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Widespread dispersal of the microsporidian *Nosema ceranae*, an emergent pathogen of the western honey bee, *Apis mellifera*

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Abstract

The economically most important honey bee species, *Apis mellifera*, was formerly considered to be parasitized by one microsporidian, *Nosema apis*. Recently, [Higes, M., Martín, R., Meana, A., 2006. *Nosema ceranae*, a new microsporidian parasite in honeybees in Europe, J. Invertebr. Pathol. 92, 93–95] and [Huang, W.-F., Jiang, J.-H., Chen, Y.-W., Wang, C.-H., 2007. A *Nosema ceranae* isolate from the honeybee *Apis mellifera*. Apidologie 38, 30–37] used 16S (SSU) rRNA gene sequences to demonstrate the presence of *Nosema ceranae* in *A. mellifera* from Spain and Taiwan, respectively. We developed a rapid method to differentiate between *N. apis* and *N. ceranae* based on PCR–RFLPs of partial SSU rRNA. The reliability of the method was confirmed by sequencing 29 isolates from across the world ($N = 9$ isolates gave *N. apis* RFLPs and sequences, $N = 20$ isolates gave *N. ceranae* RFLPs and sequences; 100% correct classification). We then employed the method to analyze $N = 115$ isolates from across the world. Our data, combined with $N = 36$ additional published sequences demonstrate that (i) *N. ceranae* most likely jumped host to *A. mellifera*, probably within the last decade, (ii) that host colonies and individuals may be co-infected by both microsporidia species, and that (iii) *N. ceranae* is now a parasite of *A. mellifera* across most of the world. The rapid, long-distance dispersal of *N. ceranae* is likely due to transport of infected honey bees by commercial or hobbyist beekeepers. We discuss the implications of this emergent pathogen for worldwide beekeeping.

Keywords: Microsporidia; *Nosema apis*; Exotic; Small subunit ribosomal RNA gene; 16S rRNA; PCR; RFLP; Beekeeping

1. Introduction

Microsporidia are obligately intracellular organisms that are common parasites of insects and other invertebrates (Larsson, 1986). The western honey bee, *Apis mellifera*, is economically the most important of the nine currently described members of the genus *Apis*. It has been transported throughout the world for beekeeping purposes from its native range in Europe, Africa and the Near East (Ruttner, 1988). Associated with it throughout its former and its expanded global range is the microsporidian, *Nosema apis* (Fig. 1, see also Matheson, 1993). It is a parasite of the ventriculus of adult honey bees that is

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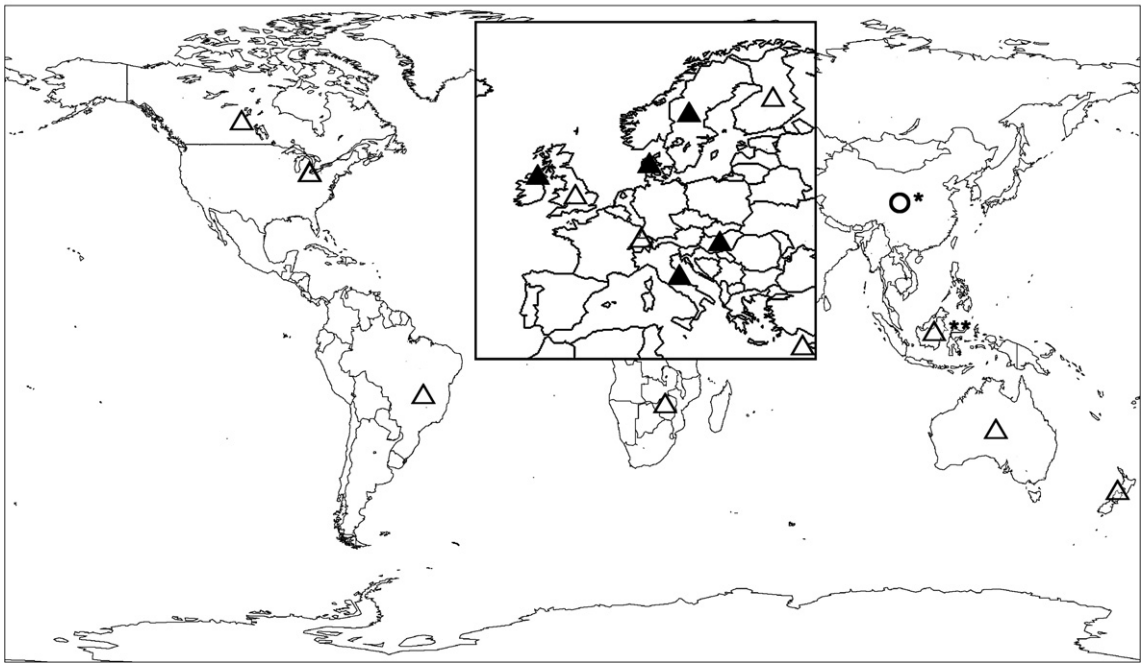

Fig. 1. Pre-2003 world distribution of *N. apis* (triangles) and *N. ceranae* (circle) in honey bees based on (i) our own RFLP analysis (filled symbols, $N = 5$ isolates; see Table 1) and (ii) sequenced partial rRNA from other sources (open symbols, $N = 26$ *A. mellifera* isolates and $N = 2$ *A. cerana* isolates; see Table 2). The host is *A. mellifera* except for *(*A. cerana*) and **(*A. mellifera* and *A. cerana*).

transmitted *per os* (i.e. horizontally) (Fries, 1997). Though not generally considered highly virulent (*sensu* Thomas & Elkington, 2004), *N. apis* is nevertheless thought to be pandemic, to lead to shortened worker lifespan and considerable weakening of heavily infected colonies, and to cause significant economic damage (Fries et al., 1984; Anderson and Giacon, 1992).

In 1994, a new microsporidian, *Nosema ceranae*, was first detected from Beijing, China, in an Asiatic honey bee, *Apis cerana* (Fries et al., 1996; see Fig. 1). Though morphologically similar to *N. apis* by light microscopy (Fries et al., 2006), *N. ceranae* could be clearly separated from its congener based on 16S small subunit (SSU) rRNA gene sequences and ultrastructural features (Fries et al., 1996). Although experimentally infective in *A. mellifera*, *N. ceranae* was thought to be restricted in host range and geography to *A. cerana* in East Asia (Fries, 1997). On the basis of SSU rRNA gene sequences, Huang et al. (2007) recently discovered *N. ceranae* in a naturally infected *A. mellifera* colony in Taiwan, where this honey bee is not native. Also based on SSU rRNA gene sequences, Higes et al. (2006) have similarly demonstrated the presence of *N. ceranae* in Spanish colonies of *A. mellifera*. There has been considerable concern expressed across Southern Europe, where beekeeping with the native *A. mellifera* is an important economic activity, of honey bee colony losses, some of which have been associated with microsporidian infection (e.g. Greece: Hatjina and Haristos, 2005; Spain: Higes et al., 2005). Higes et al. (2007) have, moreover, recently demonstrated that *N. ceranae* is highly virulent for experimentally caged honey bee (*A. mellifera*) workers. The threats posed to beekeeping by a potentially virulent emergent pathogen give impetus to the development of a rapid method to allow the differentiation of *N. apis* from *N. ceranae*.

As species of microsporidia like *N. apis* and *N. ceranae* are often difficult to distinguish morphologically (Larsson, 1986; Weiss and Vossbrinck, 1999), our first aim was to develop a rapid PCR–RFLP method to distinguish between *N. apis*, *Nosema bombi* and *N. ceranae*, the only three members of the large genus *Nosema* that have been detected in bees. Our second aim was to use the method to screen microsporidian isolates from *A. cerana* and *A. mellifera* from across the world to reveal their past and current distribution.

2. Materials and methods

2.1. Samples

Naturally infected *A. mellifera* were collected from 11 European countries, where the species is native, as well as Brazil (= Africanized honey bees), New Zealand, the USA and Vietnam, where the species is introduced ($N = 111$ colonies, see Table 1). In addition, infected Asian honey bees (*A. cerana*) were sampled in Vietnam ($N = 4$ colonies, see Table 1). Samples were stored in 99% ethanol. Reference DNA extractions of *N. apis* and *N. ceranae* were provided by Dr. N. Pieniazek (CDCP-USA). Frozen (−20 °C) abdomens of heavily infected *Bombus terrestris* from The Netherlands (provided by Jozef van der Steen,

Table 1
The location, date of sampling, host species, sample size of our analyzed samples and results of *Nosema* screening based on RFLP identification

Region and country	Year	Location within country	Host species	*N* of colonies	***N*** Individuals	*Nosema* species detected by RFLP	Sequences
The Americas							
Brazil	07/2006	São Paulo State	*A. mellifera*	3	5*	*N. ceranae*	*N. ceranae*[a]
USA	2004	Washington DC	*A. mellifera*	1	4	*N. ceranae*	*N. ceranae*
Asia							
Vietnam	03/2006	Gia Lai Province	*A. mellifera*	2	3	*N. ceranae*	*N. ceranae*
	04/2006	Hung Yen Province	*A. cerana*	2	3	*N. ceranae*	*N. ceranae*
	04/2006	Ha Nam Province	*A. cerana*	2	5	*N. ceranae*	*N. ceranae*
Europe							
Denmark	1988	Seeland	*A. mellifera*	1	Spore extract	*N. apis*	*N. apis*
	2004	Læsø	*A. mellifera*	1	Spore extract	*N. ceranae*	
	2006	Seeland	*A. mellifera*	1	Spore extract	*N. ceranae*	
	2006	Funen	*A. mellifera*	1	Spore extract	*N. ceranae*	*N. ceranae*
	2006	Jutland	*A. mellifera*	4	Spore extracts	*N. apis*/ *N. ceranae*	
	2006	Rømø	*A. mellifera*	1	Spore extract	*N. ceranae*	
Finland	2006	Jokioinen	*A. mellifera*	2	Spore extracts	*N. ceranae*	*N. ceranae*[a]
Germany	03-04/2006	Mecklenburg-Vorpomern	*A. mellifera*	1	5*	*N. ceranae*	
	03-04/2006	Brandenburg	*A. mellifera*	1	5*	*N. apis*	
	03-04/2006	Brandenburg	*A. mellifera*	3	5*	*N. apis*/ *N. ceranae*	
	03-04/2006	Saxony-Anhalt	*A. mellifera*	1	5*	*N. apis*	
	03-04/2006	Saxony-Anhalt	*A. mellifera*	1	5*	*N. apis*/ *N. ceranae*	
	03-04/2006	Saxony	*A. mellifera*	1	5*	*N. ceranae*	
	03-04/2006	Saxony	*A. mellifera*	2	5*	*N. apis*/ *N. ceranae*	
	03-04/2006	Northrhine-Westphalia	*A. mellifera*	4	5*	*N. apis*	
	03-04/2006	Northrhine-Westphalia	*A. mellifera*	1	5*	*N. apis*/ *N. ceranae*	
	03-04/2006	Berlin	*A. mellifera*	5	5*	*N. ceranae*	
	03-04/2006	Hesse	*A. mellifera*	2	5*	*N. apis*	
	03-04/2006	Hesse	*A. mellifera*	3	5*	*N. ceranae*	
	03-04/2006	Hesse	*A. mellifera*	2	5*	*N. apis*/ *N. ceranae*	
	07/2006	Baden-Wuerttemberg	*A. mellifera*	7	22	*N. ceranae*	
Greece	11/2005	Chalkidiki	*A. mellifera*	1	5*	*N. ceranae*	*N. ceranae*
	02/2006	Komotini	*A. mellifera*	1	5*	*N. ceranae*	*N. ceranae*
	08/2006	unspecified	*A. mellifera*	1	5*	*N. ceranae*	
Hungary	05/1994	unspecified	*A. mellifera*	1	Spore extract	*N. apis*	
Ireland	2005	Tipperary	*A. mellifera*	1	Spore extract	*N. apis*	*N. apis*
Italy	1994	unspecified	*A. mellifera*	1	2	*N. apis*	*N. apis*
	11/2005	Sardinia	*A. mellifera*	14	5*	*N. ceranae*	*N. ceranae*
	02/2006	Bologna	*A. mellifera*	8	5*	*N. ceranae*	
	04/2006	Milano	*A. mellifera*	2	5*	*N. ceranae*	
	04/2006	Verona	*A. mellifera*	1	5*	*N. ceranae*	*N. ceranae*
	04/2006	Ascoli Piceno	*A. mellifera*	1	5*	*N. ceranae*	*N. ceranae*
Serbia	2006	Lipovac (Aleksinac)	*A. mellifera*	1	5*	*N. ceranae*	*N. ceranae*
	2006	Stanci (Aleksinac)	*A. mellifera*	1	5*	*N. ceranae*	
	2006	Slatina (Leskovac)	*A. mellifera*	1	5*	*N. ceranae*	*N. ceranae*
	2006	Leskovac	*A. mellifera*	1	5*	*N. ceranae*	
Spain	2005/06	Huelva	*A. mellifera*	3	10	*N. ceranae*	*N. ceranae*
	2004/05	Sevilla	*A. mellifera*	4	13	*N. ceranae*	*N. ceranae*
	2005	Salamanca	*A. mellifera*	2	11	*N. ceranae*	*N. ceranae*
	2005	Zamora	*A. mellifera*	1	3	*N. ceranae*	*N. ceranae*
Sweden	1998	Kungsör	*A. mellifera*	1	2	*N. apis*	*N. apis*
	2003	Vallentuna	*A. mellifera*	1	3	*N. apis*	*N. apis*
	2005	Tystberga	*A. mellifera*	1	3	*N. apis*	*N. apis*
	2005	Uppsala	*A. mellifera*	1	Spore extract	*N. apis*	
	2006	Nässjö	*A. mellifera*	1	3	*N. apis*	*N. apis*
	2006	Grästorp	*A. mellifera*	1	3	*N. apis*/ *N. ceranae*	*N. apis*

(*continued on next page*)

Table 1 (*continued*)

Region and country	Year	Location within country	Host species	*N* of colonies	*N* Individuals	*Nosema* species detected by RFLP	Sequences
UK	2001	Northern Ireland	*A. mellifera*	1	5*	*N. apis*	
	2005	Northern Ireland	*A. mellifera*	1	5*	*N. apis*	*N. apis*
	2006	Northern Ireland	*A. mellifera*	1	5*	*N. apis*	
Australasia							
New Zealand	03/2006	Auckland	*A. mellifera*	5	Spore extract	*N. apis*	
			Total	$N = 115$colonies	$N = 235$ inds $N = 11$ spore extracts	*N. apis* = 25*N. ceranae* = 76 Both species = 14	*N. apis* = 9*N. ceranae* = 20

5*, five bees per colony homogenized together and analyzed; spore extract, multiple bees per colony homogenized in water and the supernatant sent to Belfast for analysis.

Partial SSU rRNA gene was sequenced in some samples ($N = 29$ sequences) to determine the reliability of RFLP identification. Total of *Nosema* sp. detected by RFLP is for the 115 colonies.

[a] $N = 2$ sequences.

Wageningen University, The Netherlands) were used as the reference source of *N. bombi*.

2.2. Nucleic acid preparation

Either single bee abdomens were homogenized in 200 μl ddH_2O or five abdomens of bees from the same colony were homogenized together in 2 ml ddH_2O and checked by light microscopy (400×) for microsporidian infections. The former infected homogenates were directly used for DNA extraction while the latter were filtered through a syringe filled with glass wool and washed twice with ddH_2O by centrifugation (5 min, 16,100*g*). Homogenates were centrifuged (5 min, 16,100*g*) and the supernatant discarded. Pellets were frozen in liquid nitrogen and crushed using sterile sealed pipette tips. DNA was extracted with a DNeasy Plant Mini Extraction Kit (Qiagen). Extracts were kept at −20 °C until needed as DNA template in PCRs.

As negative controls, DNA was also extracted from ethanol-washed legs of bees, parts of the anatomy that are considered not to be infected with *Nosema* (Fries, 1997), and from honey bee abdomens visually showing no sign of *Nosema* infection (10 extracts) using the above methods, with the order of infected samples and negative controls randomized to control for potential contamination with *Nosema* DNA across extracts. These negative controls were used as template in >40 PCRs, as described below, but never generated an amplicon, indicating that our methods of DNA extraction did not lead to contamination of extracts with DNA from another sample.

2.3. PCR primers

By aligning the sequences from the GenBank database for *N. apis*, *N. bombi* and *N. ceranae* (respectively: Gatehouse and Malone, 1998, Accession No. U97150; Tay et al., 2005, Accession No. AY741110; Huang et al., 2007, Accession No. DQ078785), conserved regions for all three species of the 16S or SSU rRNA gene were selected for primer design using PrimerSelect 6.1 (Lasergene). Additionally, *N. apis* sequence U26534 (Fries et al., 2006) was compared with U97150 to resolve unknown sites; it differed from U97150 at site 49 by one SNP that does not impact upon the molecular genetic identification described below. The primers we developed, SSU-res-f1 (5′-GCCTGACGTAGACGCTATTC-3′, positions 16–35 in U97150) and SSU-res-r1 (5′-GTATTACCGC GGCTGCTGG-3′, positions 399–417 in U97150), amplify ca. 400 bp of partial 16S rRNA gene.

2.4. Polymerase chain reaction

PCRs were performed using a MJ Research DYAD thermal cycler in 25 μl volumes containing 5 μl of template DNA, 2.5 μl of 10× PCR buffer, 2.5 mM $MgCL_2$, 200 μM of each dNTP (Abgene), 0.625 U *Taq* polymerase (Promega) and 0.5 μM of each forward and reverse primer. PCR parameters for amplification were: initial DNA denaturation of 4 min at 95 °C followed by 45 cycles of 1 min at 95 °C, 1 min at 48 °C and 1 min at 72 °C, and terminated with a final extension step at 72 °C for 4 min. Amplification products (5 μl DNA) were electrophoresed on 1.4% agarose gels (1× TBE), stained with ethidium bromide, and visualised under UV light. A commercial 100 bp ladder was used as a size marker. For each PCR, positive (reference *N. apis* and *N. ceranae* DNA extracts as template) and negative (ddH_2O or DNA extracts from legs of bees or those in which we did not visually observe *Nosema* as template) controls were run along with DNA extracts of isolates as template.

Repetition of the PCR protocol using an ABI Biosystems/Perkin-Elmer thermal cycler and Qiagen HotStar*Taq* polymerase demanded a 'touchdown' PCR profile to generate a clean amplicon. In the 'touchdown' PCR profile, the annealing temperature dropped from 53 to 49 °C by 1 °C every second cycle, followed by 32 cycles at 48 °C. Results using the two PCR methods were identical for 25 DNA extracts tested using both protocols.

2.5. Species identification by restriction fragment length polymorphism (RFLP)

To differentiate between the species *N. apis*, *N. bombi* and *N. ceranae*, discriminating restriction endonuclease sites were identified in the PCR amplicon with the help of SeqBuilder version 6.1.3 (Lasergene) and sequences of *N. apis* (Gatehouse and Malone, 1998, Accession No. U97150; Fries et al., 1996, Accession No. U26534), *N. bombi* (Tay et al., 2005, Accession No. AY741110) and *N. ceranae* (Huang et al., 2007, Accession No. DQ078785). Based on these sequences, the 402 bp amplicon of *N. apis* obtained with primer pair SSU-res-f1/r1 differs from *N. bombi* and *N. ceranae* by 30 SNPs/27 SNPs and 3 INDELS/11 INDELS, respectively, whilst *N. bombi* differs from *N. ceranae* by 16 SNPs and 8 INDELS. The restriction endonuclease *Pac*I was selected because it provides one unique digestion site for *N. ceranae* whilst the enzyme *Nde*I only digests *N. apis*. *Msp*I digests *N. apis*, *N. bombi* and *N. ceranae* and is used as a control for successful restriction digestion of PCR products (Fig. 2).

The predicted restriction fragments produced from digestion of the PCR amplicons are illustrated in Fig. 2. Amplicons were digested with *Msp*I, *Pac*I and *Nde*I (New England Biolabs) in one reaction at 37 °C for 3 h. Digests were performed in a 12.5 μl volume with 10 μl of the amplified DNA and 1.5 U of each enzyme. The 1× NEBuffer 2 (provided by NEB with *Msp*I) was used as buffer for the reactions. *Msp*I and *Nde*I show 100% activity with this buffer whilst *Pac*I performs to 75% in this buffer and therefore may result in partial digestion, though undigested bands migrating around 225 bp do not interfere with species designation based on bands migrating between 90 and 140 bp. Fragments were separated in a 3% NuSieve agarose gel (Cambrex Bio Science) in 1× TBE buffer with a 20 bp ladder as size marker at 100 V for 1 h 45 min. Gels were stained with ethidium bromide and visualized under UV light. For samples in which both *N. apis* and *N. ceranae* specific fragments were detected, amplicons were digested separately with each distinguishing restriction enzyme combination, *Msp*I/*Nde*I and *Msp*I/*Pac*I, respectively, to confirm the presence of each *Nosema* species.

2.6. Sequence analysis

For sequencing, PCR products were initially purified with a MinElute PCR Purification Kit (Qiagen). PCR products were then sequenced in both directions in an Applied Biosystems Genetic Analyser 3100 automated sequencer in a commercial laboratory (Fusion Antibodies, Belfast, UK). The sequence data were aligned and visually checked using SeqMan 6.1 (Lasergene), followed by a BLAST database search to test sequence similarities. In addition to sequencing the reference DNA extracts of *N. apis*, *N. bombi* and *N. ceranae*, 29 additional sequences from 27 locations were generated (Table 1).

3. Results

3.1. RFLP patterns for N. apis, N. bombi and N. ceranae

PCR products of the reference samples of *N. apis*, *N. bombi* and *N. ceranae* were sequenced and found to be identical to those previously published in GenBank (*N. apis*: Gatehouse and Malone, 1998, Accession No. U97150, Fries et al., 1996, Accession No. U26534; *N. bombi*: Tay et al., 2005, Accession No. AY741110; *N. ceranae*: Huang et al., 2007, Accession No. DQ078785). As these sequences were used for designing the PCR–RFLP technique, reference samples were useful positive controls in test PCRs.

As expected, the PCR products of the reference samples with primer pair SSU-res-f1/r1 were approximately 400 bp (Fig. 3) and gave RFLP patterns as predicted for the three *Nosema* species: *N. apis*, *N. bombi* and *N. ceranae* (Fig. 4).

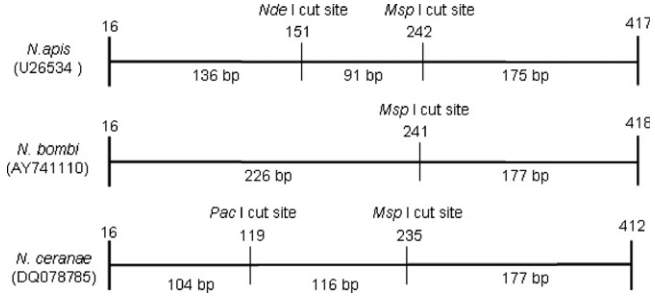

Fig. 2. Diagram showing the total length and size (in base pairs) of expected restriction fragments of *N. apis*, *N. bombi* and *N. ceranae* partial SSU rRNA PCR products amplified with the primer pair SSU-res-f1 and SSU-res-r1, and digested with *Pac*I, *Msp*I and *Nde*I. Base pair cut sites based on GenBank sequences: Accession No. U26534 (Fries, 1996) for *N. apis*, Accession No. AY741110 (Tay et al., 2005) for *N. bombi*, and Accession No. DQ078785 (Huang et al., 2007) for *N. ceranae*.

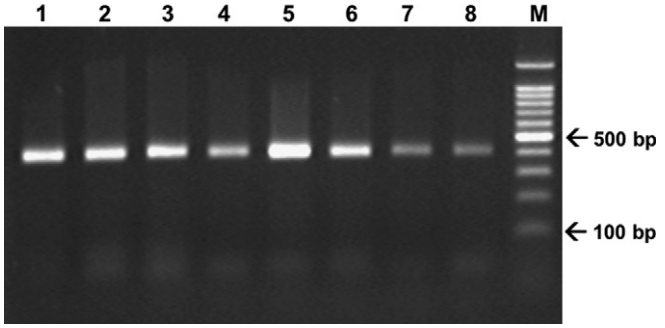

Fig. 3. Ethidium bromide stained agarose gel showing examples of PCR products for the fragment of SSU rRNA amplified with the primer pair SSU-res-f1 and SSU-res-r1. Lane 1, *N. ceranae* reference DNA extract; lane 2, *N. apis* reference DNA extract; lane 3, *N. bombi*; lane 4, Spain (Huelva); lane 5, Spain (Sevilla); lane 6, Sweden (Uppsala); lane 7, Vietnam (Hung Yen Province); lane 8, Vietnam (Ha Nam Province); lane M, 100 bp ladder. All samples are listed in Table 1.

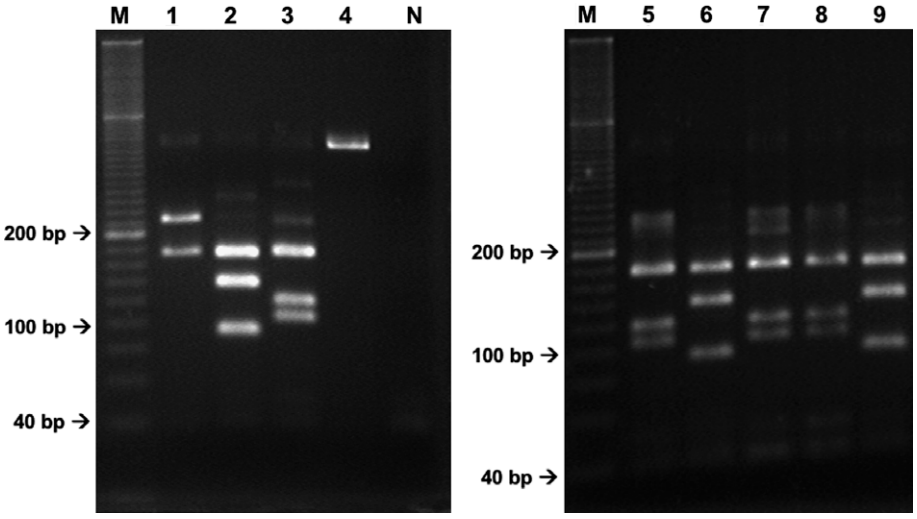

Fig. 4. Ethidium bromide stained agarose gel giving examples of RFLP analysis of partial SSU rRNA of *N. apis*, *N. ceranae* and *N. bombi* digested with the enzymes *Msp*I, *Pac*I and *Nde*I. Lane M, 20 bp ladder; lane 1, *N. bombi*; lane 2, *N. apis* reference DNA extract; lane 3, *N. ceranae* DNA extract; lane 4, uncut PCR product; lane N, negative control; lane 5, Italy (Verona); lane 6, UK (Northern Ireland 2001); lane 7, Brazil (sample 1); lane 8, Brazil (sample 2); lane 9, Sweden (Uppsala). All samples are listed in Table 1.

The sum of the sizes of the restriction bands was equal to the size of the undigested PCR products.

All analyzed samples from the different localities (Table 1) produced PCR products of approximately 400 bp in size. Only RFLP patterns either of *N. apis* or *N. ceranae* (or both) were revealed with the three restriction enzymes (e.g. Fig. 4). The presence of additional faint bands on the gel suggests incomplete restriction digestion or may indicate unspecific amplicons, but it was always possible to see clearly the specific restriction bands for *N. apis* or *N. ceranae* (or both e.g. Fig. 5). For samples in which both *N. apis* and *N. ceranae* specific fragments were seen following triple restriction digestion with the three endonucleases, amplicons were digested separately with *Msp*I/*Nde*I and *Msp*I/*Pac*I. In all apparently co-infected samples, separate digestion with either *Msp*I/*Nde*I or *Msp*I/*Pac*I revealed the presence of both *N. apis* and *N. ceranae*, respectively, even in an individual honey bee worker (Fig. 5).

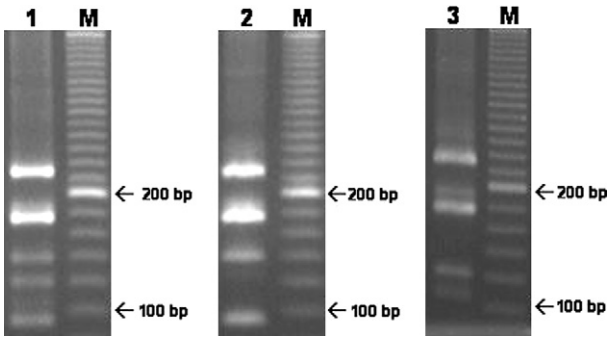

Fig. 5. Ethidium bromide stained agarose gel giving an example of the RFLP analysis of partial SSU rRNA of one isolate (one bee from the sample 03-04/2006, Hesse; see Table 1) co-infected with both *N. apis* and *N. ceranae*. Lane M, 20 bp ladder; lane 1, sample digested with the enzymes *Msp*I, *Pac*I and *Nde*I; lane 2, sample digested with the enzymes *Msp*I and *Nde* I (cuts *N. apis* only); lane 3, sample digested with the enzymes *Msp*I and *Pac*I (cuts *N. ceranae* only).

In total we sequenced amplicons from 29 isolates from across the world (from 29 different colonies, see Table 1). In every case, sequences were identical to either *N. apis* (Gatehouse and Malone, 1998, Accession No. U97150; Fries et al., 1996, Accession No. U26534) or *N. ceranae* (Huang et al., 2007, Accession No. DQ078785). RFLP patterns of these 29 isolates always matched those expected for the *Nosema* species (Fig. 4). Thus our RFLP technique can reliably distinguish *N. apis* from *N. ceranae* in 100% of cases.

3.2. Change in the distribution of N. apis and N. ceranae

The results of the PCR-RFLP analyses for all 115 samples are given in Table 1. To visualize the worldwide change in the distribution of *N. apis* and *N. ceranae*, RFLP data were mapped together with confirmed literature references and personal communications that were based on sequencing of partial rRNA and GenBank submissions ($N = 36$ samples, see Table 2) onto world maps (pre-2003 in Fig. 1, based on $N = 33$ molecular genetically confirmed samples; 2003 onwards in Fig. 6, based on $N = 118$ molecular genetically confirmed samples).

In older (pre-2003) samples, we detected *N. apis* in *A. mellifera* in Italy (1994), Hungary (1995), Denmark (1988), Sweden (1998) and Northern Ireland (2001) by RFLPs (2 samples) or sequencing (3 samples, see Fig. 1). In addition, all pre-2003 literature references and other sources that we could trace, confirmed through DNA sequences, demonstrate the past universal presence of *N. apis* in *A. mellifera* across its world distribution

Table 2
All literature references, GenBank Accession details or personal communications of natural infections of *N. apis* and *N. ceranae* in host honey bees *A. cerana* and *A. mellifera* based on SSU rRNA gene sequences, with location and date of sample collection ($N = 36$ sequences, of which 28 are before 2003 and 8 are after 2003)

Location of sampling	Microsporidian taxon	Honey bee host	Date of sampling	Literature reference or Accession No.
Africa				
Zimbabwe	*N. apis*	*A. mellifera*	2002	Fries et al. (2003)
Americas and Caribbean				
Brazil	*N. apis*[*]	*A. mellifera*	1997	Gatehouse and Malone (1999)
Canada	*N. apis* (2 isolates)[*]	*A. mellifera*	1996	Gatehouse and Malone (1999)
Canada	*N. apis*	*A. mellifera*	1994	Rice (2001)
Martinique	*N. ceranae*	*A. mellifera*	2005	Huang and Wang (pers. comm.)
USA	*N. apis*	*A. mellifera*	1995	Fries et al. (1996)
USA	*N. apis*[*]	*A. mellifera*	1996	Gatehouse and Malone (1999)
Asia				
China	*N. ceranae*	*A. cerana*	1994	Fries et al. (1996)
Indonesia	*N. apis*	*A. cerana*	1994	Rice (2001)
Indonesia	*N. apis*	*A. mellifera*	1994	Rice (2001)
Taiwan	*N. ceranae*	*A. mellifera*	2004	DQ078785 (Huang et al., submitted)
Taiwan	*N. ceranae*	*A. cerana*	2005	Huang and Wang (pers. comm.)
Europe and Near East				
Finland	*N. apis* (2 isolates)[*]	*A. mellifera*	1996	Gatehouse and Malone (1999)
France	*N. ceranae*	*A. mellifera*	2005	DQ374655 (Higes et al., submitted)
Germany	*N. ceranae*	*A. mellifera*	2005	DQ374656 (Higes et al., submitted)
Israel	*N. apis*[*]	*A. mellifera*	1997	Gatehouse and Malone (1999)
Italy	*N. apis*[*]	*A. mellifera*	1997	Gatehouse and Malone (1999)
Spain	*N. apis*	*A. mellifera*	2004/2005	Higes et al. (2006)
Spain	*N. ceranae*	*A. mellifera*	2004/2005	Higes et al. (2006)
Sweden	*N. apis*	*A. mellifera*	1994	Fries et al. (1996)
Sweden	*N. apis*[*]	*A. mellifera*	1996	Gatehouse and Malone (1999)
Switzerland	*N. apis* (2 isolates)[*]	*A. mellifera*	1997	Gatehouse and Malone (1999)
Switzerland	*N. ceranae*	*A. mellifera*	2005	DQ673615 (Higes et al., submitted)
UK	*N. apis*∗	*A. mellifera*	1996	Gatehouse and Malone (1999)
Australasia				
Australia	*N. apis* (4 isolates)	*A. mellifera*	1996	Rice (2001) and U76706 (Rice, submitted)
New Zealand	*N. apis*	*A. mellifera*	1996	Gatehouse and Malone (1998)
New Zealand	*N. apis* (3 isolates)[*]	*A. mellifera*	1995/1996	Gatehouse and Malone (1999)
New Zealand	*N. apis*	*A. mellifera*	1994	Rice (2001)

[*] Partial sequence of SSU, all ITS and partial LSU.

($N = 26$ partial sequences) and even in *A. cerana* in its native range of Indonesia ($N = 1$ sequence; Rice, 2001).

Pre-2003, *N. ceranae* was only once recorded using DNA-based data, namely in the original description of the species in Chinese samples of *A. cerana* (Fries et al., 1996; see Fig 1). But from 2003 onwards, *N. ceranae* appears to have become widespread in Europe and other continents (the Americas and Asia) in *A. mellifera* (Fig. 6). For example, all 26 colonies of Italian *A. mellifera* sampled in 2005/2006 that we analyzed by RFLPs harbored *N. ceranae* but not *N. apis* (Table 1). Our recent Vietnamese samples of both native *A. ceranae* and non-native *A. mellifera* also harbored only *N. ceranae* (Table 1).

We found exclusively *N. apis* in post-2003 samples of *A. mellifera* analyzed by RFLPs in Ireland (the Republic of Ireland and Northern Ireland) and New Zealand (Table 1 and Fig. 6). In Denmark, Germany, and Sweden, we detected both *N. apis* and *N. ceranae* in the same colony of *A. mellifera* (Table 1) and even in the same bee (Fig. 5).

4. Discussion

Our results suggest that *N. ceranae* has jumped host from *A. cerana* to *A. mellifera* and become distributed almost worldwide. Given the limited historical sampling, we can state that this host range expansion by *N. ceranae* probably occurred at some time within the last decade.

A quick and accurate molecular genetic method of detection of microsporidia is important for identification (e.g. Klee et al., 2006) because species of microsporidia are often difficult to distinguish using morphological criteria (Larsson, 1986; Weiss and Vossbrinck, 1999). Our RFLP method offers such a quick and accurate method. When infected by *Nosema*, a single honey bee worker usually contains several million spores (Fries et al., 1984). We have therefore not attempted to develop a very sensitive molecular marker, which would probably require amplification of a much shorter fragment than the ca. 400 bp generated in our assay (see Klee et al., 2006, for an example).

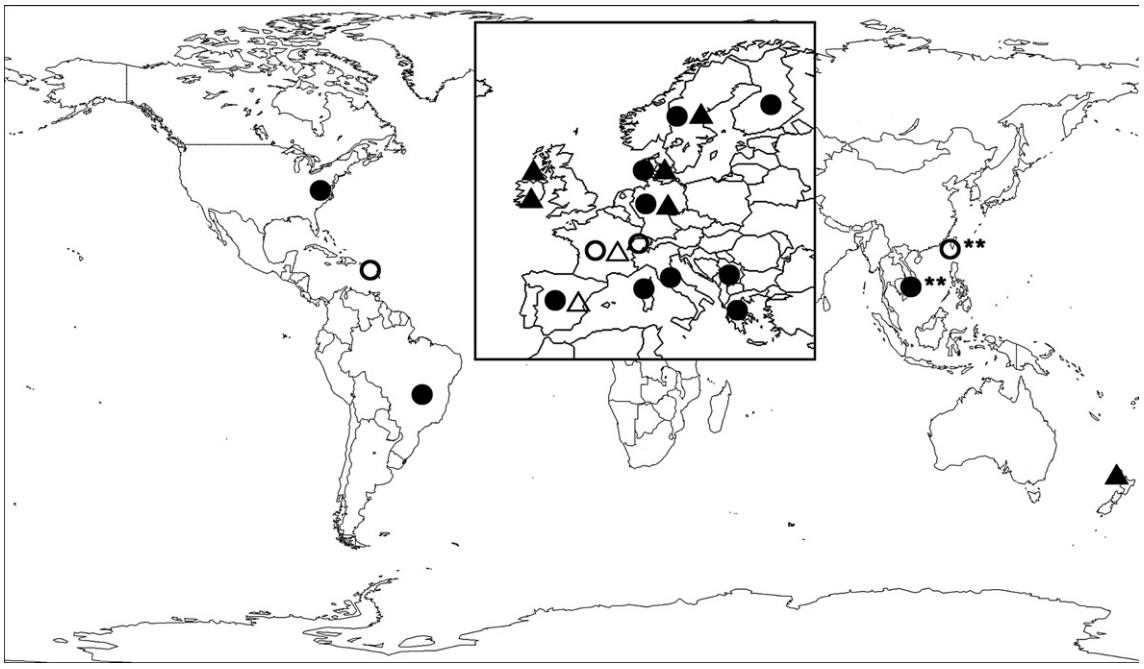

Fig. 6. Post-January 2003 world distribution of *N. apis* (triangles) and *N. ceranae* (circles) in honey bees based on (i) our RFLP analysis (filled symbols, $N = 110$ isolates; see Table 1) and (ii) sequenced partial rRNA from literature references (open symbols, $N = 8$ isolates; see Table 2). The host is *A. mellifera* except for **(*A. mellifera* and *A. cerana*).

Former reports of the distribution of nosema disease (Matheson, 1993) assumed that only one *Nosema* species, *N. apis*, infected honey bees (*Apis* spp.). The discovery of a second species, *N. ceranae*, in an Asian honey bee, *A. cerana*, in China (Fries et al., 1996) means that nosema disease may potentially be caused by this additional species. Subsequently Huang et al. (2007) discovered *N. ceranae* in *A. mellifera* in Taiwan in 2004, where this host bee is non-native but within the range of *A. cerana*. Thereafter, Higes et al. (2006) detected *N. ceranae* in Spain in 2005 and Chauzat et al. (Proc. 2nd Europ. Conf. Apidology 2006, p. 30) found it to be widespread in France in *A. mellifera* collected between 2003 and 2005; in Europe, *A. mellifera* is native whereas *A. cerana* is absent. These new data might suggest that there needs to be a re-consideration of the historical (pre-2003) host associations of these two microsporidian parasites of honey bees. Yet our own molecular genetic analyses (RFLPs or sequences) of older (pre-2003) isolates of *Nosema* from *A. mellifera* ($N = 5$ sequences) and published sequences of older (pre-2003) isolates from the same host ($N = 26$ sequences, for a total of $N = 31$ molecular genetically confirmed isolates from across the world) reveal only *N. apis* in *A. mellifera*. It seems highly likely, therefore, that *A. mellifera* was formerly parasitized by *N. apis* alone and not *N. ceranae* and that *N. ceranae* should consequently be considered an exotic parasite of *A. mellifera*. Our data support the view that *N. bombi*, on the other hand, is not a parasite of honey bees (van den Eijnde and Vette, 1993).

The current incidence of *N. ceranae* in *A. mellifera* provides strong support for the view that this microsporidian is an emergent pathogen of the western honey bee, most probably due to a host jump from *A. cerana* to *A. mellifera*. Pathogen emergence has often been linked to host species jumps (Woolhouse et al., 2005), and a precedent has already been set within world beekeeping with *Apis* species. *Varroa destructor* (previously *jacobsoni*, see Anderson and Trueman, 2000) is a parasitic mite formerly associated with *A. cerana* that has now jumped species to *A. mellifera* and spread worldwide to become the major scourge of beekeeping with the western honey bee, directly through host feeding and indirectly through transmission of viruses (Ball, 1989; Bowen-Walker et al., 1999; Shen et al., 2005a,b; Yue and Genersch, 2005). We cannot state the exact time of the host jump or *N. ceranae*'s transmission route from *A. cerana* to *A. mellifera* because of limited pre-2003 samples, though we suggest it occurred within the last decade. Nevertheless, *N. ceranae*'s relatively recent worldwide range expansion seems clear. Its great ability to spread through the *A. mellifera* population is exemplified by the presence of *N. ceranae* on the island of Læsø, isolated from the Danish mainland by 20 km and to which there is very limited movement of honey bees. *Nosema ceranae* is now present on four continents, North America, South America, Asia and Europe, in *A. mellifera*.

Our data suggest that *N. apis* is becoming limited to northern and western Europe and Australasia, possibly because *N. ceranae* is displacing *N. apis* in *A. mellifera* populations. However, we lack data on the incidence of natural infection of the two microsporidia with which to support this contention. That we found individual bees harboring both microsporidian species suggests they could co-exist in a population. Higes et al. (2006) found one of twelve *Nosema* infected *A. mellifera* colonies in Spain harbored

N. apis whereas the others harboured *N. ceranae*. Infection experiments with each parasite and with both parasites together are sorely needed to determine their competitive abilities and population dynamics in sympatry.

The main reason for such a rapid expansion in the range of *N. ceranae* in *A. mellifera* is probably the world trade in living honey bees, a reason which also explains the rapid spread of *V. destructor* around the world (Bailey and Ball, 1991). Microsporidia have also been implicated in the demise of native North American bumble bees (*Bombus* spp.) through so-called pathogen spill-over from commercially reared bumble bees used in greenhouse pollination (Colla et al., 2006); in this instance, one of the pathogens concerned is *N. bombi*, a microsporidium widespread in European *Bombus* spp. (Tay et al., 2005). Trade in honey bee hive products may also play a role in the dispersal of *N. ceranae*. The increased mobility of people, goods and livestock is thought to account for the geographical and host range expansion of numerous pathogens in human and animal populations or ecosystems (Daszak et al., 2000; Kolar and Lodge, 2001; Woolhouse, 2002). Recent reports of honey bee colony losses in southern and central Europe (e.g. Greece: Hatjina and Haristos, 2005; Switzerland: Imdorf et al., 2006) combined with Higes et al.'s (2006) observations of *N. ceranae* in dying Spanish colonies and Higes et al.'s (2007) experimental demonstration of the virulence of *N. ceranae* for caged *A. mellifera* give cause for concern. Over the past few years, many beekeepers in Europe have noticed a change in the course of nosema disease in their colonies, especially loss of honey bees the whole year around and quick die off of colonies in the winter time (Faucon et al., 2002; Higes et al., 2005). *Nosema apis*, on the other hand, generally affects colonies during spring (Fries, 1997). It is unknown if these differences are due to the emergent parasite, *N. ceranae*, as the relationship between recent south European honey bee colony losses and nosema disease is largely correlational (e.g. Hatjina and Haristos, 2005; Higes et al., 2005, 2006). Analyses of colonies with and without apparent nosema disease combined with experimental infection trials are needed to determine if there is a causal link between *N. ceranae* and colony collapse.

Honey bee populations worldwide are considered to have decreased substantially over recent years for a variety of reasons, with a concomitant decrease in the essential ecosystem service of pollination (Ghazoul, 2005; Steffan-Dewenter et al., 2005). Clearly there is a pressing need for studies on the epidemiology of *N. ceranae* in *A. mellifera* and on methods to control *N. ceranae* if its potential contribution to further honey bee losses worldwide are to be avoided.

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