first 25% trees of each run was discarded as burn in and a 50% majority-rule consensus tree was constructed from the post-burn-in trees.

**Table 2:** Primer sequences were used to identify β-tubulin and ochratoxin metabolic genes.

| Target gene | Primer DNA sequences                  | Size   | Reference                  |
|-------------|---------------------------------------|--------|---------------------------|
| β-Tubulin   | Bt1a F’5- TTCCCCCGTCTCCACTTCTCATG-3   | 537 bp | (Glass and Donaldson 1995)|
|             | Bt1b R’5-GACGAGATCGTTCATGTTGAAC-3    |        |                           |
| Aopks       | Aopk1 F’5-CAGACCATCGACACTGCATGC-3    | 549 bp | (Reddy et al. 2013)       |
|             | Aopk2 R’5-CTGGCGTTCAGTACCAGTGAG-3    |        |                           |

**Aopks: Ser/Thr protein kinase gene**

**Extraction and identification of mycotoxin from pure Aspergillus section Nigri colonies:** A flask containing 100 ml of Yeast Sucrose Broth (YES) was inoculated with spores and mycelia of mold isolates from 5-7 days old culture. The broth was incubated for 6-8 days at 25°C. After incubation, the broth was put in a blender to grinding all contents (100 ml of chloroform was added to the broth in a clean flask). This mixture was put on a vertical shaker for 24 h. Then the mixture was poured in a separator funnel to separate the mixture into a high layer consisting of spore and mycelia, where the lower layer consisted of chloroform with extracted toxin. Subsequently, the lower layer (final extract of chloroform) was transferred to a clean dry flask and was evaporated at water bath (50°C). Then dry powder remained in a flask (Samson et al. 2014). From the extracted powder dissolved with 1 ml of chloroform in a clean flask, 20 ul of the dissolved extract was spotted on activated TLC plates and then air-dried. Dried plates were put in a glass tank with the solvent mixture chloroform : methanol (97:3 v/v) until the solvent came near to the top of a plate. The TLC plates were removed from the tank, dried and examined under UV-light (360 nm, blue color). TLC was used for estimation of ochratoxin A (OTA) presence in extracts. The toxin must have the
same retention factor (Rf) and fluorescence as reference material of the toxin to identify this toxin (here OTA) (El-Shanawany et al. 2005; El Khoury and Atoui 2010; Lin et al. 1998).

**RESULTS**

*Mycological analysis reveals Aspergillus isolates in feed*

Forty feed samples were collected and were screened by visual mycological analysis. Among the 40 samples, on average 16 samples were contaminated with *Aspergillus section Nigri* (Table 2). The percentage of positive isolates were 40% (4 out of 10 samples) in Nag-Hammady samples, regardless to the feed-type. However, 20% (2 of 10 samples) and 60% (6 of 10 samples) were identified as positive isolates in commercially prepared and mixed poultry feed (Elhemadat) samples, respectively (Table 2). However, no significant differences were reported between collection points and feed-type included in poultry ration (Table 2). Thus, feed samples are contaminated with *Aspergillus section Nigri* regardless to the feed source and type.

*Molecular analysis to further classify the identified fungal contamination*

Due to high resemblance between species of *Aspergillus section Nigri* (Supplemental Figure 1), molecular characterization was performed. In detail, PCR analysis was achieved in 16 positive isolates (Table 2). Unfortunately, one positive isolate showed no band at 537 bp, therefore, it was excluded from further analysis. Further, all samples were contaminated with *Aspergillus section Nigri* (Table 3). However, it was noticed that the mixture feed was more contaminated with *Aspergillus niger* than pellets (Table 3). In detail, according to blasting the sequence of amplified region of β-tubulin gene on gene bank, the results showed that our isolates (n=15) belonged to one genera (*Aspergillus section Nigri*) and two species: 11 isolates were identified as *A. niger* and 4 isolates as *A. tubingensis* (Table 3). The phylogeny analysis was conducted on 14 isolates only (n=11 and 3 isolates of *A. niger* and *A. tubingensis*, respectively). Because of a short sequence, one isolate belonging to *A. tubingensis* (C7) was excluded. Using parsimony and Bayesian analyses, it appeared that coding regions of the β-tubulin gene comprised of 479 total characters: 173 characters were constant, 273 variable characters were parsimony-uninformative, and 33 characters were parsimony-informative. From parsimony analyses, 50,000 most parsimonious (MP) trees of 4845 steps with a CI 0.9938 (excluding uninformative characters), RI 0.9905, and RC 0.9844 were saved. In β-tubulin trees, *A. niger* and *A. tubingensis* isolates were divided into two clades (Figure 1 and 2). Clade A considered a major clade, comprised of *A. niger* isolates and was divided into two subclades based on their sequence divergences. These subclades showed identified isolates ranked using reference strains (similar nucleotide sequence) of the gene bank. For example, the first subclade grouped with *A. niger* AY820005, GQ376121 and MF150890 (PP=0.96 BS=68). The second subclade grouped with *A. niger* were HQ632662 and MG701893 (BS=99; Figure 1 and 2). Furthermore, clade B was ranked for *A. tubingensis* isolates, and divided into three subclades. These *A. tubingensis* isolates were MG387175 (first group), KY990210, JX463309 and MG387171 (second group) as well as KT965701 (third group). The tree was rooted by *Cheatomium globosum* MG885809 (Figure 1 and 2).
In conclusion, two species of *Aspergillus section Nigri* (*A. niger* and *A. tubingensis*) are molecularly characterized in the positive isolates of contaminated poultry feed.

**Table 3. Accession number of black Aspergillus isolates.**

| No. of isolate | Spp.         | source       | Accession number |
|----------------|--------------|--------------|------------------|
| 1              | *A. niger*   | Mixture grains | MH621313        |
| 2              | *A. tubingensis* | Pellets      | MH621314        |
| 3              | *A. tubingensis* | Pellets      | MH621315        |
| 4              | *A. niger*   | Mixture grains | MH621316        |
| 5              | *A. niger*   | Pellets      | MH621317        |
| 6              | *A. niger*   | Mixture grains | MH621318        |
| 7              | *A. niger*   | Mixture grains | MH621319        |
| 8              | *A. tubingensis* | Pellets      | MH621320        |
| 9              | *A. niger*   | Mixture grains | MH621321        |
| 10             | *A. niger*   | Mixture grains | MH621322        |
| 11             | *A. niger*   | Mixture grains | MH621323        |
| 12             | *A. niger*   | Mixture grains | MH621324        |
| 13             | *A. tubingensis* | Pellets      | MH621325        |
| 14             | *A. niger*   | Mixture grains | MH621326        |
| 15             | *A. niger*   | Pellets      | MH621327        |

*Isolates have ochratoxin metabolic gene; #Isolates have ochratoxin metabolic gene and produce ochratoxin A in YES.

**Ochratoxin A producing Aspergillus section Nigri**

To further investigate the mycotoxicogenicity of the isolated *Aspergillus section Nigri*, ochratoxin metabolic genes and produced ochratoxin A were analyzed by PCR and by thin layer chromatography (TLC), respectively. Using a specific primer for ochratoxin metabolic genes, we observed that 12 out of 15 isolates showed amplicon at 549 bp (Table 3, Figure 3A). Further, the TLC-based analysis revealed that ochratoxin A was produced by 4 out of 12 identified *Aspergillus species*, namely *A. niger* and *A. tubingensis* (Table 3, Figure 3B, Supplemental Table 1). Apparently, the isolated *Aspergillus species* from poultry feed can produce ochratoxin A *in vitro*. 
Figure 1: Bayesian 50% majority rule phylogram of \(\beta\)-tubulin showing the phylogenetic relationships among the studied strains of *Aspergillus section Nigri*. The newly generated sequences of *Aspergillus niger* are preceded by blue square, and those of *A. tubingensis* are preceded by red circles. The GeneBank accessions used in this study are preceded by black triangles. Numbers above the branches represent Bayesian posterior probabilities (\(\geq 0.90\)), and the maximum parsimony bootstrap support values are given below the branches (\(\geq 70\%\)).

DISCUSSION

Poultry feed is rich in microbial growth promoters i.e. nutrients and additives which under optimal conditions can lead to fungal growth (Okoli et al. 2006). Therefore, periodical assessments of fungal contamination and mycotoxin production are crucial for animal safety. In the present study, a high incidence of fungal contamination by black aspergilli was recorded in poultry feed (40% on average). These results were in agreement with Rosa and co-workers (Rosa et al. 2006) where fungal contamination and ochratoxin A production were reported in commercially-available poultry feed. Not only commercial feed was contaminated with mycotoxin producing fungi but also mixed feed was more infected. In this line, high incidence of *Aspergillus spp* isolated from maize - main component in poultry feed - were reported by Kpodo et al. (Kpodo et al. 2000) and Battilani et al. (Battilani et al. 2003) from two different continents. Therefore, no major differences were reported in the incidence of black Aspergilli in poultry feed collected from Nag-Hammady and Elhemadat (Qena governarate, Egypt). This indicates that periodical fungal and mycotoxins surveys in raw materials and storage practices of poultry feed are needed.
Figure 2: Strict consensus cladogram of 50,000 Most Parsimonious (MP) trees found, using the β-tubulin sequence data, showing the phylogenetic relationships among the studied strains of Aspergillus section Nigri. The newly generated sequences of Aspergillus niger are preceded by blue square, and those of A. tubingensis are preceded by red circles. The GeneBank accessions used in this study are preceded by black triangles. Values above the branches indicate Bayesian posterior probabilities (≥ 0.90), and the maximum parsimony bootstrap support values (≥70%) are given below the branches.

Members of Aspergillus section Nigri are not easily distinguishable by morphological characterizations e.g. culture characteristics, colonies and incubation temperature (Samson et al. 2007). Therefore, molecular characterization was applied to identify different spp of Aspergillus section Nigri as described by Samson and co-workers (Samson et al. 2014). Principally, it was reported that DNA sequences of β-tubulin can accurately differentiate between various Aspergillus section Nigri spp., i.e. A. niger, A. tubingensis, A. foetidus, A. carbonarius, and A. awamori. The morphologically identified 16 isolates have been shown to belong to section Nigri, based on DNA sequences of β-tubulin. Further cladogram analysis of DNA sequences of β-tubulin revealed that A. niger and A. tubingensis isolates were identified. However, no apparent sister group relationships existed, so it is not possible to infer any further intergroup phylogenetic analysis. This genetic variation between A. niger and A. tubingensis isolates was previously reported by...
Samson et al. (Samson et al. 2014) and Geiser et al. (Geiser et al. 2007). Moreover, phylogenetic analysis revealed that our isolates were grouped with gene bank-based reference strains for confirmatory purposes (Min and Hickey 2007). It was previously shown that A. niger and A. tubingensis isolated from patients and environmental samples i.e. soil, air, and plants were grouped with gene bank-based reference strains (Astoreca et al. 2011) and reviewed by Kumar and co-workers (Kumar et al. 2016). This might explain the source of fungal contamination of poultry feed. Therefore, poor crop harvesting, inappropriate drying, handling, packaging, storage, and transport conditions for raw materials and formulated rations must be avoided to minimize mycotoxin production (Logrieco et al. 2007).

Figure 3: A) Amplification profile of ochratoxin metabolic genes at (549 bp) of all Aspergillus section Nigri positive samples. Lane 1 is a positive control, lanes 2, 3, 4, 7, 8, 9, 10 are 11 positive isolates and lanes 5 and 6 are negative isolates. B) Thin layer chromatography (TLC) is applied to assess the mycotoxicogenicity of isolated Aspergillus section Nigri extracts in developing spotted silica gel plates. Lane (1) is positive for gliotoxin and lanes (4, 5 and 7) positive for ochratoxin A production.

In the present study, ochratoxin metabolic genes (Aopks) were detectable in the majority of isolates (12 out of 15 isolates). However, only four isolates, A. niger (n=2) and A. tubingensis (n=2), were able to produce a detectable amount of ochratoxin A. Most frequently, detection of Aopks is well associated with mycotoxin production, however, several factors affect this step i.e. water availability (activity) and temperature stimulating germination and growth as well as sporulation
In line with our data, several reports suggested that ochratoxin A is produced by *A. niger* (Accensi et al. 2001), however, Vankudoth and co-workers (Vankudoth et al. 2016) reported that *A. niger* is not an ochratoxin A producer. In contrast, Moslem and colleagues (Moslem et al. 2010) reported a high production of ochratoxin A from *A. niger* strains. In contrast to our report, Samson et al. (Samson et al. 2014) reported that ochratoxin production from isolates is considered a variable character and is strain dependent. However, in agreement with our data, it has been shown that *A. tubingensis* produces ochratoxin A (Wicklow et al. 1996). Moreover, sclerotium production was not necessarily correlated with ochratoxin A production because no *A. niger* strain has been able to produce sclerotia. Generally ochratoxin A induces a wide range of health effects including nephrotoxicity, mutagenicity, teratogenicity and immunotoxicity as reviewed by O’Brien and Dietrich (O’Brien and Dietrich 2005). Furthermore, prolonged feeding of poultry on ochratoxin A contaminated feed decreases egg production and reduces animal performance (Hassan et al. 2012).

**CONCLUSION**

Fungal contamination of poultry feed poses a considerable economical and health risk to food safety and animal husbandry. Morphological and molecular identification and characterization of mycotoxin (here OTA) producing fungi in poultry feed has been achieved. Special attention should be paid to the raw materials and commercially available poultry feed to avoid mycotoxin contamination in animal practices and markets.

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Supplemental Figure 1: Morphological-based analysis of Aspergillus niger. A) Macroscopic examination shows colonies consisted of grayish white colonies, with a yellow basal reverse on Malt Extract Agar (MEA). B) Microscopic analysis shows Aspergillus niger conidial head. Further, it has dark brown to black, radiate metulae with long phialides, conidia brown and rough walls.

Supplemental Table 1. Ochratoxin A production by isolated Aspergillus species.

| Plate No. | Sample No. | Positivity | Level (ug/L) |
|-----------|------------|------------|--------------|
| 1         | 1          | -          | -            |
|           | 2          | -          | -            |
|           | 3          | -          | -            |
|           | 4          | +          | 50-100       |
|           | 5          | +          | 50-100       |
|           | 6          | -          | -            |
|           | 7          | +          | 50-100       |
|           | 8          | -          | -            |
| 2         | 1          | -          | -            |
|           | 2          | -          | -            |
|           | 3          | -          | -            |
|           | 4          | +          | 50-100       |
|           | 5          | -          | -            |
|           | 6          | -          | -            |
|           | 7          | -          | -            |
|           | 8          | -          | -            |