Short Communication

FIRST DETECTION OF *NOSEMA CERANAE* INFECTING *APIS MELLIFERA* IN INDIA

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Abstract

***Apis mellifera* colonies were sampled for over twelve months to study the *Nosema* infection in different apiaries of Himachal Pradesh, India. We found that the infection incidence was highest in the winter season (48.40 %) followed by autumn and rainy months. The infection was diagnosed conventionally through the microscopic analysis detection of *Nosema* species spores. However, with the recent findings that both *N. ceranae* and *N. apis* affect western honey bees, more sensitive and species specific molecular techniques are required that reliably differentiate between species of microsporidia. Therefore, molecular studies were conducted to precisely differentiate between *Nosema* species infecting honeybee colonies in India. Species specific multiplex PCR amplification using previously reported primers derived from 16S rRNA fragments were performed in a single reaction mixture. A single diagnostic band size of only 143 bp for *N. ceranae* was observed in the agarose gel. Furthermore, a diagnostic band size of 252 bp was observed with primer NoS, thus reconfirming the presence of *N. ceranae*. This study appears to be the first to report the detect of *N. ceranae* species from India in samples of *A. mellifera*.**

Keywords: *Apis mellifera*, India, microscopy, Multiplex PCR, *Nosema ceranae*

INTRODUCTION

Nosema apis (Zander, 1909) and *N. ceranae* (Fries et al., 1996; Higes et al., 2006), two microsporidians responsible for *Nosema* infection in both *A. cerana* and *A. mellifera,* are known to cause substantial economic losses to apiarists and beekeepers worldwide. *N. apis* was the only microsporidian identified in *A. mellifera* until Higes et al. (2006) and Huang et al. (2007) found bees positive for *N. ceranae* in Europe and Taiwan, respectively. The morphological features of microsporidia have been studied by Fries et al. (1996) and Shimanuki & Knox (2000) under both light and transmission electron microscopes but morphological distinction of the spores at the species level remains subtle. Species specific microsporidia identification of both *N. ceranae* and *N. apis* can be detailed by advanced electron microscopic techniques, biochemical analysis and sensitive molecular methods (Fries et al., 2006). *Nosema* infection in India was first scientifically documented by Singh (1974) in the Kumaon region of Uttarakhand. Microscopic examination of adult bee samples revealed the presence of spores of *Nosema* sp., which was at those times considered as *N. apis.* The occasional occurrence of *Nosema spp.* was also reported from other states of India (Kshirsagar, 1982; Rana & Katna, 2011) but the species was not identified with the use of molecular biology. The present study aims to identify the probable *Nosema* species infection of European honey bees in India.

MATERIAL AND METHODS

For these preliminary studies, samples of *A. mellifera* bees were collected during the

summer (April- May), rainy (July- August), autumn (October- November) and winter (December- January) months of the year 2014-2015 from five apiaries of Himachal Pradesh, India (Fig.1). Fifty colonies were examined in each apiary to calculate the percentage of infected colonies whereas one-hundred forager bees from each colony to calculate the percentage of infected bees. To count the average number of *Nosema* spores per bee (Cantwell, 1970), the abdomens of fifty honey bees sampled from colonies with highest level of infection were crushed in 50 ml of water. Suspension was visualized under light microscope (400x). To extract the DNA, abdomens of ten honey bees of each collected sample were homogenized in 10 ml distilled water for five minutes and centrifuged for three minutes at 22000 g. Supernatant was discarded and the pellet was suspended in sterile water. The solution was then vortexed with glass beads at 3000 rpm for 1 min. The DNA was isolated with a DNeasy Plant Mini Kit (Qiagen). The extracted DNA was quantified through nanodrop (Thermo scientific U.S.A) and qualitatively checked on 0.8% agarose gel with a horizontal electrophoresis unit (Biorad). The previously reported primers derived from 16S rRNA (SSU rRNA) were used to differentiate and identify putative *Nosema* spp (Tab. 1). PCR reaction was performed in 0.2 ml tubes in thermocycler Biorad (U.S.A) under conditions described in Tab. 2. DNA isolated from healthy honey bees was used as negative controls in all PCR experiments. Amplification products were separated through electrophoresis in 2 to 2.5 % (w/v) agarose gels. The species specific marker Mnceranae was also confirmed with the more sensitive chip-based Multina electrophoresis system (Shimadzu, Japan). The samples for Multina were prepared through the use of DNA-500 Kit with prepackaged chemical reagents (25 to 500 bp resolution) according to the manufacturer's protocol.

Fig. 1. Map of Himachal Pradesh (India) with the study area.

Table 1.

Details of primer sets used for detection of *Nosema* species

Marker Name	Size of amplified product (bp)			*Annealing temp (°C)	Reference
	N. apis	*N. bombi*	*N. ceranae*		
Nosema	208		212	50.7	Chen et al., 2008
Nos-16S	483		488	50.7	Stevanovic et al., 2011
BOMBICAR		101		55.0	Plischuk et al., 2009
Mnceranae- F			143	55.0	Fries et al., 2013
Mnapis-F	224			55.0	Fries et al., 2013
Mnbombi-F		171		55.0	Fries et al., 2013
Muniv-R				55.0	Fries et al., 2013
NOS	240		252	60.0	Higes et al., 2006
NosA	209			60.0	Webster et al., 2004

* Annealing temperature for particular primer sets as standardized using different gradient temperature range

Table 2.

PCR master mix and general thermal cycling conditions

Contents	Quantity (µl)	Thermal cycling conditions
10x PCR buffer (10mM Tris HCL)	2.0µl	Initial denaturation at 95°C for 2 min for all primer pairs except 12 min for Nosema and NoS- 16S primer pairs. *Annealing at 35 cycles of (95°C-30 s., 50.7°C for 30 s., 72°C-60 s.) Final extension at 72°C-60 s. and hold at 4°C forever
$Mgcl_2$ (50 mM)	0.4 µl	
dNTP mix (10mM)	0.3 µl	
Forward primer	0.4 µl	
Reverse primer	0.4 µl	
Gold Taq polymerase	0.2 µl	
Distilled water	5.3 µl	
DNA sample	1.0 µl	
Total	10 µl	

*Annealing temperature for each primer set is mentioned in Table 1

RESULTS AND DISCUSSION

Nosema infection occurred during autumn, winter and rainy seasons in all the studied apiaries. The average percentage of infected colonies and bees was high during winter (48.4, 55.6 %) followed by autumn (38.4, 42.5%) and rainy (25.2, 29.6 %) months (Fig. 2). The incidence of *Nosema* infection was negligible in the colonies during the summer. In contrast, Lotfi et al. (2009) reported that the infection was of its highest level in the spring (59.5%) but was considered to be low in the fall (0%) and in the summer (3.33%). Rana & Katna (2011) found about 30% *Nosema* infected *A. mellifera* bees on cold, foggy and rainy days due to their long confinement in hives. Langridge (1961) showed that during summer months, colonies generally carry less than 1% *Nosema* infected bees. The average number of spores per bee was counted

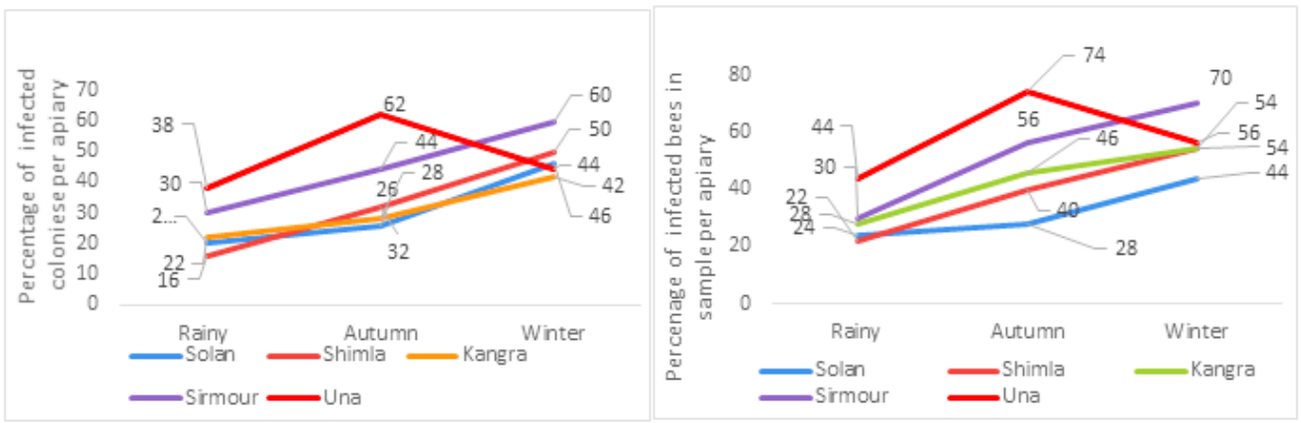

Fig. 2. Percentage of *Nosema* infected *A. mellifera* colonies and mean percentage of bees infected in colony in different apiaries of Himachal Pradesh determined by spore analysis.

to be 1,25,000±5000. The presence of generic primer pairs Nosema and Nos-16S with diagnostic band sizes 212 bp and 488 bp, respectively was confirmed in the samples from Sirmour and Una apiaries (Fig. 3A). A band size of 252 bp was observed with primer Nos (Fig. 3B) diagnostic for the presence of *N. ceranae* in the studied samples from Sirmour and Una. Multiplex PCR reaction was set using species specific pairings of Mnceranae, Mnapis and Mnbombi forward sequences paired with Muniv-Reverse primer derived from partial 16s rRNA gene fragments. An amplicon size of 143 bp diagnostic for *N. ceranae* species was present in one sample

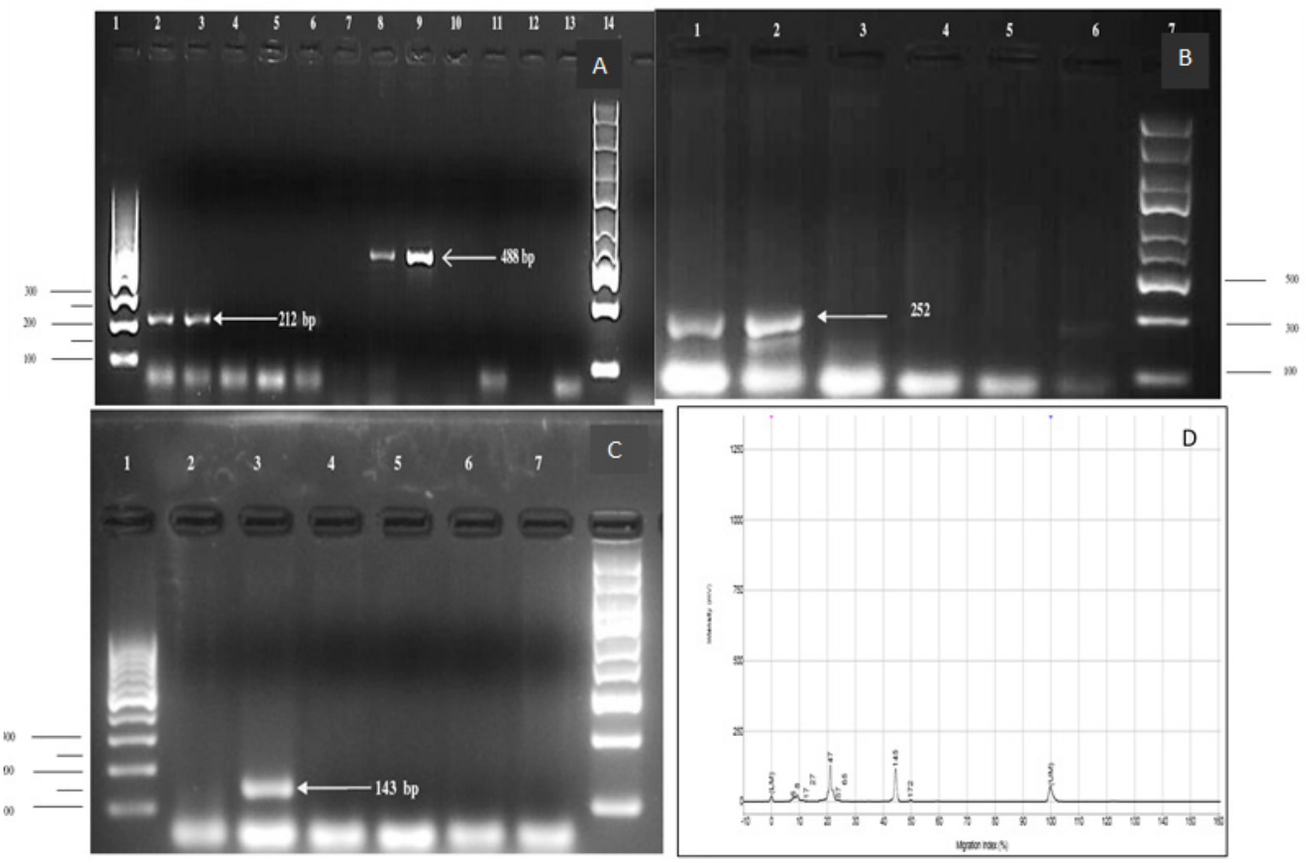

Fig. 3 Gel images of PCR products of *Nosema* species specific primers: (A) Nosema and Nos-16S with diagnostic band size of 212 and 488 bp, (B) Nos with diagnostic band size of 252 bp, (C) Mnceranae primer with diagnostic band size of 143 bp and (D) Electropherogram of Mnceranae in chip-based Multina system showing amplification of 143 bp diagnostic band.

from Sirmour as depicted in Fig. 3C. The same product was also resolved in a more sensitive automated chip-based electrophoresis system (Multina) (Fig. 3D).

Amplification of BOMBICAR and NosA primers was not observed in the PCR reactions, ruling out the possibility that *N. apis* and *N. bombi* were present in the investigated samples. *N. ceranae* was detected only in the samples from Sirmour and Una apiaries with 44.67 - 48% colonies with *Nosema* infection but not detected in the rest of investigated apiaries. This could be due to low DNA concentration in the samples of Solan (1.4 ng/ml), Shimla (28.4 ng/ml) and Kangra (30.2 ng/ml) colonies that were not amplified in the PCR assay as compared to Una (128.4 ng/ml) and Sirmour (45.6 ng/ml) samples. Previous reports by Giersch et al. (2009) indicated that seven bee samples from Australia tested negative using PCR-RFLP but were positive using microscopy and contained very few *Nosema*-like spores. *N. ceranae* has already been reported from other Asian countries (Chen & Huang, 2010), but the precise occurrence of *Nosema ceranae* infecting *A. mellifera* in India has not been identified accurately yet. Based on molecular data, our studies confirm that the microsporidia isolated from Sirmour and Una samples of *A. mellifera* are indeed *N. ceranae*. Although we could not detect spores of *N. apis* and *N. bombi* in the investigated samples, we are not completely sure of their absence or presence as the positive controls using DNA of these species have not been used in PCR reactions. However, our results can pave a way forward in the diagnosis and identification of *N. ceranae* and further investigations in distribution pattern and pathogenicity in Indian races of *A. mellifera*.

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REFERENCES

Cantwell, G.E. (1970). Standard methods for counting *Nosema* spores. *American Bee Journal, 110*, 220-223.

Chen, Y.P., Evans, J.D., Smith, J.B., & Pettis, J.S. (2008). *Nosema ceranae* is a long-present and wide-spread microsporidian infection of the European honey bee (*Apis mellifera*) in the United States. *Journal of Invertebrate Pathology, 147,* 1027-1028. DOI: 10.1016/j.jip.2007.07.010

Chen, Y.P., & Huang, Z.Y. (2010). *Nosema ceranae*, a newly identified pathogen of *Apis mellifera* in the USA and Asia. *Apidologie, 41,* 364-374. https://doi.org/10.1051/apido/2010021

Fries, I., Feng, F., Silva, A.D., & Slemenda, S.B. (1996). *Nosema ceranae* n sp. (Microspora, Nosematidae), morphological and molecular characterization of a microsporidian parasite of the Asian honey bee *Apis cerana*. *European Journal of Protistology, 32*, 356-365. https://doi.org/10.1016/S0932-4739(96)80059-9

Fries, I., Martin, R., Meana, A., Garcia, P., Higes, M. (2006). Natural Infections of *Nosema ceranae* in European honey bees. *Journal of Apicultural Research, 47,* 230-233. https://doi.org/10.3896/IBRA.1.45.4.13

Fries, I., Chauzat, M.P., Chen, Y.P., Doublet, V., Genersch, E., Gisder, S., Willams, G.R. (2013). Standard methods for *Nosema* research. *Journal of Apicultural Research, 52*(1), 110-115. https:// doi.org/10.3896/IBRA.1.52.1.14

Giersch, T., Berg, T., Galea, F., & Hornitzky, M. (2009). *Nosema ceranae* infects honey bees (*Apis mellifera*) and contaminates honey in Australia. *Apidologie. 40*, 117-123. https://doi.org/10.1051/apido/2008065

Higes, M., Martin, R., & Meana, A. (2006). *Nosema ceranae*, a new microsporidian parasite in honey bees in Europe. *Journal of Invertebrate Pathology, 92*(2), 93-95. https://doi.org/10.1016/j.jip.2006.02.005

Huang, W.F., Jiang, J.H., & Wang, C.H. (2007). A *Nose-*

ma ceranae isolate from the honey bee *Apis mellifera*. *Apidologie, 38*, 30-37. https://doi.org/10.1051/apido:2006054

Kshirsagar, K.K. (1982). Current incidence of honey bee diseases and parasites in India. *Bee World, 63*, 162-164. https://doi.org/10.1080/0005772X.1982.11097890

Langridge, D.F. (1961). *Nosema* disease of the honeybee and some investigations into its control in Victoria, Australia. *Bee World, 55*, 36-40. https://doi.org/10.1080/0005772X.1961.11096838

Lotfi, A., Jamshidi, R., Shahryar, H.A., & Yousefkhani, M. (2009). The prevalence of nosemosis in honey bee colonies in Arasbaran region (Northwestern Iran). *American-Eurasian Journal of Agricultural and Environmental Science*, *5*(2), 255-257.

Plischuk, S., Martin, H.R., Prieto, L., Lucia, M., Botias, C., Meana, A., ... Higes, M. (2009). South American native bumblebees (Hymenoptera: Apidae) infected by *Nosema ceranae* (Microsporidia), an emerging pathogen of honeybees (*Apis mellifera)*. *Environmental Microbiology Reports*, *1*, 131-135. https://doi.org/10.1111/j.1758-2229.2009.00018.x

Rana, B.S., & Katna, S. (2011). Incidence of *Nosema* disease in *Apis mellifera* L. of the north India. *Insect Environment, 17*(3), 143-144.

Shimanuki, H., & Knox, D.A. (2000). Diagnosis of Honey Bee Diseases. In *U.S. Department of Agriculture, Agriculture Handbook No. AH- 690*, 61 pp.

Singh, Y. (1974). *Nosema* in Indian honey bees. *Indian Bee Journal, 36,* 16.

Stevanovic, J., Stanimirovic, Z., Genersch, E., Kovacevic, S.R., Ljubenkovic, J., Radakovic, M., Aleksic, N. (2011). Dominance of *Nosema ceranae* in honey bees in the Balkan countries in the absence of symptoms of colony collapse disorder. *Apidologie, 42*, 49-58. https://doi.org/10.1051/apido/2010034

Webster, T.C., Pomper, K.W., Hunt, G., Thacker, E.M., Jones, S.C. (2004). *Nosema apis* infection in worker and queen *Apis mellifera*. *Apidologie, 35*, 49-54. https://doi.org/10.1051/apido:2003063

Zander, E. (1909). Tierische Parasitenals Krankenheitserregerbei der Biene. *Leipziger Bienenzeitung, 24,* 164-166.