Parasitol Res
DOI 10.1007/s00436-011-2566-2

ORIGINAL PAPER

Genetic variation and widespread dispersal of *Nosema ceranae* in *Apis mellifera* apiaries from Argentina

Sandra Karina Medici · Edgardo Gabriel Sarlo · Martín Pablo Porrini · Martín Braunstein · Martín Javier Eguaras

Received: 20 May 2010 / Accepted: 19 July 2011
© Springer-Verlag 2011

Abstract Using molecular techniques, we documented the presence of *Nosema ceranae* in honeybees (*Apis mellifera*) from Argentina. Samples were collected from *A. mellifera* colonies in 38 districts of Buenos Aires province, Argentina. Molecular characterization was achieved with a multiplex PCR-based method, which allows parallel diagnosis of *N. ceranae* and *N. osema apis*. *N. ceranae* was identified in all the samples analyzed. Moreover, coinfections with *N. apis* were detected in Balcarce and Maipú districts. We identified three rRNA sequence variants of *N. ceranae*, which may represent diverse sources of bee importation. The results suggest that *N. ceranae* is widely distributed in Argentina and that the genetic variation observed between the different isolates could be related with the difference in the symptomatology found previously by our work group. Our results highlight the need to re-assess the health protocols currently in force so that they recognize *N. ceranae* as the main causal agent of *Nosemosis* in this country.

S. K. Medici · E. G. Sarlo · M. P. Porrini · M. J. Eguaras
Laboratorio de Artrópodos,
Universidad Nacional de Mar del Plata,
Funes 3350,
7600 Mar del Plata, Argentina

M. Braunstein
Apícola Malka S.R.L.,
La Plata, Argentina

S. K. Medici (✉)
Fares Taie Instituto de Análisis,
Rivadavia 3331,
7600 Mar del Plata, Argentina
e-mail: skmedici@mdp.edu.ar

S. K. Medici · M. J. Eguaras
Comisión Nacional de Investigaciones Científicas (CONICET),
Mar del Plata, Argentina

Introduction

Microsporidia are intracellular parasites commonly present in invertebrates. Taxonomic classification is mostly restricted to morphologic and physiologic data. Since many attempts to amplify large rDNA subunits have not been successful across all microsporidian species (Vossbrinck et al. 1987), 16S SSU rDNA has become the standard sequence for molecular characterization of microsporidia (Weiss and Vossbrinck 1999).

In *Apis mellifera*, nosemosis is a disease that affects adult honeybees. It is caused by two members of *Nosema* genus (Microsporidia, Nosematidae) with worldwide distribution: *Nosema apis* Z. (1909) and *Nosema ceranae* (Fries et al. 1996; Higes et al. 2006; Klee et al. 2007). Nosemosis has been studied by our work group since 1999. In Argentina, symptoms have differed from those reported for *N. apis*, but have been consistent with those described for *N. ceranae*, including mortality without dysentery (Faucon et al. 2002), greater pathogenicity (Fries et al. 2006), and a massive depopulation of honeybee colonies (Higes et al. 2006) with heavy losses in winter (Higes et al. 2007).

Shafer et al. (2009) found that *N. ceranae* was more related to *N. bombii* than *N. apis* and suggested that these variations could be related to the different degrees of virulence observed in apiaries with similar parasitic intensities.

Widespread distribution of *N. ceranae* and its symptoms have been reported in Argentina (Sarlo et al. 2008). The aims of this work were to study the distribution of *N. ceranae* in Argentina and to determine if there is genetic variation in this parasite among the different apiaries that explains the difference in the recorded symptoms.

Published online: 02 August 2011

Material and methods

Bee samples collection

Samples were collected from honeybee colonies in 38 districts of Buenos Aires province (Fig. 1) from March to August 2008. One hundred returning flight bees were collected from five randomly selected colonies. Each sample was individually fixed in 96% ethyl alcohol.

Spores collection

Fifty bees from each sample gathered from the apiaries were macerated using a tissue grinder (Universal grinding Fw100, Hangzhou Chincan Tranding Co., Ltd). Then, each suspension was filtered through a 1-mm^2 metallic mesh. Each sample was examined by microscopy to confirm the presence of *Nosema* sp. spores. The spores obtained were purified using the “triangulation” method described by Cole

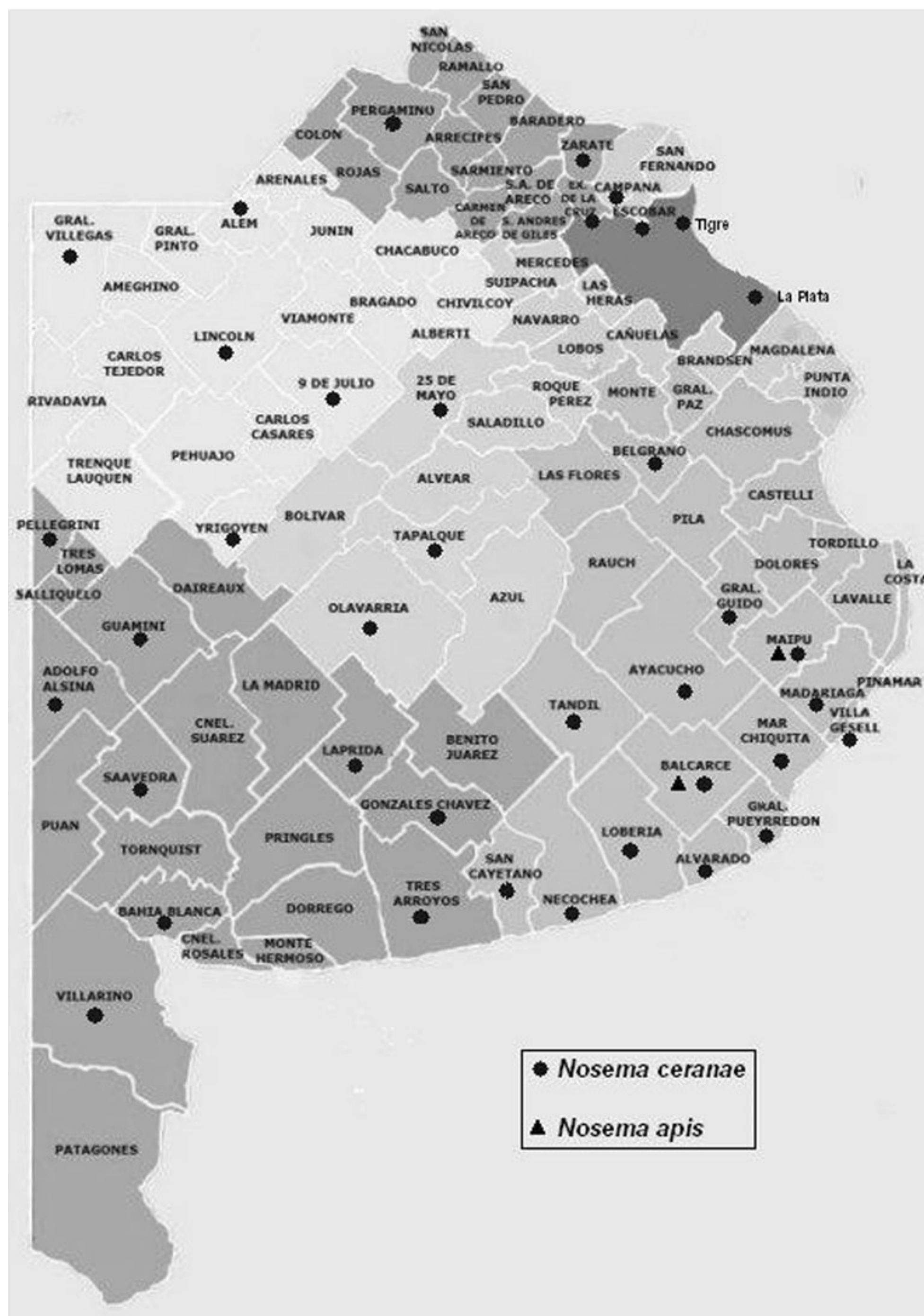

Fig. 1 Distribution of Nosema species detected in the districts comprising Buenos Aires province. *N. apis* (*filled triangle*) and N. ceranae (*filled circle*)

(1970), transferred to Eppendorf tubes and centrifuged at 800 rev/min for 3 min. The supernatant was replaced by 96° alcohol until covering the pellet, and stored at 4°C until DNA extraction, yielding a total of 76 samples.

DNA extraction

Purified spores were washed twice in 200 µl of distilled water and centrifuged at 800 rev/min for 6 min each, with the supernatant removed in each case. The final precipitate was resuspended in 200 µl of germination buffer (De Graaf et al. 1993) and incubated at 37°C for 30 min.

A High Purelink Total Plant DNA Purification Kit (Invitrogen®) was used to extract the DNA, and the resulting product was stored in 10:1 Tris/EDTA buffer at −20°C.

Multiplex PCR assay

Nosema spores were molecularly characterized according to a multiplex PCR-based method (Martín-Hernández et al. 2007).

Amplifications were carried out with a Thermal Cycler Perkin Elmer Model 9600. Forty-five microliters (12.5 U) of Platinum PCR Supermix (Invitrogen®), 0.2 µM of each primer, and 2.5 mM of Cl_2Mg were added to the 50 µl reaction mix, which consisted of 10 µl template DNA. The PCR protocol employed was 94°C for 2 min for initial denaturation, followed by 35 cycles of 15 s at 94°C, 30 s at 55°C, 50 s at 72°C, and 2 min at 72°C for final extension.

The PCR products obtained in these reactions were visualized in 2% agarose gel using a size ladder (100 bp, Invitrogen®). All fragments whose length coincided with those expected for *N. ceranae* (218 bp) were purified and sequenced afterward.

DNA sequencing

The PCR fragments obtained were purified with the ExoSap-IT kit (Amersham, Biosciences) and sequenced in an automatic MegaBACE Sequence Analyzer (Amersham, Biosciences). The sequences, in turn, were aligned in accordance with those published in the GenBank (NCBI) for *N. ceranae* and *N. apis* using T-Coffee software (Poirot et al. 2003) and including *Vairimorpha cerevisae* as outgroup reference.

Phylogenetic analysis

The taxonomic status of the microsporidia under study was determined by analysis of phylogenetic relationship, length, G + C content, identity, and divergence of the SSU rRNA sequences. BioEdit software (Hall 1999) was used to manually edit the sequences after the multiple alignment. For the relationship analysis between taxons, the Phylogeny.fr software package (Dereeper et al. 2008) was used. This included BioNJ neighbor-joining among related taxa (Gascuel 1997; Felsenstein 1989; Elias and Lagergren 2007), Phyml for maximum likelihood (Guindon and Gascuel 2003; Anisimova and Gascuel 2006) and MrBayes for Bayesian inference determinations (Huelsenbeck and Ronquist 2001). The phylogenetic tree was constructed by means the TREEVIEW phylogeny display program (Page 1996).

Results

All samples of bees containing microsporia spores were positive for *N. ceranae*, whereas only 2.6% of samples were positive for *N. apis* spores (Fig. 1).

The alignment of the sequences obtained from all samples (Fig. 2) allowed us to differentiate three haplotypes with either insertions (G) or deletions (A, T), which were consistent with those described by Huang et al. (2005) and Williams et al. (2008).

The haplotypes were published in GenBank and identified as FJ545267 (Strain 1), FJ545268 (Strain 2), and FJ545269 (Strain 3), respectively.

A consensus sequence was derived for *N. ceranae* presence in Buenos Aires province based on the sequences obtained in the different districts, and deposited in the GenBank database (http://www.ncbi.nlm.nih.gov, Accession No. FJ425736) (Benson et al. 2005).

The fragment obtained corresponded to the 16S rRNA gene subunit of *N. ceranae*, which includes nucleotides 676 to 853, and was 98% similar to those published for *N. ceranae* in the GenBank database (Accession No. EF584422, EF458657, FJ452785, DQ374656, DQ673615, DQ286728, and AF141130) and 83% similar to those of *N. apis* (Accession No. DQ235446, X73894, EU545140, EF458660, and EF584423) (Fig. 3).

The haplotype variation and divergence analyses performed using the neighbor-joining and maximum likelihood methods presented very similar topology. The Hasegawa–Kishino–Yano (HKY) sequence evolution model was implemented to conduct such analyses.

The Bayesian logistic regression, which evidences the evolutionary relationships between the haplotypes generated by Single Nucleotide Polymorphism (SNP), was used to explain the variation and history of the haplotypes found. This model incorporates environmental factors along with the relationships between SNPs. The logical tree was constructed (Fig. 3) on the basis of this analysis, using *V. cerevisae* as the outgroup reference.

Based on the phylogenetic tree obtained (Fig. 3), it could be observed that the sequence of *N. ceranae* parasitizing *Apis ceranae*, as was described in China, is phylogeneti-

```
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      605        615        625        635        645        655
N.cer Aleman CGACGATGTG A-TATG-AAA ATATTAATTT GTATTACAT- AATAGAAATT TGAGTTTTTT
N.cer FranciaCGACGATGTG A-TATGGAAA ATATTAATTT GTATTACAT- AATAGAAATT TGAGTTTTTT
N.cer Canada CGACGATGTG A-TATG-AGA ATATTAATTT GTATTACAT- AATAGAAATT TGAGTTTTTT
Madariaga    ---------- ---------- -------CTT GTATACTA-- -ATAGAAATT -GAGTTTTTT
Delta        ---------- ---------- -------CTT GTATACTAT- AATAGAAATT TGAGTTTTTT
Ayacucho     ---------- ---------- -------CCC TTGATACAT- AATAGAAATT -GAGTTTTTT
Mar del Plat ---------- ---------- -------CTT GTATAACATG AATAGAAATT TGAGTTTTTT
Consensus    ---------- ---------- ---------- -----ACATG AATAGAAATT TGAGTTTTTT
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      665        675        685        695        705        715
N.cer Aleman GGCTCTGGGG ATAGTATGAT CGCAAGATTG AAAATTAAAG AAATTGACGG AAGAATACCA
N.cer FranciaGGCTCTGGGG ATAGTATGAT CGCAAGATTG AAAATTAAAG AAATTGACGG AAGAATACCA
N.cer Canada GGCTCTGGGG ATAGTATGAT CGCAAGATTG AAAATTAAAG AAATTGACGG AAGAATACCA
Madariaga    GGCTCTGGGG ATAGTATGAT CGCAAGATTG AAAATTAAAG AAATTGACGG AAGAATACCA
Delta        GGCTCTGGGG ATAGTATGAT CGCAAGATTG AAAATTAAAG AAATTGACGG AAGAATACCA
Ayacucho     GGCTCTGGGG ATAGTATGAT CGCAAGATTG AAAATTAAAG AAATTGACGG AAGAATACCA
Mar del Plat GGCTCTGGGG ATAGTATGAT CGCAAGATTG AAAATTAAAG AAATTGACGG AAGAATACCA
Consensus    GGCTCTGGGG ATAGTATGAT CGCAAGATTG AAAATTAAAG AAATTGACGG AAGAATACCA
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      725        735        745        755        765        775
N.cer Aleman CAAGGAGTGG ATTGTGCGGC TTAATTTGAC TCAACGCGAG GTAACTTACC AATATTTTAT
N.cer FranciaCAAGGAGTGG ATTGTGCGGC TTAATTTGAC TCAACGCGAG GTAACTTACC AATATTTTAT
N.cer Canada CAAGGAGTGG ATTGTGCGGC TTAATTTGAC TCAACGCGAG GTAACTTACC AATATTTTAT
Madariaga    CA-GGAGTGG ATTGTGCGGC TTAATTTGAC TCAACGCGAG GTAACTTACC AATATTTTAT
Delta        CA-GGAGTGG ATTGTGCGGC TTAATTTGAC TCAACGCGAG GTAACTTACC AATATTTTAT
Ayacucho     CA-GGAGTGG ATTGTGCGGC TTAATTTGAC TCAACGCGAG GTAACTTACC AATATTTTAT
Mar del Plat CA-GGAGTGG ATTGTGCGGC TTAATTTGAC TCAACGCGAG GTAACTTACC CATATTTTAT
Consensus    CA-GGAGTGG ATTGTGCGGC TTAATTTGAC TCAACGCGAG GTAACTTACC AATATTTTAT
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      785        795        805        815        825        835
N.cer Aleman TATTTTGAGA GAACGGTTTT TTGTTTGAGA ATGATAATAG TGGTGCATGG CCGTTTTCAA
N.cer FranciaTATTTTGAGA GAACGGTTTT TTGTTTGAGA ATGATAATAG TGGTGCATGG CCGTTTTCAA
N.cer Canada TATTTTGAGA GAACGGTTTT TTGTTTGAGA ATGATAATAG TGGTGCATGG CCGTTTTCAA
Madariaga    TATTTTGAGA GAACGGTTTT TTGTTTGAGA ATGA------ ---------- ----------
Delta        TATTTTGAGA GAACGGTTTT TTGTTTGAGA ATGA------ ---------- ----------
Ayacucho     TATTTTGAGA GAACGGTTT- ---------- ---------- ---------- ----------
Mar del Plat TATTTTGAGA GAACGGTTTT TTGTTTGAGA ATGA------ ---------- ----------
Consensus    TATTTTGAGA GAACGGTTTT TTGTTTGAGA ATGA------ ---------- ----------
```

Fig. 2 Haplotypes of *N. ceranae* recorded in Buenos Aires province and aligned in agreement with sequences described in other countries (GenBank)

cally separate from that found parasitizing *Apis mellifera* worldwide. The haplotypes of *N. ceranae* corresponding to Buenos Aires province differed from those described in Europe. Likewise, it is evident that the haplotypes from Ayacucho (GenBank Accession No. FJ545269) and Mar del Plata (GenBank Accession No. FJ545268) were phylogenetically distant from the others, suggesting that microsporidia could have entered to the country from different directions.

Discussion

The detection of *N. ceranae* in the 38 surveyed districts indicates the wide distribution of this parasite in Buenos Aires province.

Three rRNA sequence variants of *N. ceranae* were found based on the sequences obtained. Even though the 16S/SSU rRNA gene exists in multiple copies in other microsporidia (O'Mahony et al. 2007), this might suggest the existence of different entrance sites to the country, which is consistent with the continuous and varied importation sources of bees.

These results could become the platform for future research as to whether the different haplotypes influence the virulence in *N. ceranae*, as suggested by Tay et al. (2005) for *N. apis* and by Williams et al. (2008) for *N. ceranae*. We agree with Fries (2010), who suggests field studies to confirm this as a general phenomenon.

N. apis is at a much lower frequency and present only in coinfections with *N. ceranae.* This tendency of *N. ceranae* to replace *N. apis* has been described by Fries (2010) in populations of European honeybees. Recently, Burgher-

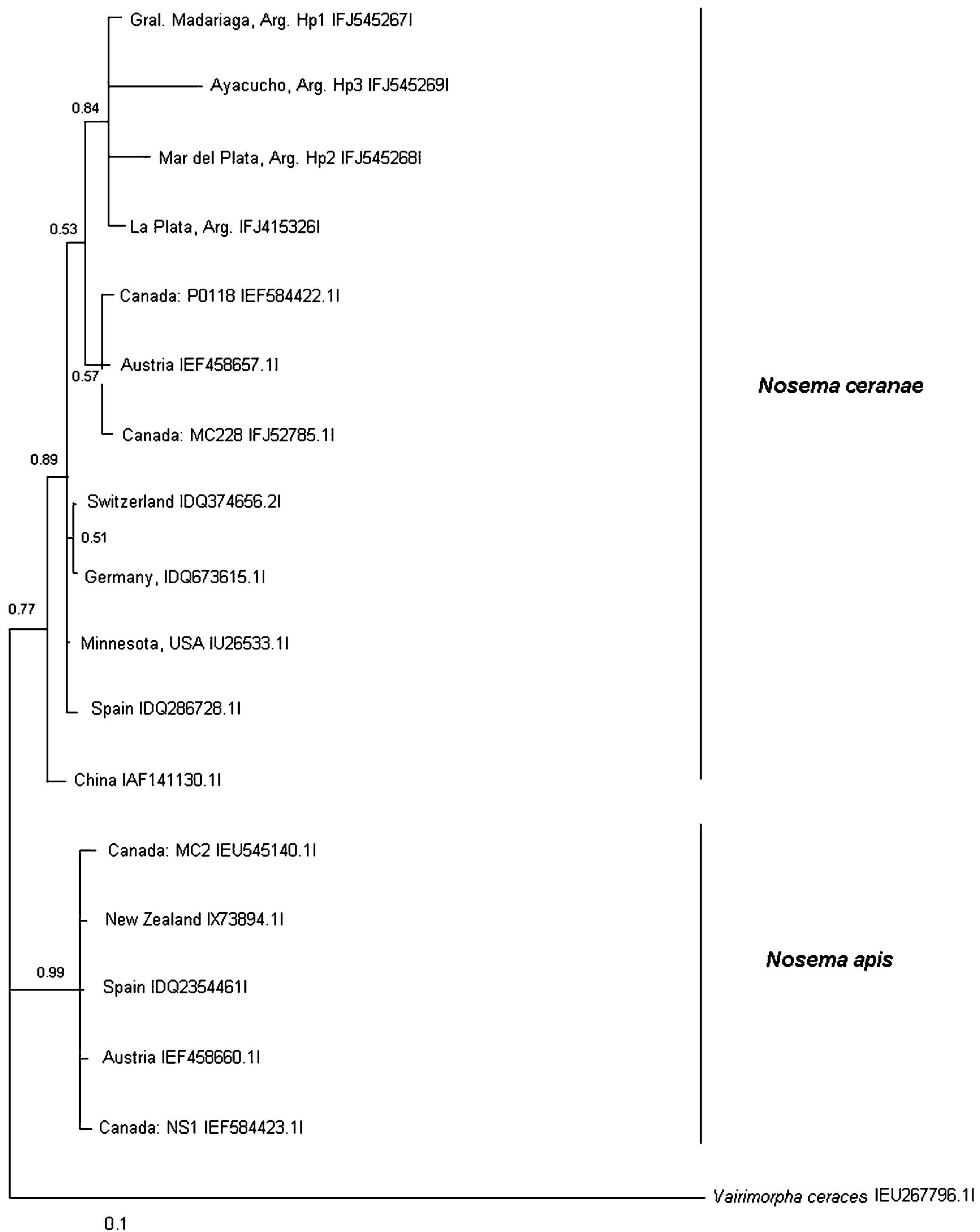

Fig. 3 Phylogenetic tree obtained (MrBayes). Position of *N. ceranae* sequences present in Buenos Aires province, Argentina

MacLellan et al. (2010) have improved a methodology to detect coinfections of *Nosema* sp in honeybees using Real Time PCR with High Resolution Melting curve. This method is more sensitive than end point PCR in detecting *N. apis* and perhaps will expedite detection of this parasite in samples where we could only see the band corresponding to *N. ceranae*.

Our results highlight the need to reassess the health protocols currently in force such that *N. ceranae* is reconsidered as the main causal agent of Nosemosis in Argentina.

Acknowledgements We thank the Comisión de Investigaciones Científicas (CIC) and PICT 890 ANPCyT for financial support. We also want to thank the beekeepers who collected and sent us the samples of honeybees, because without their collaboration this work would not have been possible: Edelmar Abratte, Muselli Carlos, Delpiano Jorge, Baquero Alcides, Meschino Horacio, Paravicini Mauricio, Ormazabal, Fabi, Bartuche, Gonzalez Alfredo, Arévalo, Balbi, Poffer, Defeudis Leonardo, Hoffer, Robenaker, Schafen Ana, Izaguirre, Machín marta, Aguirre, Espié Jorge, Torchelli Mauricio, Quies Roberto, Lliteas Gustavo, Colombani Martín, Grupo Apibal, Frígoli Juan Carlos, Serritela, Tambascia.

References

Anisimova M, Gascuel O (2006) Approximate likelihood ratio test for branches: a fast, accurate and powerful alternative. Syst Biol 55:539–552

Benson D, Karsch-Mzrachi I, Lipman DJ, Ostell J, Wheeler DL (2005) GenBank. Nucleic Acids Res 33:D34–D38

Burgher-MacLellan KL, Williams GR, Shutler D, MacKenzie K, Rogers REL (2010) Optimization of duplex real-time PCR with melting-curve analysis for detecting the microsporidian parasites *Nosema apis* and *Nosema ceranae* in *Apis mellifera*. Can Entomol 142:271–283

Cole R (1970) The application of the "triangulation" method for the purification of *Nosema* spores from insect tissues. J Invertebr Pathol 15:193–195

De Graaf D, Masschelein G, Vandergeynst F, De Brabander H, Jacobs F (1993) In vitro germination of *Nosema apis* (Microspora: Nosematidae) spores and its effect on their αα-Trehalose/D-Glucose ratio. J Invertebr Pathol 62:220–225

Dereeper A, Guignon V, Blanc G, Audic S, Buffet S, Chevenet F, Dufayard J, Guindon S, Lefort V, Lescot M, Claverie J, Gascuel O (2008) Phylogeny.fr: robust phylogenetic analysis for the non-specialist. Nucleic Acids Res 36:w465–w469

Elias I, Lagergren J (2007) Fast computation of distance estimators. BMC Bioinforma 13:89

Faucon J, Mathieu I, Ribiére A, Drajnudel P, Zeggane S, Aurieres C, Aubert M (2002) Honey bee winter mortality in France in 1999 and 2000. Bee World 83:14–23

Felsenstein J (1989) Phylip—phylogeny inference package (version 3.2). Cladistics 5:164–166

Fries I (2010) *Nosema cerane* in European honey bees (*Apis mellifera*). J Invertebr Pathol 103:S73–S79

Fries I, Feng F, Da Silva A, Slemenda S, Pieniazek J (1996) *Nosema ceranae n.* sp. (Microspora, Nosematidae), morphological and molecular characterization of a microsporidian parasite of the Asian honey bee *Apis cerana* (Hymenoptera, Apidae). Eur J Protistol 32:356–365

Fries I, Martín R, Meana A, García-Palencia P, Higes M (2006) Natural infections of *Nosema ceranae* in European honey bees. J Apic Res 45:230–233

Gascuel O (1997) BIONJ: an improved version of the NJ algorithm based on a simple model of sequence data. Mol Biol Evol 14:685–695

Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 52:696–704

Hall T (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. Nucl Acids Sym Ser 41:95–98

Higes M, Martín R, Meana A (2006) *Nosema ceranae*, a new microsporidian parasite in honeybees in Europe. J Invertebr Pathol 92:93–95

Higes M, García-Palencia P, Martín-Hernández R, Meana A (2007) Experimental infection of *Apis mellifera* honeybees with *Nosema ceranae* (Microsporidia). J Invertebr Pathol 94:211–217

Huang W, Jiang J, Chen Y (2005) Complete rRNA sequence of the *Nosema ceranae* from honeybee (*Apis mellifera*). Dissertation, National Taiwan University, Taipei

Huelsenbeck JP, Ronquist F (2001) MrBayes: Bayesian inference of phylogenetic trees. Bioinforma 17:754–755

Klee J, Besana A, Genersch E, Gisder S, Nanetti A, Tam D, Chinh T, Puerta F, Ruz J, Kryger P, Message D, Hatjina F, Korpela S, Fries I, Paxton R (2007) Widespread dispersal of the microsporidian *Nosema ceranae*, an emergent pathogen of the western honey bee, *Apis mellifera*. J Invertebr Pathol 96:1–10

Martín-Hernández R, Meana A, Prieto I, Martínez Salvador A, Garrido-Bailón E, Higes M (2007) Outcome of colonization of *Apis mellifera* by *Nosema ceranae*. Appl Environ Microbiol 73:6331–6338

O'Mahony EM, Tay WT, Paxton RJ (2007) Multiple rRNA variants in a single spore of the microsporidian *Nosema bombi*. J Eukaryot Microbiol 54:103–109

Page R (1996) TreeView: an application to display phylogenetic trees on personal computers. Comput Appl Biosci 12:357–358

Poirot O, O'Toole E, Notredame C (2003) Tcoffee@igs: a web server for computing, evaluating and combining multiple sequence alignments. Nucleic Acids Res 31:3503–3506

Sarlo EG, Medici SK, Porrini MP, Eguaras MJ (2008) Presencia y distribución de *Nosema ceranae* en la región Sudeste de la provincia de Buenos Aires. 2do Congreso Argentino de Apicultura

Shafer AB, Williams GR, Shutler D, Rogers RE, Stewart DT (2009) Cophylogeny of *Nosema* (Microsporidia: Nosematidae) and bees (Hymenoptera: Apidae) suggests both cospeciation and a host switch. J Parasitol 95:198–203

Tay W, O'Mahoney E, Paxton R (2005) Complete rRNA gene sequences reveal that the microsporidian *Nosema bombi* infects diverse bumblebee (*Bombus* spp.) hosts and contains multiple polymorphic sites. J Eukaryot Microbiol 52:505–513

Vossbrinck CR, Maddox JV, Friedman S, Debrunner-Vossbrinck BA, Woese CR (1987) Ribosomal RNA sequence suggests microsporidia are extremely ancient eukaryotes. Nature 326:411–414

Weiss IM, Vossbrinck CR (1999) Molecular biology, molecular phylogeny, and molecular diagnostic approaches to the microsporidia. In: Wittner M, Weiss IM (eds) The microsporidia and microsporidiosis. ASM Press, Washington, DC, pp 129–171

Williams GR, Shafer A, Rogers R, Shutler D, Stewart D (2008) First detection of *Nosema ceranae*, a microsporidian parasite of European honey bees (*Apis mellifera*), in Canada and Central USA. J Invertebr Pathol 97:189–192