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Molecular diagnosis of different isolates of *Aspergillus niger* isolated from grapes

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Abstract
This study was carried out in the Plant Virology laboratory of the Plant Protection Department at the College of Agriculture, University of Karbala, Iraq in order to isolate and diagnose ten isolates of *Aspergillus niger* isolated from stems, branches, leaves, and small grape clusters showing symptoms of black mold disease. These fungal isolates were identified using the polymerase chain reaction (PCR) technique and determining the nucleotide sequences of the amplified-PCR products using the primer pair ITS1 and ITS4. PCR amplification of DNA extracted from these *A. niger* isolates showed the possibility of amplifying DNA products with sizes ranging between 600bp and 700bp. Analysis of the nucleotide sequences of the PCR products amplified from *A. niger* isolates using the Basic Local Alignment Search Tool (BLAST) showed that all fungal isolates identified in this study belong to the fungus *A. niger*.
A phylogenetic tree constructed using nucleotide sequences of the ten *A. niger* isolates was composed of three main clades. Among the *A. niger* isolates, the isolate (2) appeared in a separated clade with similarity percentage of nucleotide sequence ranged between 87-91% with other *A. niger* isolates. It was also demonstrated that the *A. niger* isolate (3) appeared in a separated clade along with the isolates 6 and 8 with nucleotide sequence similarity reached 99 %.

Keywords: Molecular, *Aspergillus niger*, isolated

Introduction
Grape (*Vitis* spp.) is one of the most economically important plant species due to its uses in the production of wine, grape juice, and other products derived from grape (Ali *et al*., 2010; Cosme *et al*., 2018) [4-5]. According to data published by the Food and Agriculture Organization (FAO), it was found that global grape production has a monetary value reach $55 million; most grapes are utilized for wine-making (71%) and 27% are consumed fresh, as well as only a minor portion (2%) are used as fresh and dried fruits (FAOstat, 2016) [6]. Grape is grown in all continents with the temperate regions that have dry summers, adequate rain, warm, however; relatively moderate winters are natural climatic patterns (Vivier and Pretorius, 2000; Georgiev *et al*., 2014) [21, 7].

Contamination with *Aspergillus* spp. is a worldwide problem, especially in grapes used for importing and exporting across the world as fungal contamination can reduce the quality and nutritional value of the grapes as well as lead to mycotoxin contamination (Gil-Serna *et al*., 2018) [8]. Contamination with mycotoxin produced by different isolates *Aspergillus* spp. usually happens due to the high moisture level during processes of storage and in nature as well as the presence of mites and insects (Pattron, 2006; Zulkifli and Zakaria, 2017) [13, 25].

Different *Aspergillus* species, e.g. *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus*, and *Aspergillus terreus* are well-known as mycotoxin producers. Therefore, an accurate and correct identification of the species of *Aspergillus* is very important because it will give evidence to the types of mycotoxin produced. Black aspergilli are known to be difficult to classify and the taxonomy of this section is still unclear (Zulkifli and Zakaria, 2017; Tsang *et al*., 2018) [25, 20]. In previous studies, the identification of section Nigri had mainly been depending on morphological characteristics. Morphological identification is still difficult and insufficient for identifying because of some of the characteristics of the species are very similar as the characteristics in black *Aspergillus* species (Sørensen *et al*., 2011) [18].

For accurate identification, different molecular methods have been successfully used, although the taxonomy of *Aspergillus* section *Nigri* is not completely resolved, especially within the *A. niger* species (Samson *et al*., 2007; Perrone *et al*., 2007) [14, 17]. Among the molecular techniques used, the polymerase chain reaction (PCR) has been successfully applied to diagnose *Aspergillus* species using the internal transcribed spacer (ITS) that is the universal barcode of fungi and can be used for identification of *Aspergillus* spp. ITS sequences are also mainly used for phylogenetic analysis for fungal identification (Samson *et al*. 2014) [16]. Combination of morphological identification and sequencing of ITS can reliably identify *Aspergillus* isolates to the species level. Therefore, the present study was conducted to identify *Aspergillus* species, isolated from contaminated grapes, using morphological and molecular characteristics.

Materials and Methods
Plant materials and isolation of *A. niger*
Samples were collected from stems, branches, leaves, and small grape clusters at the beginning of their formation from grape trees showing symptoms of black mold disease. The samples were placed in polyethylene bags and transferred to

the laboratory of Diseases/Colege of Agriculture/ University of Kufa. Collected samples were washed with distilled water to remove any suspended soil, then cut into small pieces (0.5-1 cm) using a sterile sharp scalpel. The pieces were surface sterilized in sodium hypochlorite (1% NaOCl) for two min, rinsed in two changes of sterile distilled water and dried with filter papers to remove any excess water. The pieces were then transferred to Petri-dishes containing Potato Dextrose Agar (PDA), supplemented with Chloramphenicol antibiotic at a concentration of 200 mg/ L. Petri dishes were incubated at a temperature of 2±25˚ for four days. Colonies of grown *A. niger* were separately purified on the same medium (PDA) and initially identified on the basis of the morphological characteristics. The same *A. niger* isolates were also molecularly identified using polymerase chain reaction (PCR) technology and determining the nucleotide sequences, according to the method described below.

Genomic DNA extraction

PCR amplification and DAN sequencing of rDNA-ITS region

The partial ITS region of each DNA extracted from each *A. niger* isolate was PCR-amplified using the universal primer pair: ITS1 (TCCGTTGGTGAACCAGCGG) and ITS4 (TCCTCCGC TTATGATATGC) (White *et al.*, 1990) [22]. PCR amplification was done using *Taq* DNA polymerase (Roche, Cat. No. 11 146 173 001) in a final volume of 20 µl PCR reaction mixture containing 2 µ1 10X PCR buffer, 1 µl each primer (10 pmol), 2 µl dNTPs (2 mM), 3 µl template DNA (30 ng/µ1), and 1 unit *Taq* polymerase. Each sample volume was then completed to 20 µl by adding nuclease-free water.

PCR amplification was performed using the following conditions: initial denaturation at 94°C for 1 min followed by 35 cycles each consisting of final denaturation at 94 °C for 30 sec, annealing temperature at 55℃ for 30 sec, initial extension for 1 min, and final extension at 72°C for 5 min (Zhang *et al*., 2012) [24]. PCR-amplified products were electrophoretic ally separated on a 1% agarose gel for 140 min at 80 V, 400 mA and visualized with ethidium bromide staining under UV illumination and images were captured using Vilber Lourmat, Taiwan gel documentation system.

For DNA sequencing, the PCR-amplified products were gel-purified using the Favor Prep PCR Purification Kit (Cat. No. FAGCK 001, Favorgen, Tawan) and sent along with the primer pair (ITS1 and ITS4) to the Macrogen DNA sequencing service in Korea. PCR products were directly sequenced in both directions using the respective forward and reverse primers. The obtained nucleotide sequences were then aligned and compared with the sequences belonged to the other *A. niger* isolates and previously published in NCBI database using the Basic Local Alignment Search Tool (BLAST). Multiple sequence alignment of the nucleotide sequences were carried out and phylogenetic trees were constructed by the MEGA6 software (Tamura *et al.*, 2013) [19], using the Neighbor-joining method.

Results and Discussion

PCR amplification and nucleotide sequencing of the ITS region of fungal isolates

As shown in Fig.1, ten *Aspergillus* isolates were isolated in this study from diseased stems, branches, leaves, and small clusters of grape trees and initially morphologically identified as *A. niger* based on black colony, biseriate conidial heads and small conidia, which were similar with the morphological characteristics of *A. niger* reported by Klich (2002) [12], and Samson *et al.* (2002) [15].

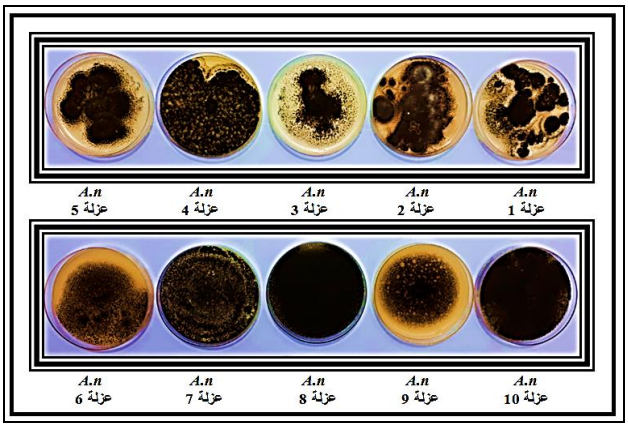

Fig 1: Colony morphology of the *A. niger* isolates as grown on PDA.

PCR amplification of DNA extracted from these isolates showed the possibility of amplifying DNA products with sizes ranging between 600 and 700bp using the universal ITS1-ITS4 primers (Fig. 2). The PCR product (ITS1, 5.8S rDNA and ITS4) amplified from each fungal isolate was sequenced with both directions and the generated nucleotide sequences were subjected to a BLAST search. Confirming the morphological identification, all obtained sequences belonged to *A. niger*.

Fig 2: DNA products amplified by polymerase chain reaction (PCR) from *A. niger* isolates (1-10) isolated from stems, branches, leaves, and small clusters of grape trees. M= 1Kbp DNA ladder marker. NC: Negative control (no template DNA added).

Results of the nucleotide sequence analysis of the identified *A. niger* isolates using MEGA7 software showed distinct differences in some nucleotide positions of their nucleotide sequences as shown in Figure 3 and Table 1.

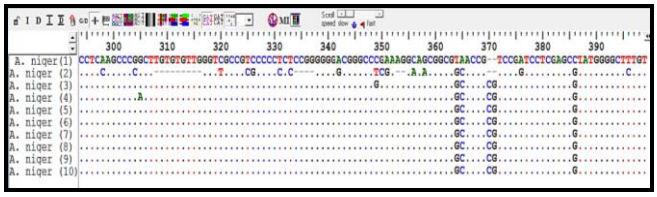

Fig 3: A graphical view of the similarity and difference in the ITS1, 5.8S rDNA and ITS4 sequences generated from the ten *A. niger* isolates obtained in this study.

Table 1: Comparison of the similarity percentages of nucleotide sequences of *A. niger* isolated in this study from stems, branches, leaves, and small clusters of grape trees.

									-	(1) A. niger
								-	88	(2) A. niger
							-	89	99	(3) A. niger
						-	99	87	100	(4) A. niger
					-	100	99	88	100	(5) A. niger
				-	100	99	99	91	100	(6) A. niger
			-	100	100	100	99	88	100	(7) A. niger
		-	100	100	100	99	99	88	100	(8) A. niger
	-	100	100	100	100	100	99	88	100	(9) A. niger
-	100	100	100	100	100	100	99	*88*	100	(10) A. niger
A. niger (10)	A. niger (9)	A. niger (8)	A. niger (7)	A. niger (6)	A. niger (5)	A. niger (4)	A. niger (3)	A. niger (2)	A. niger (1)	

A phylogenetic tree was constructed using nucleotide sequences of ten *A. niger* isolates. The obtained phylogenetic tree was composed of three main clades (Fig. 4). Among the analyzed *A. niger* isolates, the isolate 2 appeared in a separate clade with similarity percentage of nucleotide sequence ranged between 87-91% with other *A. niger* isolates. It was also found that the *A. niger* isolate 3 appeared in a separated clade along with the isolates 6 and 8 with nucleotide sequence similarity reached 99 %.

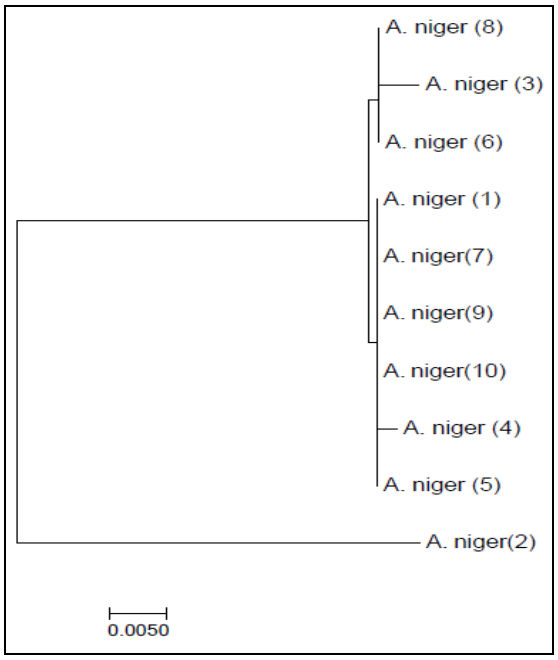

Fig 4: A phylogenetic tree generated using the neighbor-joining method based on a comparison of the ITS1, 5.8S rDNA and ITS4 sequences generated from *A. niger* isolated in this study.

Aspergillus species is generally diagnosed by colony characteristics on an agar plate e.g. CzapekDox agar, and microscopic morphology and many of *A. niger* isolates are morphologically similar; therefore their fina identification can be incorrect (Sørensen *et al*., 2011) [18]. Therefore, PCR was used in this study to diagnose different isolates of *A. niger* because of its high due to its high accuracy in the diagnosis of many organisms, including pathogenic and non-pathogenic fungi such as *F. solani*, *R. solani*, *Alternaria alternata* and *Aspergillus* spp. (Huang *et al*., 2006; AL-Abedy, 2018; Al-Fadhal *et al*., 2018; Khan *et al*., 2018) [10, 1, 2, 11] to eliminate the diagnostic problems based on morphological characters. Despite the usefulness of morphological characteristics in the identification of fungi under study and in putting the fungal isolates into smaller groups before applying the other methods of identification. From previous studies, it was found that many problems can be occurred with the morphological diagnosis of fungi such as the need a high level of expertise, especially in the fungal species exceedingly related to each other, such as *Fusarium* spp., in addition to the need for time and effort (Yang *et al*., 2007; Hsuan *et al*., 2011) [23, 9]. There are also other factors that affect the morphological characteristics, including the type and nature of growth medium, humidity, and lighting which can affect the color, forms, and sizes of spores. In previous studies, identification of some fungi such as *Fusarium verticillioides* and *Fusarium subglutinans* mainly depending on morphological characters may lead to incorrect species identification after their re-diagnosis by PCR (Alhussaini *et al*., 2016) [3].

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