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First molecular detection and geographical distribution of *Nosema apis* & *Nosema ceranae* in indigenous honey bees reared in Algeria

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Abstract

The aim of the present study was to study with the molecular tools the presence and the geographical distribution of Nosema spp. in native honey bees reared in Algeria (Apis mellifera intermissa). The study was carried out on 51 samples of adult honey bees from 11 different regions of Algeria. Total DNA was extracted from the abdomen of honeybees, for each sample, pure genomic DNA was rehydrated in millipore filtered and deionized dH2O and stored at 4 °C. DNA samples were subjected to molecular detection by multiplex PCR using primers specific for a region of the 16S rRNA gene of the Nosema species. The results of the PCR show that Nosema apis (N. apis) and Nosema ceranae (N. ceranae) were detected in the bee samples examined. 22 samples (43.13%) were positive for N. ceranae of which three samples (5.88%) were positive as co-infection with N. apis and N. ceranae, while 29 samples (56.87%) are unscathed from nosemosis infection. Infection with N. apis has always appeared as a co-infection with N. ceranae and apiaries established in western Algeria. The climate is considered to be one of the main factors in the spread of Nosema species. This is the first report on detection of N. ceranae and N. apis, and the level of contamination and distribution pattern in Algeria. This work suggests that our A. mellifera populations may be genetically resistant to these substances, and nevertheless, these findings require further molecular genetics research to be confirmed.

Keywords: Geographical distribution, multiplex PCR, Apis mellifera intermissa, Nosema apis, Nosema ceranae, Algeria.

الملخص:

الهدف من هذه الدراسة هو الاستكشاف بالطرق الجزيئية عن تواجد و طريقة التوزيع الجغرافي لـ مرض النوزيما في نحل العسل الجزائري Apis mellifera intermissa أجريت الدراسة على 51 عينة من نحل العسل موزعة على 11 منطقة مختلفة من الجزائر. تم استخلاص الحمض النووي الكلي من بطن النحل، لكل عينة، تمت إعادة ترطيب الحمض النووي الجيني النقي و تخزينه في البرودة عند 4 درجات مئوية. تم إخضاع عينات الحمض النووي للكشف الجزيئي عن طريق تفاعل البلمرة PCR باستخدام بادئات خاصة بمنطقة من مورثة الحمض النووي الريبوزي الريبوزومي S rRNA16 لمعرفة أنواع النوزيما. أظهرت نتائج اختبار تفاعل البلمرة المتسلسل أن نوزيما أبيس N. apis و نوزيما سيراني (N. ceranae) قد تم اكتشافهما في عينات النحل التي تم فحصها. 22 عينة (43.13٪) كانت موجبة لنوع N. ceranae منها ثلاث عينات (5.88٪) كانت إيجابية كعدوى مشتركة N. apis و N. ceranae بينما لم تتأثر 29 عينة (56.87٪) من عدوى نوزيما. جدير بالذكر أن الإصابة بـ N. apis كانت دوما كعدوى مشتركة مع N. ceranae بالمناحل الموجودة في الغرب الجزائري. كما يعتبر المناخ أحد العوامل الرئيسية في انتشار أنواع نوزيما. هذا هو التقرير الأول الدي يتحدث عن الكشف عن N. ceranae و N. apis ومستوى التلوث ونمط التوزيع في الجزائر. تُظهر هذه الدراسة أيضًا إمكانية وجود مقاومة وراثية لمجموعات النحل المتواجدة لدينا لهذا المرض، ومدى الحاجة إلى مزيد من التحقيقات الجينية الجزيئية لإثبات ذلك.

الكلمات الرئيسية: نحل العسل الجزائري. التوزيع الجغرافي. تفاعل البلمرة المتسلسل. نوزيما أبيس N. apis. نوزيما سيراني N. ceranae .

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Introduction

Nosemosis is a devastating disease of honeybee colonies (Higes *et al*., 2007; Huang *et al*., 2015) caused by one of two species of microsporidia of the genus *Nosema* (Traver & Fell, 2011), *Nosema apis* and *Nosema ceranae*. Zander, (1909) reported that the European bee *Apis mellifera (A. mellifera*) was infested only by *Nosema apis*. However, in Europe, a new species of the same genus, Nosema ceranae, he has been described in Apis ceranae (Fries et al., 1996) and was found in A. mellifera (Higes et al., 2006).These two *Nosema* species can co-infect European bee *A. mellifera* (Martín-Hernández *et al*., 2007; Botías *et al*., 2013).Currently, *N. ceranae* and *N. apis* are reported from four continents as Europe (Martín-Hernández *et al*., 2007;Gisder *et al.,*2010;Odnosum,2017; Shumkova *et al.,*2018; Matović *et al.,*2020), Asia (Yoshiyama *et al.,*2011;Ivgin *et al.,* 2016; Ansari *et al.,* 2017; Khezri *et al.,* 2018).North and South of America (Chen *et al.,*2010; Emsen *et al.,*2016; Pacini *et al.,*2016; Guerrero-Molina *et al.,*2016). In Africa, it has been detected in North Africa (Higes *et al*., 2009; Chahbar *et al*., 2016) and in Benin (Cornelissen *et al*., 2011) on *A. mellifera adansonii*. On the other hand , in Ghana, no species have been detected (Llorens-Picher *et al*., 2017). A third new species, *N. neumani*, was declared in 2017 in Uganda (Chemurot *et al.,* 2017).

N. apis is known to have these clinical symptoms which manifest as diarrhea (Fries, 1993), but this isn't the case in *N. ceranae* (Paxton, 2010). However, some authors report that these two species have no clinical signs of infection (Higes *et al.,* 2010; Maiolino *et al.,* 2014). The infection levels of these two *Nosema* species are different between bee colonies (Chen *et al*., 2009; Mulholland *et al*., 2012; Epilobee *et al.,* 2016; Martin-Hernandez *et al*., 2018). A significant number of differences has been found between the two Nosema species, in morphology (Fries *et al.,* 2013; Ptaszynska *et al*., 2014),in genome size (Chen *et al.,* 2013 ; Gómez-Moracho *et al.,*2014), in spore production (Huang *et al.,* 2013; Martin-Hernandez *et al.,* 2018; Sinpoo *et al.,* 2018),in virulence and impact on bee health(Paxton *et al.,*2007; Higes *et al.,*2008; Martin-Hernandez *et al.,*2011).

Despite these differences, morphological discrimination between these two species by optical microscopy is nearly impossible (Fries et al., 1996), and molecular tools are required to distinguish between the two species of Nosema spp (Klee *et al.,* 2007; Chen *et al.,* 2013; Papini *et al.,* 2017). The conventional diagnosis of the disease is based on the detection of the spores during a microscopic examination. Recently in Algeria, Nosemosis has been reported by the presence of Nosema spores through optical microscopy, assuming *N. ceranae* to be the causal agent (Chahbar *et al.,* 2016). These findings have led to a demand for PCR (polymerase chain reaction) especially multiplex PCR based research that determine, which species of Nosema have been detected in native honeybees. The multiplex 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 (L M. Weiss, C R. Vossbrinck., 1999; Papini *et al.,* 2017). In this study, all samples were screened using a multiplex PCR assay based on species-specific primers targeting the 16S rRNA gene to distinguish between N. ceranae and N. apis.

Methods

Sampling

We sampled adult worker bees from 11 regions in Algeria (Figure 1, Table 1). A total of 51 samples were collected and sampling was performed according to the protocol proposed by the International Organization for Animal Epidemiology (OIE, 2013).

Table 01. Samples distribution.

Region	Tlemcen (1)	Sidi Bel Abbes (2)	Chlef (3)	Tipaza (4)	Alger (5)	Blida (6)	Tissemsilt (7)	Medea (8)	Setif (9)	Batna (10)	Khenchela (11)	**Total**
Number of tested samples	10	10	2	2	4	3	2	3	7	4	4	51

The colonies sampled showed no signs of disease at the time of sampling and had not been treated for infection with Nosema spp. Collected bees were placed in falcon tubes, placed in insulated bags, and stored at −20 °C until laboratory processing.

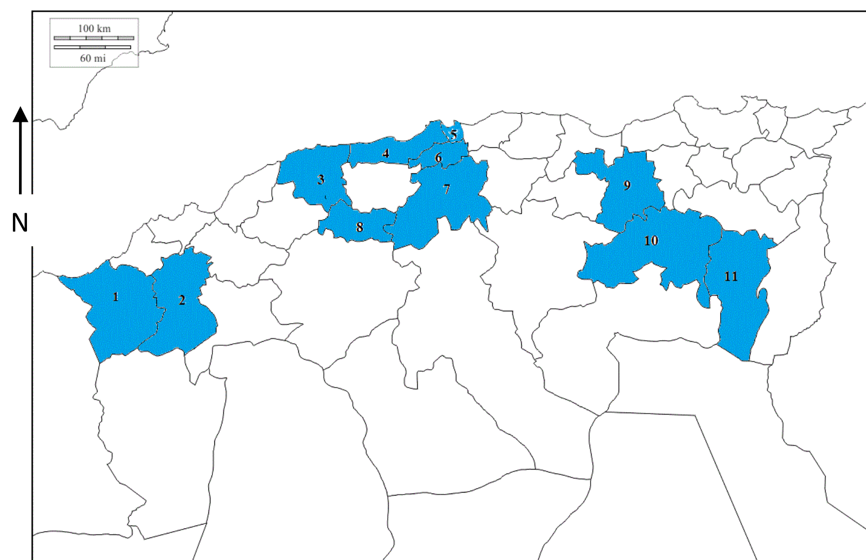

Figure 1. Map of Algeria with the sampled locations. Sampling sites names corresponding to the numbers are shown in Table 1.

DNA Extraction

Total DNA was extracted from the abdomen of honeybees (Higes *et al.,* 2006), from each sample were macerated in 1000µL of distilled water. The suspension was filtered and further 5ml of water used to rinse. The suspension was centrifuged for 6min at 800*g*. The homogenates (200µl) were diluted in 0.3% hydrogen peroxide to induce spore germination (Van Laere, 1976) to easily obtain the DNA. After 15min at room temperature, 0.1 g of glass beads (1mm diameter) was added to each tube and vortexed at 3000 rpm for 1min.Lysis Buffer (100 mM Tris pH 8.0, 10 mM EDTA pH 8, 1% SDS) and 100 mg 1 mm glass beads were then added to each sample and homogenized for 3 min at speed 8 in a Bullet Blender (Next Advance, Inc., Averill Park, NY) and then treated with 80 µL Proteinase K (10 mg/mL) at 70 °C for 10 min. After Proteinase K treatment, 7.5 mM NH4OAc was added for protein precipitation, followed by isopropanol precipitation, 2x70% EtOH washes, and lyophilization (Bourgeois *et al.,*2006). Pure genomic DNA was rehydrated in millipore filtered and deionized dH_2O and stored at 4 °C.

PCR amplification

After the DNA extraction, the DNA samples were submitted to duplex-PCR (Martin-Hernández *et al.,* 2007; Hamiduzzaman *et al.,* 2010). For the diagnosis of two Nosema species, we used two types of primers. The primer sequences used to amplify the 224 bp fragment corresponding to the 16S ribosomal gene of *N. apis* were 224 Mnapis-F: 5'-GCATGTCTTTGACGTACTATG-3' and 224 Muniv-R5'-GACTTAGTAGCCGTCTCTC-3 ' (Fries *et al*., 2013).

The primer sequences utilized to amplify the 143 bp fragment corresponding to the 16S ribosomal gene of *N. ceranae* were 143 Mnceranae-F: 5'-CGTTAAAGTGTAGATAAGATGTT-3' andMuniv-R5'-GACTTAGTAGCCGTCTCTC-3' (Fries *et al*., 2013).

PCR was performed using a thermal MyCycler T100 (Biometra professionnel) in a reaction volume of 20 µL containing 1µL of template DNA, 1µl PCR buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.2 µM of each forward and reverse primer and 1U Taq polymerase. The PCR conditions were 2min at 95 °C (initial denaturation), 35 cycles of 1 min at 95° C, 1 min at 50 °C and1min at 72 °C, and finally 5min at 72 °C (final extension). The PCR products were separated on agarose gels (1.5%) stained with ethidium bromide, visualized and photographed on an UV transilluminator.

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Statistical analysis

Statistical analyzes were performed on 51 observations (colonies) using SAS. 9., the GENMOD procedure of the SAS is used, in order to specify the binomial distribution of the variable. The fixed factors are regions and apiaries within regions. The variable factor chosen for this analysis is the presence of the disease. In other words, we want to know the effects of different regions and different apiaries within regions on the frequency and distribution of the disease across the country. The significance is chosen for a value of p = 0.05.

Results

Nosomosis is a serious bee disease that causes economic losses worldwide. For this purpose, highly reliable diagnostic techniques are required. Multiplex PCR is performed using primers specific for *N. apis* and *N. ceranae*. The results revealed (fig 2) that 22 samples (43.13%) were positive for *N. ceranae* and three samples were positive (5.88%) for *N. apis* (table 2). It is important to emphasize co-infection with *N. apis* /*N. ceranae* (fig 2), originates from apiaries in western Algeria. These results are consistent with those of Chahbar *et al.,* (2016).

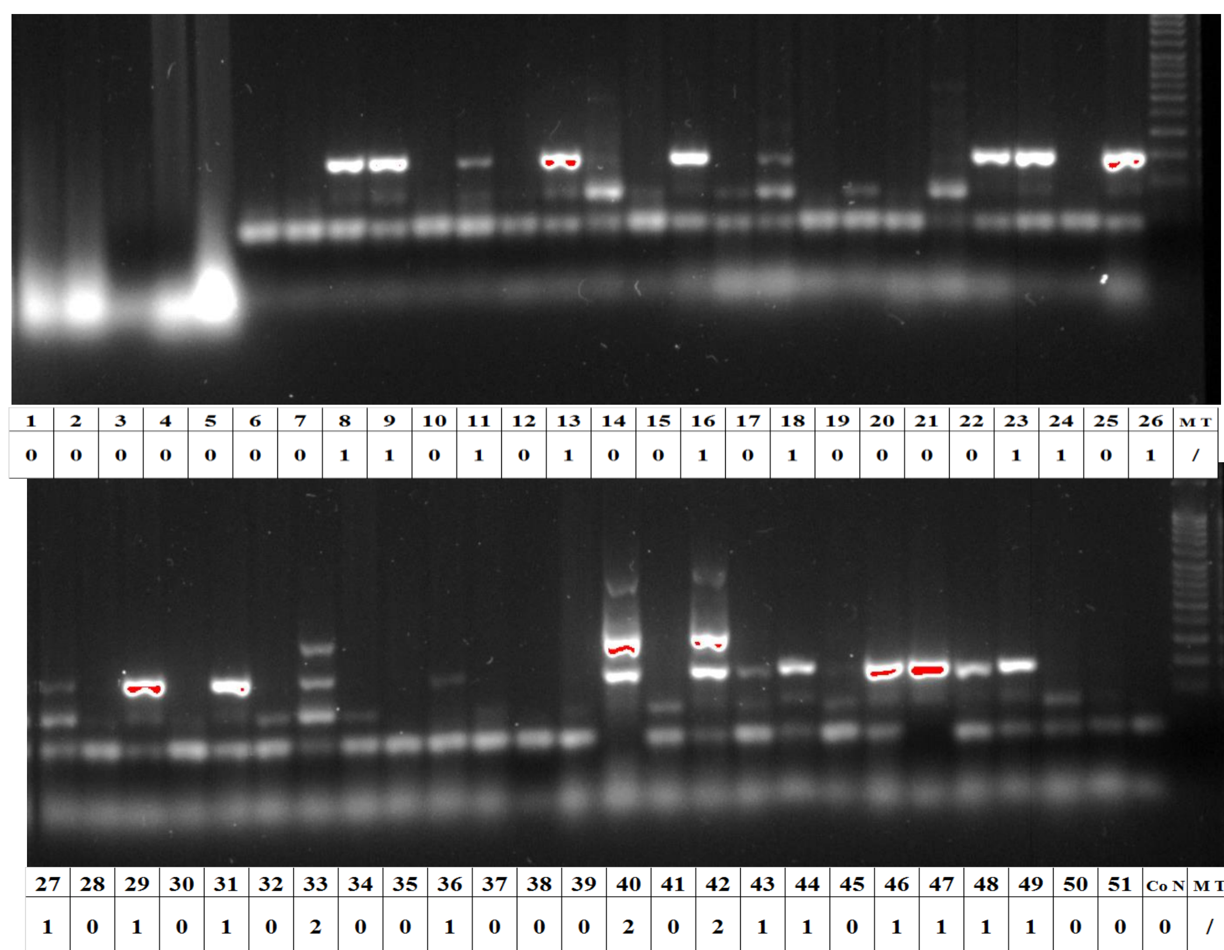

Figure 2 - Agarose gel showing the results of amplification of the 16S gene using the primer Mnapis 224 Pb and Mnceranae 143 Pb.

Lane MT: molecular weight marker, arrowhead indicates the position of 100 pb band:

Lane Co N: Negative control (distilled water)

Samples from central regions of Algeria are the most affected by N. cerenae. A prevalence of 100% has been observed in the regions of Blida and Chlef. The region of Algiers recorded a prevalence of 75% as well as the region of Medea with a prevalence of 66.66%. The lowest prevalence apiaries are located in the mountainous area of Khenchela with 25% followed by Sidi Bel Abbes with 30% and

Sétif with 42.85% with Tlemcen with 50%. No infection was reported in the regions of Batna, Tissemsilt and Tipaza (p <0.05), Beekeeping practices and the trade in queens and workers can be a source of infection in certain regions of Algeria. In addition, transhumance is widely practiced in central regions. (fig 3).

The study region component has statistical significance (p <0,05) when the distribution of the disease is assessed using a generalized linear model with variable response followed by a binomial distribution, adjusted using the SAS software's GENMOD procedure (Table 03). Table 02 shows that the disease frequency differs significantly across the study regions (Chi 2 = 22.16; DF = 10; P = 0.0143), but not among the different beehives inside the regions (Chi 2 = 21.38; DF = 13; P = 0.0658).

Table 02. Prevalence of colonies infected with *N. apis* , *N. ceranae* and co-infection (*N. apis* and *N. ceranae*) in apiaries.

Region	Number of tested samples	Number of positive samples	Prevalence	*N. ceranae*	*N. apis*	Co-infection
Tlemcen (1)	10	5	50%	5	1	1
Sidi Bel Abbes (2)	10	3	30%	3	2	2
Chlef (3)	2	2	100%	2	\	\
Tipaza (4)	2	\	\	\	\	\
Alger (5)	4	3	75%	3	\	\
Blida (6)	3	3	100%	3	\	\
Tissemsilt (7)	2	\	\	\	\	\
Medea (8)	3	2	66,66%	2	\	\
Setif (9)	7	3	42,85%	3	\	\
Batna (10)	4	\	\	\	\	\
Khenchela (11)	4	1	25%	1	\	\
Total	**51**	**22**	**43,13%**	**22**	**3**	**3**

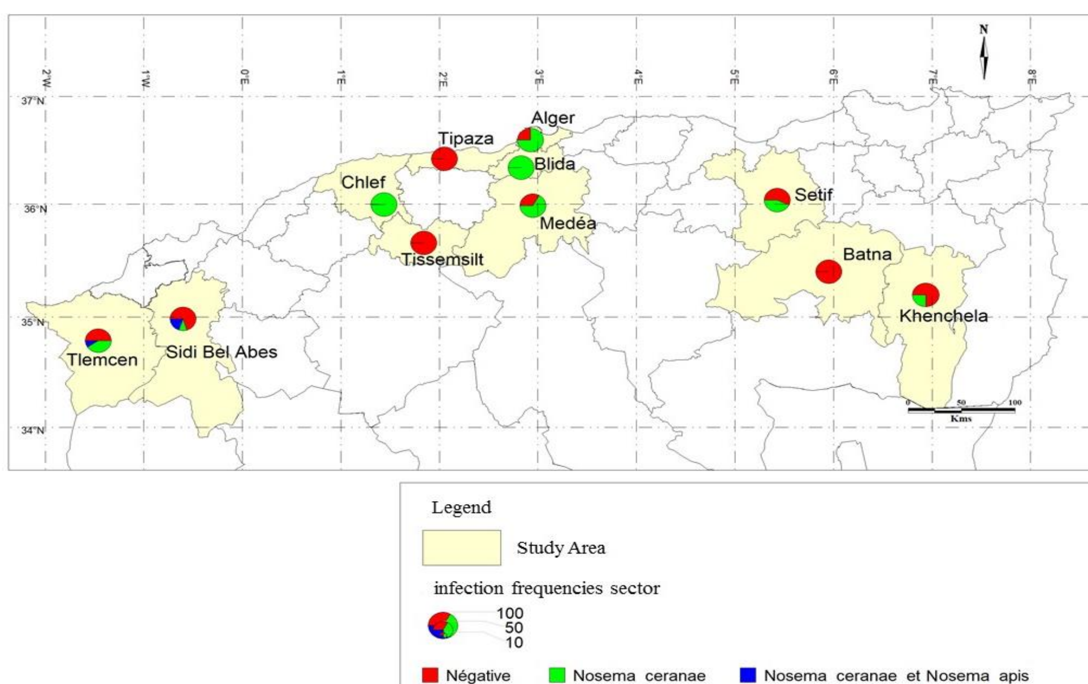

Figure 3- Prevalence of *Nosema* species in apiaries Algerian honeybees. Bee colonies not infected by *Nosema* are indicated in red. Bee colonies infected by *N. ceranae* are indicated in green, bee colonies corresponding to infection by mixed *Nosema* infection are indicated in blue. Sectors in circles indicate representation cases (existence/absence) of an infection frequency.

Table 3: Results of a nosemosis distribution evaluation using SAS GENMOD procedure.

Source	Deviance	DF	Khi 2	Pr > Khi 2
Intercepte	70,2100			
Regions	48,0547	10	22,16	0,0143
Beehives (regions)	26,6794	13	21,38	0,0658

So, while geography has an impact on disease frequency, there is no significant difference in disease incidence among beehives in the same region.

Among the study regions, Batna (Khi 2 = 314.14; DDL = 1; P <0.0001), Bouira (Khi 2 = 6.86; DDL = 1; P = 0.0088), Khenchela (Khi 2 = 286.94; DDL = 1; P <0.0001), Sidi-Belabbes (Khi 2 = 470.41; DDL = 1; P <0.0001), Tlemcen (Khi 2 = 5.06; DDL = 1; P = 0.0245) and Tipaza (Chi 2 = 173.51; DDL = 1; P <0.0001) are characterized by significant differences between the frequencies of onset of the disease. In terms of the rest of the areas, there is no significant difference in the disease's incidence rates. However, no difference are detected bettwen aprairies under each region (P>0.05).

Discussions

The results show that 43.13% of bees were infected with *N. cerenae* and 5.88% were N. apis positive but still co-infected with *N. apis* /*N. ceranae (*Figure 1; Table 2). N. ceranae was found to predominate in almost all areas investigated. This result contradicts the results of his Higes *et al.,* (2009). Furthermore, numerous studies in Europe show that *N. ceranae* replaces *N. apis* (Klee et al., 2007; Gisder et al., 2010; Stevanovic et al., 2011). The climate is considered to be one of the main factors in the spread of *Nosema* species (Fries *et al.,* 2010; Gisder *et al*., 2010, Gisder *et al*., 2017). In warmer climates *N. ceranae* is more competitive than *N. apis*. In contrast, in cold climates spores of *N. ceranae* appear to be much more vulnerable than spores of *N. apis* (Fries *et al.,* 2010, Higes *et al*., 2010,Papini *et al*., 2017). Laboratory studies also suggest that the spread of *N. ceranae* across the globe is reduced in colder climates, as *N. ceranae* spores are able to survive at high temperatures and desiccation, but are intolerant of cold (Gisder *et al.,* 2010, Ansari *et al.,*2017). On the other hand, the impact of meteorological conditions on the distribution of Nosema, mainly *N. ceranae,* in the field, is poorly understood (Fries *et al.,* 2010).

Our study it's the first work using molecular tools to detect the presence of two species of *nosemosis*, *Nosema ceranea* and *Nosema apis,* in the Algerian honey bees. Although Higes *et al*., (2009) reported the presence of *Nosema ceranea* in *Apis mellifera intermissa.* The study based on microscopic observation of Nosemosis in Algeria was made by Chahbar *et al.,* (2016). Given the many similarities of the spores of these two species, their differential diagnosis by microscopic observation is very difficult and sometimes impossible. Therefore, molecular techniques should be used in this regard, who was used in this study.

Differences in prevalence may be due to differences in the number of apiaries examined, sampling methods, geographic areas, characteristics of the honey bee population, diagnostic techniques and other biotic and abiotic factors. In a survey, Higes *et al.,* (2007) reported that none of the 22 *N. ceranae* positive beehives showed no clinical sign of CCD at the time of sampling and / or had a history of signs related to nosemosis caused by *N. ceranae*. Also, Chahbar *et al.,* (2016), reported low infection levels. These results suggest that the species *N. ceranae* could be less virulent than what has been reported by the literature or that the species of *N. ceranae* disseminated in apiaries could be less pathogenic. The populations of Apis mellifera studied can be developed resistance to *N. ceranae*. A combination of these factors could be behind this result.

Conclusion

This is the first molecular study on the presence of *Nosema ceranea* and *Nosema apis* in the Algerian honey bees. The study showed that nosemosis in Algerian honeybees can be caused by *N. ceranae*, alone or in combination with *N. apis*. Also, this study could also indicate that *N. ceranae* replaced *N. apis* in Algeria depending on the sampling region. This study shows also a possibility about a genetic

resistance of our *A. mellifera* populations to these agents how need more molecular genetics investigations to prove it.

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