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Molecular Detection of *Vairimorpha ceranae* and Determining the Incidence Rate of Honey Bee Hives and Workers of *Apis mellifera* L.

M S Manjy* and K W Shaher

Department of Plant Protection, College of Agricultural Engineering Sciences, University of Baghdad, Baghdad, Iraq

Corresponding author's e-mail: kamila.shaher@coagri.uobaghdad.edu.iq.

Abstract *Vairimorpha (Nosema* formerly *) ceranae* is one of the most serious diseases affecting adult honey bees worldwide, and is referred to as the silent killer because it has no distinct symptoms. Therefore, a multiplex PCR (polymerase chain reaction) was used to identify rRNA for *Vairimorpha ceranae* in 7,200 samples of *Apis mellifera* L. honey bees collected from four province (Anbar, Babil, Al-Qadisiyah, Karbala) in Iraq over a 12-month period in 2023. Sequence analysis revealed DNA methylation of *V. ceranae* It turned out that there was no difference in the samples in the 219 bp 16s SSU of *V. ceranae* from Iraq, and 3 strains were registered in NCBI GenBank under (OR762237.1 in Karbala province), (OR762238.1 in Qadisiyah province) and (OR762239.1 In Babylon province) respectively all isolates were registered in the gene bank in the name of Baghdad province.Samples were examined using an optical microscope that were collected from the provinces of Iraq, Anbar, Babylon, Al-Qadisiyah, and Karbala, where the Anbar province was free of infection with the pathogen. The highest infection rate in honey bee colonies was found in the winter of 2023 in the Karbala governorate, reaching 21.00% in the month. December and the lowest infection rate in Babil province was 4.00% in July. The highest infection rate for honey bee workers in Karbala was 88.50% in the month of December, and the lowest infection rate for workers bees in Babylon was 19.50% in July.

1. Introduction

The global *Apis mellifera* L. honey bee is the strongest common, widespread, and economically important species in the world, providing an necessary serving to the ecosystem by being pollinators that in pollinating plants play a important role for crop production, reproduction of wild plants, and food integrity. They are also a major source of honey, beeswax, propolis, and royal jelly.[1]. Moreover, studies have shown that the abundance and diversity of insect-pollinated wild plant species decreases in areas where the number of honey bees is low [2] , and honey bees have also been found to be the most valuable pollinators in terms of The economic impact of crops in the world is that the pollination of some types of fruits, nuts, and seeds decreases by more than 90% in the non existent of honey bees [3]. About 80% of blossoming plants depend on animal pollination, so the loss of pollinators poses a major risk of decline or extinction of various pollinator-dependent species. Moreover, the impairment of has direct economic conclusion ecosystem services and puts sustainable food production at risk, and the Although honey bee hives have high annual losses
intense global efforts by researchers to uncover the causes. In the last few years, the loss of honey bee colonies has increased, and in some countries and regions losses usually reach more than 30%. It has been revealed that it reaches more than 50% [4] . Different pests and pathogens belonging to diverse groups, including fungi, bacteria , viruses, archaea, mites and insects can cause harm to honey bees, however, remain unexplained most honey bee hives losses, Due to the complexity of different abiotic factors and biotic [5]. In Europe and the United States of America, it was observed that the mortality rate the of global honey bee *A. mellifera* was abnormally high in honey bee colonies (especially in the

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winter). The reasons behind this were attributed to including of factors numbers, food shortages (flower resources), climate change, and poor beekeeping practices. And chronic exposure to pesticides and, most importantly, parasites, viruses, and pathogens, including *Nosema ceranae*, Founds two microsporidian species, *Vairimorpha* (*Nosema*) *apis* and *Vairimorpha* (*Nosema*) *ceranae*, that infect *Apis mellifera* L.of honeybee the European [6] .The pathogen *Vairimorpha ceranae* (formerly *Nosema*), which causes Nosemosis, important diseases is one of the most that affect honey bees around the world, as it is known as the silent killer because it does not show distinct symptoms that can be observed upon infection, It infects the middle intestine of honey bees, which leads to the degeneration of epithelial tissues, poor cell regeneration, lack of immune responses, altered metabolism, decreased production of honey and pollen, increased bee mortality and thus sudden colony collapse , The disease was recorded for the first time in Iraq in 2018 and caused an infection rate of 50-100% [7] . *Vairimorpha ceranae* spores have an oval shape and their spores are about 2.7-4.7 micrometers long, making them smaller than Nosema apis spores, which are about 3-6 micrometers long , *V.ceranae* affects hormonal and immune responses and behavior and affects the number of flights made by bees as well as the average flight time , It also infects the tissues of the hypopharyngeal glands (HPGs) and leads to atrophy of the gland and a decrease in its activity, which greatly affects brood rearing and colony growth [8] . The number of dead cells from this disease reached 2,915 cells in one of the Chilean provinces and represents This number represents 47.3% of the region's hives , and the infection with this disease in Venezuela reached 60% , high levels of colony deaths were recorded in the summer and spring seasons in Canada, and the infection led to the collapse of bee colonies , and the infection rate reached 60% in the city of Saskatoon in Canada , and the rate of infection of communities with the pathogen was Canada reached 75% [9]
In Iraq, there are some studies on Nosema disease, where the of bee hives infection rate with the pathogen *Vairimorpha ceranae* in the month of October in Diyala Governorate reached 96% [10] , and in Baghdad Governorate the infection rate reached 100% in the month of November [11] . In a study on the pathogen *Virimorpha ceranae* in the first two publications of 2018, the rate of infection of communities in Iran was 46.52%, in the widespread view of spread of the pathogen *Vairimorpha ceranae* recently in some Provinces of Iraq and its cause of many deaths in honey bee colonies, the study therefore aimed to conduct a survey of the spread of the pathogen in different apiaries of the governorates of Iraq, diagnose the pathogen molecularly and morphologically, and determine the rate of infection of the bee and worker colonies bee.

2. Materials and Methods

2.1 Estimating infection rates with the pathogen Vairimorpha ceranae

2.1.1 Infection rates of Apis mellifera L.honey bee hives.

Samples of honey bee workers were collected from three sites (apiaries) in each of the governorates of Iraq (Anbar, Babil, Al-Qadisiyah, Karbala) on a monthly basis and for a period of one year, starting from 1/1/2023 until 1/1/2024. The number of hives in each was The apiaries range between 20-25 hives. Worker samples were taken from at least 6 hives from each apiary at a rate of (25 + 3 workers/hive), with 150 bees/hive being collected per month. Samples were collected from the wandering bees after they returned from the hive and gathered them in front of the hive gate (hiveentrance) after it was closed, where it was collected in sterilized plastic containers with 70% ethyl alcohol to protect the samples from damage. Information was recorded for each sample (collection location, date of collection). The samples were examined in the laboratory, and the infected hives were identified on the basis of the infected workers. The contagion rate was calculated on the basis of the infected cells and the examined cells In accordance with the following equation:

Percentage of cells infected = number of infected cells / number of hives examined x 100

2.1.2 Infection rates of honey bee workers

A laboratory examination was conducted for all the workers collected (25+3 workers/hive) from each hive and from each apiary to determine the percentage of workers infected with the pathogen *V.ceranae*. Each worker was taken individually and crushed and mashed in a small ceramic mortar using a pestle after adding 1 ml of Water Distilled, and after it was completely crushed and the solution was obtained, a drop of the solution was transferred on a glass slide and a cover slide was placed on it. The examination was conducted using an Optika light microscope with a magnification of 40_X. The infected workers were recorded on the basis of the presence of spores of the pathogen *V.ceranae* upon examination and were identified. The percentage of female workers infected In proportion to the following equation:

Percentage of workers infected = number of infected workers / number of workers examined x 100.

2.2 Isolation of spores of the pathogen V.ceranae from honey bee workers

The method of Fries et al. [12] was adopted in isolating spores of the pathogen from honey bee workers collected from the hive gates, where the worker was held by the thoracic area and the worker's digestive system was withdrawn from the back area using forceps. The worker's digestive system was transferred to a small ceramic mortar and 1 ml was added of distilled water and crushed with a mortar and pestle in a circular motion for the purpose of releasing all the spores from the digestive system. Then it was filtered with two layers of gauze (thin cotton cloth). The suspended solution It has been placedto in 5 ml plastic tube. Two layers of the solution were formed, a clear layer and a sediment layer, where the liquid was removed and 1 was added. ml of distilled water to the sediment, and after mixing and stirring, of the solution I took a drop using a pipette, placed it on a glass slide, covered the slide cover quietly to prevent the formation of bubbles, and examined it with an optical microscope with a magnification of For hives.

2.3 Identification of V.ceranae spores using optica microscopy

Spores were identified based on the method of Human et al.[13] where each bee sample (the digestive system to the honey bee) was mashed with 1 ml of distilled water for the purpose of releasing the spores. Then the mixture was centrifuged, the odorant was removed, and a drop of the sediment was taken on a slide and examined with a microscope. Scanning under the 40_X objective lens.

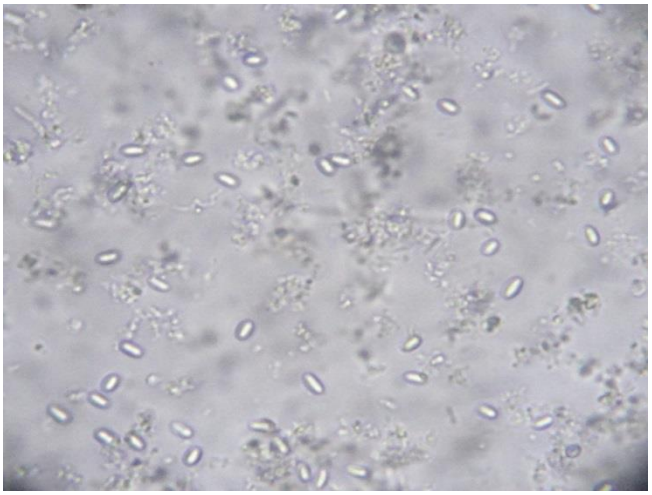

Fig 1. *Vairimorpha ceranae* spores (40X) magnification.

2.4 Molecular diagnosis of the pathogen V.ceranae

For the purpose of confirming microscopic diagnosis of results the *Vairimorpha* species diagnosed in this study, which were collected from some governorates of Iraq, polymerase chain reaction

technology was adopted and sent the samples to the National Taqadum Laboratory for the purpose of molecular diagnosis.

2.5 Counting spores of the pathogen V.ceranae

The Human et al.[41] method was used to calculate the numbers of spores by calculating the approximate number of spores for the examined samples using a glass slide used to count red blood cells called the Hemocytometer slide, and it was calculated using the equation:

$C= n/80x4x\ 10^6$ as:

C = number of spores/ml

n=sum of total spores counted in 5 squares

I used a fine pipette to withdraw 1 ml of solution suspension the spores that had been prepared, and I placed a drop in the cavity of the glass counting slide of the Hemocytometer. I covered the slide cover quietly to prevent the formation of bubbles as the spore suspension entered the counting cavity, filling it with a volume of 1 ml. I placed the slide under the optical microscope and the lens was set to 40_X So that the spores appeared clearly, 5 squares were chosen, and each square contained 16 small distributed squares in the center of the slide and the four sides surrounding the center of the square. The numbers of spores were calculated in the five squares containing 80 squares, and touched spores the bottom and right double line of the slide were excluded.

2.6 Samples preparation for PCR

Each apiary's 25 adult, dead honey bees' abdomens were macerated in 10 mL of distilled water (PCR grade), and the resulting suspension was filtered before being centrifuged at 800g for six minutes. 200 µL of newly made germination buffer (containing 0.50 M sodium chloride, 0.50 M sodium hydrogen carbonate, and or-tho-phosphoric acid to adjust the pH to 6.0) was used to promote spore germination , which was then incubated at 37 °C for 15 min 30.

2.7 DNA Extraction :Polymerase chain reaction (PCR)

Genomic DNA was isolated from plant root samples according to the protocol of ABIOpure Extraction.

According to the OIE Terrestrial Manual 2008 for *V.ceranae*, 16S rRNA was am-plified using a PCR kit (Macrogen Korea) in a Thermal cycler (Thermo Fisher Scientific, USA). *V.ceranae*. The first 50 µL reaction mixture for multiplex PCR amplification of incomplete 16S rRNA gene fragments contains 5 ng genomic DNA, 3 mM MgCl2, 200 µM of each deoxyribonucleotide triphosphate, 100 ng of primers, 5 µL of 10X PCR buffer (100 mM Tris/HCl, pH 8.3; 15 mM MgCl2; 500Mm KCL) and 1 Unit of Taq polymerase. The conditions of amplification are an initial denaturation at 95 °C for 5 minutes, followed by 35 cycles of denaturation at 95 °C for 30 sec, annealing at 56 °C for 30 extension at 72°C for 30 sec, and a final extension step of 7 minutes at 72 °C. 1.5 % agarose gel electrophoresis was used to separate the PCR products. Safe stain (Bromega, USA) was used to stain the products, and UV transillumination was used to see what was left behind. PCR products were separated by electrophoresis on a 1.00% agarose gel stained with a safe stain (Bromega, USA). It was visualized by ultraviolet radiation. In this study, primers targeting the 16S rRNA gene of the small subunit of *V.ceranae* were used 13.

2.8 Statistical Analysis

Statistical analyzes were conducted using a completely randomized block design (RCBD) [14] to determine the differences between the three governorates in the rate of infection of colonies, the rate of infection of workers, and the numbers of spores of the pathogen *V.ceranae* in the different apiaries. compared The results to using the Least Significant test. Difference (LSD) at the level of (5%) and the results were analyzed using the statistical program Genstat 12.1.

3. Results and Discussion

3.1 Gel electrophoresis

The total number of apiaries from which honey bees were collected was 12, (500) samples of bees were tested in order to discover the disease(*Vairimorpha ceranae*), the results showed that (9) honey bees apiaries were infected, also the ratio of infection was between (20% to 90%), where as noted existing of spores of the disease by microscope, DNA was tested for samples which have previously diagnosed microscopically (morphology and size of spores) that give positive results. For molecular test(PCR) specific primer for *Vairimorpha ceranae* (IDT) used and after laboratory steps required show compliance completely. The two positive *Vairimorpha ceranae* samples showed no intraspecific DNA sequence differences among the (253bp) (Fig.1).

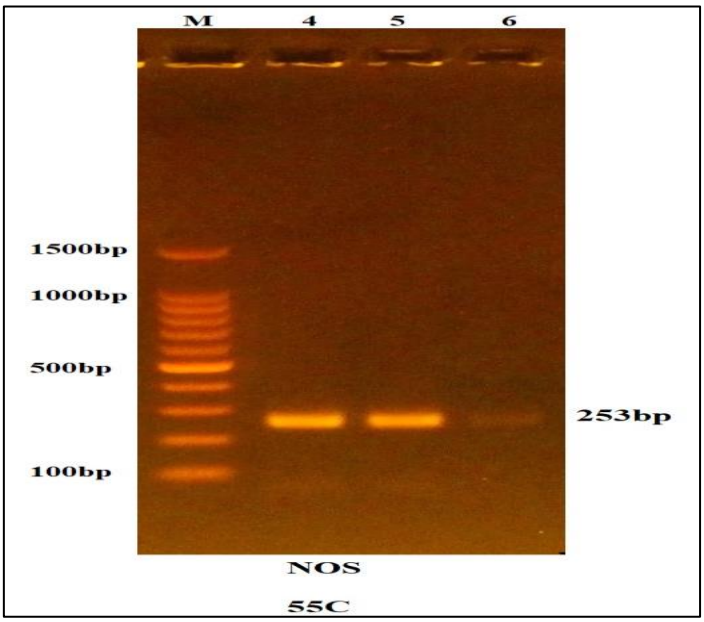

Fig 2. Gel electrophoresis result

3.2 Percentage of hives honey bee infected with the pathogen Vairimorpha ceranae in some governorates for the 2023 season

It is clear from Table (1) that Anbar Governorate was free of infection with the pathogen *Vairimorpha ceranae* and there was a variation in infection rates between different governorates and different months of the year. Revealed Statistical analysis there were significant differences between infection rates for hives of bee colonies in the different governorates of Anbar, Babylon, and Al-Qadisiyah,Karbala, which reached 00.00, 9.90, 11.58, and 13.54%, respectively. The results indicate founds significant differences between the infection rates of sects in the different months of the year, as the highest infection rate reached 13.94% in the month of December and the lowest infection rate reached 3.56% in the month of July. Likewise founds significant differences between the infection rates of sects in different governorates and different months of the year, with the highest infection rate reaching 21.00% in the month of December in Karbala Governorate and the lowest infection rate reaching 4.25% in the month of July in Babil Province.

Many studies have indicated the reason is due to honey bee colony losses is linked to infection with *Vairimorpha ceranae*, as it causes asymptomatic infections and its non-seasonal and rapid spread, which leads to a continuous high level of infection and infection throughout the year [15,16,17].

N. ceranae prefers warm conditions for growth and spore formation, as it was noted that the highest infection in beehives was at a temperature of 33°C, and *Nosema* spores are very sensitive to low temperatures and tolerate drought and high temperatures that reach 60°C [18,19]. The presence of *N.*

cerana in Iraq was recorded for the first time by Abd Alhameed and Hadi [20] specifically in three governorates (Baghdad, Diyala, and Babil), with an infection rate ranging between 50-100% ,in THE study the seasonal relationship between *Nosema* infection and bee density in Canada the rate of infection of bee colonies with the pathogen *N.ceranae* reached 77%.

Abdul Karim [21] indicated to the highest infection rate with *Nosema* in Baghdad Governorate was recorded in the month of November and amounted to 71%. that the pathogen was present in areas of Nineveh and Diyala Governorates, and the infection rate reached 8 and 65%, respectively, that the rate of infection of bee colonies in the fall reached 28% and 20% in the spring during his study on the effect of the season and the age of the workers on their infection with the pathogen *N.ceranae*. In a previous study, it indicated that the infection rates of *Nosema* in Baghdad, Diyala, Najaf, and Wasit amounted to 66, 83, 69, and 67%, respectively [22]. [23,24] reported that the average infection rate of bee colonies in the governorates of Baghdad, Babylon, Karbala, and Najaf amounted to 71, 67, 69, and 67%, respectively.

Table 1. Percentage infection of bees Colonies with the pathogen *V. caranae* in different apiaries in some provinces in during season 2023.

Month	**Provinces**				**Mean**
	Al-Anbar	**Babil**	**Al-Kadiseya**	**Karbala**	
Jan.	00.00	15.00	16.25	18.25	12.38
Feb.	00.00	13.25	14.00	16.25	10.88
Mar.	00.00	12.00	12.75	15.50	10.06
Apr.	00.00	10.00	11.00	13.50	8.63
May	00.00	8.75	10.75	11.75	7.813
Jun.	00.00	5.75	6.50	8.50	5.19
Jul.	00.00	4.00	4.50	5.50	3.56
Aug.	00.00	4.50	5.25	6.75	4.13
Sep.	00.00	7.00	11.75	13.00	7.94
Oct.	00.00	9.75	13.75	15.50	9.75
Nov.	00.00	12.00	14.25	17.00	10.81
Dec.	00.00	16.50	18.25	21.00	13.94
Mean	00.00	9.90	11.58	13.54	
L.S.D. (0.05)	**Month 3.40**	**Provinces 1.50**	**Interaction 4.60**		

3.3 Percentage of honey bee workers infected with the pathogen Vairimorpha ceranae in some governorates for the 2023 season

It is clear from Table (2) that Anbar Province was free of infection with the pathogen *Vairimorpha Ceranae* and there was a variation in infection rates between different governorates and different months in the year. Statistical analysis demonstrated the existence of significant differences between infection rates for bee colony hives in the different governorates of Anbar, Babylon, Al-Qadisiyah and Karbala which reached 00.00, 42.19, 54.54, and 64.42%, respectively. The results indicate to the existence of significant differences between the infection rates of the sects in the different months of the year, as the highest infection rate reached 55.06% in the month of December and the lowest infection rate reached 21.44% in the month of July. Founds also significant differences between the infection rates of sects in different governorates and different months of the year, with the highest infection rate reaching 88.50% in the month of December in Karbala Governorate and the lowest infection rate reaching 19.50% in the month of July in Babil Province.

The rapid spread of the *Vairimorpha* parasite in beehives in the colonies included in the study may be due to the spread of *Vairimorpha* spores through honey, pollen, bee bread, and even through the

theft of sick beehives, as well as the exchange of oral food (Trophyllaxis), which that means of transmitting and sharing *Nosema* spores between bees. Flowers fed by bees infected with *Nosema* are a source of infection and injury [25] . That weakness beekeeping management and the use of random applications contribute to increasing the damage caused by the pathogen, and the infection rate of worker bees reached 28% [26]. The decrease the density of honey bee colonies infected with the pathogen *Vairimorpha ceranae* , the infection rate was 74% ,and in the study on the seasonal relationship between *Vairimorpha* infection and bee density in Canada the rate of infected honey bee workers with the pathogen *N.ceranae* reached 73.9% in the spring and 68.0% in the summer. [27] found that the infection rate of worker bees in the fall was 24% and 33% in the spring during his study on the effect of the season and the age of the workers on their infection with the pathogen *N.ceranae*. Hadi [22] reported that the infection rate of honeybee workers with the pathogen *N.ceranae* in Diyala, Najaf al-Ashraf, and Wasit governorates reached 96, 72, and 62%, respectively.

Table 2. Percentage of worker bees infected with the pathogen *Vairimorpha ceranae* in some governorates for the 2023 season.

Month	Provinces				Mean
	Al-Anbar	Babil	Al-Kadiseya	Karbala	
Jan.	00.00	49.50	72.75	79.00	**50.31**
Feb.	00.00	47.50	71.00	75.50	**48.50**
Mar.	00.00	45.75	56.50	72.75	**43.75**
Apr.	00.00	44.00	54.75	56.00	**38.69**
May	00.00	39.50	41.50	44.50	**31.38**
Jun.	00.00	22.50	33.50	39.75	**23.94**
Jul.	00.00	19.50	30.00	36.25	**21.44**
Aug.	00.00	37.00	39.50	43.00	**29.88**
Sep.	00.00	46.25	58.5	75.75	**45.13**
Oct.	00.00	48.75	58.75	77.50	**46.25**
Nov.	00.00	51.25	60.75	84.50	**49.13**
Dec.	00.00	54.75	00.77	88.50	**55.06**
Mean	00.00	42.19	54.54	64.42	
L.S.D. (0.05)	**Month 5.42**	**Provinces 7.35**	**Interaction 20.25**		

3.4 Numbers of spores of the pathogen Vairimorpha ceranae in worker bees in some governorates for the 2023 season

It is clear from Table (3) that Anbar Governorate was free of infection with the pathogen *V. ceranae*, and there was variation in infection rates between different Provinces and different months in the year. Statistical analysis has proven that there are significant differences between the rates of numbers of spores of the pathogen *V. ceranae* in the different Provinces of Anbar,Babylon, Al-Qadisiyah, Karbala, reaching 00.00, 25.00, 28.00,36.00×10^5 spore/bee respectively, indicated the results to founds significant differences between the spore rates of the pathogen *V. ceranae* in the different months of the year, as the highest spore rate reached 73.00 x 10^5 spore/bee in the month of December in Karbala Province and the lowest spore rate reached 13.00 x 10^3 spore/bee in the month of July in the Babil Province.

In their study of different regions in Taiwan that the increase in the severity of *Vairimorpha* infection was clearly linked to temperature unlike relative humidity which had a small effect In a study conducted in Taiwan to monitor the relationship between the pathogen *V.ceranae* and its spread season, it was found that the highest rate of spore numbers reached 6.28 x 10^6 spores/bee in April, and the lowest rate reached 5.08 x 10^5 spores/bee in November [28] **.**

Likewise, poor management of beehives and the use of random applications contribute to increasing the damage caused by the pathogen, and that the numbers of *Vairimorpha* spores reached 4 x 10^4 spores/bee , that the rate of *V.ceranae* spores reached 1.37 x 10^4 spores/bee in the month of July through an experiment on the transmission of the pathogen by wind to the floor of the apiary[29]

In the study that honey bee farming is affected by many environmental factors, including temperature and humidity, as well as geographic and seasonal changes in different growing environments, In addition to parasites and pathogens attacking honey bees, that the spores of the pathogen *N.ceranae* were preserved. In the bodies of bees and beeswax to maintain their vitality, its numbers reached 11.3 x 10^6 spores/bee in the bodies of bees and 7.67 x 10^6 spores/bee, in the study on the seasonal relationship between *Nosema* infection and bee density in Canada that the average number of spores of the pathogen *V.ceranae* reached 8 x 10^5 spores/bee in the spring and in the summer 10x 10^5 spores/bee[30]. The infection rate increases when temperatures are moderate with high relative humidity and the highest rate of spore numbers reached 168 x 10^4 spores/bee in the month of March in Baghdad Province [23]. Hadi [22] reported that the highest average numbers of spores of the pathogen *V.ceranae* in Diyala, Najaf al-Ashraf, and Wasit governorates reached 868.33, 620.00, and 556.67 x 10^4 spores/bee, respectively. Al-Hasnawi [11] reported that the average numbers of *Nosema* spores in Baghdad, Babylon, Karbala, and Najaf governorates reached 447, 191.6, 141.1, and 123.7 x 10^4 spores/bee, respectively.

Table 3. Numbers of spores of the pathogen *Vairimorpha ceranae* in workers bees in some governorates for the 2023 season.

Month	Provinces				Mean
	Al-Anbar	**Babil**	**Al-Kadiseya**	**Karbala**	
Jan.	0	3555000	3600000	6320000	3368750
Feb.	0	3230000	3480000	4420000	2782500
Mar.	0	2430000	3230000	3380000	2260000
Apr.	0	1330000	1470000	1580000	1095000
May	0	64000	75000	86000	56250
Jun.	0	15000	18000	20000	13250
Jul.	0	13000	16000	18000	11750
Aug.	0	2110000	2280000	2410000	1700000
Sep.	0	3640000	4410000	5220000	3317500
Oct.	0	4230000	4690000	5680000	3650000
Nov.	0	4540000	5220000	6950000	4177500
Dec.	0	5270000	5830000	7350000	4612500
Mean	0	2535583	2859917	3619500	
L.S.D. (0.05)	**Month 1840000**	**Provinces 450000**	**Interaction 3730000**		

The isolates recorded in NCBI with number OR762237.1، OR762238.1 and OR762239.1, respectively. (Figure 3).

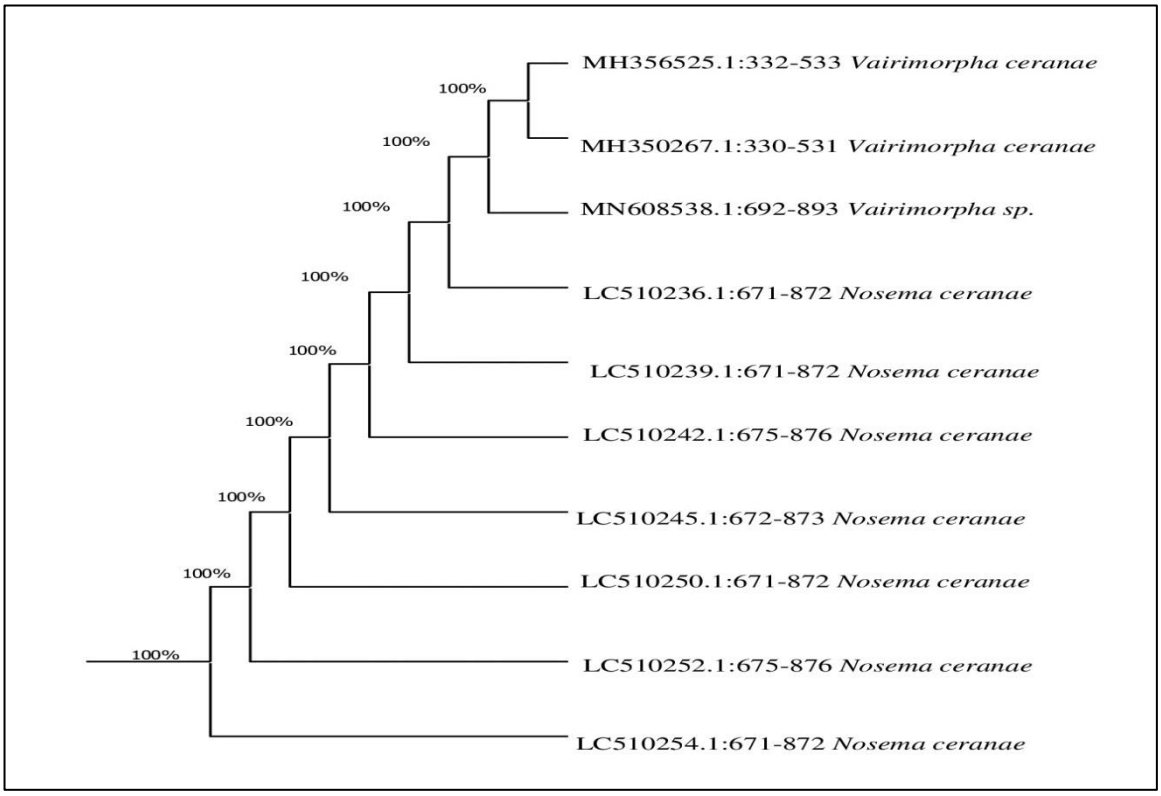

Fig 3. *Vairimorpha ceranae* genetic tree and its relation to the registrar of other isolates in NCBI.

4. Conclusion

The pathogen *V.ceranae* was recorded in three provinces (Karbala, Al-Kadiseya and Babil) with a high spread and serious damage and No cases of the pathogen have been recorded in Anbar Governorate, and it is expected to spread in most of the provinces of Iraq where bee hives are located, so it is necessary to pay attention to follow-up and control the disease to preserve the honeybee colonies from mortality.

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