



## Original article

Geographical distribution and molecular detection of *Nosema ceranae* from indigenous honey bees of Saudi Arabia

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## ARTICLE INFO

## Article history:

Received 12 January 2017

Revised 23 January 2017

Accepted 23 January 2017

Available online 1 February 2017

## Keywords:

*Nosema ceranae*

Prevalence

Molecular detection

Honeybee: Saudi Arabia

## ABSTRACT

The aim of the study was to detect the infection level of honey bees with *Nosema apis* and/or *Nosema ceranae* using microscopic and molecular analysis from indigenous honeybee race of eight Saudi Arabian geographical regions. A detailed survey was conducted and fifty apiaries were chosen at random from these locations. Infection level was determined both by microscope and Multiplex-PCR and data were analyzed using bioinformatics tools and phylogenetic analysis. Result showed that *N. ceranae* was the only species infecting indigenous honeybee colonies in Saudi Arabia. As determined by microscope, *Nosema* spores were found to be in 20.59% of total samples colonies, while 58% of the samples evaluated by PCR were found to be positive for *N. ceranae*, with the highest prevalence in Al-Bahah, a tropical wet and dry climatic region, whereas low prevalence was found in the regions with hot arid climate. Honeybees from all eight locations surveyed were positive for *N. ceranae*. This is the first report about the *N. ceranae* detection, contamination level and distribution pattern in Saudi Arabia.

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## 1. Introduction

Western honey bees (*Apis mellifera*) are a highly valued resource worldwide and are of great relevance for humans and the entire ecosystem, not only as a honey and wax producer but also as a pollinator of agricultural and horticultural crops and wild flora (vanEngelsdorp and Meixner, 2010). The total annual global economic worth of pollination amounts to 212 billion USD, representing 9.5% of the value of the global agricultural production (Gallai et al., 2009). However, very unfortunately, honeybee is facing enormous threat worldwide (USA, Europe, Middle East) (Crailsheim et al., 2009; vanEngelsdorp et al., 2009, 2010; Haddad et al., 2009; Srooker et al., 2009) including Saudi Arabia (Alattal and AlGhamdi, 2015).

Beekeeping is one of the long-standing practices in rural Saudi Arabia and is one of the most important economic activities for the communities (Al-Ghamdi and Nuru, 2013). Approximately 5000 beekeepers maintain more than one million honeybee colo-

nies and produce approximately 9000 metric tons of honey annually (Al-Ghamdi, 2007). *Apis mellifera jemenitica* Ruttner (= *yemenitica auctorum*: vide Engel, 1999), the smallest race of *A. mellifera*, is the only race of *A. mellifera* naturally found in the country and has been used in apiculture at least 2000 BC. Traditional beekeeping is mostly practiced using this race, because it is well adapted to the semi-arid to semi-desert conditions of Saudi Arabia (Alqarni et al., 2011; Al-Ghamdi and Nuru, 2013). Also, honey produced by this native bee (*A. m. jemenitica*) is sold at 10–20 times high rates than imported honeys (Al-Ghamdi personal Comm.).

Despite the great potential and multiple opportunities for beekeeping in Saudi Arabia, the bee-keeping industry is steadily growing in the country with different opportunities and, of course, many challenge. The major challenge is occurrence and distribution of honeybee disease in the country (Al-Ghamdi, 1990, 2010; Alattal and AlGhamdi, 2015; Ansari et al., 2016, 2017).

A mysterious decline in honeybee colonies has gained worldwide attention, including in Saudi Arabia. In the last decades, significant losses have been observed in indigenous honeybee colonies in Saudi Arabia (Alattal and AlGhamdi, 2015). Much attention has been given to Colony Collapse Disorder (CCD), which is a syndrome specifically defined as a dead colony with no adult bees and with no dead bee bodies but with a live queen, and usually honey and immature bees, still presents (vanEngelsdorp et al., 2009). Several causes of these large-scale losses have been reported, including honey bee parasites (*Varroa destructor*, *Acarapis*

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Peer review under responsibility of King Saud University.



woodi); pathogens (*Nosema* spp. and bee viruses); pesticides, harsh environment, use of antibiotics, poor nutrition, and migratory beekeeping practices (Kevan et al., 2007; Higes et al., 2008; Naug, 2009; vanEngelsdorp et al., 2009; Bacandritsos et al., 2010; vanEngelsdorp and Meixner, 2010; Alattal and AlGhamdi, 2015).

Nosemosis is a fungal infection of honey bees caused by either *Nosema apis* or *N. ceranae*. *N. apis* was the historic species infecting European honey bees (Matheson, 1996). *N. ceranae* was previously isolated in naturally infected *Apis cerana* worker bees in China (Fries et al., 1996) and later has been described infecting *Apis mellifera* in Europe (Higes et al., 2006). Currently, this parasite is widespread all over the world and has shown the capacity of infection in other Hymenoptera different from the honeybees (Plischuk et al., 2009), and it is now a common infection of European honeybees and is highly virulent to its new host (Chen et al., 2009a). This is problematic for beekeepers because *N. ceranae* has a different seasonal phenology than *N. apis*, causing more significant problems for beekeepers in summer months and in warm climates (Bourgeois et al., 2010). Both *Nosema* spp. can co-infect honey bees (Chen et al., 2009b; Paxton et al., 2007; Forsgren and Fries, 2010). Although co-infections occur, *N. ceranae* has become the predominant species in many regions (Chen et al., 2009a; Klee et al., 2007; Williams et al., 2008; Razmaraii et al., 2013; Haddad, 2014).

Both *Nosema* spp. invade the midgut epithelial cells of adult honeybees (*A. mellifera*), i.e. worker bees, drones, and queens, and caused honey bee disease known as nosemaiosis or nosemosis (Fries, 1988, 2010; Higes et al., 2007). This disease negatively affects productivity and survival of honeybee colonies, adult bee longevity, queen bees, brood rearing, bee biochemistry, pollen collection, and other bee behaviors (Botías et al., 2013; Huang, 2012). In contrast to the clinical symptoms of *N. apis*, such as crawling bees and dysentery (Liu, 1988), infection with *N. ceranae* is symptomless apart from reports of a massive colony depopulation and collapse (Huang et al., 2007; Paxton, 2010), reduced honey production (Higes et al., 2008). The impact of *N. ceranae* infection on colony survival is unclear and has been found in both healthy colonies (Vanengelsdorp et al., 2009; Cox-Foster et al., 2007; Gisder et al., 2010) and those undergoing sudden collapses (Higes et al., 2008, 2009; Bacandritsos et al., 2010; Martín-Hernández et al., 2007). Thus, investigation of *Nosema* species seems important.

Routine optical microscopy assessment can confirm infection with *Nosema* species, but it is impossible to distinguish between the species because the spores of the two *Nosema* species are very similar and can hardly be distinguished by light microscopy, so that in the absence of clear morphological characteristics for species recognition, other techniques using molecular markers may greatly assist in the diagnosis and identification of honeybee microsporidians. Thus, it is necessary to use molecular diagnostic tools and identification methods (Gajger et al., 2010). The PCR technique provides a very sensitive test for detecting microsporidian infection because it enables detection of the parasite even at very low levels of infection.

In some bordered countries of Saudi Arabia (Egypt, Israel, Jordan, Iraq and Iran), *Nosema* infection in honeybee colonies has been reported previously (Alzubaidy and Ali, 1994; El-Shemy et al., 2012; Nabian et al., 2011; Razmaraii et al., 2013; Aroee et al., 2016; Soroker et al., 2009). However, even though some preliminary studies have been conducted on Nosemosis in honeybee in Saudi Arabia (Al-Ghamdi, 1990; Alattal and AlGhamdi, 2015; Abdel-Baki et al., 2016). Recently, in Riyadh region of Saudi Arabia, Nosemosis has been recognized by the presence of *Nosema* spores through light microscopy, assuming *N. apis* to be the causal agent (Abdel-Baki et al., 2016). These findings have led to a demand for PCR based research that determine, which species of *Nosema* have been present in *A. m. jemenitica*, the indigenous honeybee race of

Saudi Arabia in a recent past using detailed survey and molecular characterization.

## 2. Materials and methodology

The presence of nosemosis in honeybee colonies was investigated in different beekeeping locations and eco-regions, during the spring season (March to April 2015), the active season for honeybees in Saudi Arabia. Eight different geographical localities, where beekeeping is common were included in this survey (Fig. 1): Al-Ahsa (25° 25' 46" N, 49° 37' 19" E), Abha (18° 13' 24" N, 42° 30' 26" E), Jazan (16° 53' 21" N, 42° 33' 40" E), Taif (21° 16' 0" N, 40° 25' 0" E), Al-Madinah (24° 28' 0" N, 39° 36' 0" E), Al-Bahah (20° 0' 0" N, 41° 30' 0" E), Al-Qassim (25° 49' 19.72" N, 42° 50' 6.85" E) and Riyadh (24°43'19.2"N 46°37'37.2"E). Total 50 random apiaries were visited in all locations, and 10 colonies in each apiary were inspected.

### 2.1. Sampling

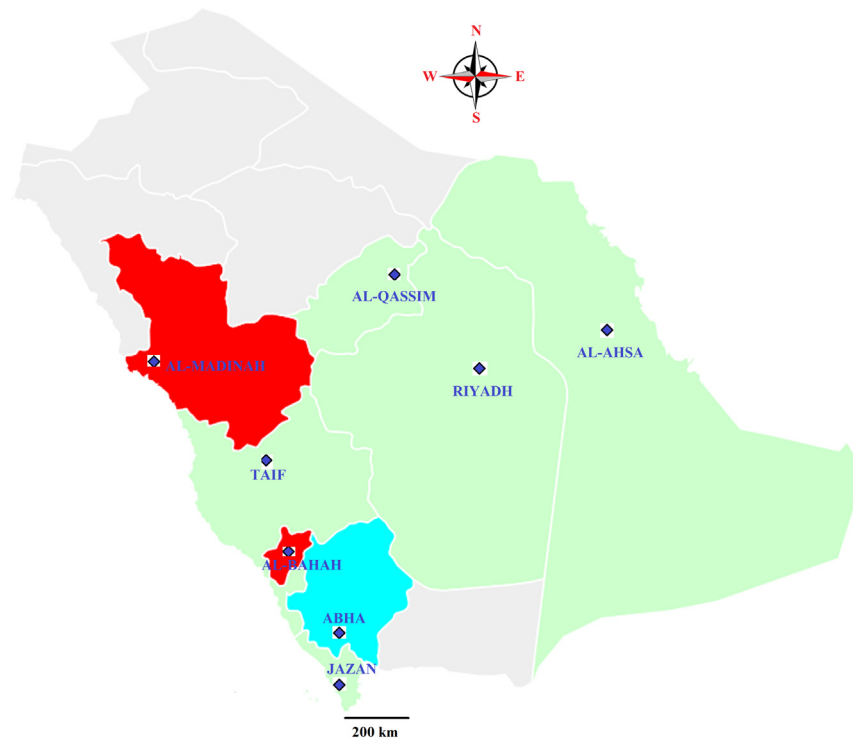
A total of fifty seemingly healthy apiaries of *A. m. jemenitica*, indigenous race of Saudi Arabia, owned by different beekeepers were randomly selected following stratified randomization procedures (Moher et al., 2010). Samples were collected from local (*A. m. jemenitica*) bee races only (10 hives from each apiary) from March to April (major nectar flow period in Saudi Arabia) during the year 2015. The samples were collected from eight major beekeeping regions of Saudi Arabia, based on, the beekeeping management schedule and the categorization of geographical regions, for instance, Riyadh, Al-Qassim, Al-Ahsa, Taif and Jazan (Hot arid climate region), Al-Madinah and Al-Bahah (Tropical wet and dry climate), and Abha (Cold semi-arid climate). The sampling hives had not been treated against *Nosema* disease for at least 6 months. In each hive, approximately 100 worker bees were collected from outer honey frames of the brood chamber, placed in falcon tube containing preservative buffer (RNA Later<sup>®</sup>), transported to the laboratory of the Bee Research Unit (BRU) at the Department of Plant Protection of the Faculty of Food and Agriculture Sciences at King Saud University and stored at –20 °C until analyzed.

### 2.2. Data collection

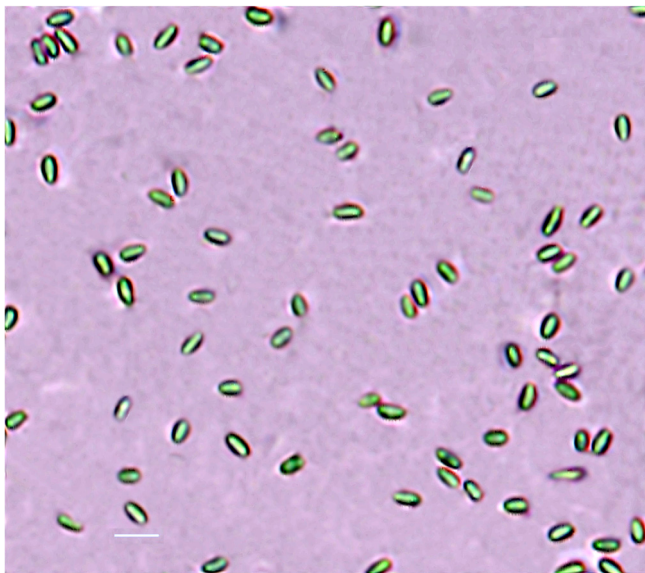
The data collected from the survey included the following information for each inspected apiary: the date of inspection, the apiary location (to facilitate repeat visits), the name of the owner, the hive type (local or modern), the honeybee race (indigenous or imported), the number of honeybee colonies and the colonies having some unusual symptoms.

### 2.3. Microscopic examination of spores

Samples were initially analyzed by phase contrast microscopy for presence or apparent absence of *Nosema* spp. spores. For each sample, the abdomens of 30 adult bees were macerated in 30 mL of ddH<sub>2</sub>O, the suspension was filtered and centrifuged for 5000 rpm for 10 min and the homogenate examined under a Phase Contrast Microscope (Olympus BX51, model BX51TF, Japan, equipped with an Olympus DP71 camera (Olympus, Japan) at 400 × magnification (Fig. 2B), and photographed (OIE, 2008). Measurements are presented in micrometers and data are expressed as the mean followed by the range in parentheses. As morphological characteristics of *N. ceranae* and *N. apis* spores are similar and can hardly be distinguished by optical microscopy, all samples were also screened by multiplex 96 polymerase chain reaction



**Fig. 1.** Sampling locations in Saudi Arabia with different eco-regions, from which *A. mellifera* were collected and tested for *N. apis* and *N. ceranae* (a) Red color: Tropical wet and dry or Tropical savanna (Aw) climate (b) Sky blue color: Cold semi-arid (BSk) climate (c) Green color: Hot arid (BWb) climate.



**Fig. 2.** Spores of *Nosema ceranae* have an oval shape with a dark border under Phase contrast microscope at 400X magnification. Scalebar = 10  $\mu$ m.

(M-PCR) assay based on 16S rRNA-gene-targeted species specific primers to distinguish between *N. ceranae* and *N. apis*. Positive samples were also used for further molecular diagnosis as discussed below.

#### 2.4. Genomic DNA isolation

For DNA extraction, the abdomen of the 30 individual honeybees was cleanly cut off with scissors, immediately put into a vial and homogenized in 100  $\mu$ l Krebs Ringer solution pH 7.4 (123 mM NaCl, 1.3 mM  $\text{CaCl}_2$ , 5 mM KCl, 100 mM HEPES, 5 mM D-glucose,

1.5% bovine serum albumin free fatty acid) with a pestle. DNA was extracted from pools using a CTAB buffer (100 mM Tris HCl, (pH 8.0); 20 mM EDTA, (pH 8.0); 1.4 M NaCl; 2% (w/v) cetyltrimethylammonium bromide; 0.2% (v/v) 2-mercaptoethanol) plus proteinase K, overnight incubation at 56 °C followed by a phenol/chloroform/isoamyl alcohol (25:24:1) extraction, and precipitated overnight at –20 °C with isopropanol, recovered by means of centrifugation, resuspended in TE 1 $\times$ , and treated with RNase (100  $\mu$ g/ml). The DNA (3–5  $\mu$ g) was loaded onto 1.2% agarose gels, which were stained with ethidium bromide after migration.

#### 2.5. Multiplex PCR (M-PCR) amplification using *N. apis* and *N. ceranae*-specific primers

Each DNA sample was subjected to molecular identification by PCR assay using the *N. apis*-specific primers (321APIS, FOR 5'-GGGGCATGTCTTTGACGTACTATGTA-3'; 321APIS REV 5'-GGGGGGCGTTTAAAATGTGAAACAACTATG-3') and *N. ceranae*-specific primer (218MITOC FOR 5'-CGGCGACGATGTGATATGAAA-ATATTAA-3'; 218MITOC REV 5'-CCCAGTCATTCTCAAACAAA-AACCG-3') described by [Martin-Hernandez et al. \(2007\)](#). The PCR reactions were performed in a total volume of 50  $\mu$ l containing 5  $\mu$ l 10 $\times$  PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 4 mM  $\text{MgCl}_2$ , 1% Triton X-100), 200  $\mu$ M of each deoxynucleotide triphosphates, 2U *Taq* DNA polymerase enzyme (Promega, USA), 100 ng of each primer and 10 ng target DNA were added. The surface of the mixture was covered with 100  $\mu$ l mineral oil. The following PCR conditions were used: initial denaturation at 95 °C (2 min), and 35 cycles of (95 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s) and a final extension cycle at 72 °C (5 min) ended the PCR. Negative controls (from DNA extraction) were included in all PCR experiments. ([OIE Terrestrial manual, 2008](#); [Martin-hernandez et al., 2007](#)). Samples of the amplicons were electrophoresed in 1.5% agarose gel.



Approximate product size was determined using the 100-bp molecular size marker (Promega, USA). The PCR product was visualized and photographed using a Gel Doc EZ system (Bio-Rad, USA) with a band at 321 bp for *N. apis* and at 218–219 for *N. ceranae*.

### 3. 16S rRNA gene sequencing and phylogenetic analysis

PCR products were purified using GenElute PCR Clean-Up Kit (Sigma–Aldrich, India) and sent to BGI Genomics Co., Ltd (Hong Kong, China) for both end sanger sequencing. The sequences obtained were manually edited using Sequencher 4.5 (Gene Codes Corp.) and were aligned using the Bioedit sequence editor software version 7.0.5.3. These sequences have been submitted to GenBank (accession number KY022481 and KY022482 for *ksuNC4* and *ksuNC6* respectively). Partial 16S rRNA gene sequences of the isolates were compared with 16S rRNA gene sequences available by the BLAST search (Altschul et al., 1990), in the National Centre for Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov/>). Multiple sequence alignments were performed using CLUSTALW version 1.8 (Thompson et al., 1994). Phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987), and the reliability of the tree topology was evaluated by bootstrap analysis using MEGA 6.06 software (Tamura et al., 2013).

#### 3.1. Data analysis

The corresponding 95% confidence intervals (95% CI) were calculated and differences among prevalence values were compared by Fisher's exact test. *P* values <0.05 were considered significant. The Cohen's Kappa coefficient was used as a measure of agreement between microscopy and M-PCR. The following ranges were considered for interpretation of the Cohen's Kappa coefficient: poor agreement = less than 0.00, slight agreement = 0.00–0.20, fair agreement = 0.21–0.40, moderate agreement = 0.41–0.60, substantial agreement = 0.61–0.80, and almost perfect agreement = 0.80–1.00.

#### 3.2. Statistical analysis

The prevalence of *Nosema* spp. contamination levels in honeybees from different geographical regions of Saudi Arabia was calculated by descriptive statistics and a confidence interval (CI) of 95%.

## 4. Results

### 4.1. Field survey

During survey presence of disease agents and parasite was monitored and results showed that *Varroa* mite is frequently infesting honeybee colonies in all the sampled locations. Presence of *Nosema* was recorded in all locations (Table 1). Majority of infection have been reported in Al-Bahah, Abha and Taif region of Saudi Arabia. These are the three major beekeeping regions in Saudi Arabia and most of the honey has been produced by these three areas. In comparison to Riyadh, Al-Qassim and Al-Ahsa, these three regions have high rainfall and moderate temperature (Table 1).

### 4.2. Microscopic examination

Spores of *Nosema* spp. were detected at 400x magnification under Phase contrast microscope (Fig. 1B). Phase contrast microscopy of the midgut content revealed the presence of large numbers of *Nosema* spp. spores. *Nosema* spores were oval shaped, measuring 3.0–4.0 µm in width and 5.0–7.0 µm in length (*n* = 30) (Fig. 2). *Nosema* infection has been found in all locations of sample

collections. As determined by microscope, *Nosema* spp. were found in 20.59% of total sampled colonies. In hot arid climatic eco-regions like Riyadh (20%), Al-Qassim (18.36%), Al-Ahsa (15%), Taif (19%) and Jazan (12.06%) of samples were found microscopically positive. Conversely, in Tropical savanna climate region like Al-Madinah (26%), Al-Bahah (31%) and Cold semi-arid climatic region, Abha (27.14%) infection was more than hot arid climatic sampling locations. A significant difference in the infection level was found in hot arid climatic eco-regions and Tropical savanna climate region together with cold semi-arid climatic region (*P* < 0.001) (Table 1).

### 4.3. Molecular characterization of *Nosema* spp

There is no previous record of presence of *Nosema* spp. using molecular identification in Saudi Arabia. Therefore, we initiate to confirm the *Nosema* spp. identification by M-PCR and DNA sequencing by collecting samples from geographically distinct locations throughout Saudi Arabia. Based on the PCR analysis, *Nosema* infections were detected in apiaries from all the eight bee-keeping regions examined. Detection by PCR using *N. apis* and *N. ceranae* specific primers found that 29 out of 50 apiaries (58%) were positive for *N. ceranae* (Table 1). But no apiaries were found to be positive for *N. apis* (Fig. 3). Infection was found in the samples collected from Tropical savanna climatic regions and cold semi-arid climatic region in comparison to hot arid climatic region. Overall, a total of 29 out of 50 (29%, 95% CI: 41.96–58.04%) apiaries tested positive for *Nosema* infection by microscopy and M-PCR. Therefore, the Cohen's Kappa coefficient for the association between results of microscopy and results of M-PCR was 1, indicating that there was a perfect level of agreement between the two diagnostic methods in all the bee samples. In hot arid climatic eco-regions, 31.6%, 32.5%, 30%, 22% and 15.51% from Riyadh, Al-Qassim, Al-Ahsa, Taif, and Jazan respectively were found to be PCR positive. Conversely, in Tropical savanna climate region, 34% (Al-Madinah), 39% (Al-Bahah) and Cold semi-arid climatic region 35.71% (Abha) were PCR positive (Table 1).

### 4.4. BLAST and phylogenetic analysis

Similar to that observed in other countries (Medici et al., 2012), we observed some intraspecific variations in the 16S SSU of *N. ceranae* in Saudi Arabia. Comparative analysis of 16S rRNA gene sequences of *ksuNC4* and *ksuNC6* isolates showed 217/218 (99%) sequence identity. Over the entire sequence range analyzed (218 bp), only one position (18th bp) was polymorphic with one gap. The BLAST search of these sequences against GenBank Nucleotide database, the highest similarity (99%) was found with *N. ceranae* 16S rRNA sequences. A Nucleotide BLAST search showed that the DNA sequence obtained from the *ksuNC4* (218 bp) isolate showed a 100% sequence identity with the 16S rDNA of some *N. ceranae* isolates (gb| KC680636.1, gb| KC680629.1 and gb| DQ329034.1). The first two close hit of *ksuNC4* closely related to the DNA sequence of *N. ceranae* isolated from honeybee samples from Lebanon (Roudel et al., 2013), and the third close hit belongs to the sequence of *N. ceranae* isolated in Spain (Higes et al., 2007). Similarly, the DNA sequence obtained from the *ksuNC6* isolate (417 bp) showed a 100% sequence identity with the 16S rDNA of other *N. ceranae* isolates (gb| KC680641.1, gb| KC680642.1 gb| KC680637.1 and gb| JF431546.1). First two closely related hits of *ksuNC6* belongs to *N. ceranae* isolates from Moroccan Honeybee, third one from Lebanon (Roudel et al., 2013), and the forth close hit belongs to the sequence of *N. ceranae* isolated in Iran (Nabian et al., 2011). This indicated that Lebanon (northern bordered country of Saud Arabia) and Iran (eastern bordered country) the close neighbors of Saudi Arabia have same type of genotype as found in Saudi Arabia.

**Table 1**  
Prevalence and estimated contamination of *N. ceranae* in various eco-regions of Saudi Arabia based on altitude, average temperature, rainfall, relative humidity, climate, monitoring time.

Sampling Area	Altitude (m)	Average Temperature (°C)		Average rainfall (mm)		Average Relative Humidity		No. of inspected apiaries/ samples collections	Microscopic Positive (%)	PCR Positive (%)	No. of infected Apiaries	Climate*
		March	April	March	April	March	April					
Riyadh	612	21	23	23	25	32	28	6/60	12 (20)	19 (31.66)	3	Hot arid(BWh) climate
Al-Qassim	753	17	22	26.7	28.8	35	33	4/40	09 (18.36)	13 (32.5)	2	Hot arid(BWh) climate
Al-Ahsa	179	20.9	25.8	16.2	10.7	44	38	2/20	3 (15)	6 (30)	1	Hot arid(BWh) climate
Al-Madinah	608	23.6	26.8	9.8	9.6	25	22	05/50	13 (26)	17 (34)	2	Tropical wet and dry or Tropical savanna (Aw) climate
Taif	1879	20	21.8	15.1	35.7	47	47	10/100	19 (19)	22 (22)	5	Hot arid (BWh) climate
Al-Bahah	2150	16.5	18.5	16.5	36.3	46	45	10/100	31 (31)	39 (39)	8	Tropical wet and dry or Tropical savanna (Aw) climate
Abha	2270	15.5	17.5	47.2	47.9	62	60	07/70	19 (27.14)	25 (35.71)	5	Cold semi-arid (BSk) climate
Jazan	5	28	30	5.7	14.6	66	64	6/60	07 (12.06)	09 (15.51)	3	Hot arid(BWh) climate

\* Köppen-Geiger climate classification.

The evolutionary relationship between the two isolates and previously reported isolates were constructed using MEGA6.06 software (Tamura et al., 2013). The results illustrate the degree of evolutionary relatedness between the two Saudi Arabian isolates and other previously reported isolates (Fig. 4). From our study, the ksuNC4 isolate showed greater relatedness to three previously reported *N. ceranae* isolates {gb| KC680629.1 (Lebanon), gb| KC680636.1 (Lebanon) and gb| DQ329234.1 (Spain)}, while isolate ksuNC6 formed a separate clade by itself with gb| KC680637.1 (Lebanon), gb| JF431546.1 (Iran) and gb| KC680642.1 (Morocco). This indicates that the genotypes of the two isolates differ. The ksuNC4 genotype was isolated from the apiary located in Al-Bahah and the isolate ksuNC4 was isolated from Jazan, both are two different geographical regions of Saudi Arabia.

## 5. Discussion

During the last decade, an increase infection by *Nosema* parasite in the honeybee (Emsen et al., 2016) followed by increased numbers of honeybee colony death and decreased honey production has been reported worldwide. One of the main reasons that might explain these problems is the recent introduction of *N. ceranae* in honeybee in Europe (Higes et al., 2006) and appeared to be highly virulent (Paxton et al., 2007; Higes et al., 2007). Environmental conditions also strongly influence many parasitic relationships and, regardless of the effects of altitude, flora and colony management, in warm countries like Spain the influence of temperature on the consequences of *N. ceranae* has been observed (Martín-Hernández et al., 2012).

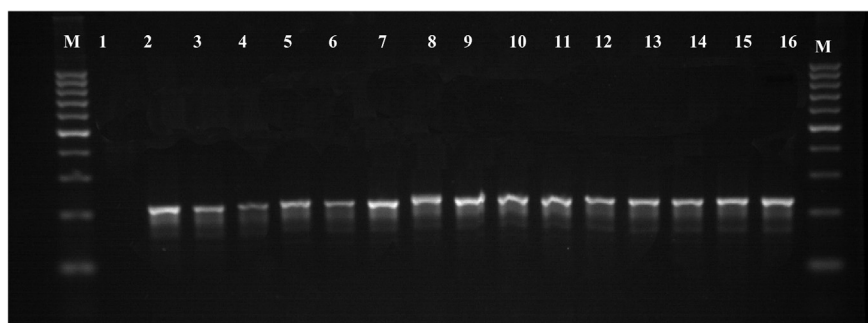
The prevalence of nosemosis disease has been proven to vary among regions and years (Mulholland et al., 2012). Although, *N. apis* has a world-wide distribution, it is not considered an important problem in tropical and sub-tropical regions (Wilson and Nunamaker, 1983). However, in temperate regions *N. apis* infections typically peak in the spring, decrease during the summer and then increase again in the fall before declining during the early winter months (Pickard and El-Shemy, 1989). *N. ceranae* was the most prevalent microsporidia found in *A. mellifera* in hotter regions (Mediterranean regions) and is reduced in colder climate (Fries, 2010; Gisder et al., 2010). Since *N. ceranae* infection appears to be more common in warmer climates and in specific geographical areas as *N. ceranae* spores are capable of surviving high temperature (60 °C) (Fenoy et al., 2009), this should be considered when importing bees from such areas (Fries, 2010).

In Sweden, 83% of colonies had *N. apis* only and 17% had both *N. apis* and *N. ceranae* (Fries, 2010). In Scotland, 70.4% of colonies revealed the presence of both *N. ceranae* and *N. apis* (Bollan et al., 2013). In east Azerbaijan province of Iran, an eastern bordered country of Saudi Arabia, 67.1% of colonies revealed the presence of *N. ceranae* (Razmaraii et al., 2013). Nabian et al. (2011) and Aroee et al. (2016), also reported the presence of *N. ceranae* in Iran. In Jordan, situated on northern border of Saudi Arabia, 23.9% of colonies found to be infected by *N. ceranae* and *N. apis* infection was not detected by PCR (Haddad, 2014). More evidences are available for prevalence of *Nosema* infection in some more cross bordered countries of Saudi Arabia, For instance, Iraq (Alzubaidy and Ali, 1994), Egypt (El-Shemy et al., 2012); Kuwait (OIE, 2004) and Oman (Matheson, 1993). Symptoms of nosemosis have been reported before among honey bees in Saudi Arabia (Al-Ghamdi, 1990; Matheson, 1993; Alattal and AlGhamdi, 2015). Recently, Abdel-Baki et al. (2016), observed the presence of *Nosema* spores using optical microscope from Honeybee in Riyadh region of Saudi Arabia.

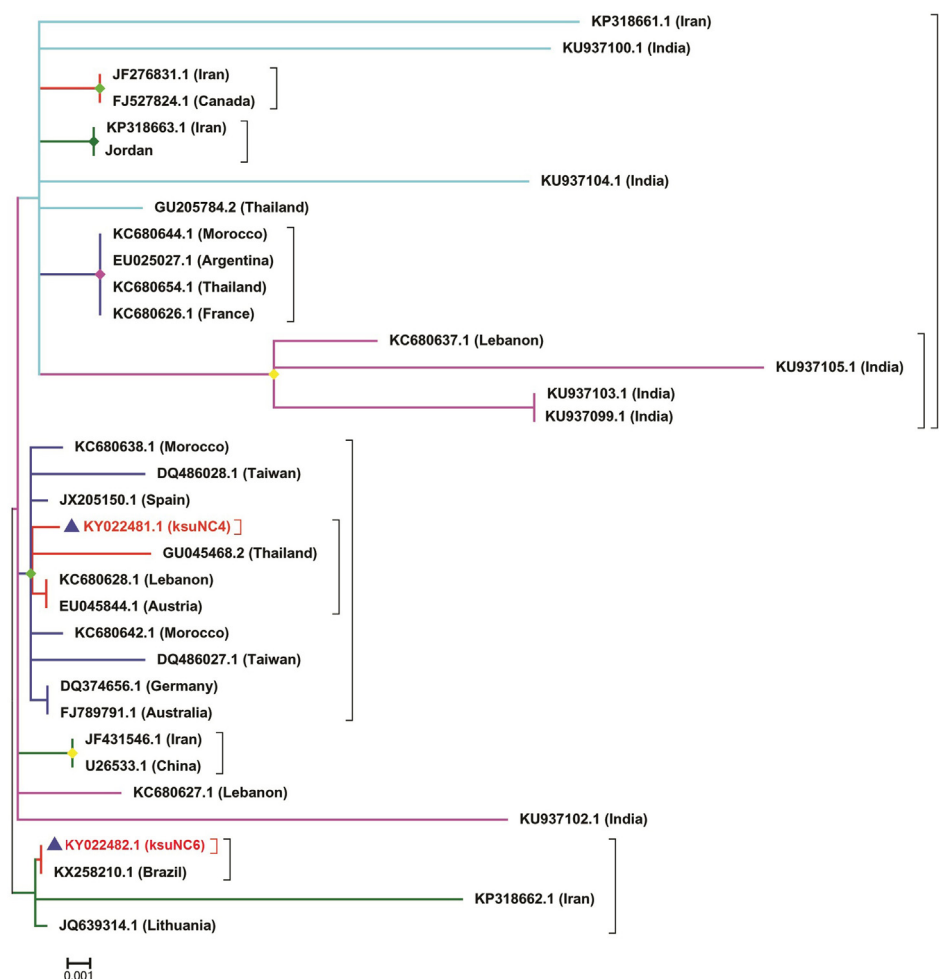
The identification of *Nosema* in the Eight provinces surveyed in Saudi Arabia was expected, given that this *Nosema* species symp-

toms and microscopic examinations of spores has previously been reported in Saudi Arabia and cross border countries prevalence of *Nosema* infection. *N. ceranae* is recent fungal pathogen for Saudi Arabian honeybees as reported in this study. since Al-Ghamdi, 1990 and Abdel-Baki et al., 2016, suspects the symptoms of *N. apis* in honeybee of Saudi Arabia by morphological symptoms and microscopy. In this study, the high prevalence (58%) of *N. ceranae* together with the absence of *N. apis* infection in the present survey corroborates the findings of other authors that *N. ceranae* is definitely spread in Saudi Arabia and has basically replaced *N. apis*

(Klee et al., 2007). High thermotolerance at 60 °C and 35 °C, resistance to desiccation, significant decrease in viability after freezing, and rapid degeneration of *N. ceranae* spores maintained at 4 °C were observed under experimental conditions (Fenoy et al., 2009). Therefore, it has been proposed that *N. ceranae* may be more prevalent in warmer climates (Fries, 2010) such as the typical middle east countries climate that occurs in the different study area. The present prevalence is close to values as high as 67% observed in Iran (Razmaraii et al., 2013) but considerably higher than, 23.9% and 49.2% as previously reported in Jordan (Haddad, 2014)



**Fig. 3.** Agarose gel showing amplification of the part of small subunit of ribosomal RNA by using primer 218MITOC and 321APIS. Lane M: DNA Marker 100 bp ladder; Lane 2: No amplification in negative control; Lane 1–14: Honeybee samples positive for *N. ceranae*; Lane 15: PCR product of positive control of 218 bp for *N. ceranae*.



**Fig. 4.** Neighbor-joining phylogenetic tree of the two *N. ceranae* isolates (ksuNC4 and ksuNC6) based on 16S rRNA gene sequence comparisons and closest NCBI (BLASTn) strains based on the 16S rRNA gene sequences (neighbor-joining tree method). The scale bar indicates 0.001 nucleotide substitutions per nucleotide position. The numbers at node show the bootstrap values obtained with 500 resampling analyses.

and Thailand (Chupia et al., 2016), respectively. Different prevalence values reported in the literature may be due to differences in the number of apiaries examined, sampling methods, geographical areas, characteristics of honeybee population, diagnostic techniques, and other biotic and abiotic factors.

Based our findings, microscopy is still a valuable, relatively cheap and simple method to screen for the presence of *Nosema* infection in apiaries since a perfect agreement between microscopy and M-PCR was observed in the experiments. Unfortunately, very strong morphological similarities occur between *N. apis* and *N. ceranae* spores, resulting in a high risk of misdiagnosis. Both *Nosema* species spores are smooth and darkly outlined with elongated-elliptical shape and bright centre. *N. apis* spores end rounded and measure 6.0 µm in length and 3.0 µm in width. *N. ceranae* spores end sharper and measure 4.4 µm in length and 2.2 µm in width (Huang, 2012; Michalczyk et al., 2011). The main differences are noted with respect to the length of the polar filament, and they can be detected only under an electron microscope (Fries, 2010; Paxton, 2010).

In this scenario, molecular techniques such as M-PCR are needed for a reliable identification of *Nosema* to species level (Michalczyk et al., 2011). Indeed, the advent of new highly sensitive and specific molecular tools has played a key role for detection of *N. ceranae* in *A. mellifera* and for retrospective analyze of samples, showing that *N. ceranae* is not a new microsporidian agent in *A. mellifera* but it has infected this host during the last two decades (Guerrero-Molina et al., 2016). It is likely that the delay in a correct identification of *N. ceranae* in *A. mellifera* is attributable to the routine use of microscopy as a diagnostic technique for the identification of *Nosema* spores (Higes et al., 2010). Therefore, accurate identification of *Nosema* spores to species level by molecular tools should be especially useful for Saudi Arabian beekeepers.

A Nucleotide BLAST search showed that the DNA sequence obtained from the ksuNC4 (218 bp) isolate showed a 100% sequence identity with the with the other *N. ceranae* isolates from Lebanon (Roudel et al., 2013), whereas, ksuNC6 (217 bp) isolate showed a 100% sequence identity with *N. ceranae* isolates from Morocco and Lebanon. This indicated that Lebanon and Morocco, which are close neighbors of Saudi Arabia, have the same type of genotype as found in Saudi Arabia. It is unlikely that the primary infestation of *N. ceranae* in Saudi Arabia is due to human facilitated transportation of bee packages or bee products between both countries, but it seems that a third party is the main source of this type of infestation in both countries, which could be Egypt, where bee exportation is common to most of the African and middle east countries (Alattal and AlGhamdi, 2015).

*Nosema* spores are primarily spread to neighboring bees through fecal matter contaminating the environment (fecal-oral pathway) or, alternatively, they can also reach the crop and be regurgitated to other colony members during food exchange (oral-oral pathway) (Smith, 2012). Therefore, infections by both *Nosema* species can be transmitted among bees via ingestion of environmentally resistant mature spores from contaminated wax, combs, other hive interior surfaces, and water (OIE, 2013). Other potential routes of transmission include contamination of pollen, beekeeping material, and honey as well as cleaning activities and trophallaxis (Higes et al., 2010). Auto-infections can also occur (Higes et al., 2007). In our survey, we did not attempt to identify any source of infection. However, all these routes of spread and transmission of infective spores may have played a role in the presence of *N. ceranae* in the *A. mellifera* colonies investigated.

In contrast to nosemiasis caused by *N. apis*, *N. ceranae* affected bees that do not exhibit defecation near or inside the hive with evident dysentery but the main clinical symptom is dwindling, i.e. the progressive reduction in the number of bees in a colony with no apparent cause, until the point of collapse (Huang, 2012; Paxton,

2010). Sometimes dwindling may affect the whole apiary and other times only specific colonies may show symptoms. The disease sometimes occurs rapidly but may also occur over several months (vanEngelsdorp et al., 2009).

In a recent study in Argentina, an increasing gradient of infection and counts of *Nosema* spp. were observed from warmer to colder regions (Pacini et al., 2016). In accordance to these results of Pacini et al., 2016, in our study, maximum infection found in Al-Bahah (Tropical savanna (Aw) climate), Abha (Cold semi-arid (BSk) climate) and Al-Madinah (Tropical savanna (Aw) climate), whereas comparatively little infection has been observed in the samples collected from the apiaries situated in hot arid (BWh) climate (Riyadh, Al-Qassim, Al-Ahsa, Taif and Jazan) (Table 1). This uneven distribution pattern of *Nosema* spp. and infection level, may be caused by a diversity of yet unknown factors that have to be identified in future investigations as certain environmental conditions, beekeepers management practices and genetic background of honey bees that influences *Nosema* distribution. Alternatively, an ongoing displacement process of *N. apis* by *N. ceranae* may also result in the observed pattern yet such a chronological process can only be confirmed in a long-term study.

Our results show that *N. ceranae* is the only *Nosema* spp. found to infect honey bees in the different geographical regions of Saudi Arabia. None of the samples was infected with *N. apis*. From the available literature, we can understand that *N. apis* has been present in some Middle east countries (Al-Ghamdi, 1990; Alzubaidy and Ali, 1994; OIE, 2004; Matheson, 1993) as well as in the Europe and America (Paxton et al., 2007; Chen et al., 2009b) for the past decade. Recently *N. ceranae* emerging as new microsporidian infection in middle east and north African countries as already reported in Europe, USA, Canada, and China (El-Shemy et al., 2012; Roudel et al., 2013; Haddad, 2014; Aroee et al., 2016; Williams et al., 2008; Higes et al., 2006). This indicates that *N. ceranae* is a new emerging pathogen for honey bees, and has presumably been transferred from its original host *A. ceranae* to *A. mellifera* (Klee et al., 2007) much earlier than previously recognized (Guerrero-Molina et al., 2016). The evidence, when *N. ceranae* appeared and started to parasitize Saudi Arabian bees is unknown, and it is difficult to investigate the past incidence because of a lack of bee samples. However total absence of *N. apis* might be due to better adaptation of *N. ceranae* to warm climate of Saudi Arabia (Forsgren and Fries, 2013; Martín-Hernández et al., 2012).

This is the first report of molecular detection of *N. ceranae* in Saudi Arabia. Further research and analysis of more colonies are needed to determine the actual prevalence of this new agent in the country. Intensive survey and further research are thus necessary to determine the distribution and prevalence of *Nosema* spp. in the Kingdom of Saudi Arabia and their Preventive measure. This report is an alarm for beekeeping industry of Saudi Arabia and protection from honeybee pathogens. Beekeepers must pay attention when moving their colonies in different season to void the pathogens including *Nosema*.

## 6. Conclusions

Overall, our results provide evidence that *N. ceranae* infection occurs frequently in the cohort of apiaries examined despite the lack of clinical signs. This suggests that colony disease outbreaks might probably be caused by other factors, both known and unknown, that singly or in combination may lead to higher susceptibility of honeybees to *N. ceranae*. The results confirmed the colonization of *N. ceranae* in Saudi Arabia and need further molecular study at a more extensive monitoring level in order to elucidate possible links between infection by *N. ceranae* and colony losses in Saudi Arabia.



## Conflict of interest statement

None of the authors of this article has any conflict of interest.

## Acknowledgements

The project was financially supported by King Saud University, Vice Deanship of Research Chairs. We are grateful to the beekeepers who allowed us to sample their hives.

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