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Molecular detection of three Coleopteran species found in stored rice from some provinces in Iraq

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***Abstract*---**Insect pest infestations on stored rice cause both quantitatively and qualitatively significant damage and this affects the economy and food security of the country. The samples were collected from different geographic regions which represented by some of the provinces in Iraq; (Mosul, Baghdad, Saladin, Diyala and Dhi Qar). The current study showed three main beetles that infect stored rice: *Oryzaephilus surinamensis, Tribolium castaneum and Sitophilus oryzae.* Showed the highest abundance of *Oryzaephilus surinamensis* in all stores that investigated, while *S. oryzae* was the less abundant. The genetic relationships among the local species were studied using molecular techniques. The study was performed using a phylogenetic tree, with the diagnosis confirmed using sequencing of mtDNA COI and 16s rRNA genes. The results showed differences in the nitrogenous base sequences of both genes when comparing the local sample with the reference sequences that published in NCBI as well, there was genetic diversity of species within and between in different provinces appeared in the form ofsubstitution mutations. The tree of genetic relationships between local species was drawn and the results were compared with the standard samples. The analysis showed that *T. castaneum* and *S. oryzae* clustered in one group, while *O. surinamensis* originated in another group. Thus through DNA sequencing, the current study provides a useful reference backbone for those pest research based on some selected mtDNA genes sequences. And the information obtained can help to develop a pest

management program. The aim of the current study is to reveal the evolutionary structure of three pests that infest stored rice.

***Keywords*---**pest, stored rice, mtDNA, COI, 16s rRNA.

Introduction

Rice, *Oryza sativa* L. (Graminae), is the world's second most important crop after wheat,and Asia is the main producer and consumer Al-Salim, (2016). Aftab, *etal.* (2022). Pests of this important crop attack not only field crops during the growing season, but also grains and other food commodities kept in granaries. This will cause a significant damage both quantitatively and qualitatively on rice products. Syarifah *etal.* (2018) Cao *etal.*(2019). Experts and researchers announced that that between a quarter and a third of the global cereal crop is lost each year during storage, which causes big financial loss annually as a result of the destruction of large quantities of contaminated stored grains, which causes. When consumed health problems such as intestinal disorders and allergic diseases. (Tyagi, *etal.*, 2019) .(Banga *etal.*, 2018). The information on the variety of insect pests found in rice warehouses is extremely valuable for implementing insect pest infestation management and control in stored rice grains Syarifah *etal.* (2018). Molecular approaches are widely utilized to quickly and reliably detect insect pests, allowing for large-scale study of many samples. Liu *etal.* (2017). These important methods uses DNA barcoding and short DNA sequences from a known section of the genome as a reference sequence for species identification to detect the pests inside grain kernels after ovulation and during the early larval stages. Abels and Ludescher, (2003) ; Dasmahapatra,(2010). The phylogenetic tree was used to investigate the genetic relationships between the studied species from various geographical regions,especiallyby using mtDNA to confirm the diagnosis

Materials and Methods

Samples collection

The pest specimens were collected from five province of Iraq (Baghdad, Diyala, Dhi Qar, Mosul and Saladin) for the period from 1-12-2020 to 1-12-2021, from infected rice grains collected from silos, local markets, stores, and directly preserved in alcohol 70%.

Molecular study

DNA was extracted from specimens using Geneaid™ DNA Isolation Kit Tissue (Taiwan). The work was done according to the protocol leaflet. In order to amplify a portion of the COI gene The universal primer pairs used was LCO1490 (5′ -GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCA AAAAATCA-3′) were chosen, The program of COI gene portion amplification is : initial denaturation step, 4 min 94°C, denaturation step 30 sec 94°C, annealing step 30 sec 58°C , extension step 1 min 72 °C and Final extension 1min 72 °C .While the 16srRNA gene portion was amplified using the

primer pairs that named LR-N-13398 (5′-TTTAATCCAACATCGAGG-3′) and LR-J-12961 (5′-CGCCTGTTTAACAAAAACAT-3)′.

The molecular genetic techniques have been used to study a wide range of invertebrates. (Folmer *etal.* 1994; Olson,*etal.* 2014). The program of 16s rRNA gene is: Initial Denaturation step, 4 min 94°C, Denaturation step 30 sec 94°C, Annealing step 30 sec 55°C ,Extension step 1 min 72 °C and Final extension 1min 72 °C. The PCR amplification reaction contained 1 µL forward and reverse from each primer solution added to 5 µL PCR master mix 5 µL DNA template, and 13µl DDW. PCR products were verified by using 1.5% agarose gel electrophoresis in 1 X TBE buffer and stained with Red safe (Bioneer/ Korea) to visualize in UV light ultraviolet light for 90 min at 70 V.

Results and Discussion

PCR were successfully amplified COI and 16s rRNA genes fragments using their specific primers, and then agarose gel electrophoresis was used to indicate PCR amplification of the COI gene fragment, which yielded 700 bp, (Fig. 1), and 16srRNA gene fragment which yielded a 500 bp product (Fig. 2). At species level, these amplifications were used to diagnose the species and provide comparative data for developmental taxonomic studies and family development research

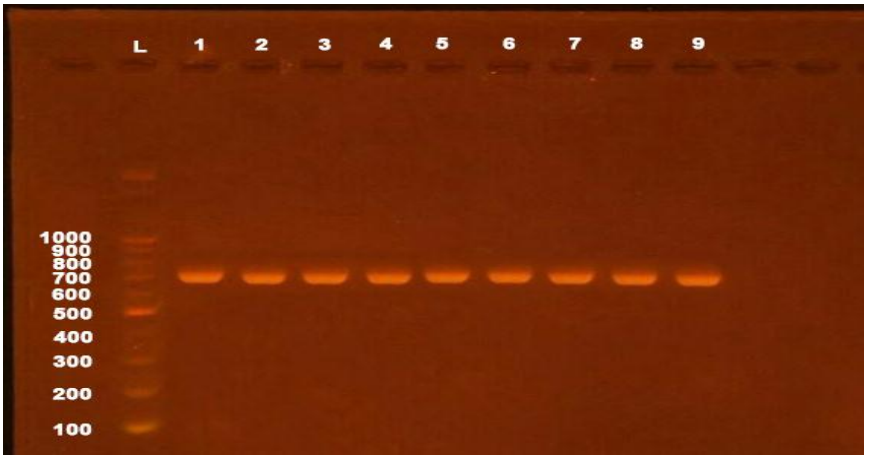

Fig (1) Gel electrophoresis for PCR product of COI gene (710 bp) samples in lane 1-9 The amplicons were run on agarose gel 1.5% in 70% Volts , 90 min and visualized with Trans illuminator, M: Marker (ladder) ranged 100-1000 bp. *T. castaneum* (1: Baghdad, 2: Diyala, 3:Dhi Qar, 4: Mosul 5: Saladin) *O.surinamensis*(6*:* Baghdad) *S. oryzae* (7: Baghdad 8: Dhi Qar 9: Diyala)

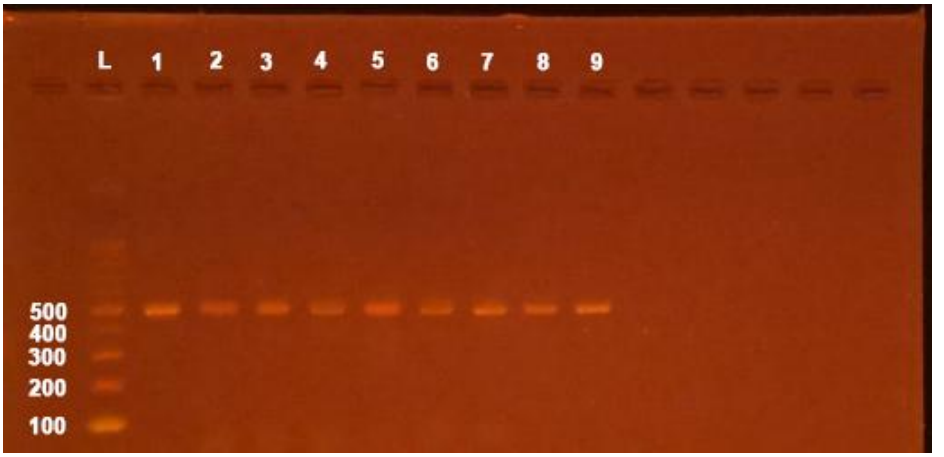

Fig (2) Gel electrophoresis for PCR product of 16s gene (500bp) samples in lane 1-9 The amplicons were run on agarose gel 1.5% in 70% Volts , 90 min and visualized with Transilluminator, M: Marker (ladder) ranged 100-1000 bp.*T. castaneum* (1: Baghdad, 2: Diyala, 3:Dhi Qar, 4: Mosul 5: Saladin) *O.surinamensis*(6*:* Baghdad) *S. oryzae* (7: Baghdad 8: Dhi Qar 9: Diyala)

DNA sequencing

One of the most important tools that has contributed to the quick diagnosis of species and aids in determining the degree of heterogeneity across species in various geographic locations is DNA sequencing (Pons, *etal.* 2006). The sequencing of DNA was studied by analysing the results of PCR products that came from foreign company (Macrogen / Korea) which sent to this company with their forward primers .The results were evaluated by using Bioedit computer program version7; 2013; https://bioedit.software.informer.com/7.2/. The process of alignment and comparison between the sequences that obtained from sequencing results were also compared with the data of the same organism and genes in the gene bank taken from the National Centre for Biotechnology (NCBI) website, which has been studied previously in different countries around the world and analysed by using Basic Local Alignment Search Tool (BLAST) in this website. The results had been showed that there was 99-100% agreement with NCBI. The local species sequences were recorded in NCBI as shown in table (1). The developmental tree were drawn for the three species studing using phylogeny program http://www.phylogeny.fr/.

COI Sequencing

The COI Gene partial fragment sequences of the three species studied that taken from different regions were compared with the reference sequences of the same gene fragment took from the NCBI. The current study showed a genetic varation in the form of substitution mutations in the sequence of nitrogenous bases among the studied samples, and there were many differences between the samples studied in different provinces as expected, and many differences found in the sequence of nitrogenous bases of the samples under study compared to the reference samples that studied in India ID: KR054738.1 for *Tribolium castaneum*

and reference samples that studied in India ID:MH910048.1 for *Sitophilus oryzae*, indicating the presence of genetic varation figs. (3and4) Except that of *Oryzaephilus surinamensis*, which showed that the sequencing results were identical compared with the reference sequences ID:MK649855, that studied in Thailand as no mutations were observed in the same fragment results of COI gene fig. (5). The presence of many mutations in the gene confirms the genetic polymorphism of these species, and these differences indicate the degree of the geographical isolation the effect of geographical distance or geographical isolation, or indicates that rice was not imported from that country.

16srRNA Sequencing

This study showed a high genetic polymorphism in the form of substitution mutations in the sequence among the studied samples. The 16srRNA Gene fragment sequences of the three species taken from different provinces were compared with the reference sequences of the same gene fragment. It has been observed that there were many substitution mutations in intra and inter species figs. (6,7,8,) by comparing the samples under study with the reference samples that studied in France ID:KJ003060.1 for *Tribolium castaneum* also reference samples that studied in USA ID: KX373615.1 for *Sitophilus oryzae* , and reference samples that studied in USA ID: KP133981 for *Oryzaephilus surinamensis*. Indicating the presence of genetic varation figs. (6,7and8).The current study provides a useful reference backbone for pest research based on the mtDNA genes sequences. These information could help in the development of a pest management programs. The results suggest a difference in distance from its peers, which could be attributed to the development of living patterns found in the store's insect pests, or the transfer of infected seeds over vast distances. The key motivation for the emergence of similar patterns within the population is the geographical component

Table 1
The Accession number in NCBI of the studied samples of COI gene and 16srRNA fragments and Collection regions of three species

Species	Collection regions	Accession number in NCBI of studied sample of COI gene	Accession number in NCBI of studied sample of 16s gene
Tribolium castaneum	Baghdad	OM977094.1	OM812088.1
	Mosul	OM010349.1	OM665391.1
	Dhi Qar	ON328324	OM812071.1
	Diyala	OM993285.1	OM654410.1
	Saladin	OM993286.1	OM674727.1
Oryzaephilus surinamensis	Baghdad	OM141127.1	OM971060.1
Sitophilus oryzae	Baghdad	OM033550.1	OM801304.1
	Dhi Qar	OM758331.1	OM811685.1
	Diyala	OM991872.1	OM674913.1

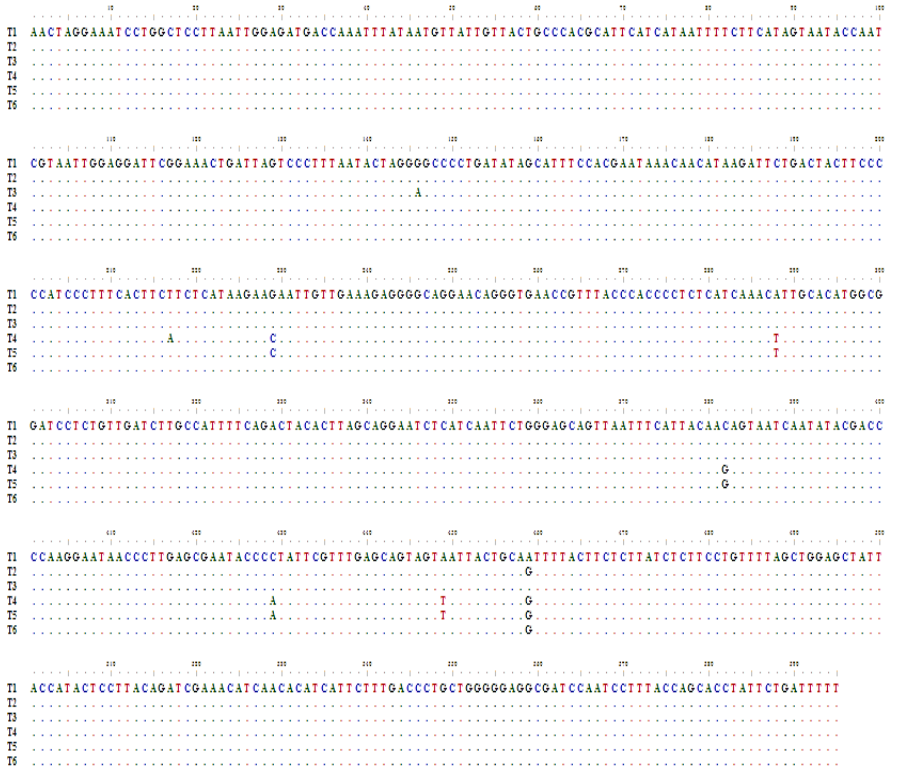

Fig 3. Comparison of the sequences alignment of five *Tribolium castaneum* samples from five provinces in a fragment of the COI gene

T1 =KR054738.1 (India), T2 =Mosul ,T3= Dhi Qar , T4 =Baghdad , T5= Diyala , T6 = Saladin

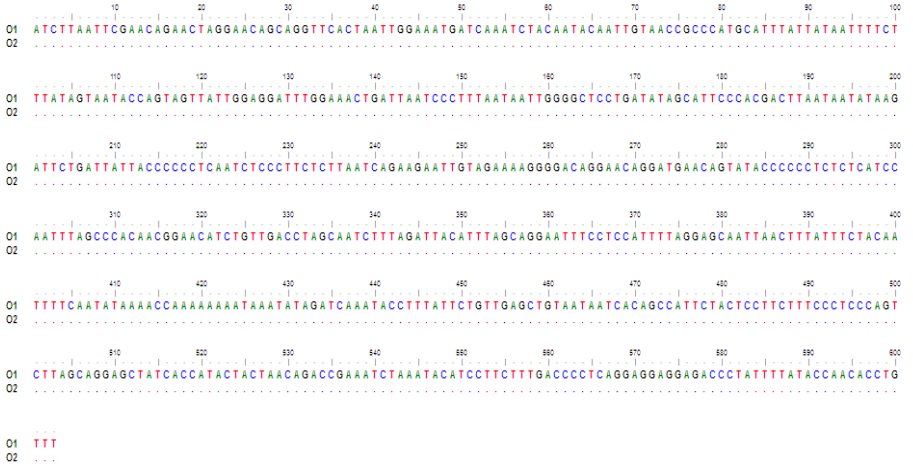

Fig 4. Comparison of the sequences alignment of *Oryzaephilus surinamensis* samples in a fragment of the COI gene

O1: MK649855.1 (Thailand) O2: Baghdad

Fig 5. Comparison of the sequences alignment of *Sitophilus oryzae* samples in a fragment of the COI gene

S1: MH910048.1 (India), S2: Baghdad S3: Dhi Qar S4: Diyala

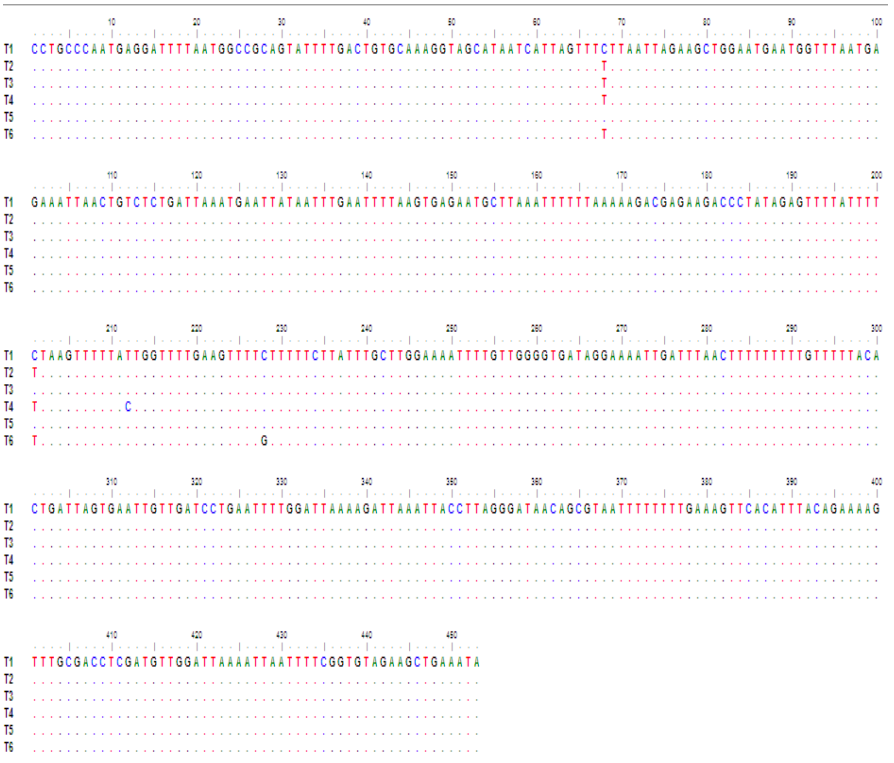

Fig 6. Comparison of the sequences alignment of five *Tribolium castaneum* samples in five provinces in a fragment of the 16s gene

T1 = EU048285.1 (USA) , T2 =Saladin , T3= Mosul, T4 Baghdad, T5= Dhi Qar, T6= Diyala

Fig7. Comparison of the sequences alignment of *Oryzaephilus surinamensis* samples in a fragment of the 16S gene

O1: KP133981(USA) O2: Baghdad

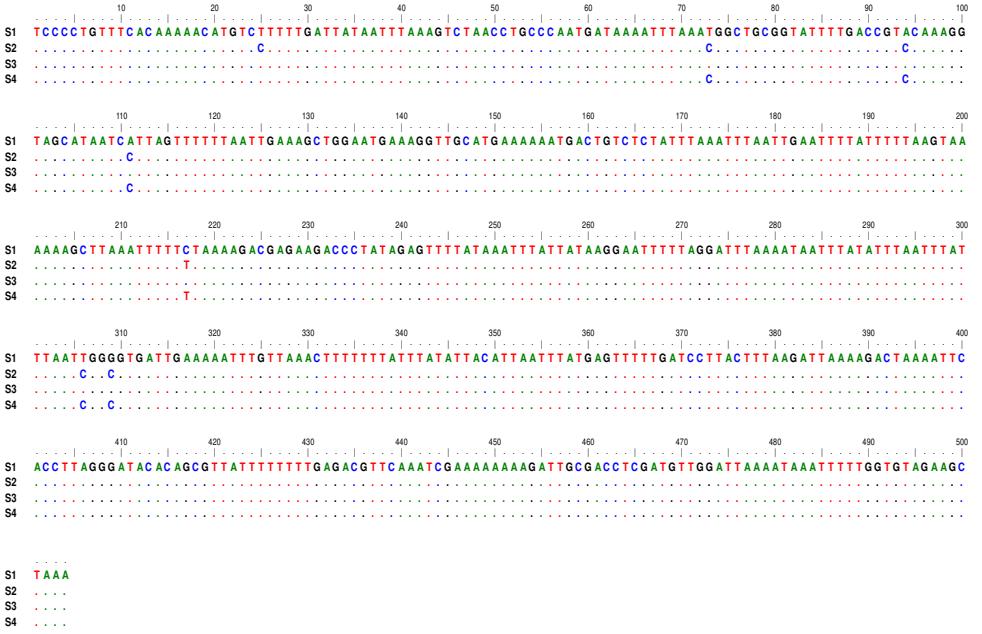

Fig 8. Comparison of the sequences alignment of *Sitophilus oryzae* samples in a fragment of the 16S gene

S1: KX373615 (USA) S2: Baghdad S3: Diyala S4: Dhi Qar

The phylogenetic tree

The phylogenetic tree of the studied samples was drawn and compared with the standard samples fromNCBI for the two genes studied, COI, 16srRNA by using the website http://www.phylogeny.fr/simple_phylogeny.cgi. The tree of genetic relationships between local species of the two genes studied were drawn and compared with the standard samples that took online from NCBI, and the results showed that both genes which studied in *T. castaneum* and *S. oryzae* were clustered in one group, while *O. surinamensis* located in another group.

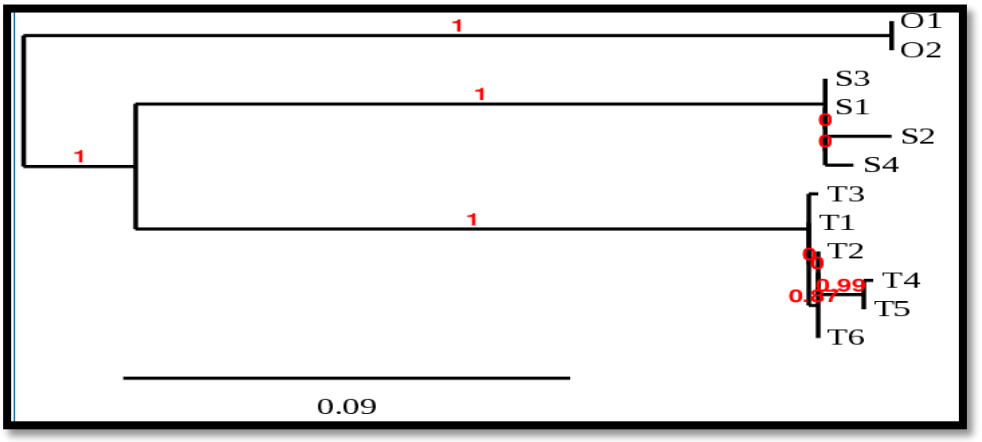

Fig 13. The Tree of genetic relationships of the three species studied using sequencing data of COI gene fragment

T1: FM877932.1 (India), T2: Mosul T3: Dhi Qar T4: Baghdad T5: Diyala T6 : Saladin S1: MH910048.1(India), S2: Baghdad S3: Dhi Qar S4: Diyala
O1: MK649855.1 (Thailand) O2: Baghdad

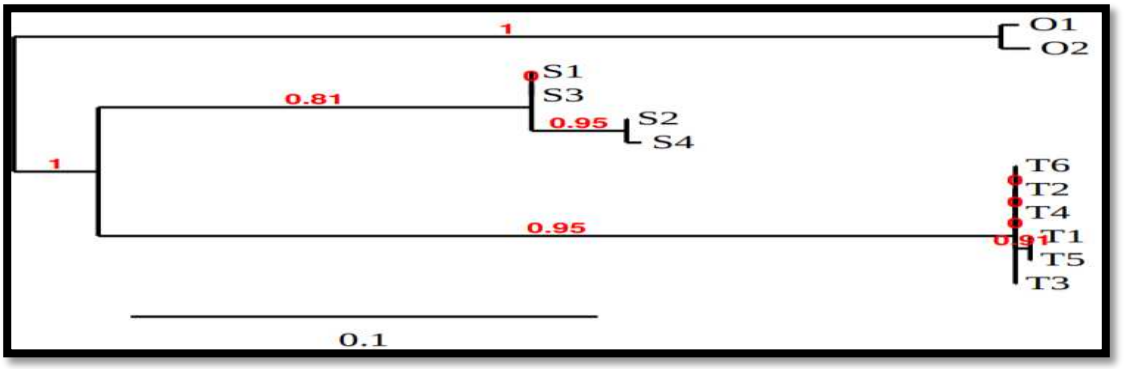

Fig 14. The Tree of genetic relationships of three species using Sequencing data of 16srRNA gene fragment

T1: KJ003060.1(France) T2: Saladin T3: Mosul T4 :Baghdad T5: Dhi Qar T6: Diyala
S1: KX373615 (USA) S2: Baghdad S3: Diyala S4: Dhi Qar
O1 : KP133981 (USA) O2:Baghdad

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