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وحماية المستهلك

MOLECULAR CONFIRMATION OF THE MITE *Varroa destructor* ISOLATED FROM HONEY BEES IN 15 IRAQI PROVINCES

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ABSTRACT

***Varroa destructor* was identified based on the cytochrome C oxidase subunit I (COI) gene. DNA was extracted from 15 Varroa mite samples collected from 15 provinces in Iraq. Polymerase chain reaction (PCR) was performed using primer set targeting COI gene. Nucleotide sequence analyses DNA amplified confirmed that all Varroa mite isolates belong to the species *V. destructor*. Neighbor-Joining tree grouped the mite isolates into different clades indicating they are variable. Sequence comparison showed Varroa isolates 1 and 10 were highly diverged from the equivalent GenBank isolates suggesting they are new, To the best of knowledge, *V. destructor* is the dominant species in Iraq.**

Key words: Honey bee parasite, Varroa isolates, Colony collapse disorder.

التأكيد الجزيئي لحلم *Varroa destructor* المعزول من نحل العسل في 15 محافظة عراقية

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الخلاصة:

تم التعرف على *Varroa destructor* على أساس جين السيتوكروم cytochrome C oxidase subunit I (COI) I. تم استخراج الحمض النووي DNA من 15 عينة من الفاروا تم جمعها من 15 محافظة في العراق. تم إجراء تفاعل البلمرة المتسلسل (PCR) باستخدام مجموعة بادئات تستهدف جين COI. أكد تحليل تسلسل النيوكليوتيدات DNA المضخم أن جميع عزلات الفاروا تنتمي إلى النوع *V. destructor* . أظهرت شجرة التحليل الوراثي تجمع عزلات الفاروا في فروع مختلفة مما يشير إلى أنها متغيرة. أظهرت مقارنة التسلسل أن عزلات الفاروا 1 و10 كانت متباعدة بشكل كبير عن عزلات البنك الجيني المكافئة مما يشير إلى أنها جديدة. وعلى حد علمنا، فإن *V. destructor* هو النوع السائد في العراق.

الكلمات المفتاحية: طفيل نحل العسل، عزلات الفاروا، اضطراب انهيار الطائفة.

INTRODUCTION

The Varroa mite is a honeybee pest, native to the *Apis cerana* species in Asia **(FAO,2015; Al-Jourani *et al*., 2004).** It attacks honeybee worldwide causing serious losses. The major injury of varroa infection is weakening honeybee colonies and resulting in a significant reduced of honey production **(Abdulhay & Yonius,2020; Al-Badri, 2017)**. The Varroa mites:*Varroa destruction* Anderson and Trueman (Arachnida: Acari: Varroidae) can causes huge economic losses to the beekeeping sector because it is prevalent and high tolerated to treatments. This mite feeds on hemolymph both in broods and adults bees making them

susceptible to other diseases, mainly viral diseases. Stronger colonies with the largest brood number are the most targeted due to the high probability of mite breeding at the brood level (**Hamed *et al.,* 2016; Mohammed & Nawar, 2020; Hadi *et al.,* 2019**). There are four types of Varroa mites: *Varroa jacobsoni*, *Varroa underwoodi, Varroa rindereri*, and *V.destruction*, the most dangerous species for honeybee colonies (**Oldroyd, 1999; Loeza-Concha *et al*., 2018; Tantillo *et al*., 2015**) . *V.destruction* has crossed from its original host *A.cerana* to *A.mellifera* in the mid-20th century, and has become the most devastating parasite on *A.mellifera* since then (**Lanzi *et al*., 2006; Shen *et al*., 2005; Yang & Cox-Foster, 2007).** The Varroa can transmit a number of viruses including the deformed wing virus DWV through feeding on the internal tissues of both honeybees and pupae, and in the process can cause an extra damage (**Yang & Cox-Foster, 2005; Awad, 2023; Martin, 2001).** Research showed that the Varroa parasite was a major factor in the phenomenon of bee collapse (CCD Colony Collapse Disorder) in the United States, Canada and Iran (**Ongus *et al*., 2004; Yang, 2004).**

The Varroa parasite can be affected by the geographical location and natural climatic conditions, especially temperature, humidity, and others. This may resulted in a genetic variation that can change some morphological, physiological, and behavioral characters of mite (**Hou *et al*., 2016).** Therefore, the aim of our study was to identify the species present in Iraq and the different isolates based on morphology and molecular approaches**.**

V. destructor are relatively large external parasites that feed on the body fluids of adult and developing honey bees. *V. destructor* cause physical damage, weaken bees and transmit a variety of pathogens, particularly viruses. In almost all cases, when varroa infestations are not effectively managed they will eventually result in the death of the entire honey bee colony. It is crucial that beekeepers manage the health of their honey bees by suppressing the population of varroa in all of their honey bee colonies throughout the beekeeping season (**Shegaw *et al*., 2022). Mahdi *et al*. (2020)** were able to diagnose 17 different isolates of the Varroa collected from different regions of southern, central and northern Iraq and some Middle Eastern countries such as Iran, Turkey, Syria, Egypt and Jordan using polymerase chain reaction (PCR) technology, determine the sequence of nitrogen bases of the cytochrome oxidase 1 gene zone in mitochondria (mtCOI) and diagnose two isolates of Varroa (*V. destructor*) (MK482687 and MK509767) are genetically different from other isolates registered at the National Center for Biotechnology Information (NCBI), **Muntaabski *et al*. (2020)** also showed, by evaluating the genetic diversity of *V. destructor* isolates in Argentina the existence of a difference between isolates is closely related to geographical latitude.

MATERIAL AND METHODS

Varroa sampling:

Varroa samples were collected from 15 provinces in Iraq (Erbil, Diyala, Maysan, Anbar, Nineveh, Baghdad, Najaf, Basra, Dhi Qar, Al-Qadisiyah, Sulaymaniyah, Babylon, Karbala, Kirkuk, Salah al-Din) (Table1and figure1). Each sample contained 15 -20 Varroa individuals and were preserved in collection tubes containing ethyl alcohol with the collection place and date.

Table (1): Varroa samples collection in this study.

Sample number	Location	Collection date
1	Erbil	12/3/2021
2	Diyala	20/4/2021
3	Maysan	15/3/2021
4	Anbar	12/5/2021
5	Nineveh	21/5/2021
6	Baghdad	18/3/2021
7	Najaf	19/3/2021
8	Basra	1/3/2021
9	Dhi Qar	3/4/2021
10	Qadisiyah	21/3/2021
11	Sulaymaniyah	26/4/2021
12	Babylon	24/3/2021
13	Karbala	20/3/2021
14	Kirkuk	19/4/2021
15	Salahaddin	12/3/2021

Morphology identification

Random samples were taken from Varroa samples collected from the provinces in Iraq (Table 1) and examined using optical and electron microscopy to identify varroa species. Optical microscopy was performed in the Graduate, under magnification (×20) using the LOMO LABOROSCOPE™ AL-2000 Binocular Microscope, BF. Field Emission Scanning Electron Microscopy (FESEM) electron microscopy was performed in the Al-Khora Laboratory for Nanoscale Research, using the Inspect F-50 electron microscope, of Dutch origin, produced by the FEI Company. The varroa sample is placed on a glass slide, then the slide is placed on a metal base containing an adhesive substance that fixes the slide on it ،The metal base is then transferred with the slide to Plasma sputter Coatings for coating by depositing gold nanoparticles on the sample, as gold provides high-quality microscopic images due to its high reflectivity under a microscope, and then transferred for examination under an electron microscope . The diagnosis (optical and electron microscopy) was based on the following characteristics (size, colour, shape of the shield, number of hairs on both sides of the shield, rhombic shape of the body inside the shield, shape of the iliac crest of the leg, length of the leg) following the classification key established by **(Zhang, 2000).**

Molecular diagnosis of Varroa parasite

Varroa samples were transferred to the Plant Virus Laboratory / College of Agriculture/ University of Karbala for molecular diagnosis. DNA was extracted from the samples using the G-spin Total DNA Extraction Kit (iNtRON Biotechnology, South Korea) supplied by Intron biotechnology/Korea and following the steps described by the manufacturer. Polymerase chain reaction (PCR) was carried out using the kit (Maxime PCR PreMix (i-Taq)) supplied by iNtRoN (South Korea).

PCR was carried out in a volume of 25 µL containing 17 µL of sterile distilled water (Nuclease-free water) and 1 µL each of forward (LCO 14905'-GGTAACAAATCATAAAGATATTGG-3') and the reverse (HCO 2198 5'-TAAACTTCAGGGTGACCAAAAAATCA- 3') primer **(Mahdi *et al*., 2020).** 1 µL of DNA template were add to 0.2 the tube containing PCR pre supplied **(Folmer *et al*., 1994).**The PCR amplification was carried out using 1 initial denaturation for five minutes at of 98 °C, followed by 35 cycles of denaturation for 40 seconds at 94 °C, primer annealing for 40 seconds at 58 °C, for one minute at 72 °C, and final extension for 10-min at 72 °C **(Zhang *et al*., 2012).**

Electrophoresis

The agarose gel layer was prepared by dissolving 1 gram of agarose powder in 100 ml of 1×TBE buffer solution (Tris-boric acid EDTA buffer). After the mixture turned into a clear solution and its temperature decreased, 3 micro A liter of Ethidium tincture and mix well.Pour the melted agarose into an Agarose gel tray that has a comb at one end for the purpose of making holes inside the gel layer. After the agarose layer had hardened at room temperature, the comb was carefully lifted, the mold was placed in place in the electrophoresis tank, and the 1×TBE buffer solution was added to cover the agarose gel layer by approximately 70 mm. Approximately 5 microliters of agarose gel was added to each hole. DNA multiplied by polymerase reaction (PCR products), and 5 microliters of DNA size marker (Molecular-weight size marker) was placed in the hole located on the left side of the added samples. Then I connected the electrodes of the power supply to the electric current and ran it for one hour at 150 mA After completing the transillumination process for the samples, I examined the agarose gel layer containing the duplicated DNA products under ultraviolet (UV transillumination) and took pictures of it.

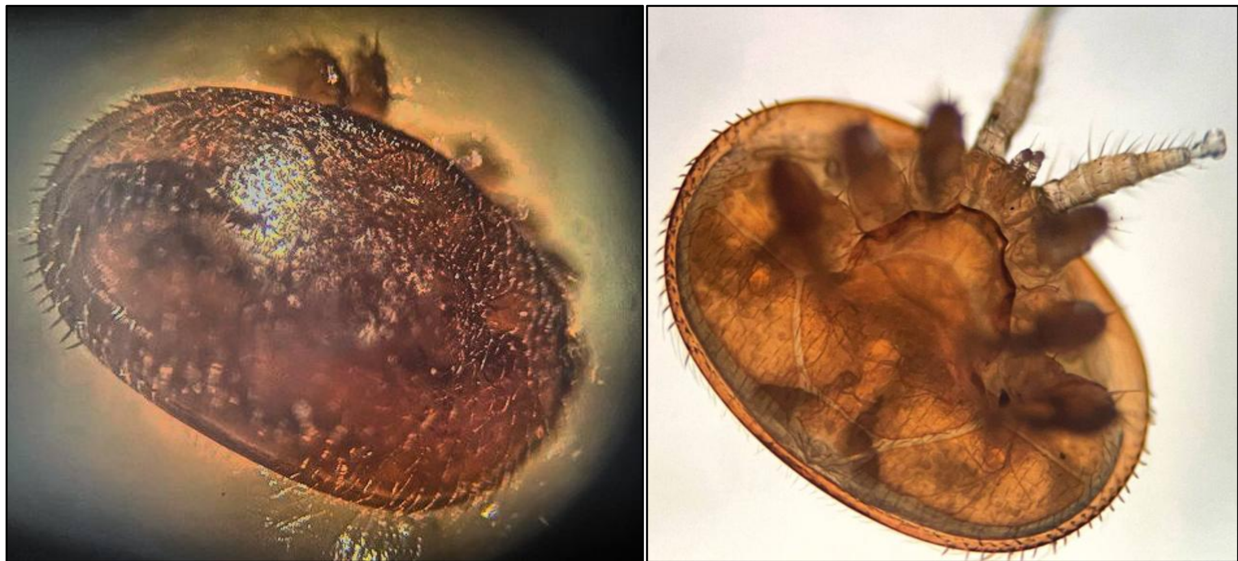
Sequence analyses.

DNA fragment amplified from Varroa isolates were sequenced in Macrogen (South Korea). Sequence analyses were performed using MEGA-X software **(Kumar *et al*.,2018).**

RESULTS AND DISCUSSION

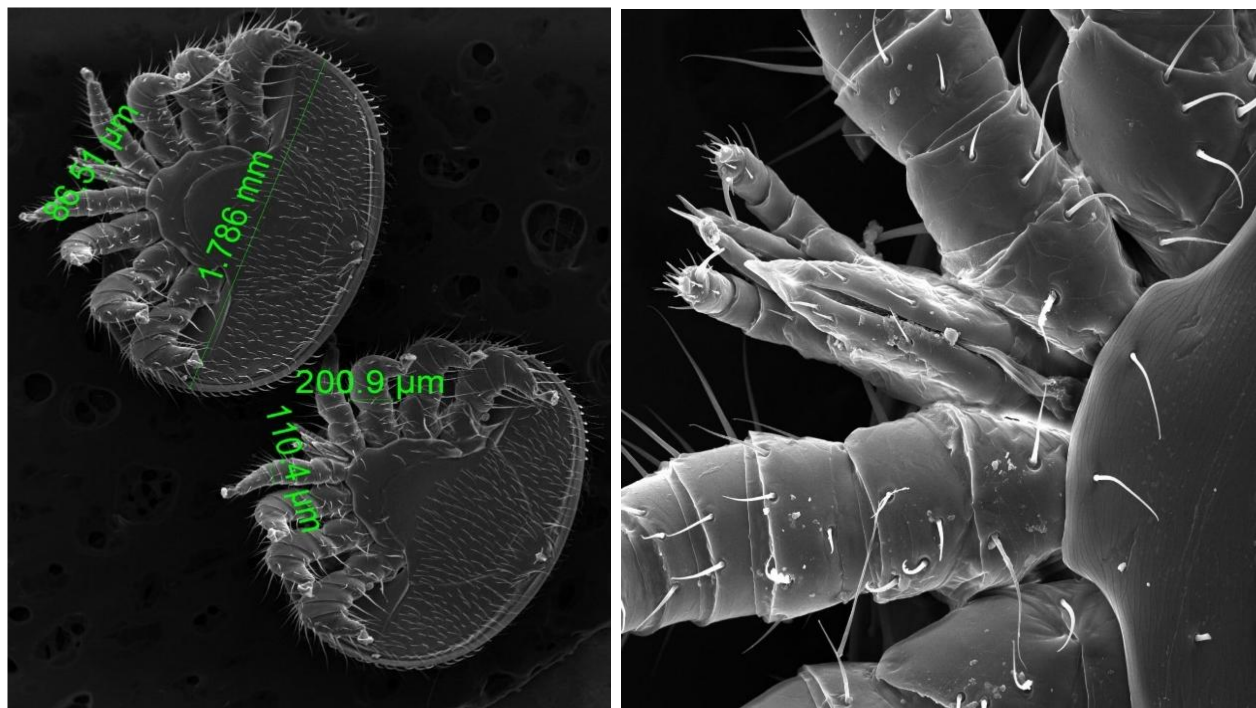
Varroa morphology identification

Microscopic examination of the Varroa samples showed that the females were 1.5 mm long and 1.7 mm wide, with flattened, oval shapes, and they appeared to be transverse rather than longitudinal, and they had eight short legs, and were brown to reddish brown in color. Their bodies, mouth parts, and legs were covered with hairs. On both sides of the body or shield, there were hard hairs ranged 19-22, resembling a sickle. The abdominal plates bore fine hairs (Fig.1and2). The males, were distinguished through their spherical body, has smaller size than the females, and with a pale-yellow color.

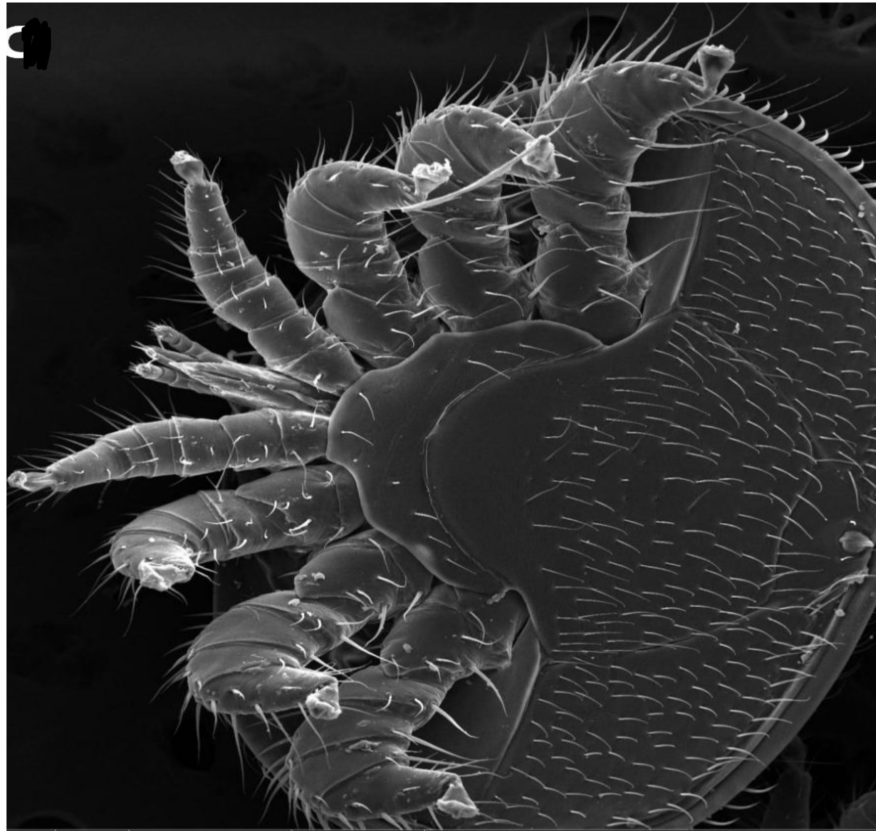


B A

Figure (1): Varroa female at 20x magnification. A: Dorsal view, B: Ventral view.



A B



C

Figure (2): Field Emission Scanning Electron Microscopy (FESEM) ahowing Varroa female A: Ventral view (70x). B: Mouth parts (500x). C: Ventral view (130x).

Molecular confirmation of the mite *Varroa destructor* isolates in Iraq

Gel electrophoresis of PCR products confirmed PCR using LCO1490 and HCO2198 primer set could amplify 720 bp. DNA fragments from all 15 samples tested Figure (3).

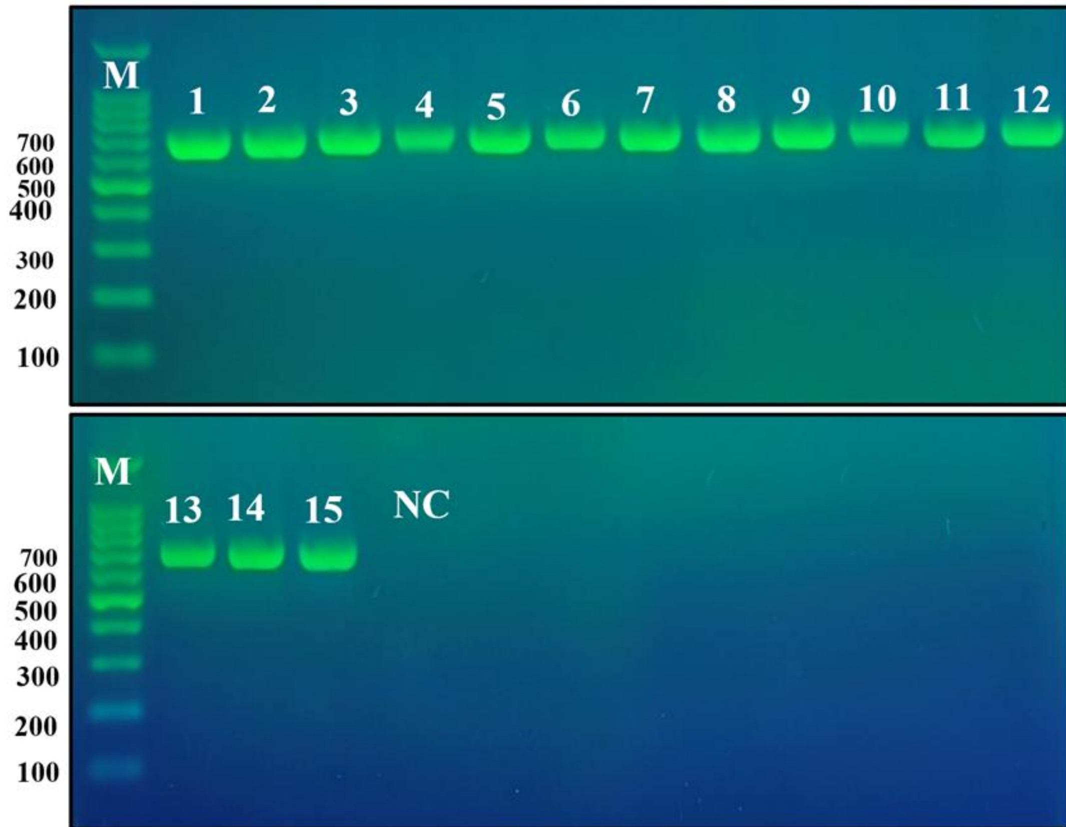


Figure (3): Gel electrophoresis patterns showing 720 bp DNA fragments amplified using polymerase chain reaction (PCR) and LCO1490 and HCO2198 primer set from Varroa samples (*V. destructor*) (lanes 1-15) isolated in this study. M = 100 bp DNA marher ,NC;negative control.

Nucleotide sequence analysis of the DNA fragments amplified from Varroa samples confirmed all 15 isolates belong to *V. destructor* when shared 100% highest nucleotide sequence identity with the equivalent sequences,retrieved from the NCBI. Nucleotide sequences from Varroa parasite isolates 7, 8, 9, 13, 14 and 15 shared the highest similarity up to 100% with those inform Canada (MN360198), South Korea (MW725321) and America (AY163547) suggesting common origin. These isolates were collected from Najaf, Basra, Dhi Qar, Karbala, Kirkuk, Salah al-Din, respectively, indicating a homologues population of *V. destructor* infecting honeybees in these locations. Whereas Varroa isolates 1, 2, 3, 4, 5, 6 and 11 shared 100% similarity when compared to each other them and 99% maximum identify with equivalent from NCBI, including those reported in Taiwan (MW599147), Argentina (MT462468) and Taiwan (MW599128), while It was most similar (98%) to that isolate from Taiwan (MW599122) and Germany (KR528383). Based on these results, isolate (1) was chosen to be representative of the other isolates (2, 3, 4, 5, 6 and 11) for the purpose of completing the comparison and analysis with isolates registered globally in the National Center for Biotechnology Information (NCBI) (Fig.4and 5).

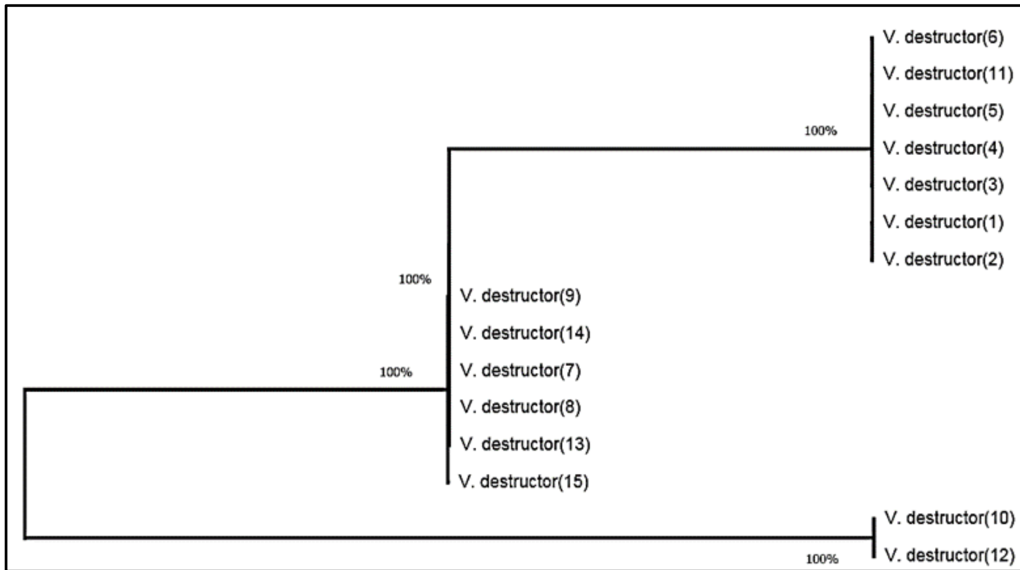


Figure (4) phylogenetic tree shows the relationship between the sequences of the nitrogenous bases of the doubled DNA products of Varroa parasite (*V. destructor*) (1-15) isolated in this study.

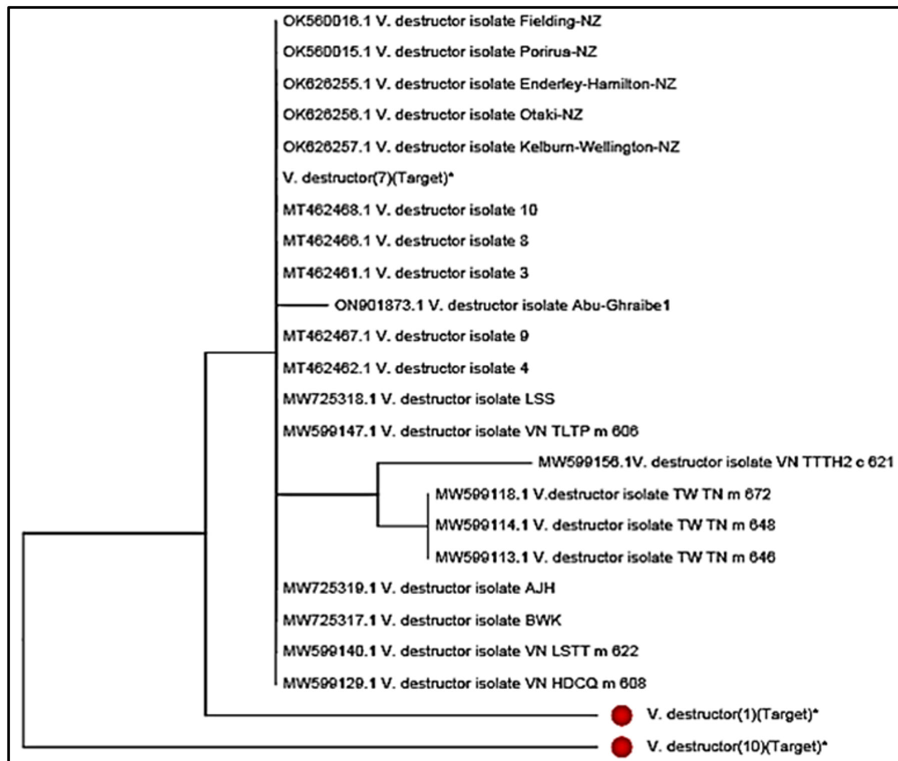


Figure (5) Neighbor-Joining tree shows the genetic relationship between Varroa parasite (V. destructor) isolates 1, 7 and 10 isolated in this study and other isolates previously registered in the National Center for Biotechnology Information (NCBI).

The results of this study show that there is genetic diversity among varroa samples collected from different regions in Iraq, and it is believed that one of the reasons for this difference indicates the entry of varroa-infected beehives or the occurrence of genetic mutations due to changing geographical locations, pressures or climatic changes, especially temperature and humidity, which contribute to morphological and behavioral changes that allow the adaptation, growth and natural reproduction of varroa **(Maggi *et al*.,2009; Dadgostar & Nozari,2018).** It is concluded from the molecular diagnosis of Varroa parasite isolates isolated in this study that isolates 1 and 10 are new isolates previously unknown in the world and as shown in Figure (5), so they were registered in the US National Center for Biotechnology Information (NCBI) under entry numbers OP984055. 1 and P984078.1, respectively.

CONCLUSION

The molecular diagnostic and base sequence determination of the DNA products from 15 isolates of Varroa collected from different provinces of Iraq (Erbil, Diyala, Maysan, Anbar, Nineveh, Baghdad, Najaf, Basra, Dhi Qar, Qadissiya, Sulaymaniyah, Babil, Karbala, Kirkuk, and Salahaddin) showed that isolates 1 and 10 (Erbil and Qadissiya) were not previously identified in the world, so they were registered in the National Center for Biotechnology Information (NCBI) under deposit numbers OP984055.1 and OP984078.1, respectively

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