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Short Communication

Distribution and prevalence of *Nosema apis* and *N. ceranae* in temperate and subtropical eco-regions of Argentina

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ABSTRACT

A total of 361 colonies from 59 apiaries located in two temperate and three subtropical eco-regions were examined during the post-harvest period to determine distribution and prevalence of *Nosema* spp. Apiaries from subtropical eco-regions showed a lower spore count than those from temperate eco-regions. Pure *N. ceranae* and co-infection were detected in apiaries from all regions. In contrast, pure *N. apis* infection was exclusively observed in the subtropical study region. The predominant detection of *N. apis* in a subtropical region joining a southern temperate region where mainly co-infected apiaries were identified is in contrast to previous reports.

1. Introduction

Nosema apis (Fries, 1993) was the only known etiological agent of Nosemosis in *Apis mellifera* L. until 2005, when additionally *N. ceranae* was identified in Europe (Higes et al., 2006). Both microsporidia were found under different environmental conditions in the northern and southern hemisphere. However, it has been put forward that *N. ceranae* displaces *N. apis* especially under temperate climatic conditions (Giersch et al., 2009).

Evidence of genetic differentiation of the *N. apis* and lack of differentiation of the *N. ceranae* parasite population suggests that the spread of *N. ceranae* throughout *A. mellifera* colonies worldwide is a relatively recent event (Maside et al., 2015). This finding corresponds to previous studies that support the idea that *N. apis* has been replaced by *N. ceranae* in different regions of the world (Klee et al., 2007).

Nosemosis caused by *N. ceranae* in *A. mellifera* may be more virulent than Nosemosis produced by *N. apis*, as the former showed a rapid autoinfective capacity and produced higher mortality rates in worker bees (Higes et al., 2008). Accordingly, it has been suggested that *N. ceranae* could be the cause of the elevated colony losses registered in Europe and United States (Cepero et al., 2016).

Information on the distribution of both species in South America conditions is scarce (Medici et al., 2012; Teixeira et al., 2013). In order to address this knowledge gap, the present study analyzes the current distribution and prevalence of *N. apis* and *N. ceranae*

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in selected temperate and neighboring subtropical climate eco-regions in the post-harvest period.

2. Materials and methods

2.1. Study design and sample size

A cross sectional study was carried out during the post-harvest period from February to June 2015, in north-central Argentina.

A total of 361 colonies from 59 apiaries owned by different beekeepers were sampled. The sample size was calculated based on the fact that there are an estimated number of 5300 apiaries in the study area and an expected prevalence of *Nosema* spp. of 86.1% (Giacobino et al., 2016), with a 95% confidence level and a precision <10%. Five eco-regions were defined based on the nectar flow period, the beekeeping management schedule, the categorization of regions (Burkart et al., 1999; Riveros, 2009), and agricultural practices (RIAN, 2010): South and Central Santa Fe (regions of temperate climate) as well as Warm, Transition and Semi-arid Chaco (regions of subtropical climate) (Table 1).

Apiaries were randomly chosen following stratified randomization procedures (Moher et al., 2010). The proportion of apiaries was distributed according to the officially registered number of apiaries in each eco-region. Within each apiary, a minimum of six colonies or 10% of the total colonies (Lee et al., 2010) have been randomly selected for counting of spores.

2.2. Nosema spore count

A minimum of 60 worker honey bees were randomly collected from the hive entrance using a portable vacuum device and stored in labeled plastic flasks containing ethyl alcohol 96°. Individual bee abdomens were crushed in a morter and 60 ml of distilled water added to prepare a spore suspension. Nosema spores/bee were determined using a light microscope (400× magnification) and a haemocytometer. For each sample the number of spores in 80 hemocytometer squares (5 groups of 16 squares) were counted (Cantwell, 1970 modified by Del Hoyo and Rodríguez, 1997) allowing to detect up to 5% of infected bees with a confidence of 95% (Fries, 1988).

2.3. Determination of Nosema species

Genomic DNA was isolated from samples of Nosema spores obtained from the 59 apiaries using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. Species-specific PCR was carried out as described in Chen et al. (2009).

2.4. Determination of Apis mellifera haplotypes

In order to analyze the genetic variability of *A. mellifera*, a random sampling of individual honeybees from a representative number of apiaries from each eco-region was performed. Total DNA was isolated from the posterior legs of two honey bees derived from 116 colonies from 20 apiaries, as described in Baruffi et al. (1995). The cytochrome oxidase I-cytochrome oxidase II intergenic region (COI-COII) of the mitochondrial genome of *A. mellifera* was amplified using the primers described by Crozier and Crozier (1993) and Lobo Segura (2000). PCR products were digested with *HinfI* (Promega) as described by Agra et al. (2014).

2.5. Statistical analysis

Differences in the presence of *Nosema* spp. and spore counts per bee were compared using a multivariable generalized linear mixed model (GLMM) including region as fixed effects and the apiary as random effect. All statistical analyses were carried out using InfoStat (Di Rienzo et al., 2014).

3. Results

As determined by spore count, *Nosema* spp. was found in 64% of the sampled colonies. In temperate eco-regions, 91.5% and 70% of the colonies resulted positive for *Nosema* spp. from South and Central Santa Fe, respectively. Conversely, in subtropical eco-regions *Nosema* spp. was found in 30%, 35%, and 30% of the colonies from Warm, Transition, and Semi-arid Chaco, respectively. A significant difference in the infection level was found between temperate and subtropical eco-region ($P < 0.001$).

Differential PCR diagnostics demonstrated an exclusive *N. ceranae*-infection in 37.8% of all studied apiaries whereas 26.7% of apiaries were only infected by *N. apis*. The remaining 35.6% apiaries were found to be co-infected by both *Nosema* spp. (Fig. 1).

A distribution analysis showed that apiaries exclusively infected by *N. ceranae* and co- infections were found in all regions (Warm (29.4% and 5.9%), Transition (44.4% and 22.2%), and Semi-arid Chaco (11.1% and 11.1%) and South (37.5% and 62.5%) and Central Santa Fe (25% and 43.8%), respectively). Co-infection was more prevalent in regions with temperate (77.9%) as compared to those of subtropical climate (22.1%) ($P < 0.001$). In contrast, apiaries only infected with *N. apis* were exclusively identified in subtropical eco-regions (Warm (29.4%), Transition (11.1%)), with a predominance in Semi-arid Chaco (66.7%) (Table 1).

A highly significant association ($P < 0.001$) between *Nosema* spp. and spores/bee was found. Infection level in colonies exclusively infected with *N. apis* was estimated to be 9×10^4 spores/

Table 1
Eco-region characterization based on annual mean temperature and precipitation, land use, floral resources, apiaries surrounding vegetation, monitoring time and infection level (Nosema spores/bees).

Eco-region (no. of colonies included)	Annual temperature (°C)	Annual precipitation (mm)	Main land use	Nectar/pollen flow	Apiaries surrounding vegetation	Monitoring time	Infection level mean (SD)
South Santa Fe (47)	18	600–1100	Soy, corn, and wheat	Short (less than three months)	Forest-crops	February and March	9.92×10^5 (1.57×10^6)
Central Santa Fe (101)	17–18	800–900	Dairy farms and wintering animals on alfalfa pastures	Intermediate (three –four months)	Forest-crops	February, March and April	6.24×10^5 (1.81×10^6)
Warm Chaco (89)	23	>1200	Small farmstead, livestock or forest and rice production	Long(between 9 and 10 months)	Forest-grassland	March, April, May and June	4.56×10^4 (2.62×10^5)
Transition Chaco (63)	23–24	<1000	Cereals, oleaginous, and cottonseed crops mixed with livestock production	Long(between 9 and 10 months)	Forest-grassland	March, April, May and June	7.58×10^4 (2.32×10^5)
Semi-arid Chaco (61)	23	550–800	Forest production	Long(between 9 and 10 months)	Forest-grassland	March, April, May and June	7.99×10^4 (3.54×10^5)

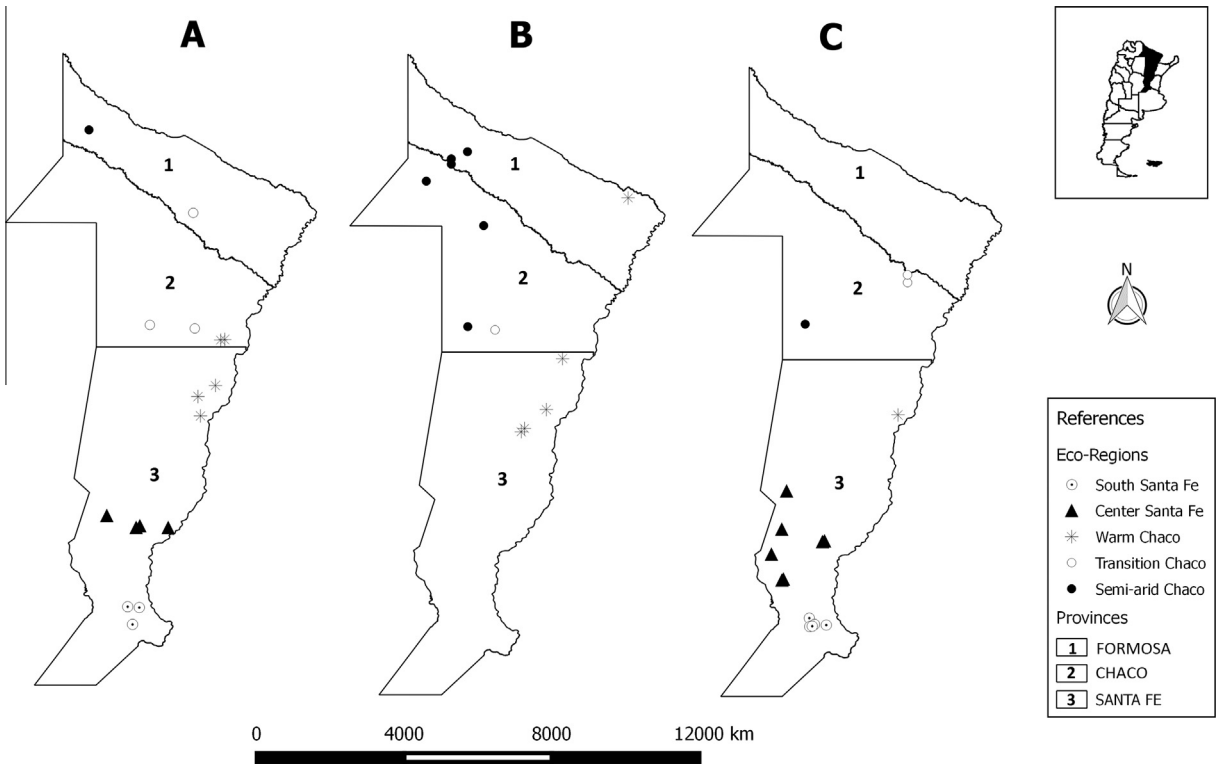

Fig. 1. Distribution of monitored apiaries within eco-regions. Location of apiaries with co-infection (A), pure *N. apis*-infection (B), and pure *N. ceranae*- infection (C).

bee whereas colonies where only *N. ceranae* or co-infection was detected showed higher numbers of 6.5×10^5 and 7.8×10^5 - spores/bee, respectively.

The European lineage C (C1 and C2j haplotypes) and the African lineage A (A1 and A4 haplotypes) were observed in *A. mellifera*. A significant association was found between climatic regions and *A. mellifera* haplotype distribution ($P = 0.004$). European-derived haplotype lineages were found in 84% of the samples from temperate eco-regions whereas 79.5% of samples from subtropical eco-regions were identified as African derived haplotype lineages. No association between *Nosema* spp. and *A. mellifera* haplotype was found ($P = 0.238$).

4. Discussion

The geographic distribution of *Nosema* spp. presented three core features: (i) the presence of pure *N. ceranae*-infected colonies in all studied regions, (ii) a significantly higher proportion of co-infection in the temperate study region, and (iii) the presence of colonies with pure *N. apis*-infection exclusively in the subtropical regions. Furthermore, our results show a higher proportion of apiaries infected with *N. ceranae* than *N. apis*, as has been documented in many regions worldwide (Martin-Hernandez et al., 2012).

However, contrary to recent publications (Medici et al., 2012; Bravo et al., 2014), we could demonstrate a strong occurrence of *N. apis* in an adjacent subtropical region during the post-harvest period. This finding seems to be in contrast with the idea that *N. ceranae* is more competitive than *N. apis* in warmer climates (Forsgren and Fries, 2012). The high prevalence of *N. ceranae* and *N. apis* co-infection in the studied temperate eco-regions may suggest that *N. ceranae* is moving from southern temperate to northern subtropical climate regions. However, this hypothesis would need to be tested in future studies.

It has been put forward that the genetic origin of bees might be a factor that explains observed distribution patterns of *Nosema* spp. (Mendoza et al., 2014). We found that Africanized haplotypes of *A. mellifera* were more common in subtropical than in temperate eco-regions, as suggested by a previous study (Agra et al., 2014). However, in our study, no significant associations between honey bee haplotypes and *Nosema* spp. could be found. Nonetheless, we do not thoroughly discard the possibility that using higher sample numbers and an improved study design such an association of bee genetics and *Nosema* spp. may be revealed.

An increasing gradient of co-infection and counts of *Nosema* spp. were observed from warmer (Warm, Semi-arid and Trasition Chaco) to colder (Central and South Santa Fe) regions in Argentina whereas a strong presence of *N. apis* was found in the subtropical study region. This paradoxical 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, although, we could not find statistical evidence for this hypothesis in our study, we would not yet discard that the genetic background of honey bees may also be a factor that influences *Nosema* distribution. Alternatively, an ongoing displacement process of *Nosema apis* by *Nosema ceranae* may also result in the observed pattern yet such a chronological process can only be confirmed in a long-term study.

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