EGYPTIAN ACADEMIC JOURNAL OF
BIOLOGICAL SCIENCES
ENTOMOLOGY

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ISSN
1687-8809

WWW.EAJBS.EG.NET

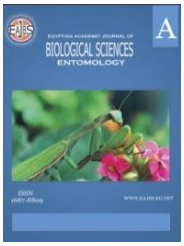
Vol. 17 No. 4 (2024)

Citation: *Egypt. Acad. J. Biolog. Sci. (A. Entomology) Vol. 17(4) pp.39-47 (2024)*
DOI: **10.21608/EAJBSA.2024.392021**

Egypt. Acad. J. Biolog. Sci., 17(4):39-47 (2024)

Egyptian Academic Journal of Biological Sciences
A. Entomology

ISSN 1687- 8809
http://eajbsa.journals.ekb.eg/



Molecular and Microscopic Detection of *Nosema ceranae* from *Apis florea* as a new Invasive Insect to The Egyptian Ecosystem

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ARTICLE INFO

Article History
Received:6/10/2024
Accepted:16/11/2024
Available:20/11/2024

Keywords:
Apis florea, *Nosema cerana*, PCR, light microscope, ecosystem.

ABSTRACT

Nosema is one of the most serious pathogens in honey bees worldwide. It is correlated that infected bees suffer both shortened lifespans and higher winter mortality. The current study aimed to determine the prevalence of *Nosema ceranae* in *Apis florea* as a new migratory insect into Egyptian ecosystem. *Apis florea* samples were collected from seven different regions in Egypt. *Apis mellifera* was used as a positive control. All samples were examined by using a light microscope and tested by polymerase chain reaction (PCR) device. In addition, all PCR products were further purified and sequenced. Moreover, we performed phylogenetic analyses with the aid of the 16S rRNA gene sequences of *Nosema ceranae* to elucidate the genetic relatedness between strains isolated from distinct honeybee species. Both microscopic examination and sequencing of PCR products determined the presence of *Nosema ceranae* in *Apis florea*. It was observed that several nucleotide' differentiations were found compared to the sequence of *Nosema ceranae* from *Apis mellifera* in Egypt. These results may indicate the presence of other species and subspecies of modern or newly introduced pathogens that may be later transmitted to honey bees (*Apis* mellifera) in Egypt. Therefore, we recommend periodic examination of bees to protect them from transmission diseases and to quickly eliminate any enemies or other pests.

INTRODUCTION

Honey bees are the most pollinator in many parts of the world. There are four main species of Apis (*Apis mellifera, Apis cerana, Apis florae* and *Apis dorsata*). The index record of insect pollinators of the genus *Apis, A.dorsata* was the highest pollinator index (19715210) followed with nearby index for *A. florae* (13888381) and *A. mellifera* (13845052), ending with *A. cerana* with the lowest pollinator index (5586381) (Bharti *et al.*, 2015)..The importance of the pollinator varies according to international laws, as it may be prohibited to import some species, such as *A. dorsata*, which are among the species prohibited from being imported into Egyptian country.

Honey bees worldwide were facing several challenges, and these issues may still persist or have evolved which we can list in the following factors: colony collapse disorder, pesticide exposure, nutrition stress, climate change, genetic factors, parasites and diseases.

Nosema, a fungal gut parasite, is another issue that can weaken bee colonies. Genetically this problem can be controlled through selective breeding for specific traits that could resist fungal infection to improve the genetic diversity of bee populations.

Various common Apis pathogens have been reported to infect both domesticated and wild honeybees. Specifically, they identified infections in *Apis mellifera*, *Apis cerana*, *Apis dorsata*, and *Apis florea.* (Lin *et al.,* 2021) In this study, we focused on *Apis mellifera* and *Apis florae*.

Apis mellifera is a beneficial insect in worldwide; it is threatened by many pests, pathogens and viral diseases **(**VanEngelsdorp *et al.,* 2009**)**, which in turn greatly negatively affects the honey bee colony population, which in turn affects the honey bee industry, which is the production of live bee packages and bee products (honey, royal jelly, propolis, pollen and venom) that interfere in the food chain effectively. Additionally, to, the dwarf bees *Apis florea* are wild, social, non-domesticated bees, unsuitable for rearing inside beehives, uneconomical for the production of honey or any other bee products and a strong competitor in food sources to the *Apis mellifera* **(**Mack *et al.,* 2000**)**. Unfortunately, Apis florae colonies migrated to Egypt it was first mentioned by (Shebl, 2017), it appeared for the first time in the Egyptian Suez Governorate and not elsewhere, meaning that it crossed the Red Sea **(**Ali *et al.*,2023**)** to reach Egyptian territory, which may indicate the possibility of human intervention, as Taiwan **(**Hsu *et al.,* 2022**).**

The most threatening pathogen is *Nosema* pathogen has two microsporidian *Nosema Apis* and *Nosema ceranae* **(**Klee *et al.,* 2007; Gisder and Genersch 2013**)**, the latter is considered the most deadly for bees and appears less uniform in shape morphologically **(**Fries *et al.,* 1996**)**. *Nosema ceranae* is an intestinal disease that causes vomiting, diarrhea, and flatulence in the abdomen which leads to colony collapse or sudden death in the honey bee colonies **(**Bailey 1955**;** Martín-Hernández *et al.,* 2007; Higes *et al.,* 2008; Higes *et al.,* 2009 and Fries *et al.,* 2013**).**

16S rRNA (ribosomal RNA) is commonly used in phylogenetic studies to analyze the evolutionary relationships among organisms, including honey bees. It's important to note that while the 16S rRNA gene is a valuable tool, researchers often use a combination of molecular markers and genomic data to gain a comprehensive understanding of phylogenetic relationships and genetic diversity in honey bee populations. Additionally, mitochondrial DNA, rather than nuclear DNA, is typically used for phylogenetic studies in eukaryotes like honey bees.

A study by **(**Chaimanee *et al.,* 2010**)** detected the fungal parasite *Nosema ceranae* in a wider range of honeybee species than previously known. This parasite wasn't just found in the European honeybee (*Apis mellifera*), but also in three Asian honeybee species: the cavity-nesting *Apis cerana*, the dwarf *Apis florea*, and the giant *Apis dorsata*. Researchers commonly utilize rRNA genes for identification, molecular characterization, and as molecular markers in phylogenetic analyses across eukaryotes. The occurrence of multiple copies of rRNA is a typical feature among microsporidia **(**Tay *et al.,* 2005; O'Mahony *et al.,* 2007**)**. In this investigation, microscopic examination and sequencing of PCR products determined the presence of *Nosema ceranae* in *Apis florea*. Furthermore, phylogenetic studies based on 16S rRNA gene sequences of *Nosema ceranae* were conducted independently to assess the genetic relationships among strains isolated from two distinct honeybee species.

MATERIALS AND METHODS

1-Samples Collection:

Seven nests of *Apis florea* bees from different areas inside Egypt were collected at the spring and summer of 2020-2021. While some of these samples are located in

residential areas and other are located in new reclamated lands. In addition, some of them are very close to the valley and the old delta (Sinai,Suez, New Qena, Qusayr, 6th of October, the 10th of Ramadan area, the Obour and the 5th District) **(**Table 1**)**. Four pools of adult worker honey bees (*Apis mellifera* L.) as a positive sample (control) of *Nosema ceranae* were collected.

Table 1. Code of the samples, addresses and location coordinates.

Code	Title	Address	Latitude	Longitude
A	Sinai	Arish, Qesm Thaleth Al Arish, North Sinai Governorate, Egypt	31.13209	33.80328
B	Suez	Suez, Suez Governorate, Egypt	29.96683	32.5498
C	Qena	New Qena, Qena Governorate, Egypt	26.21622	32.77459
D	Qusayr	Al Qusiyyah, Senbo, El Qusiya, Assiut Governorate, Egypt	27.43931	30.81713
E	6th of October	6th of October City, Giza Governorate, Egypt	29.96111	30.9296
F	10th of Ramadan	10th of Ramadan City, Al-Sharqia Governorate, Egypt	30.30321	31.7403
G	Al Obour	Al Obour City, Al Obour, Al-Qalyubia Governorate, Egypt	30.19381	31.46016
H	5th District	The 5th District, New Cairo 1, Cairo Governorate, Egypt	30.00849	31.42847

2-Microscopical Examination:

Four midguts from *Apis florea* and *Apis mellifera* the7 experimental localities were homogenized with 4 mL of distilled water (1mL/bee) in sterile mortar and 10µl was taken on a new glass slide then examined by light microscopy (MEIJI TECHNO CO., LTD., Japan) and measured with 400X according to (Cantwell 1970*;* Fries *et al.*, 1996).

3-Genomic DNA Extraction from Adult Bees:

Due to the difficulty of determining infected bees by using routine diagnosis light microscopy so, it was necessary to use PCR device to detect the presence of *Nosema ceranae* with specific primers.

4-Samples Preparation and DNA Extraction:

Four abdomens of worker bees from each sample were homogenized using a sterile mortar. Next, 500 µl of CTAB (cetyl trimethylammonium bromide) along with 2 µl of 2-mercaptoethanol (0.2%) was added. The samples were then incubated at 55-65°C for 2 hours, with occasional inversion during incubation (every 30 minutes). After incubation, the samples were centrifuged for 1 minute at 14,000 rpm, ensuring the removal of debris that had precipitated at the bottom of a 1.5 ml Eppendorf tube. The liquid phase was carefully moved into a fresh tube, then, augmented with the same volume of phenol-chloroform. The mixture was thoroughly mixed and placed on ice for 2 minutes. Subsequently, the sample was centrifuged at full speed (~14,000 rpm) for 7 minutes at 4°C, and the upper phase was transferred to another fresh tube. Cold isopropanol (500 µl) and 3 M NaOAc (50 µl) were added, and the samples were incubated at 4°C for 30 minutes after vortexing. Following this, centrifugation was performed at 10,000 rpm for 5 min at 4°C. Finally, 1 ml of 4°C, 75% ethanol was added, and the pellet was gently loosened by tapping and treated with 5 µl of Rnase, and subsequently incubated at 37°C for 10 minutes.

The pellets were collected after centrifugation (14,000 rpm, 3 min, 4°C), washed, and resuspended in 50 µl nuclease-free water following a brief drying step (10 min) to remove residual ethanol. The resuspension was incubated overnight at 4°C.

5-Oligonucleotides Synthesis:

The specific primers were designed according to **(**Chen *et al.* 2008**)**. The forward primer (GGCAGTTATGGGAAGTAACA) and reverse primer (GGTCGTCACATTTC ATCTCT).

6-Conventional PCR:

In a 20 µl reaction volume, the amplification reaction included 10 µl of Master Mix (sigma), 1 µl of primer F, 1 µl of primer R (10 pmol), 3 µl of template DNA (10 ng), and 5

µl of diH2O. PCR amplification was conducted using a Perkin-Elmer/GeneAmp® PCR System 9700 (PE Applied Biosystems) with a program consisting of 40 cycles. The initial denaturation cycle was at 94°C for 5 minutes. Each subsequent cycle involved denaturation at 94°C for 20 seconds, annealing at 60°C for 20 seconds, and elongation at 72°C for 30 seconds. The final cycle included a 7-minute primer extension step at 72°C.

7-Detection PCR Products:

The amplified PCR products were separated via electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5 µg/ml) in 1X TBE buffer at 95 volts. Subsequently, the PCR products were visualized under UV light and captured using a Gel Documentation System (BIO-RAD 2000).

8-Sequencing of Purified Product:

After amplification, the PCR products were purified using a QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany, cat no. 28704). Subsequently, the purified samples underwent sequencing reactions using a Big Dye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, cat no. 4337455), following the manufacturer's instructions. The samples were further purified through exclusion chromatography using the DyeEX 2.0 Spin Kit (QIAGEN, GmbH, Germany, cat no. 63204). Finally, the recovered materials were sequenced using a 3500XL Genetic (DNA) Analyzer (Applied Biosystems, Germany). To confirm the presence of *Nosema ceranae*, a BLAST (Basic Local Alignment Search Tool) search was conducted. Additionally, a phylogenetic tree was constructed to identify relationships among closely related sequences retrieved from the GenBank, using the Neighbor-Joining method.

RESULTS

1-Microscopical Examination:

Microscopic examination of seven samples for *A. florea* and one sample as a positive control of *A. mellifera* showed that, all samples were positive for *Nosema cerana* **(Fig. 1)**, Using 400x magnification, *Nosema* appeared in its oval shape, individually not clustered with a dark edge, which gives indications of the explosion of the epithelial cells lining the midguts, which is the infectious stage. However, the level of pathogen infection and the degree of the immune system's response to *Apis florea* vary.

Nosema cerana appears less uniform in shape. While the spore size difference between these Nosema species is evident, it might be challenging to distinguish them using standard light microscopy during routine bee disease diagnosis. Therefore, confirming these findings with a PCR test using specific primers is crucial.

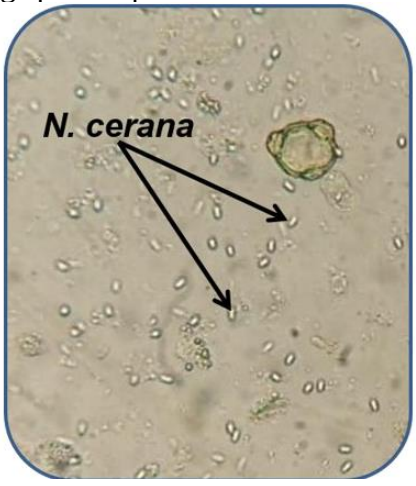

Fig. 1. Examination of *Nosema ceranae* under light microscopy.

2-Genomic DNA Extraction from Adult Bees:

Amplified fragments of *Nosema ceranae* were 220 bp **(**Fig. 2**)** from two different species of honey bees (*Apis mellifera* and *Apis florea*). The PCR products were recovered and sequenced, the homology result search from the GeneBank revealed that the sequence is similar to marker gene 16 small subunit ribosomal RNA (16sRNA) of *Nosema ceranae*.

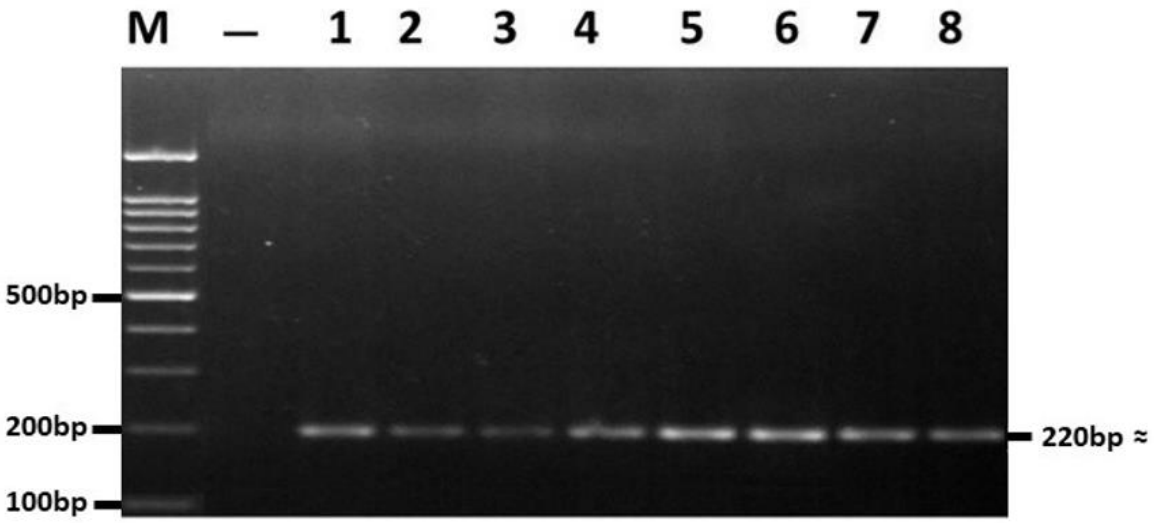

Fig. 2: Electrophoresis of PCR-amplified fragments using specific primers for *Nosema ceranae* on a 1.5% agarose gel stained with ethidium bromide. Lane M: Molecular size marker (Perkin-Elmer/GeneAmp®). Lane (-): Negative control. Lane 1: Positive control of *Nosema ceranae* from *Apis mellifera*. Lanes 2 to 8 represent *Nosema ceranae* from the abdomen of *Apis florea* collected from different areas: 2: Sinai, 3: 6th of October, 4: 10th of Ramadan, 5: 5th district, 6: Suez, 7: New Qena, and 8: Qusayr. The arrow indicates the PCR-amplified fragments (220 bp).

The phylogenetic tree analysis **(**Fig. 3**)** illustrated the relationship between *Nosema ceranae* sequences from *Apis mellifera* and *Apis florae* based on the partial sequence of 16S rRNA gene and showed two distinct clades. However, the sequence alignment between *Nosema ceranae* sequences from *Apis mellifera* and *Apis florea* showed many nucleotides differentiation and many gaps **(**Fig. 4**).**

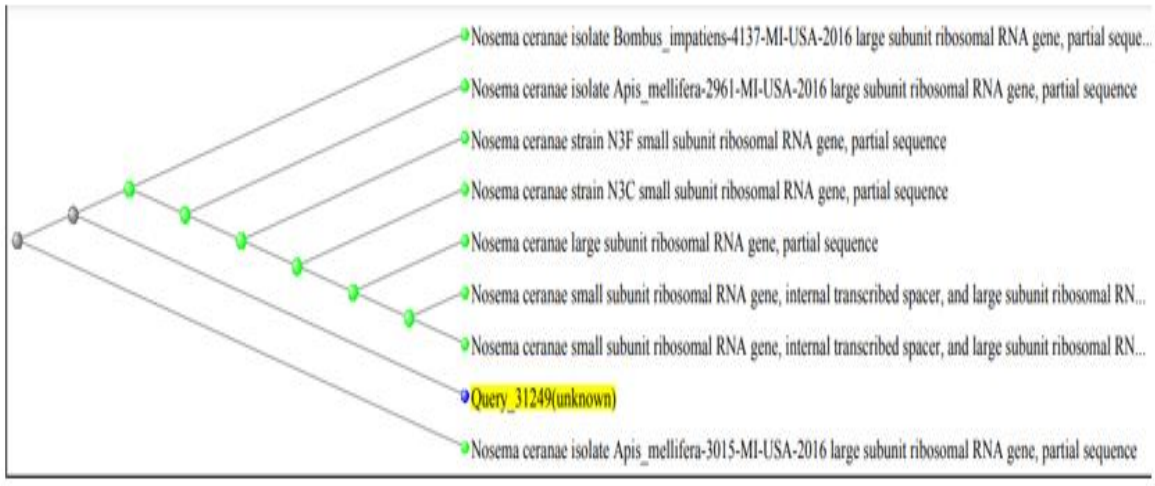

Fig. 3. Neighbor joining tree of *Nosema ceranae* sequence from *Apis florea* in Egypt.

Fig. 4. Nc: *Nosema ceranae,* nucleotides differentiations between *Nosema ceranae* from *Apis florae* and *Apis mellifera*

DISCUSSION

The previous findings indicated the co-infection of *Nosema ceranae* and other diseases in the same honey bee (*Apis spp*.) colonies **(**Phokasem *et al*., 2023**);** The alarming decline of honeybee colonies in Egypt has prompted researchers to investigate potential causes. This study identified *Nosema ceranae,* a fungal parasite, in all honeybee samples collected from both the invasive bee (*Apis florae*) and local honeybee (*Apis mellifera*) used as a positive control.

The analysis of phylogenetic relationships between *Nosema ceranae* isolated from two studied honeybee species dependent on partial 16S rRNA gene sequencing. The 16S rRNA gene exhibits conserved regions across all living organisms and serves as a commonly employed molecular genetic marker in microbial research. (Chaimanee *et al.,* 2011).

The majority of nucleotide variation occurs within individual host bees or within a single colony of *Apis mellifera*, rather than between colonies, regardless of geographical distance or host lineage **(**Blot *et al.,* 2023**)**. The results showed many nucleotides differentiations between *Nosema ceranae* in the two different studied hosts (*A. mellifera* and *A. florae*) and this might have occurred because of deletion in *Nosema ceranae* sequences infection between *Apis florea* and *Apis mellifera* **(**Pelin *et al.* , 2015**)**. In Thailand, there were variations in the nucleotide sequence between the 325th and 354th base pairs when compared to the N. *ceranae* sequence (XM_002995447) from the USA. Also, these results are consistent with **(**Chaimanee *et al.,* 2011**)** who found the isolates of *Nosema ceranae* partial 16S rRNA genes in *Apis mellifera* and *Apis florea* were varied, but on the other hand, *Nosema ceranae* isolate from *Apis mellifera* was as same as from *Apis cerana*; however, *Nosema ceranae* isolate from *Apis florae* similar to *Apis dorsata*'s isolate. Homologous recombination suggests the existence of an undisclosed cryptic sex cycle in *N. ceranae*. Notably, there are no discernible alleles associated with either Australian or European origins, or no differences between the two hosts, *A. cerana* and *A. mellifera*. This absence of biological barriers for *N. ceranae* transmission lacks a known reason or mechanism. Microsporidia diversity is substantial in both origins, and the maintenance of high heterozygosity in recently invaded European populations could potentially contribute to the heightened virulence of *N. ceranae* observed in *A. mellifera* **(**Sagastume *et al.,* 2016**)**. As a result of entrance an insect to a new Ecosystem, that needs more research. According to the pollinator index that shows proportions of both *A. florea* and *A. mellifera* converge

(Bharti *et al.,* 2015; Suwannapong *et al.*, 2018), which suggests the importance of *A. mellifera* over *A. florea* within the Egyptian ecosystem.

Conclusion

The isolation of *Nosema ceranae* from different species of honey bees (*Apis mellifera and Apis florae*) indicated the pathogen sequence may differ from one host to another and this may lead to a finding of other species and subspecies of modern or newly introduced pathogens that may later be transmitted to honey bees (*Apis mellifera*) in Egypt. Therefore, we recommend periodic examination of bees to protect them from diseases and to quickly eliminate any enemies or other pests.

Declarations:

Ethical Approval: Not applicable.

Authors Contributions: RF, AM and AA were contribution in methodology, validation, investigation, resources and data curation, RF & AA writing—original draft preparation, RF & AA writing—review and editing

Conflicts Interests: No conflict of interest was reported by the authors.

Source of Funding: This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Acknowledgements: The National Research Centre for providing the laboratory space and equipment necessary for this work, for which the authors would like to express their sincere gratitude.

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