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First record of *Nosema ceranae* detected in *Apis mellifera* at Egyptian government

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Abstract
Nosema ceranae, fungal gut parasites infect *Apis mellifera*. (NS) causes many problems to *A. mellifera* colonies and death highly infected ones. Two small-subunit rRNA regions are simultaneously amplified in a single reaction was detection of honeybee microsporidians (*Nosema apis* and *N. ceranae*). In 2019, samples from Giza region in Egypt have found in the proportions of *Nosema* spp. Significant spore-positive. During spring number of samples diagnosed as *N.* ceranae positive was found the diagnosis, while characteristic of *N.* ceranae a change in the tendency resulted increase in *Nosema*-positive samples in 2020. This is the 1STrecord of *N. ceranae* Egyptian government.

Keywords: *Apis mellifera*, 1ST record, *N. ceranae*, PCR; sequence

Introduction
Apis mellifera is commercially pollinator [1-4]. Recently finding both *N. ceranae* and *N. apis* affect *Apis mellifera* [5]. The fresh mature spores of *N. apis* are 4-6 µm in length and 2-4 µm in width, while *N. ceranae* spores are 3.3 to 5.5 µm and 2.3 to 3.0 µm, respectively [5, 6]. Spores have been distinguished under Transmission Electron Microscopy, by techniques based on PCR [7], and related: as duplex PCR [8], real-time PCR [9, 10], or LAMP [11].

Material and Method
The investigation was carried out at the apiary of Department of Apiculture research, Plant Protection Research Institute, Agriculture research center, Dokki, Egypt. during two years 2019; 2020.The bees at this site were established, locally produced hybrids of *Apis mellifera* L. Molecular analysis was conducted in the animal Health Research Institute.

A-Colony strength and its relation to fluctuations in infection and Spore detection by microscopic investigation
The studies infection levels at Dokki region, ten colonies were used at the study. Samples consisting of 20 adult worker bees were taken from hive entrance per colony per week throughout the experimentation at two year, started in 1-4-2019 until 29-4-2020. The experimental colonies (10 colonies) were nuclei and nearly were at the same strength in the beginning of experiment and headed by young mated queens. The strength of these colonies was recorded (At months intervals throughout the year) with count number of combs covered with bees.

A-The Honeybee Samples
The study was conducted with 200 adult worker bees from 10 colonies collected from 4 apiary from four different regions in Egypt (Alexandria, El fayoum, Giza, Beni- Suef) in 2019 (April and May). The colonies of bee was provided from fieldwork and the samples were determined to be distant from each other and dead bee samples were provided at specified times, which were determined with the colony owners during the study. All samples were stored at –20 °C prior to examination and were microscopically examined for presence of *Nosema* spp. Spores [12].

A-1- Microscopic Investigation
The bees were dissected and examined under a light microscope. Four apiary samples from 10 colonies were examined. Twenty adult *A. mellifera* abdomens were macerated in 3 mL of distilled water. One drops were put on a slide and examined microscopically at 40× magnification by light microscope.

B-Molecular Detection of *Nosema ceranae*
B-1- Polymerase Chain Reaction
DNA Extraction
To detection of spores of *N. ceranae* with multiplex PCR, the samples were submitted to the methodproposed [13] DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, worms were washed with 1% SDS, then 180

µl of ATL buffer was added to 25 mg of the sample and 20 µl QIAGEN protease. For homogenization of samples, tubes were placed into the adaptor sets, which are fixed into the clamps of the Qiagen tissue Lyser. Disruption was performed in two minutes high-speed (30 Hz) shaking step. Then samples incubated at 56˚C till lysis. After lysis, 200 µl of the lysate incubated with ten µl of proteinase K and 200 µl of lysis buffer at 72^{O}C for ten min. After incubation, 200 µl of 100% ethanol added to the lysate. The sample washed and centrifuged following the manufacturer's recommendations. Nucleic acid eluted with 100 µl of elution buffer provided in the kit.

Oligonucleotide Primer
Primers used supplied from Metabion (Germany) are listed in table (1).

PCR Amplification
Primers utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 5.5 µl of water, and 5 µl of DNA template. The reaction was performed in an applied biosystem 2720 thermal cycler.

Analysis of the PCR Products
The products of PCR separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 15 µl of the products was loaded in each gel slot. A generuler 100 bp ladder (Fermentas, thermofisher, Germany) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software. PCR products were purified using QIAquick PCR Product extraction kit. (Qiagen, Valencia). Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer) was used for the sequence reaction and then it was purified using Centrisep spin column. DNA sequences were obtained by Applied Biosystems3130 genetic analyzer (HITACHI, Japan), a BLAST® analysis (Basic Local Alignment Search Tool) [14] was initially performed to establish sequence identity to Gen Bank accessions. The phylogenetic tree was created by the Meg Align module of Lasergene DNA Star version 12.1 [13] and Phylogenetic analyses was done using maximum likelihood, neighbour joining and maximum parsimony in MEGA6 [16].

Table 1: Primers sequences, target genes, amplicon sizes and cycling conditions.

Target agent	Primers sequences	Amplified segment (bp)	Primary Denaturation	Amplification (35 cycles)			Final extension
				Secondary denaturation	Annealing	Extension	
N. ceranae	CGGCGACGATGTGATATGAAAATATTAA CCCGGTCATTCTCAAACAAAAAACCG	218-219	95˚C 2 min.	95˚C 1 min.	50˚C 1 min.	72˚C 1 min.	72˚C 10 min.
N. apis	GGGGGCATGTCTTTGACGTACTATGTA GGGGGGCGTTTAAAATGTGAAACAACTATG	321	95˚C 2 min.	95˚C 1 min.	50˚C 1 min.	72˚C 1 min.	

Results
A - Colony Strength and its Relation to Fluctuations in Infection
Data in tables (2) indicate that the colony no. 1 the strongest colony with a mean 4.60 throughout the experimental and the lowest bee colonies no. 7 the colonies (no.4, 5,7and 8) showed the highest mean level of infection throughout the year and Lost during winter and early spring

Table 2: Number of combs covered with bees throughout the year

Colonies / Date	1	2	3	4	5	6	7	8	9	10	Mean	mean±sd
1/4/2019	5	5.5	4.5	5	4.5	3.5	4	5	5	5.5	4.75	4.8±0.63^{c}
1/5/2019	6	5	5	4	4.5	4	3	5	5	5	4.65	4.7±0.82^{c}
2/6/2019	8	7.5	6	6	6	6	4.5	7	8	8	6.7	6.7±1.18^{a}
1/7/2019	8.5	8	7	6.5	8	7.5	6	6	8	8	7.35	7.4±0.91^{a}
1/8/2019	7.5	6	6	6	6	5.5	5	5	6	6	5.900	5.9±0.70^{b}
1/9/2019	6	5	5	5	5	3	4	3.5	4	4	4.45	4.5±0.90^{c}
1/10/2019	4	3	3.5	3.5	4	3	3	3	3.5	4	3.45	3.5±0.44^{d}
3/11/2019	3	2.5	2.5	1.5	1.5	2	2	2.5	3	2	2.15	2.2±0.54^{e}
1/12/2019	2	2	2	1	1	1.5	1	1	2	1.5	1.45	1.5±0.47^{ef}
5/1/2020	1.5	1.5	1	0	0	1.5	0	0	1	1	0.75	0.8±0.68^{f}
3/2/2020	1.5	1.5	1.5	0	0	1	0	0	1.5	1	1.33	0.8±0.71^{f}

2/3/2020	3	2.5	2.5	0	0	1.5	0	0	2	2	2.25	1.4±1.23^{f}
1/4/2020	4	3	3	0	0	3	0	0	3.5	3	3.25	2.1±1.71e^{f}
Mean	4.6	4.07	3.8	3.85	4.05	3.3	3.25	3.8	4.03	3.92		3.9±0.39
	4.6±2.3	4.1±2.1	3.8±1.8	3.0±2.5	3.2±2.7	3.3±1.9	2.6±2.1	3.0±2.5	4.0±2.2	3.9±2.4		
LSD0.05												0.803

LSD value at 0.05

Spores that belonged to *Nosema* spp. were found in the intestines and in the body cavity (Figure 1). *Nosema* spp. spores were detected in 15 from 20colonies (60%). All the bees were found a positive by microscopic examination were found a positive by the PCR technique. Following agarose gel electrophoresis and staining of the obtained products with ethidium bromide amplicons exhibited a length of 219-221bp, which corresponds to *Nosema ceranae* fig. No (2) & table (2) confections (*N. ceranae*) were found. The nucleotide sequences of amplification products from the *Nosema*-infected honeybee samples were 99% identical to the *N. ceranae* sequence deposited in the GenBank database. The detected sequence analysis data belonging to *N. ceranae* are listed in Table (2, 3), and showed variations in infection level. There was a positive correlation between levels of infection specially During Late winter. These results confirmed the results [17]. Who reported that the peak infections appeared to follow periods of brood – rearing.

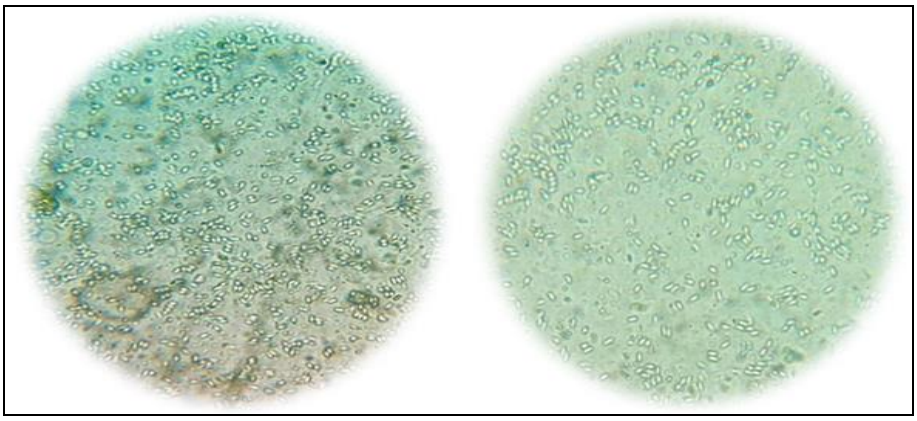

Fig 1: Nosema spores (macerated abdomen suspension of adult honeybees).

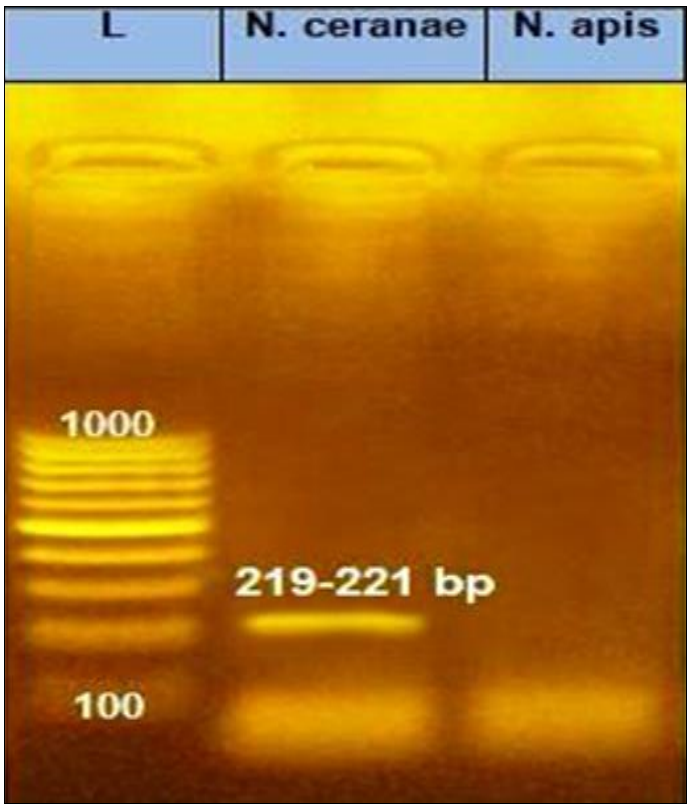

Fig 2: Analysis of PCR of extracted DNA from *Nosema* spore using primers specific for *N. ceranae* and *N. apis* derived from rRNA of protozoa. L, Gene ruler 100 bp ladder (fermentas, thermo) (100-1000 bp) and the positive results to *N. ceranae* and the negative results to *N. apis*

Table 3: positive gel electrophoresis results the PCR technique to *N.ceranae* and negative *N. apis*

Sample No.	N. ceranae	N. apis
1	+	-

Table 4: Sequence of *N. ceranae* detected in *Apis mellifera*

Sequencing Results
ATACTATAGATTTGAGTTTTTTGGCTCTGGGGATAGTATGATCGCAAGAT
TGAAAATTAAAGAAATTGACGGAAGAATACCACAAGGAGTGGATTGTGCG
GCTTAATTTGACTCAGCGCGAGGAAACTTACCAATATTTTATTATTTTGA
GAGAACGGTTTTTTGTTGAGAATGAA

Gel electrophoresis obtained PCR products, bands were detected at a molecular weight of 219-221 bp (Fig. 2). Sequence analysis and the results were recorded in GenBank (MZ044978- N._cernae_AHD). (Fig. 3)

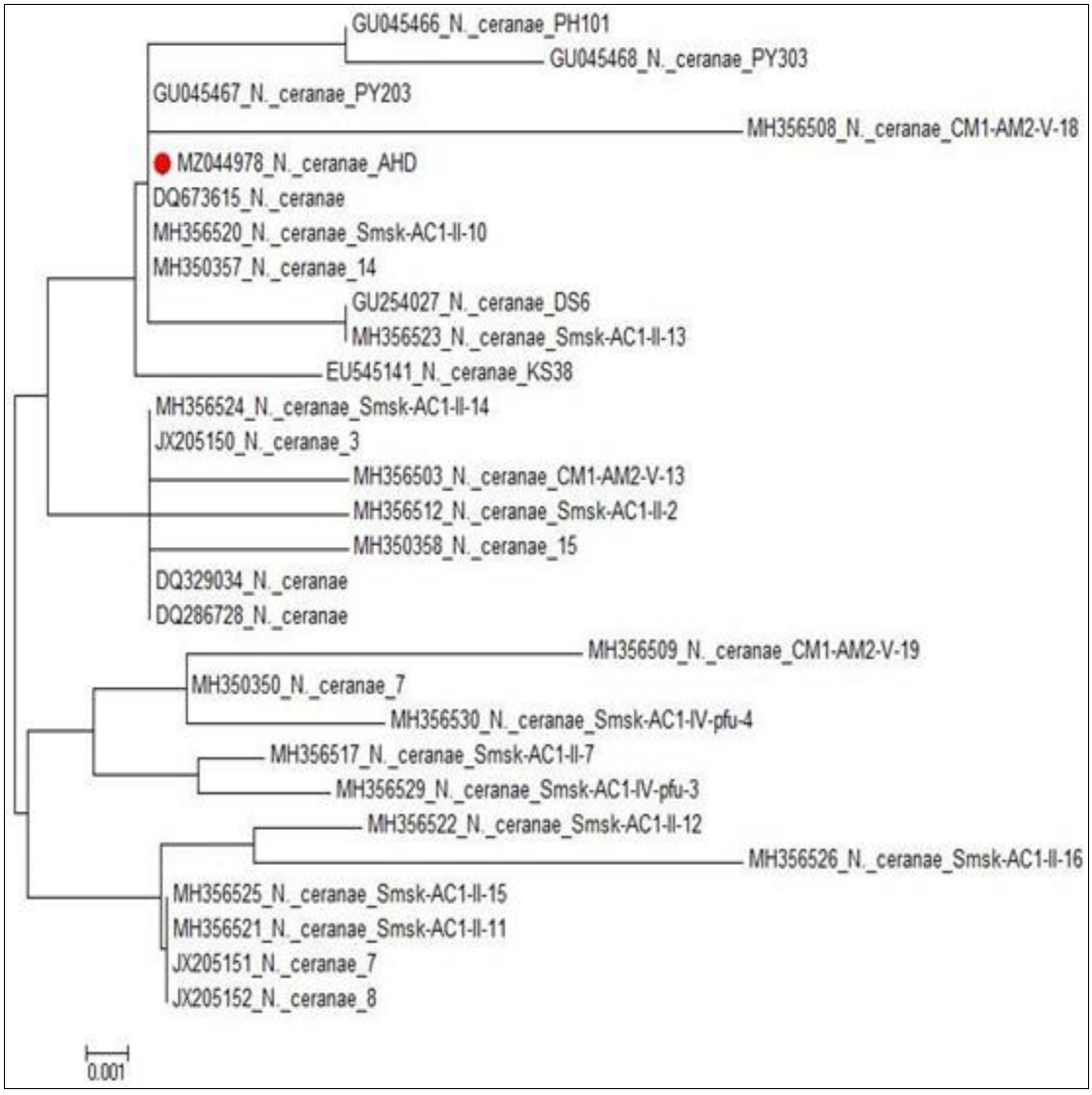

Fig 3: Phylogenetic tree showed clear clustering of the isolated Egyptian strains with *N. ceranae* strains uploaded from gene bank.

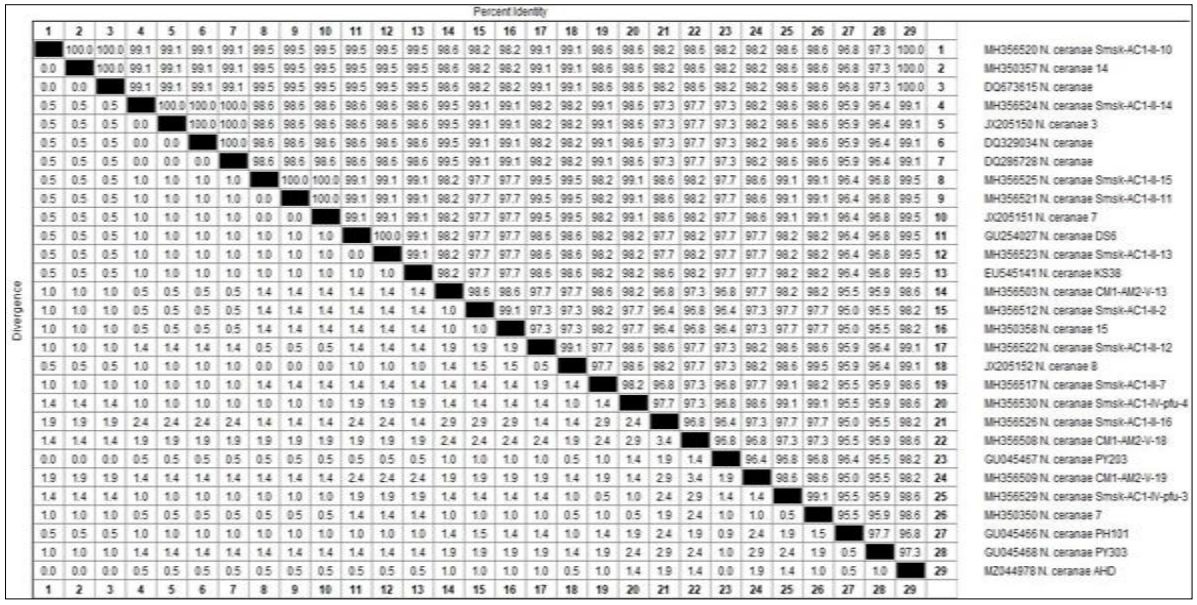

Fig 4: Sequence distance created by the Meg Align module of Lasergene DNA Star. Sequence identities of 100% homology

Discussion

These results are in agreement with [17] From the 3 region samples subjected to PCR analysis, *Nosema ceranae* was detected at two regions (Giza and Kafr el-Shekh). Honeybee disease caused by *N. apis* and *N. ceranae* [18, 19]. The diagnosis of disease is made by traditional microscopic methods and some molecular techniques [20, 21]. Using of multiplex-PCR diagnosis for *Nosema* spores and different primer pairs targeting the rRNA gene for discrimination of the species, [22].

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