PCR detection assays for the ochratoxin-producing *Aspergillus westerdijkiae* in Ras Cheese (Roomy)

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Abstract: A total of 60 samples of Roomy, Gouda and Edam cheeses samples which were consumed by different classes of consumers were collected randomly from supermarkets and groceries at Gharbia Governorate, Egypt for analyzing them for mycological examination. The lowest incidence of fungal was in Edam cheese (70%), but Ras cheese was the highest incidence of fungal (100%). The classification position of obtained fungal isolates were classified in 5 mould genera and 9 species. We conclude that the predominant isolated genera were Aspergillus spp. with prevalence of 24 (44.2%), 14 (56.0%) and 12 (52.2%) followed by Penicillium spp. 12(27.9%), 8 (32.0%) and 9 (39.1%), Cladosporium spp. 3(7.0%), 1 (4.0%) and 2 (8.7%), for Ras cheese, Gouda cheese and Edam cheese samples, respectively. While Eurotium spp. 3 (7.0%) and 2 (8.0%) detected in Ras cheese and Gouda cheese, respectively. Fusarium spp.1 (2.3%) detected only from Ras cheese. The results revealed that 4 out of 7 examined *A. ochraceus* strains were produced ochratoxin A (OTA), the minimum was 8 ppb and the maximum was 60 ppb, with a mean value of 3.0 x 10 ± 1.5 x10 ppb. In this study, two PCR specific primer pairs, AoLC35-12L and AoOTAL of the ochratoxin regulatory gene were used, four PCR products were positive on agarose gel electrophoresis. PCR products of one strain (identified as *A.ochraceus*), Sequencing of PCR product of the tested strain (*A.ochraceus*) revealed complete genome alignment, AoOTA-L and AoLc35-12L region sequences in strain of *A. westerdijkiae* isolate (Genbank accession number: MH395755) was established.
[Ashraf A. Abd El- Tawab; Eman M. El-Diasty; Fatma I. El-Hofy; Saadany A. Abd El-khalik. **PCR detection assays for the ochratoxin-producing *Aspergillus westerdijkiae* in Ras Cheese (Roomy).** *Nat Sci* 2019;17(2):77-84]. ISSN 1545-0740 (print); ISSN 2375-7167 (online). http://www.sciencepub.net/nature. 8. doi:10.7537/marsnsj170219.08.

Key word: Ras Cheese, ochratoxin A, *A. westerdijkiae*, PCR

1. Introduction

Ras cheese is a popular dairy product in Egypt. It has two common public names, Roumy or Turkey cheese. As any hard cheese, Ras cheese usually ripened for several months in relatively low temperature and high humidity rooms. Under such conditions moulds may grow on the cheese surface and may penetrate the cheese producing off flavours leading to sever economic losses. In addition, the contaminated cheese with moulds is a health hazard because some moulds are capable of producing toxic metabolites in cheese **(Dabiza and El-Deib, 2007 and Hattem *et al*., 2012).**

Mycotoxins are secondary metabolites produced by filamentous fungi that cause a toxic response when ingested by humans and animals. Fusarium, Aspergillus and Penicillium are the most abundant moulds that produce mycotoxins and contaminate human foods and animal feeds through fungal growth prior to and during harvest or during improper storage **(Binder, 2007).**

Members of Aspergillus section Circumdati (the Aspergillus ochraceus group of **Raper & Fennell 1965)** are important because of their production of several mycotoxins including ochratoxin A **(Frisvad, *et al*,2004)**.

Among them, the most important ochratoxin, due to its prevalence in foods and toxicity is ochratoxin A. It is a stable compound that is not destroyed by common food preparation procedures. Temperatures above 250 ^{0}C for several minutes are required in order to reduce the toxin concentration **(Flores-Flores *et al*., 2015).**

Frisvad *et al*., (2004) mentioned that during our mycological investigations, we have collected several new taxa of the section, including some which have formerly been identified as *A. ochraceus*. Aspergillus section Circumdati contains species with yellow to ochre conidia and non-black sclerotia that produce at least one of the following extrolites: ochratoxins, penicillic acids, xanthomegnins or melleins. The exception to this is *A. robustus*, which produces black sclerotia. Eight species consistently produce large amounts of ochratoxin A: *Aspergillus cretensis, A. flocculosus, A. pseudoelegans, A. roseoglobulosus, A. westerdijkiae, A. sulphurous, and Neopetromyces muricatus.* Two species produce large or small amounts of ochratoxin A, but less consistently: *A. ochraceus* and *A. sclerotiorum*.

The objectives of this work were to detect the ochratoxigenic Aspergillus species isolated from Ras cheese by using specific primers, molecular characterization of a polyketide synthase gene (*pks*) involved in OTA biosynthesis based on sequence information of the ITS region.

2. Material and Methods

Collection of samples:

A total of 60 samples of Roomy, Gouda and Edam cheeses were collected randomly from supermarkets and groceries at Gharbia Governorate. The samples were obtained as sold to the public and transferred as soon as possible in an icebox to the laboratory with a minimum of delay to be examined.

-Isolation of xerophilic mould according to **(Pitt and hocking, 2009).**

- Identification of isolated moulds according to **(Pitt and hocking, 2009).**

- Cultivation of ochratoxigenic Aspergillus and extraction of ochratoxin A by thin layer chromatography (TLC) according to **(Levi *et al*., 1974 and Nesheim *et al*., 1973).**

Fungal Mycelium Preparation and DNA Isolation:

Fungal mycelium was prepared from 4 isolates identified as *A. ochraceus* pure culture using 50 ml of SDA broth in 100 ml conical flasks and was incubated at 25±1°C for 7 days. Mycelia from 50 ml broth were harvested by filtration through Whatman sterile filter paper and the fungal genomic DNA was isolated **(Rohlf, 1998)**. The TLC consists of an OTA visual detection by its greenish fluorescence under long wave ultraviolet light, which changes to blue fluorescence after spraying the chromatographical plate with methanolic sodium bicarbonate solution or exposing it to ammonia fumes; scanning densitometric analysis may also be carried out.

PCR master Mix:

Dream Taq Green PCR Master Mix (2X) Fermentas Company, cat., No. K1080, USA.

-Primer selection according to (Dao *et al*., 2005).

The two primers sequences selected for *pks* gene were primers AoLc35-12L 5´ GCCAGACCATCGACACTGCATGCTC-3'; AoLc35-12R5´ CGACTGGCGTTCCAGTACCATGAGCC-3' and AoOTA-L 5´ CATCCTG CCGCAACGCTCTATCTTTC-3'; AoOTA-R5' CAATCACCCGAGGTCCAAGAGCCTCG -3' which amplified a 520 and 690 bp fragment on genomic DNA, respectively. PCR master Mix: MyFi™ Mix: Master Mix (2X) Bioline Company, cat., No. BIO-25049, England.) PCR protocol for amplification conditions of OTA producer, initial denaturation 94^0C for 4min one cycle; denaturation 94°c for 40 sec 35 cycles; annealing 58^0C for 40 sec 35 cycles; extension 72^0C for 40 sec.35 cycles and final extension 72 °C for 10 min. one cycle. PCR products were analysed for the presence of specific fragments of the expected length in a 1.5 % agarose gel electrophoresis stained with Ethidium bromide. DNA sequence polymorphisms.

The amplified fragments of one isolate were purified with Gene jet PCR purification kit, Thermoscientific company, and were sequenced by Chromogen Company, in South Korea. All the strains were sequenced in both directions. Sequences were analysed and aligned by Clustal method using the program DNA star (Laser-gene, Wisconsin, USA).

3. Results and Discussion

Mould growth on cheese is common during ripening (a complex and dynamic biochemical process that includes protein breakdown, fat hydrolysis and lactose metabolism) and during refrigeration storage and is usually accompanied by changes in the texture, smell and taste of the contaminated products due to the production of enzymes and volatile compounds. However, fungal contamination leads to unwanted cheese spoilage in the form of off-flavours, discolouration, rotting and decay of the structure **(Filtenborg, Frisvad, & Thrane, 1996).** Fungal growth on cheese is a common problem for the cheese manufacture and rendering them unfit for human consumption by retarding their nutritive value and sometimes by producing mycotoxins **(Gandomi *et al*., 2009)**.

Table (1): Statistical analytical results of incidence of mould from examined cheese samples.

Type of sample	Total number of examined	No. of +ve sample		No. of -ve Sample	
		No.	%	No.	%
Ras cheese	20	20	100	0	0
Gouda cheese	20	16	80	4	20
Edam cheese	20	14	70	6	30

Incidence of mould of Ras, Gouda and Edam cheese samples are presented in table (1) showed contaminated samples from Ras, Gouda and Edam cheeses with moulds at percentages of 100%, 80% and 70%, respectively. Nearly similar finding reported by **Lund *et al*. (1995), Taniwaki, *et al*. (2001), EL-**

bagory *et al*. (2014) and **Embaby *et al.* (2015)** recorded that contamination of semi hard cheese and hard cheese with fungal were 70.02% and 29.98% respectively.

The results shown in Table (2) declared that 5 mould genera could be isolated and identified from the examined cheese samples. We conclude that the predominant isolated genera were *Aspergillus* spp. with prevalence of 24 (44.2%), 14 (56.0%) and 12 (52.2%) followed by Penicillium spp. 12 (27.9%), 8 (32.0%) and 9 (39.1%), Cladosporium spp. 3(7.0%), 1 (4.0%) and 2 (8.7%), for Ras cheese, Gouda cheese and Edam cheese samples, respectively. While Eurotium spp. 3 (7.0%) and 2 (8.0%) detected in Ras cheese and Gouda cheese, respectively. Fusarium spp.1 (2.3%) detected only from Ras cheese. This result agree with **Abdel-All *et al*., (2008), El-Fadaly *et al*., (2015), Seddek *et al.* (2016) and Sayed-Ahmed (2016)** recorded moulds isolated from examined Ras cheese samples were *Aspergillus candidus* 4 (11.11%), *A. flavus* 1 (2.78%), *A. versicolor* 4 (11.11%), Cladosporium 4 (11.11%), Penicillium spp. 19 (52.78%) and Trichoderma spp. 4 (11.11%). While mould isolated from examined processed cheese samples were *Absidia corymbifera* 1 (3.45%), *Aspergillus candidus* 1 (3.45%), *A. flavus* 1 (3.45%), *A. fumigates* 1 (3.45%), *A. niger* 2 (6.90%), *A. ochraceus* 1 (3.45%), *A. terreus* 1 (3.45%), *A. versicolor* 3 (10.34%), chaetamium spp. 2 (6.90%), Cladosporium spp. 3 (10.34%), Mucor spp. 1 (3.45%), *Paecilomyces variotii* 1 (3.45%), Penicillium spp. 7 (24.13%), *stachybotrys chartarum* 1 (3.45%) and Trichoderma spp. 3 (10.34%).

Table (2): Frequency distribution of moulds isolated from the examined cheese samples.

Mould	Ras cheese		Gouda cheese		Edam cheese	
	No.	**%**	**No.**	**%**	**No.**	**%**
Aspergillus spp.						
A. flavus	11	25.6	7	28.0	7	30.4
A. niger	5	11.6	3	12	4	17.4
A. fumigatus	1	2.3	0	0	0	0
A.ochraceus	4	9.3	2	8.0	1	4.4
A. versicolor	3	7.0	2	8.0	0	0
Pencillium spp.	12	27.9	8	32.0	9	39.1
Cladosporium spp.	3	7.0	1	4.0	2	8.7
Eurotium spp.	3	7.0	2	8.0	0	0
Fusarium spp.	1	2.3	0	0	0	0
Total	43	100	25	100	23	100

Percentage % = according to isolated strains

Ochratoxin A (OTA) is a mycotoxin produced by secondary metabolism of many filamentous species belonging to the genera Aspergillus and Penicillium. The particularity of OTA is due to its high stability. It has been shown that it possesses a resistance to acidity and high temperatures. Thus, once foodstuffs are contaminated, it is very difficult to totally remove this molecule. Showed that the OTA is only partially degraded at a normal conditions of cooking. Moreover, this molecule can resist three hours of high pressure steam sterilization of 121 °C and even at 250 °C its destruction is not complete. The toxicological status of OTA has been have several effects such as nephrotoxic, hepatotoxic, neurotoxic, teratogenic and immunotoxic on several species of animals, and can cause kidney and liver tumour in mice and rats **(El-Khoury and Atoui, 2010).**

Table (3): Analytical result of ochratoxin A produced by Aspergillus ochraceus group isolated strains

Strains isolated from cheese	Source of isolation	No. of isolates of Aspergillus ochraceus group	Toxigenic Strain of ochratoxin A		Level of ochratoxin A (ppb) on synthetic media		
			No.	%	Min.	Max.	Mean ±SE)
A.ochraceus	Ras cheese	4	4	100	8	60	3.0 x 10 ± 1.5 x10
A.ochraceus	Gouda cheese	2	-	-	-	-	-
A.ochraceus	Edam cheese	1	-	-	-	-	-

The results revealed the presence of potentially ochratoxigenic species of the isolates studied from cheese samples, seven isolates of 4 isolates of *Aspergillus ochraceus* species were OTA producers in culture. Toxin levels ranged from 8 to 60 ppb of culture medium. *Aspergillus westerdijkiae* was the species with the highest percentage of OTA-producing isolates **(Table 3). Moslem *et al*., (2010)** nine isolates (90%) of 10 isolates of Aspergillus species (*A. niger, A. carbonarius* and *A. ochraceus*) were OTA producers in culture. Toxin levels from *A ochraceus* ranged from 2.12 to 0.15 µg/g of culture medium.

Toxin levels ranged from 0.51 to 0.74 µg/g. **Sánchez-Hervás *et al*., (2008)** reported that all the isolates of *A. carbonarius* and *A. ochraceus* species were able to produce OTA.

Molecular characterization also facilitated proper identification as *A. ochraceus* (Aspergillus section Circumdati). Eight species consistently produce of ochratoxin A: *A. ochraceus, A. westerdijkiae, A. sclerotiorum, Aspergillus cretensis, A. flocculosus, A. pseudoelegans, A. roseoglobulosus, A. sulphurous, and Neopetromyces muricatus. A. westerdijkiae* which had been previously described as *A. ochraceus* on the basis of morphological parameters.

In the last decade, development of molecular methods for distinction of ochratoxigenic and non ochratoxigenic strains of Aspergillus section Circumdati has been focused on ochratoxin biosynthesis genes.

In this study, the PCR products from *A. westerdijkiae* (isolated from Ras chees) using the primer pair AoLc35-12L/AoLc35-12R and AoOTAL/AoOTAR were cloned and sequenced, and gave an identical sequence of 520 bp and 690 bp, respectively (Genbank accession number: MH395755) **(Figure 1)**.

Of the strain of *A. westerdijkiae* studied, *pks* could be amplified from seven strains. The *pks* genes of this *A. westerdijkiae* strain is readily distinguished from those of the other species by the 17 base insertion (TATCTTTCCAATCT GGG) at nucleotides 19–35, and their distinctive nucleotide changes (CCCTC GA) at the following positions:-9-16-18-36-47-702 and 704 **(Figure 2).**

The most important ochratoxin A producing species in this regard appears to be *A. ochraceus, A. westerdijkiae* and *A. steynii*. Isolates of these species are very common and most of the isolates in these species produce large amounts of ochratoxin A. Using *pks* to distinguish Aspergillus subgenus Circumdati section Circumdati. A detailed comparison of the *pks* gene sequences demonstrated that certain base variations can be used to differentiate *A. ochraceus, A. westerdijkiae* and *A. steynii*. A differences comprise transitions at positions (30-38-44-70-71-76-157-172-173-174-204-209-210-211). Some of these variations result in amino acid changes **figure (3)**. Phylogenetic trees were generated based on the *pks* gene and the predicted ochratoxin protein sequences. Phylogenetic analysis showed that A.west/Am.1/EG016 (Acess no: MH395755) was isolated but in the same branches, from *A.westerdijkiae-CECT-2948, A.ochraceus* and *A.steynii*-IBT23096, but in different branches, away from *P.nordicum*-DAOMC-185683, *A.carbonarius*-ITEM-5010 and *A.niger*-1062 **(figure 5). Abbas (2018**) reported that five *A. ochraceus* isolates were examined using two primer specific to AoOTA-L and AoLc35-12L of the ochratoxin regulatory gene. PCR products of one strain were positive on agarose gel electrophoresis. Sequencing of PCR product of the tested *A.ochraceus* revealed complete genome alignment of AoOTA-L and AoLc35-12L region sequences in strain of *A.ochraceus* isolate (Gen Bank Accession Number: MF094441 / MF694264) was established.

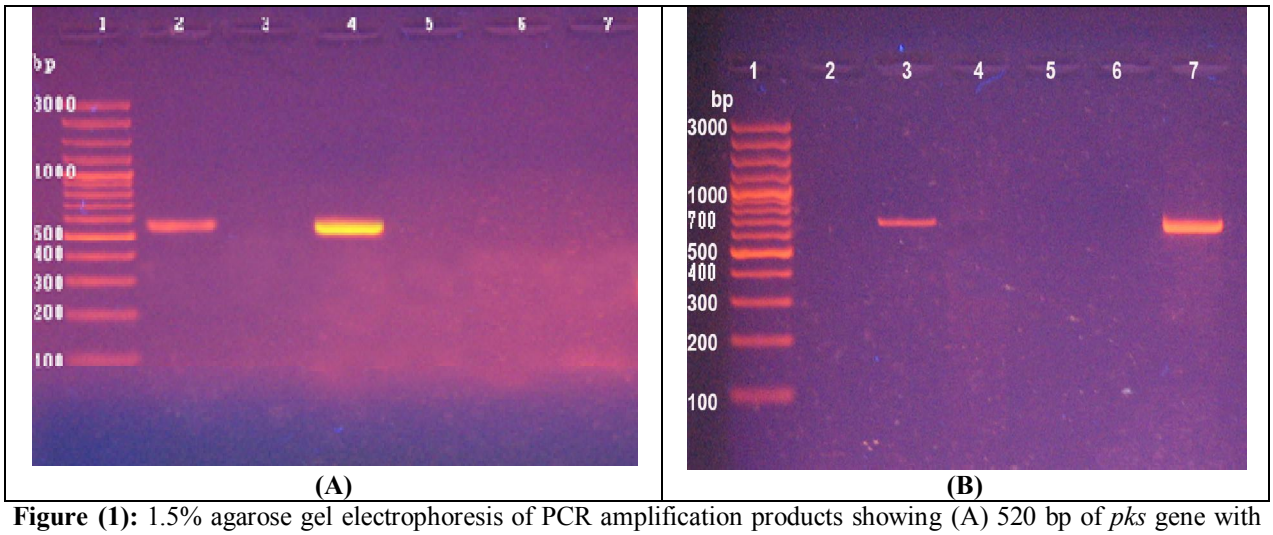

Figure (1): 1.5% agarose gel electrophoresis of PCR amplification products showing (A) 520 bp of *pks* gene with primer AoLc35 specific to the ochratoxin A regulatory gene. Lane1 100 bp DNA ladder, Lane 2 Control positive, Lane 3Control negative and Lane 4-7sample. (B) 690 bp of *pks* gene with primer AoOTA specific to the ochratoxin A regulatory gene. Lane1 100 bp DNA ladder, Lane 2 Control negative, Lane 3 control positive and lane 4-7 samples.

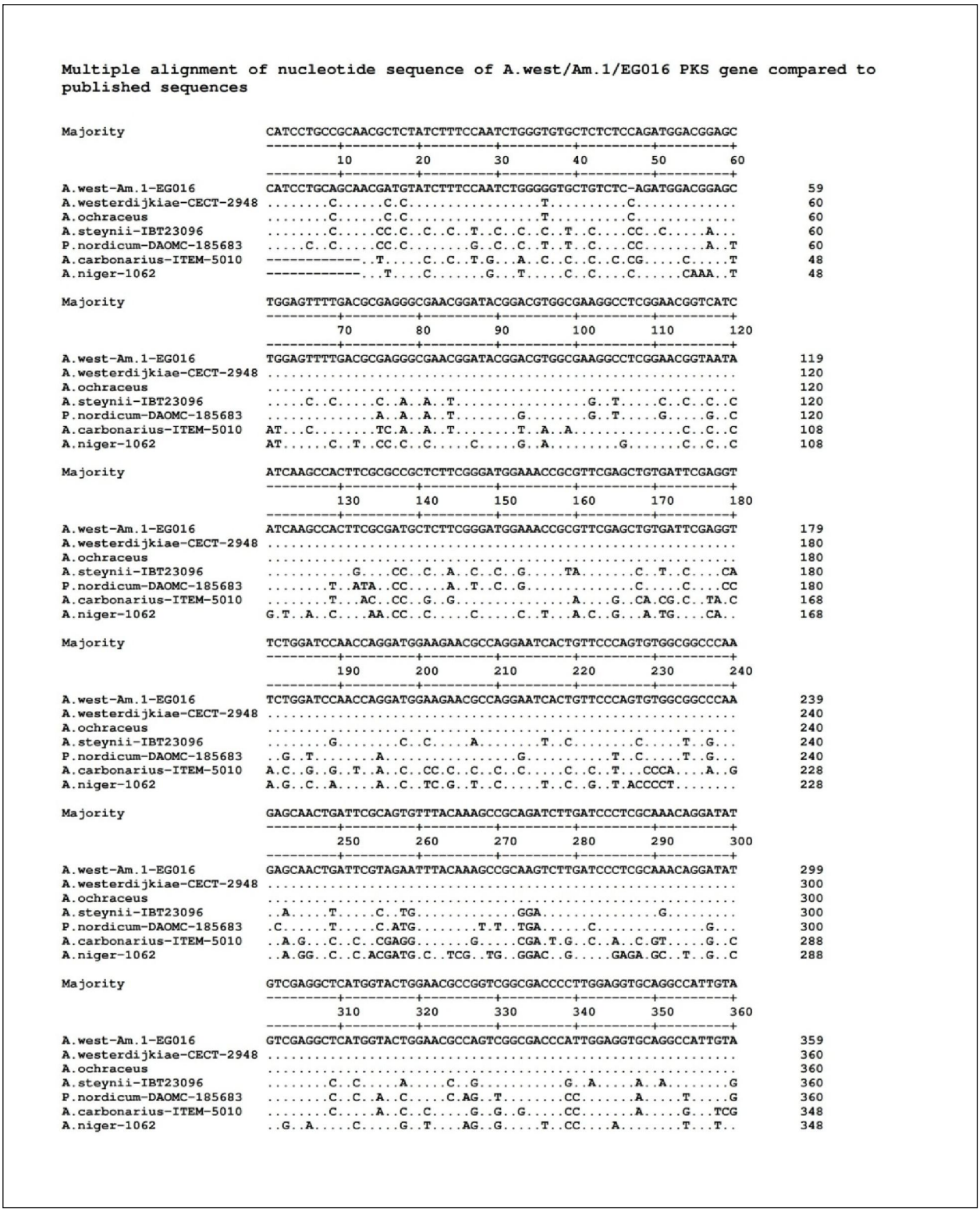

Figure (2): Multiple alignment of nucleotide sequence of A. west/Am.1/EG016 PKS gene compared to published sequence

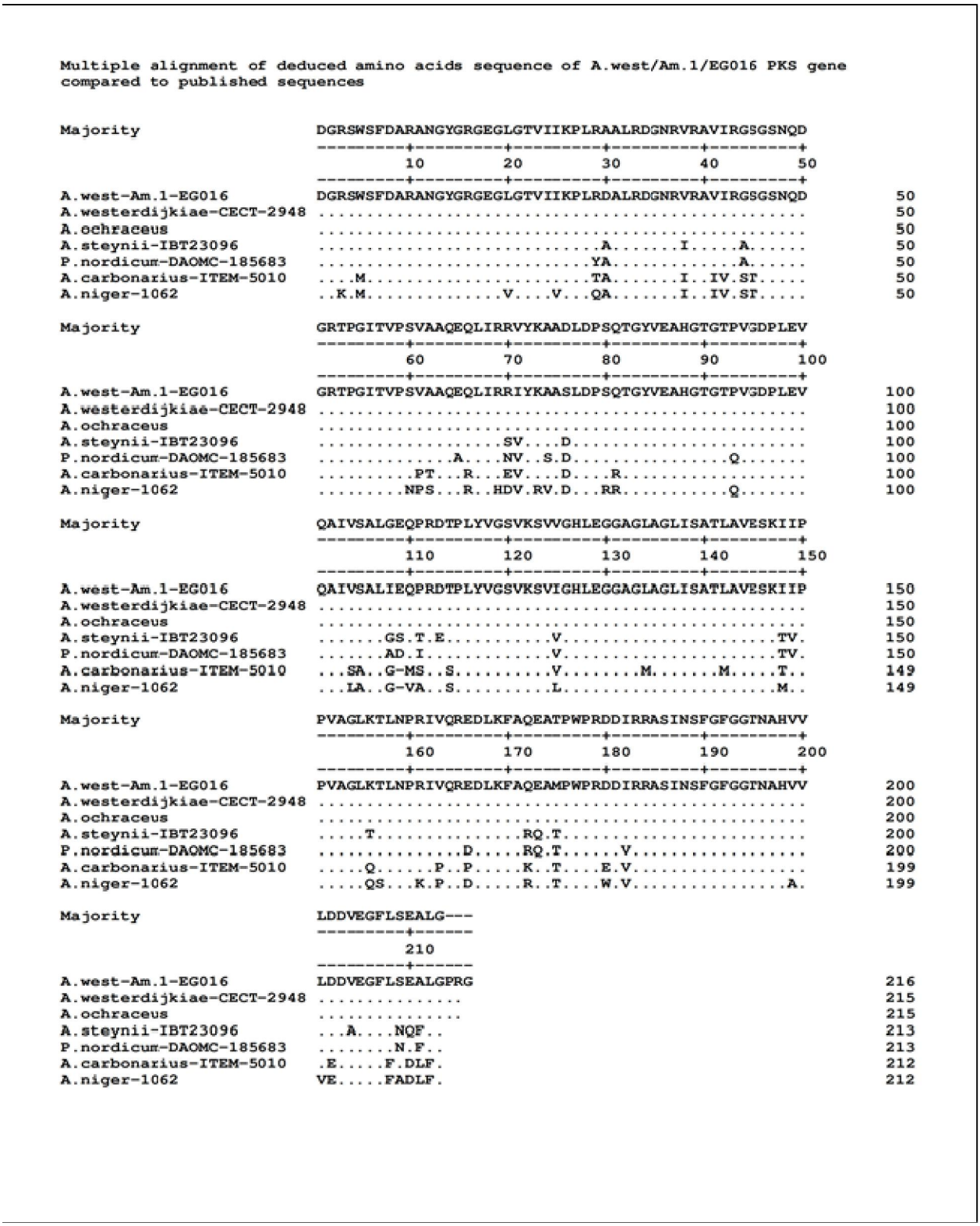

Figure (3): Multiple alignment of deduced amino acids sequence of A. west/Am.1/EG016 PKS gene compared to published sequences

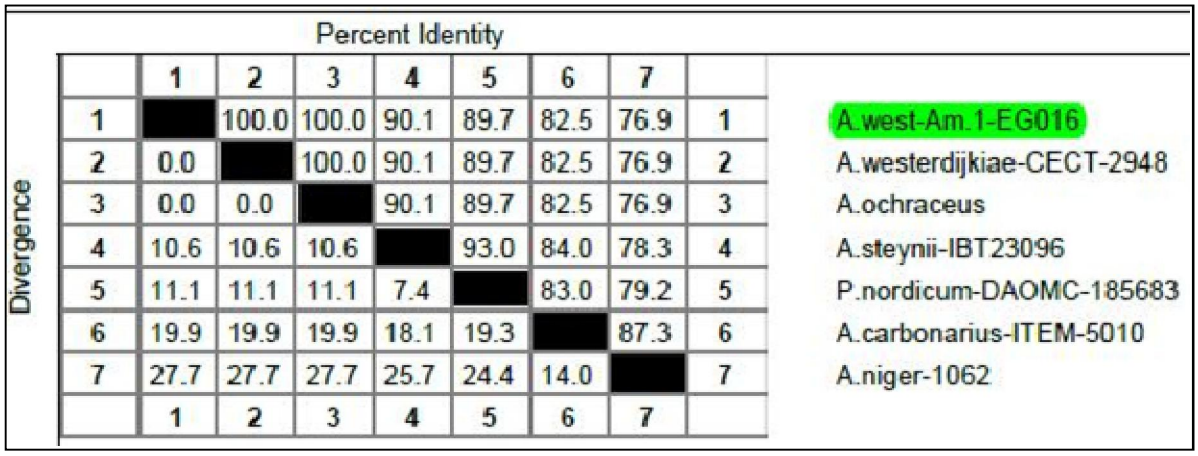

Figure (4): Amino acid identity of A. west/Am.1/EG016 pks gene sequences of compared to published sequences

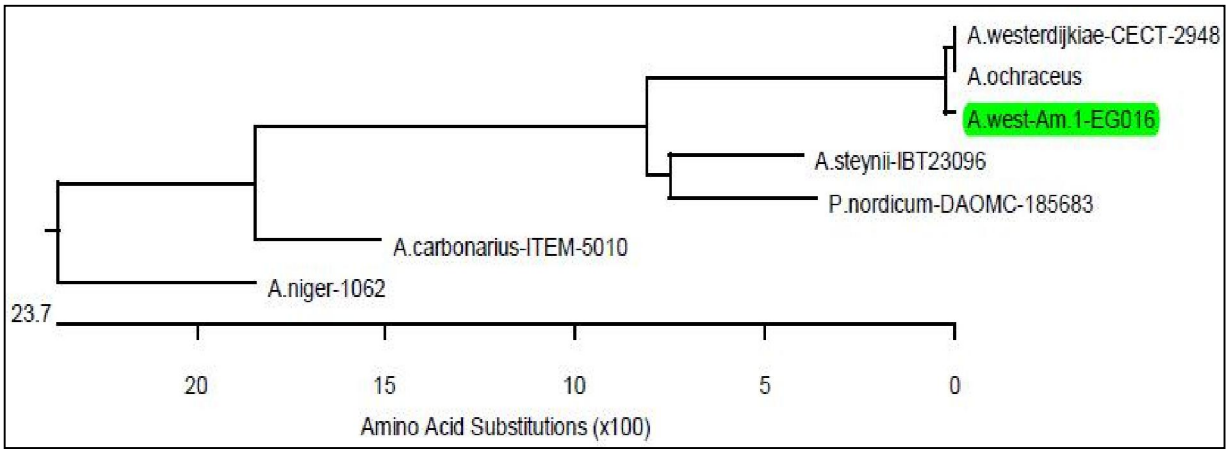

Figure (5): Phylogenetic tree of Amino acid sequences of A. west/Am.1/EG016 pks gene of compared to published sequences.

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12/16/2018