



**Department of Physiology
Laboratory of Zoophysiology**



“Nosemosis: old story, new issue”

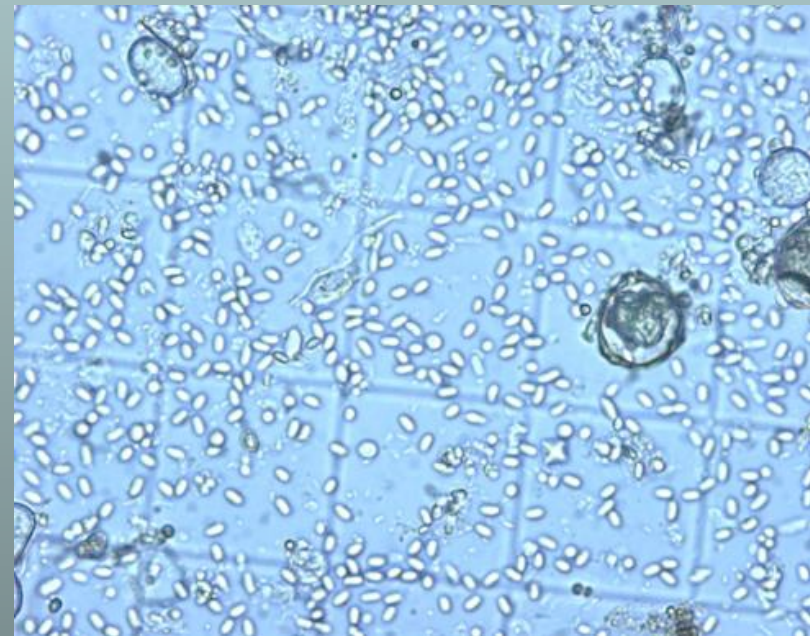
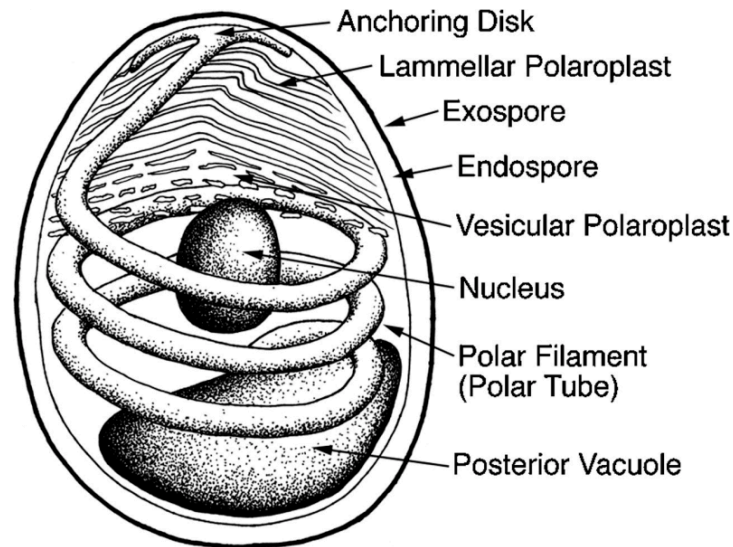
prof. dr. Dirk C. de Graaf

overview

- **The old story: *Nosema apis***
- ***Nosema ceranae*, parasite of the Asian honeybee**
- **A new issue: *Nosema ceranae***
- **Diagnostics according to the OIA Manual**
- **Beyond OIE Manual**

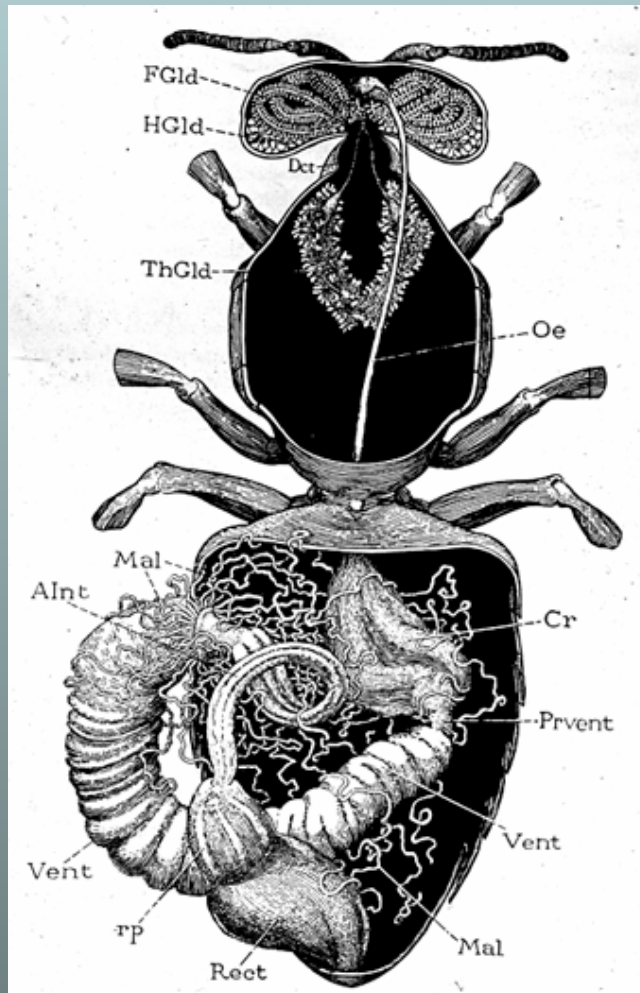
The old story: *Nosema apis*

- microsporidia (fungi)
- spores: characteristic internal structure
- first description in bees: Dönhoff & Leukart (1857)
- classification by Enoch Zander in 1909



The old story: *Nosema apis*

- tissue specific: midgut



Tissue Specificity of *Nosema apis*

Nosema apis (Microsporidia, Nosematidae), a midgut parasite of the honeybee, *Apis mellifera*, is tissue specific (T. P. Liu, *J. Invertebr. Pathol.* 44, 282-291, 1984). In contrast, many other Nosematidae develop in several tissues of the host (L. A. Malone, and P. J. Wigley, *J. Invertebr. Pathol.* 38, 315-329, 1981; J. D. Sedlacek, L. P. Dintenfass, G. L. Nordin, and A. A. Ajlan, *J. Invertebr. Pathol.* 46, 320-324, 1985). *Nosema algerae* sporoplasms are found in virtually all tissues of *Anopheles albimanus* larvae: epidermis, gastric caeca, muscle, aorta, nerve, and neurosecretory cells (S. W. Avery, and D. W. Anthony, *J. Invertebr. Pathol.* 42, 87-95, 1983).

At present we lack any knowledge concerning the origin of tissue specificity in *N. apis* infection. Therefore we used immunoblotting as a first attempt to determine whether *N. apis* can be found in the hemolymph—a stage that should precede possible infection of other tissues. Anti-

Nosema serum was prepared and kindly provided by Dr. N. Kellner (C.T.L., Gent, Belgium). An initial experiment with homogenized midgut cells demonstrated the efficiency of this antiserum in distinguishing healthy from infected tissue by immunoblotting (Fig. 1A). Some bands were clearly of *Nosema* origin and could be used as an indicator of parasitic presence.

We collected newly emerged bees every 24 hr from a broodcomb incubated at 34°C. The honeybees were kept in Liebefeld cages for 7 days under standard conditions (F. J. Jacobs, *Apidology* 10, 75-76, 1979). On day 8, they were fed individually with 10 µl of a sucrose-water solution containing 10⁶ spores. Hemolymph was collected every 12 hr. All honeybees were bled only once.

Electrophoresis of polypeptide samples of honeybee hemolymph was performed in SDS-polyacrylamide gels, according to Laemmli (*Nature* 227, 680-685, 1970). Subsequently, polypeptides were blotted from

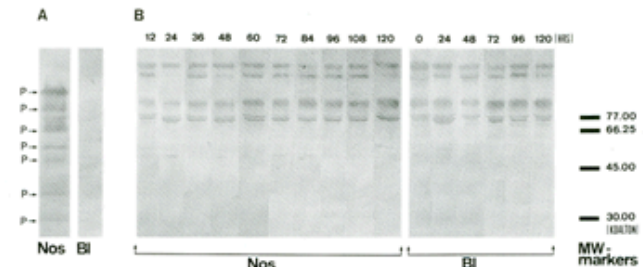
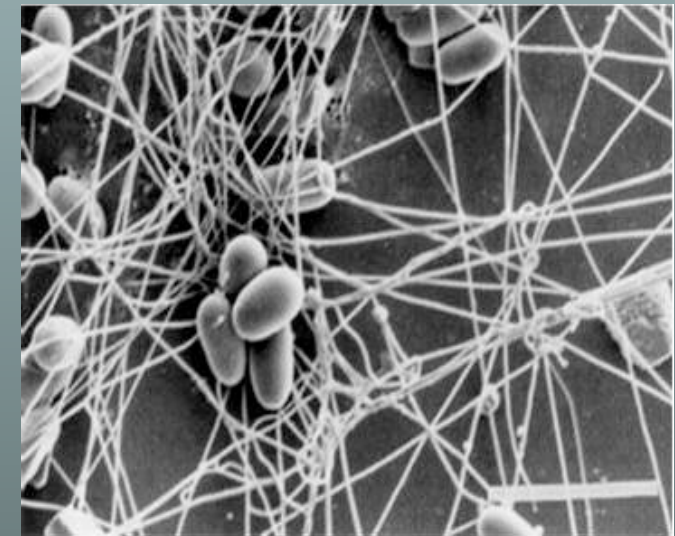
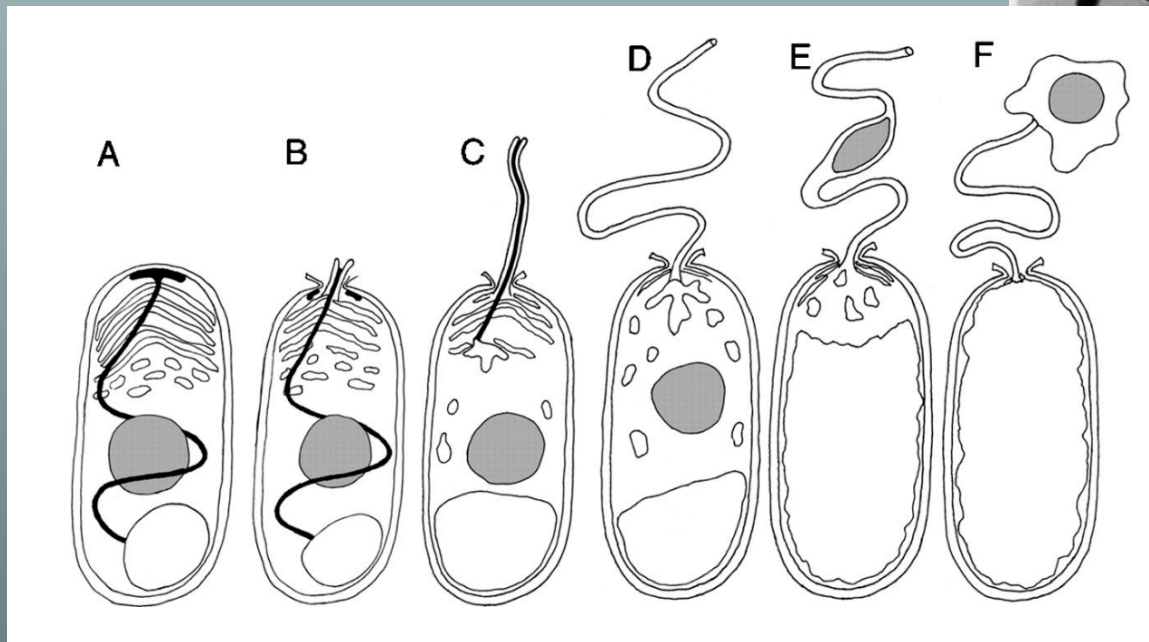
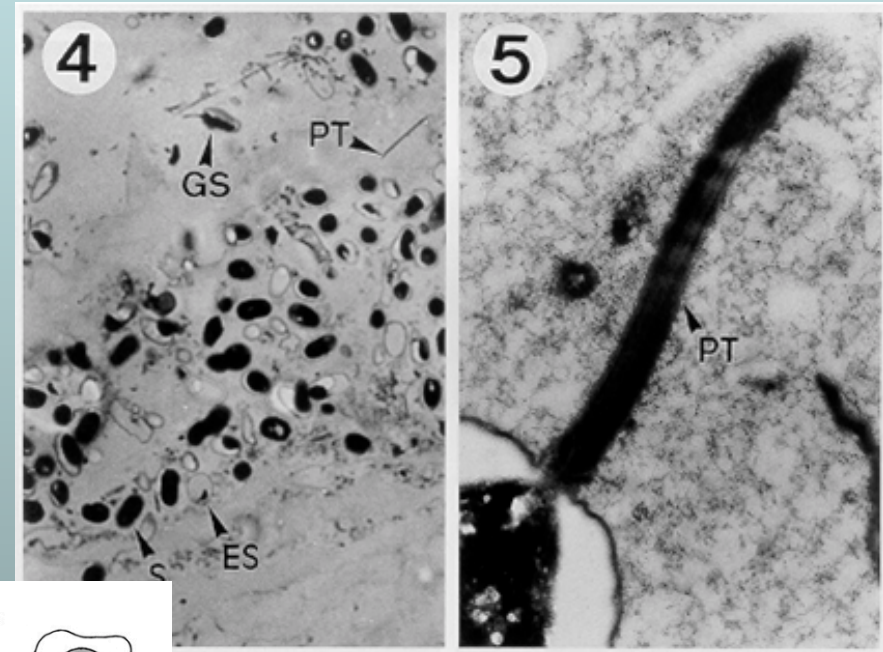


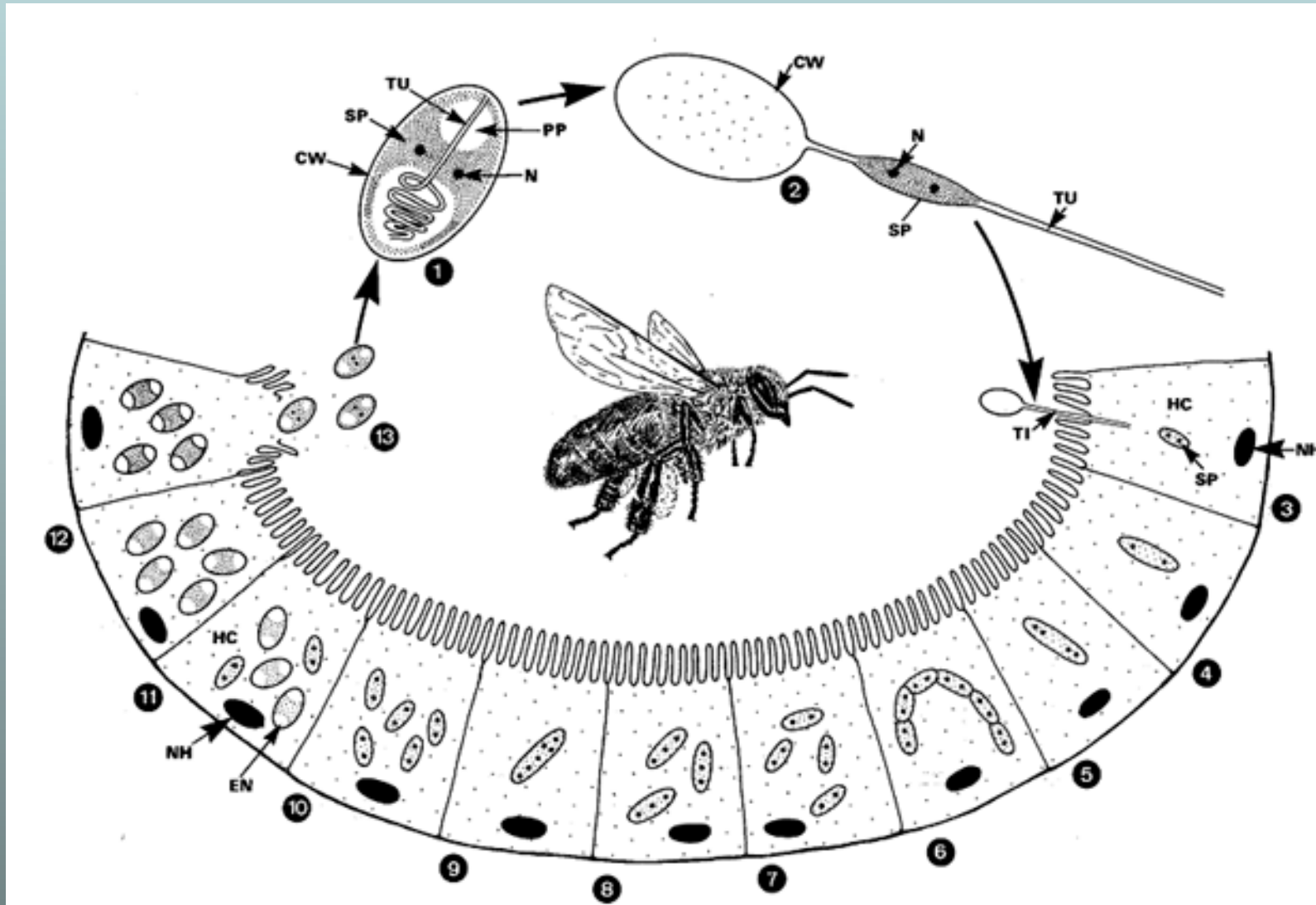
FIG. 1. Immunoperoxidase staining with anti-*Nosema* serum after SDS-PAGE (9% acrylamide). (A) Immunoblotting pattern from heavy *Nosema*-infected midguts (Nos) showing parasitic components (p). Healthy midguts (B1, blank) are unstained. (B) Immunoblotting pattern of hemolymph from *Nosema*-infected honeybees (Nos) at 12-hr interval of postinfection. Blank hemolymph samples (B1) are taken simultaneously.

The old story: *Nosema apis*

- hatching of spores
- invasion of host cells
- > polar tube
- > polaroplast
- > posterior vacuole
- > sporoplasm

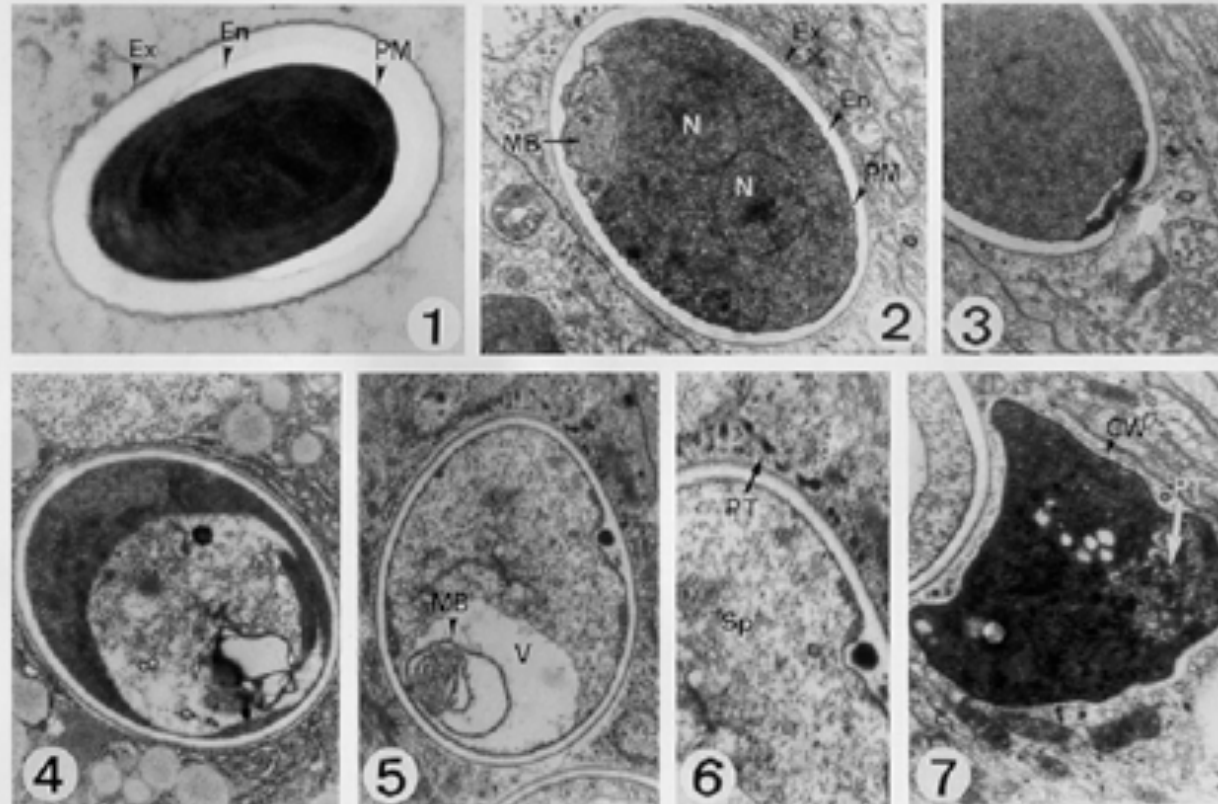


The old story: *Nosema apis*



The old story: *Nosema apis*

- thick and thin-walled spores



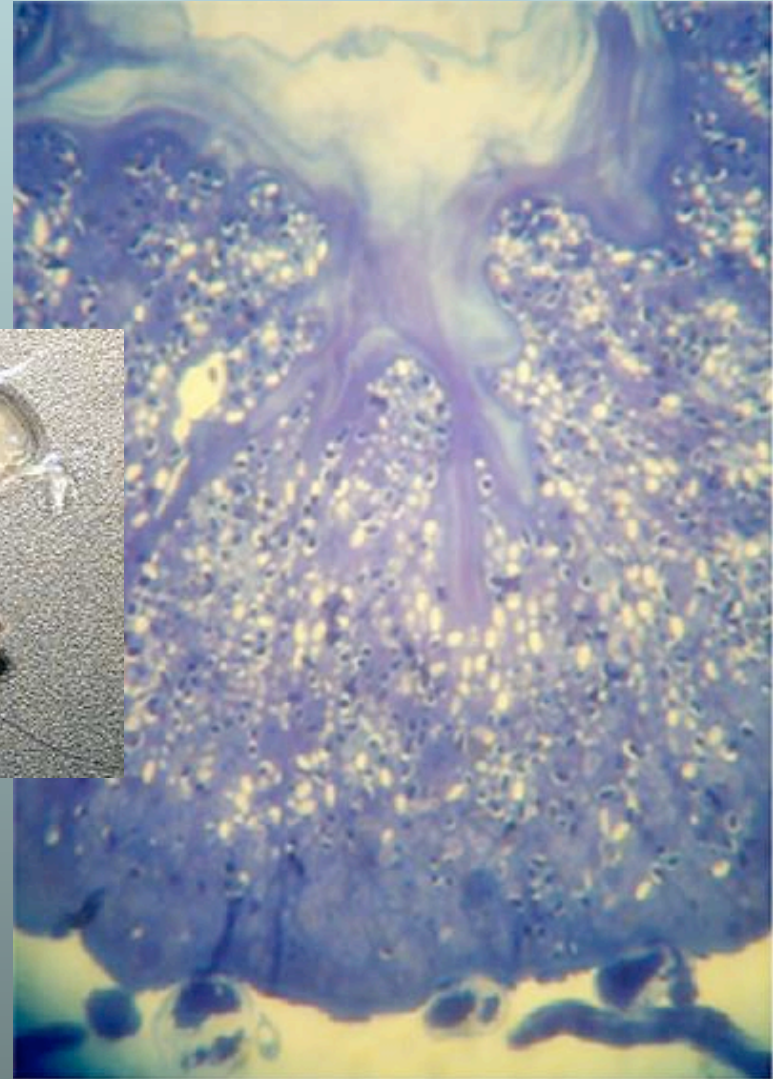
FIGS. 1-7. Abbreviations used: CW, cell wall; Ex, endospore, Ex, exospore; MB, merontic body; N, nucleus; PM, plasma membrane; PT, polar tube; Sp, spore; V, vacuole.
 FIG. 1. Ingested spore in midgut lumen, 1 hr pi (28,600 \times).
 FIGS. 2-4. Intracellular germination, 48 hr pi: Fig. 2, spore of the early population at the start of germination (19,400 \times); Fig. 3, in one of the following sections the same spore has an invaginated spore wall (19,400 \times); Fig. 4, partially germinated spore (25,600 \times); Fig. 5, excysted spore (19,600 \times); Fig. 6, detail of Fig. 5 showing the extruded polar tube (33,000 \times).
 FIG. 7. Sporoblast, 48 hr pi (28,000 \times).

The old story: *Nosema apis*

- diseased midgut

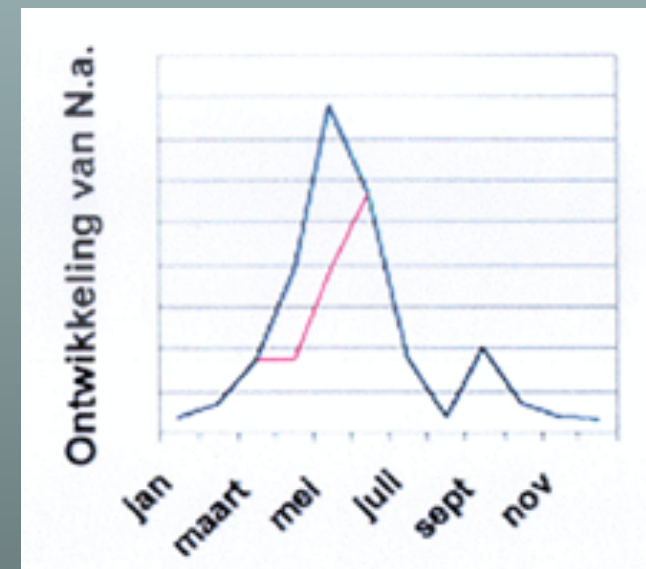
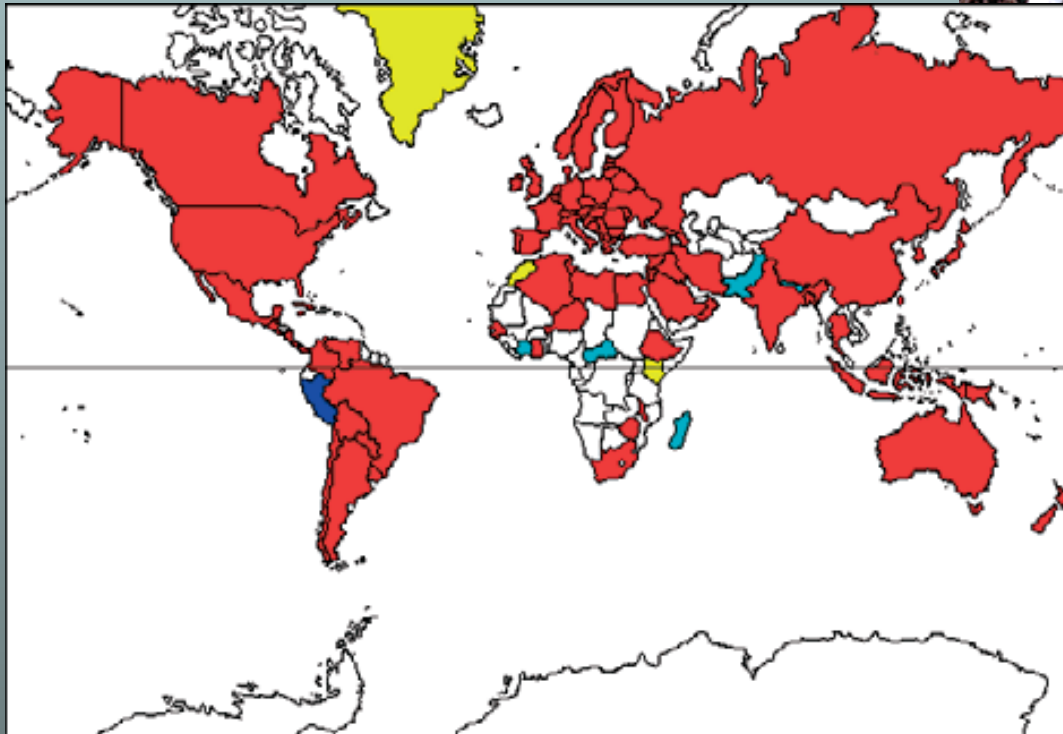
> microscopic section

> whole intestinal tract



The old story: *Nosema apis*

- diseased colonies
- seasonal course
- world-wide distribution
- economic losses in temperate climates

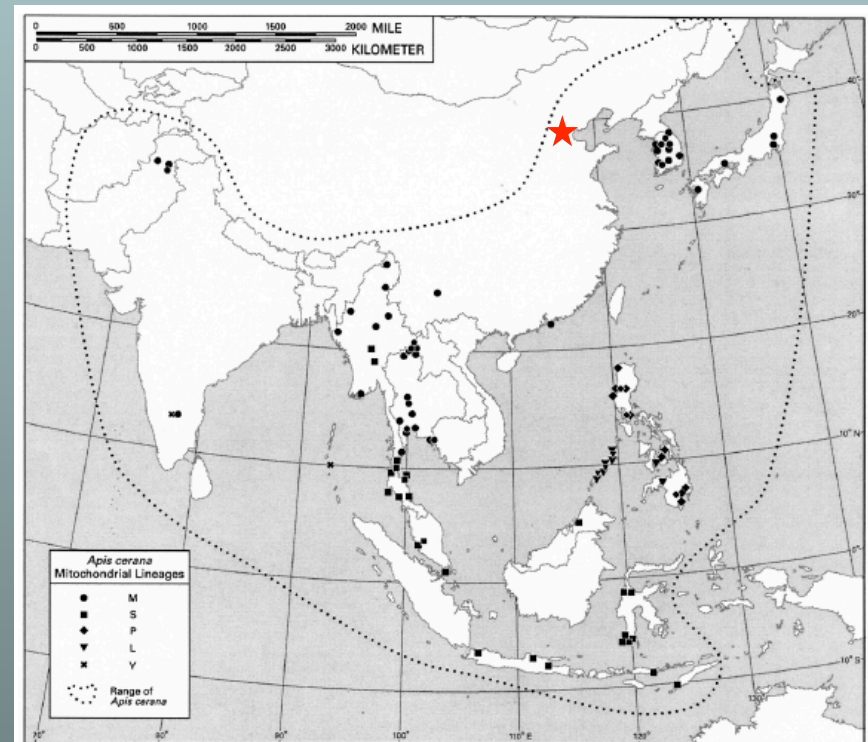


N. ceranae, parasite of Asian honeybee

Fries et al. (1996):

« *Nosema ceranae* n. sp., morphology and molecular characterization of a microsporidian parasite of the Asian honey bee *Apis cerana* »

- host: *Apis cerana*
(around Beijing, China)



N. ceranae, parasite of Asian honeybee

- spores: ovocylindrical, curved, distinctly smaller, fewer polar filament coils

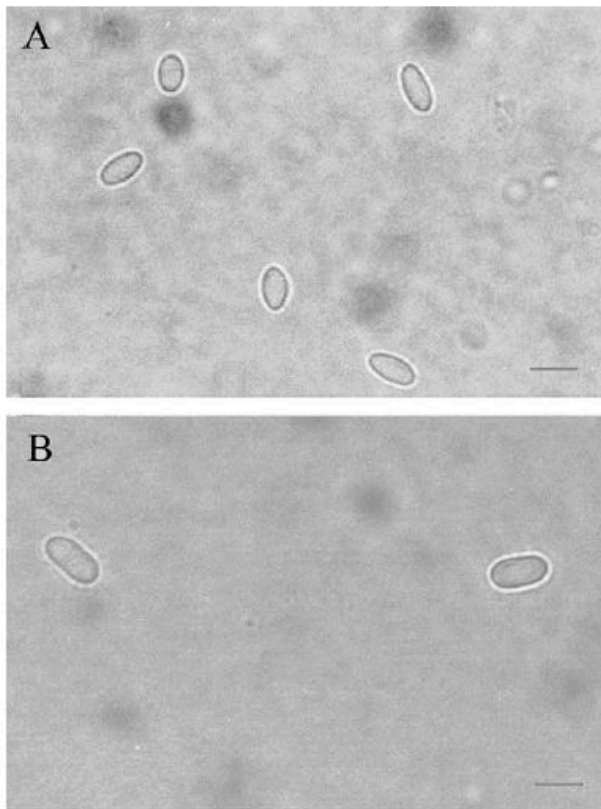


Fig. 1. Spores of *N. ceranae* (A) are distinctly smaller than spores of *N. apis* (B). Nevertheless, they can be hard to distinguish by light microscopy, in particular where mixed infections occur. Bars = 5 μ m. (From Fries et al. 2006a).

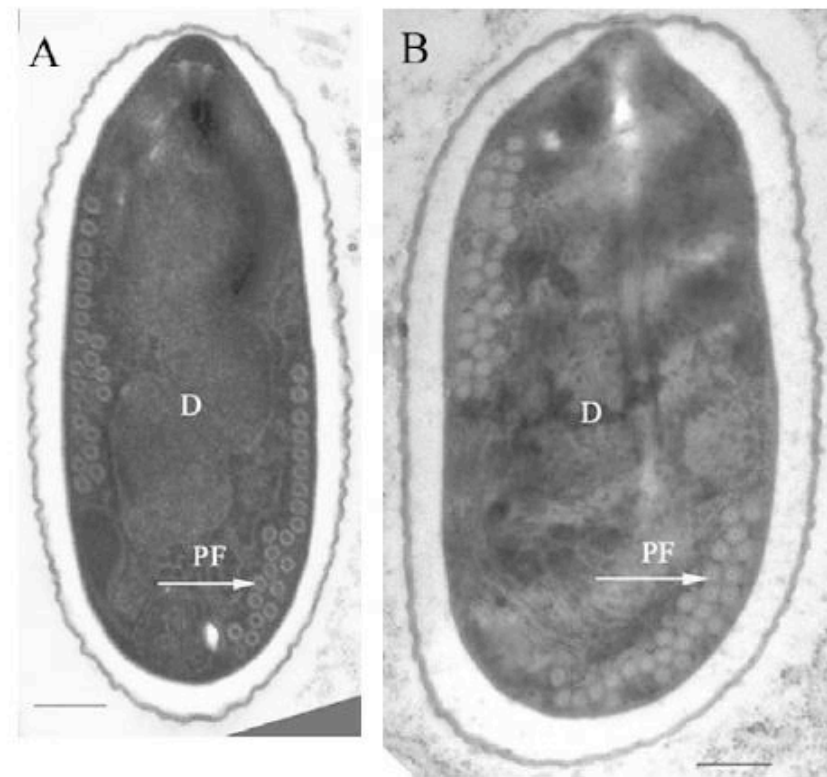


Fig. 2. The internal structures of the diplokaryotic (D) *N. ceranae* (A) and *N. apis* (B) are similar. Notably, *N. ceranae* spores contain fewer polar filament (PF) coils compared to *N. apis*. Bars = 0.5 μ m. (from Fries et al. 2006a).

N. ceranae, parasite of Asian honeybee

- ribosomes/rRNA

TYPE	SIZE	LSU	SSU
prokaryotic	70S	50S (5S, 23S)	30S (16S)
eukaryotic	80S	60S (5S, 5.8S, 28S)	40S (18S)
microspor.	70S	50S (5S, 23S)	30S (16S)

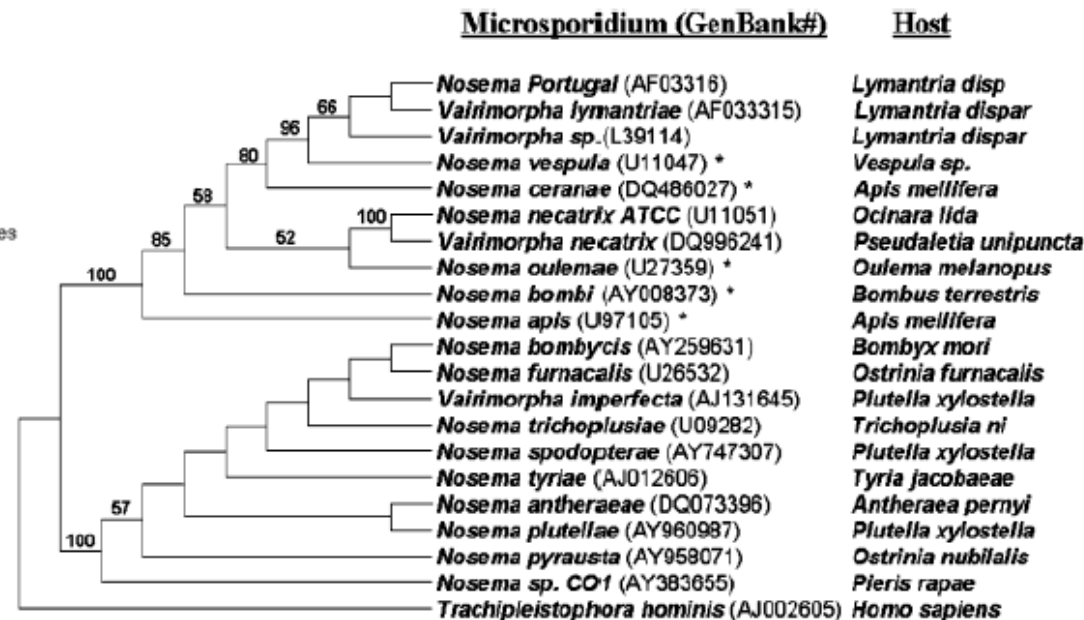
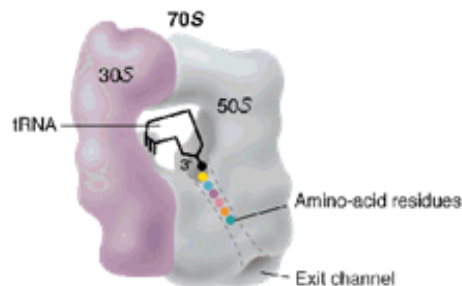


Fig. 10. Phylogenetic tree of microsporidia. Phylogenetic tree of microsporidia infecting insects based on the sequences of the small subunit rRNA gene and constructed by maximum parsimony analysis under a heuristic search. *Trachipleistophora hominis*-infecting *Homo sapiens* was used as an outgroup. The non-lepidopteran *Nosema* species are indicated by an asterisk. The reliability of the tree topology is shown by the bootstrap values located on the tree branches.



A new issue: *Nosema ceranae*

- 12 samples from different regions in Spain from colonies with signs of population depletion
- microscopy: *Nosema* spp.
- *N. apis* PCR negative (Webster 2004; 16S rRNA)
- new PCR primers + sequencing: 11 x *N. ceranae*



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Short communication

Nosema ceranae, a new microsporidian parasite in honeybees in Europe

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A new issue: *Nosema ceranae*

- rRNA sequenties
- ultrastructural characteristics

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Original article

A Nosema ceranae isolate from the honeybee *Apis mellifera**

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Received 19 December 2005 – Revised 18 April 2006 – Accepted 4 May 2006



A new issue: *Nosema ceranae*

- widespread dispersal

THE AMERICAS

Brazil, Canada, Uruguay (one sample of 1990), USA (since at least 1995)

ASIA

Taiwan, Vietnam

EUROPE

Belgium, China, Denmark, Finland (since at least 1998), France, Germany, Greece, Hungary, Ireland, Italy, The Netherlands, Serbia, Spain, Sweden, Switzerland, UK

AUSTRALASIA

New Zealand, Australia



A new issue: *Nosema ceranae*

- cage experiments (125,000 spores/bee)



Available online at www.sciencedirect.com



Journal of Invertebrate Pathology 94 (2007) 211–217

Journal of
INVERTEBRATE
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Experimental infection of *Apis mellifera* honeybees with *Nosema ceranae* (Microsporidia)

Mariano Higes ^{a,*}, Pilar García-Palencia ^b, Raquel Martín-Hernández ^a, Aránzazu Meana ^c

Table 1

Percentage of parasitized cells and mortality rate (mean \pm standard deviation) in infected and control groups

	Infected groups				Control
	3 h	3 d	6 d	7 d	
Parasite ratio (%)	0	4.4 \pm 1.2	66.4 \pm 6	81.5 \pm 14.8	0
Mortality (%)	0	0	66.7 \pm 5.6	94.1 \pm 0	5.9 (Day 7 p.i.)

Mortality was calculated as a percentage of the number of remaining caged bees at each time point.

A new issue: *Nosema ceranae*

Paxton et al. (2007):

NATURAL INFECTION

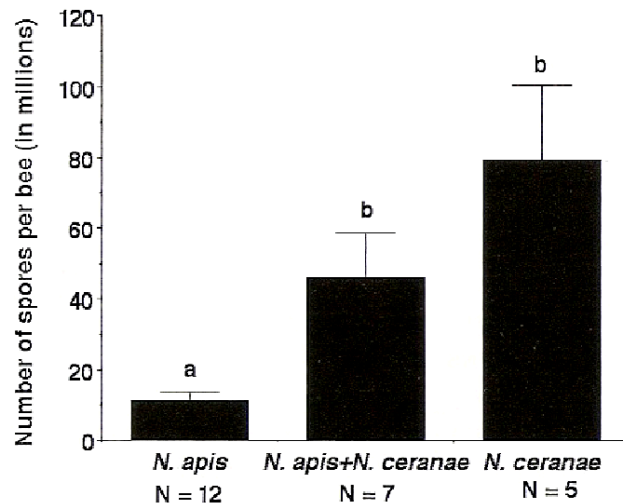


Figure 3. Numbers of spores per bee ($\times 10^6$) \pm SE for N = 24 *A. mellifera* colonies from Southern Finland, infected with either *N. apis*, a mixed infection of *N. apis*/*N. ceranae* or *N. ceranae* between 1986 and 2006 and sampled in spring. Spore identification was based on RFLPs. Different lower case letters represent means that differ significantly (ANOVA and Tukey *a posteriori* comparison of means, $P < 0.05$).

CAGE EXPERIMENT (100,000 spores/bee)

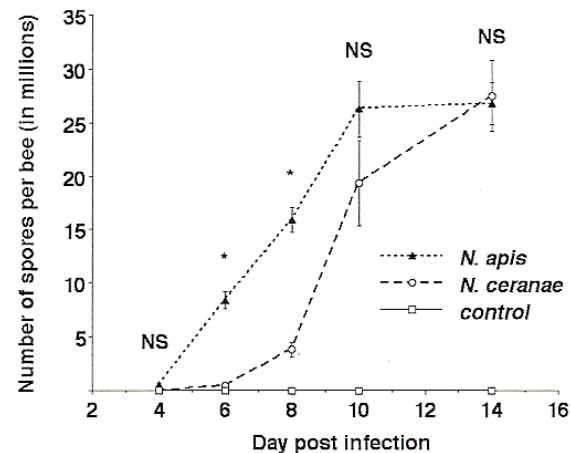


Figure 4. Number of spores per bee ($\times 10^6$) \pm SE post infection for *A. mellifera* experimentally infected with either *N. apis* (filled triangles and dotted line), *N. ceranae* (open circles and dashed line) or uninfected (control, open squares and solid line). Each point represents the mean of 5 bees. Results of the ANOVA analyses comparing the two treatments for each day post infection (with Bonferroni correction) are shown above the error bars for that day: *, $P < 0.05$; NS, not significant.

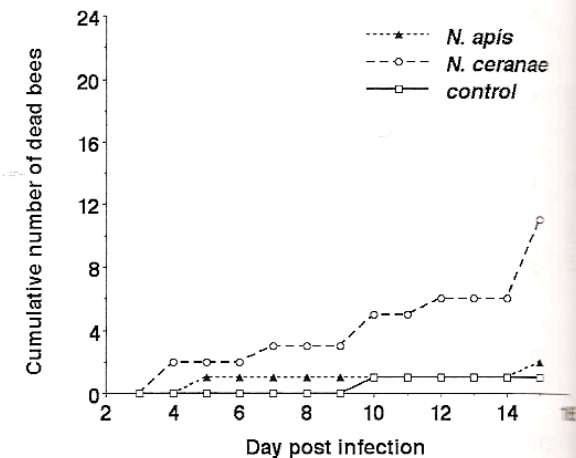


Figure 5. Mortality post infection of 25 *A. mellifera* workers experimentally infected with either *N. apis* (filled triangles and dotted line), *N. ceranae* (open circles and dashed line) or uninfected (control, open squares and solid line).

A new issue: *Nosema ceranae*

Forsgren & Fries (2010):

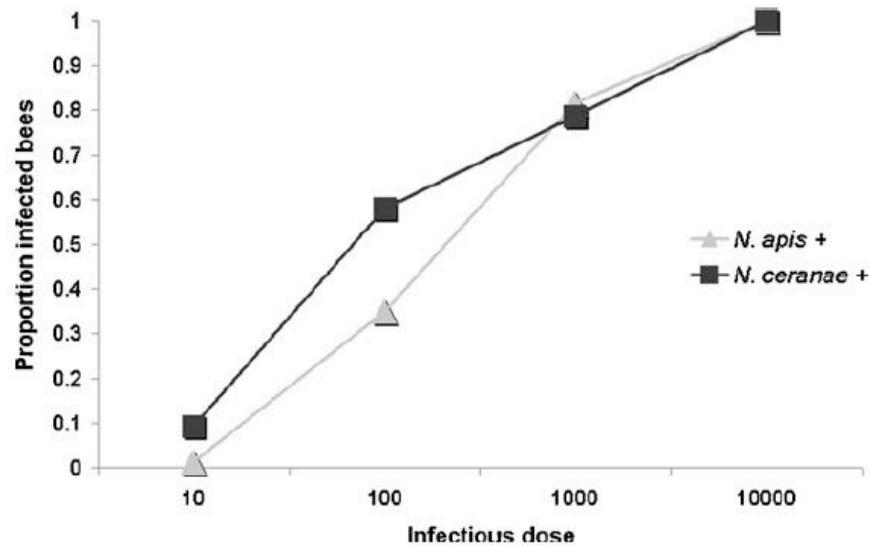


Fig. 1. The proportion of infected bees 14 days post-infection. Bees fed 10, 10², 10³ and 10⁴ spores of *N. apis* or *N. ceranae*. Results presented as a mean based on three cages with 30 bees ($n=90$) for each treatment.

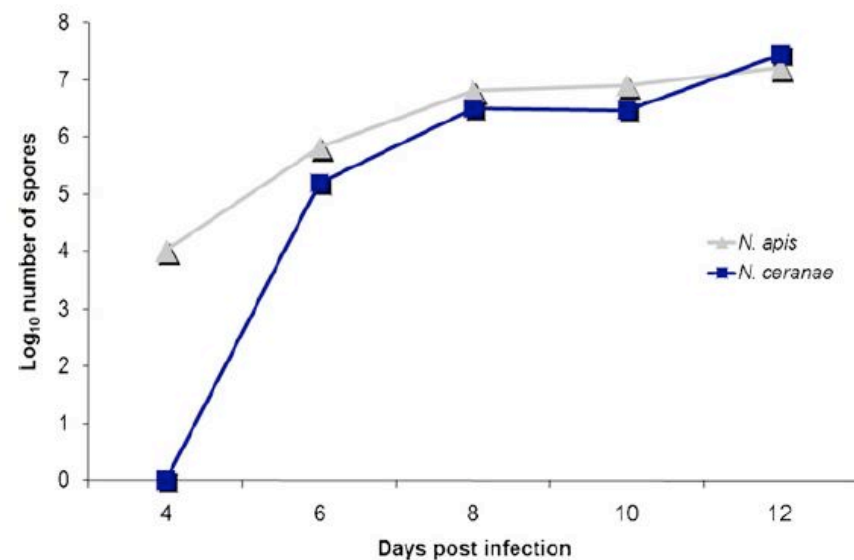


Fig. 2. The course of infection for *N. apis* and *N. ceranae*. Bees were individually infected with 10000 spores of the respective parasite. At day 12 post-infection, the infection is probably fully developed with a similar number of spores produced by both species.

A new issue: *Nosema ceranae*

Martin-Hernandez et al. (2007):

TABLE 4. Numbers of samples positive for *Nosema* spores

Year	No. of samples	% Positive
1999	154	13.0
2000	124	9.7
2001	146	24.7
2002	443	23.5
2003	484	54.5
2004	3,002	89.0
2005	1,423	95.6

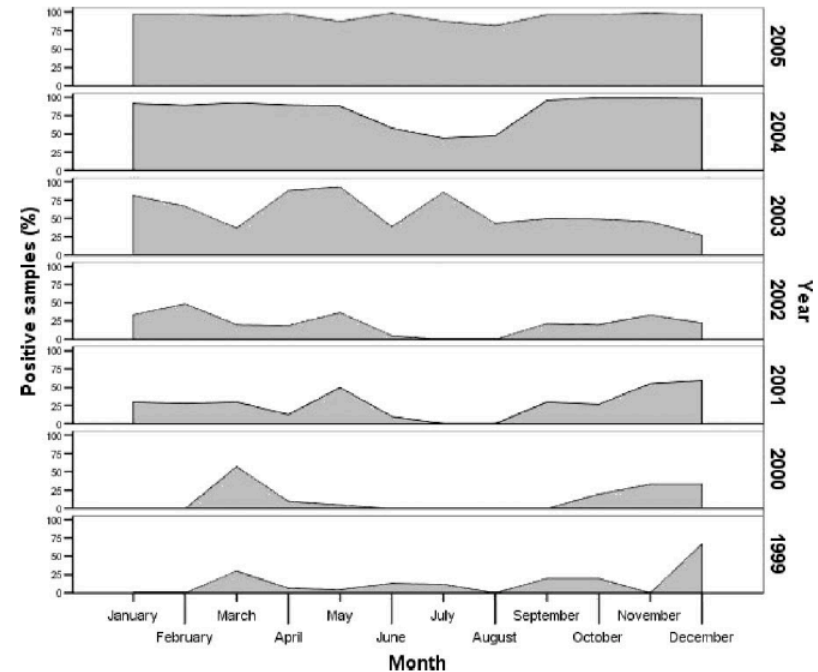


FIG. 2. Monthly distribution of *Nosema*-positive samples in every year from 1999 to 2005, obtained by comparing the number of samples positive for *Nosema* spores with the total number of samples received in the Laboratory of Bee Pathology.

TABLE 6. Association between the detection of microsporidian infection in Spanish honeybee samples and the signs observed in hives

PCR result	No. in depopulation group	No. in asymptomatic group	Total	Relative risk	95% confidence interval	P
<i>N. apis</i> + <i>N. ceranae</i>	10	1	11	5.82	3.20–10.59	0.0000
<i>N. ceranae</i>	44	8	52	5.42	3.03–9.68	0.0000
<i>N. apis</i>	2	16	18	0.71	0.17–2.92	0.4869
Negative	10	54	64			
Total	66	79	145			

A new issue: *Nosema ceranae*

Cox-Foster et al. (2007): Metagenomic survey of microbes in honeybee colonies with CCD

Table 2. Analysis of pools of bees tested for candidate pathogens. Numbers in the CCD, Non-CCD, and Total columns represent the percentage of samples found to be positive among all samples tested in each category. The positive predictive value represents the probability that a positive result for a given agent is associated with CCD. The sensitivity is the probability that test results will be positive in all CCD cases. Specificity is defined as the probability that all non-CCD samples will be associated with negative test results.

Agent	CCD (<i>n</i> = 30)	Non-CCD (<i>n</i> = 21)	Total (<i>n</i> = 51)	Positive predictive value (%)	Sensitivity (%)	Specificity (%)
IAPV	25 (83.3%)	1 (4.8%)	26 (51.0%)	96.1	83.3	95.2
KBV	30 (100%)	16 (76.2%)	46 (90.2%)	65.2	100	23.8
<i>N. apis</i>	27 (90%)	10 (47.6%)	37 (72.5%)	73.0	90.0	52.4
<i>N. ceranae</i>	30 (100%)	17 (80.9%)	47 (92.1%)	63.8	100	19.0
All four agents	23 (76.7%)	0 (0%)	23 (45.0%)	100	76.7	100

A new issue: *Nosema ceranae*

Genersch et al. (2010): German monitoring project

Table V. Effects of pathogen infection and parasite infestation in October on winter losses of honey bees.

Factor	Total No. of colonies analyzed	No. of colonies						<i>P</i> -value (χ^2)
		No. of survived colonies			No. of collapsed colonies			
		total	pathogen positive	pathogen negative	total	pathogen positive	pathogen negative	
DWV	1104	995	173	822	109	44	65	0.00001
ABPV	1104	995	75	920	109	17	92	0.0039
KBV	596	543	2	541	53	0	53	0.658
SBV	1104	995	99	896	109	6	103	0.202
<i>Nosema</i> spec.	1924	1744	317	1427	180	29	151	0.492

Table VI. Effects of beekeeping management on winter losses of honeybee colonies.

Factor	Total No. of analyzed colonies	Total No. of surviving colonies	Total No. of collapsed colonies	<i>P</i> -value
Type of beehive	4313	3889	424	0.94 (χ^2)
		wood / styrofoam (2594) / (1295)	wood / styrofoam (282) / (142)	
Starting condition ¹	4293	3876	417	0.78 (χ^2)
		O N C (2731) (724) (421)	O N C (317) (65) (35)	
Queen age years (n)	4021	3639	382	0.0052 (χ^2)
		0 1 2 3 4 (2002) (1238) (192) (5) (2)	0 1 2 3 4 (156) (181) (45) (0) (0)	
Colony strength in October (frames with bees \pm sd)	4313	3889 12.3 \pm 5.1	424 10.0 \pm 5.4	< 0.000001 (t-test)

¹ Old colony from previous year (O), newly formed colony during summer season (N), or combined colonies (C) either o + o, o + n, n + n.

A new issue: *Nosema ceranae*

Fries (2010):

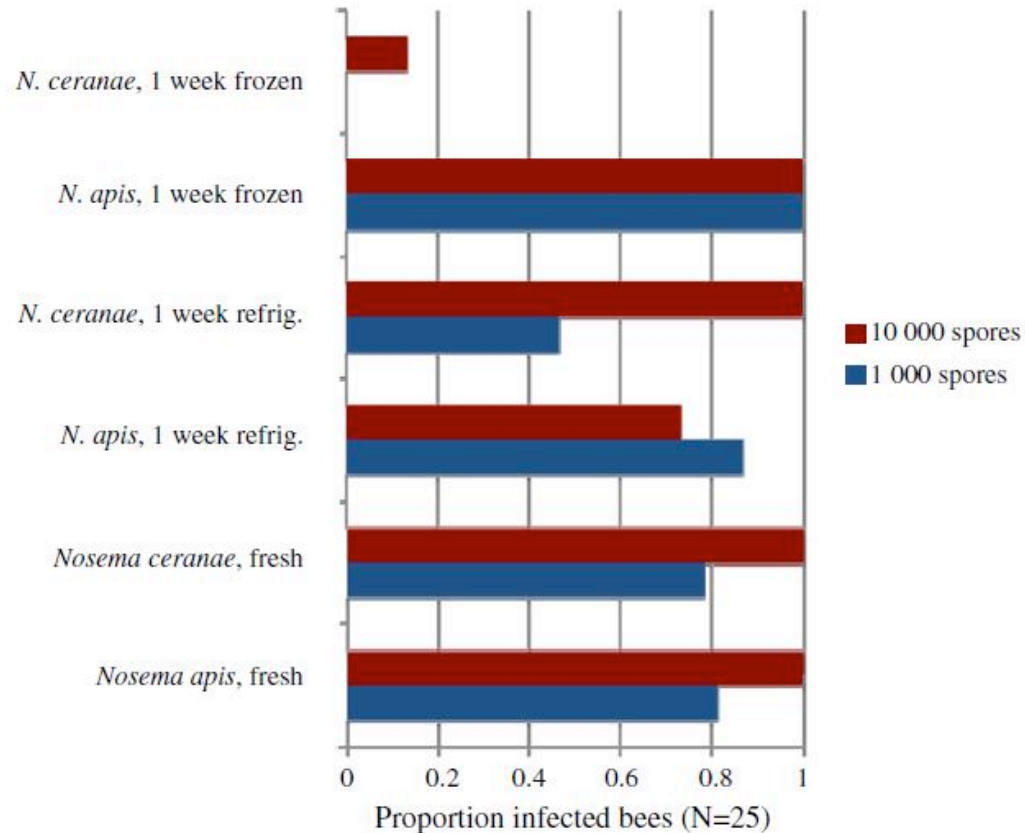


Fig. 3. The effect of freezing on *N. ceranae* spore viability is dramatic. Each bar represents 25 bees individually fed with 10 μ l sugar suspension containing 1000 or 10,000 spores of either *N. ceranae* or *N. apis*. Spores were fed from fresh infections (fresh) or (using the same suspension) after one week in a refrigerator at +8 $^{\circ}$ C (refrig.) or in a deep freezer at -18 $^{\circ}$ C (frozen). (From Fries and Forsgren, 2009).



A new issue: *Nosema ceranae*

Cornman et al. (2009):

OPEN ACCESS Freely available online

PLoS PATHOGENS

Genomic Analyses of the Microsporidian *Nosema ceranae*, an Emergent Pathogen of Honey Bees

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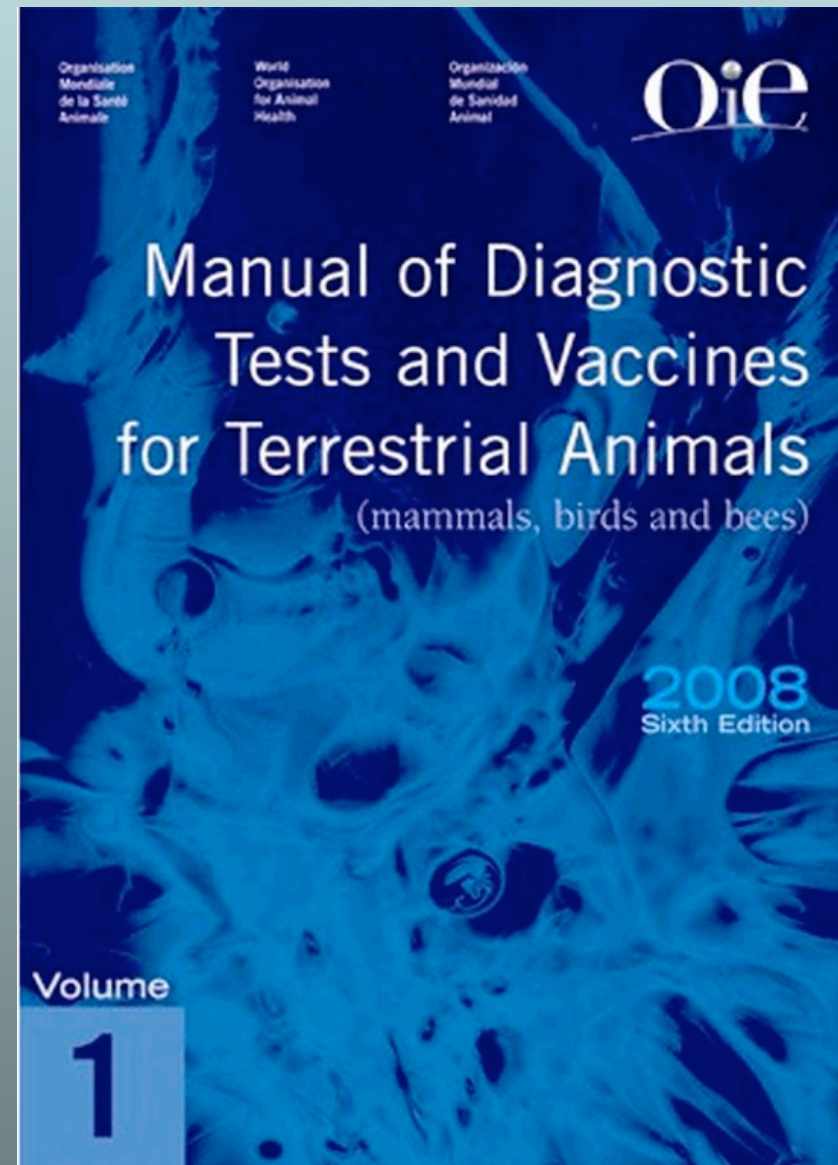
Diagnostics according to the OIA Manual

**Office International des
Epizooties**

**World Organisation for
Animal Health**

**= intergovernmental
organisation responsible
for improving animal
health worldwide**

- recognised as a reference
organisation by the World
Trade Organization (WTO)**
- 176 Member Countries
and Territories**





Diagnosics according to the OIA Manual

http://www.oie.int/eng/normes/mmanual/A_summry.htm

The screenshot shows a web browser window displaying the OIE website. The browser's address bar shows the URL http://www.oie.int/eng/normes/mmanual/A_summry.htm. The website header includes the OIE logo and the text "Organisation Mondiale de la Santé Animale", "World Organisation for Animal Health", and "Organización Mundial de Sanidad Animal". The main content area features the title "Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2010" and "Summary". A table of contents is visible, listing various chapters and sections, including "Introduction", "Part 1: General Information", and "Part 2: OIE Listed Diseases and Other Diseases of Importance to International Trade".

Health standards
Updated: 23.08.2010

*Manual of Diagnostic Tests and Vaccines
for Terrestrial Animals 2010*

Summary

-0-

Volume 1

[Introduction \(How to use this Terrestrial Manual\)](#)
[List of tests for international trade](#) (including the tests adopted in May 2009)
[Common abbreviations used in this Terrestrial Manual](#)
[Glossary of terms](#)
[Contributors](#)

Part 1
Section 1.1. Introductory Chapters
Chapter 1.1.1. [Collection and shipment of diagnostic specimens](#)
Chapter 1.1.2. [Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities](#)
Chapter 1.1.3. [Quality management in veterinary testing laboratories](#)
Chapter 1.1.4/5. [Principles of validation of diagnostic assays for infectious diseases](#) (NB: interim version, 2009)
Chapter 1.1.6. [Laboratory methodologies for bacterial antimicrobial susceptibility testing](#)
Chapter 1.1.7. [Biotechnology in the diagnosis of infectious diseases and vaccine development](#) (including text on nanotechnology adopted in May 2008)
Chapter 1.1.7.a. [The application of biotechnology to the development of veterinary vaccines](#) (NB: Version adopted in May 2010)
Chapter 1.1.8. [Principles of veterinary vaccine production](#)
Chapter 1.1.9. [Tests for sterility and freedom from contamination of biological materials](#)
Chapter 1.1.10. [Guidelines for international standards for vaccine banks](#)
Chapter 1.1.11. [The role of official bodies in the international regulation of veterinary biologicals](#)

Part 2
Section 2.1. Multiple Species
Chapter 2.1.1. [Anthrax](#)
Chapter 2.1.2. [Aujeszky's disease](#)
Chapter 2.1.3. [Bluetongue](#) (NB: Version adopted in May 2009)

Diagnostics according to the OIA Manual

Section 2.2. Apidae

- Introductory note on bee diseases**
- Ch. 2.2.1. Acarapisosis of honey bees**
- Ch. 2.2.2. American foulbrood of honey bees**
- Ch. 2.2.3. European foulbrood of honey bees**
- Ch. 2.2.4. Nosemosis of honey bees**
- Ch. 2.2.5. Small hive beetle infestation (*Aethina tumida*)**
- Ch. 2.2.6. Tropilaelaps infestation of honey bee (*Tropilaelaps* spp.)**
- Ch. 2.2.7. Varroosis of honey bees**

Diagnostics according to the OIA Manual

Microscopy

UNSTAINED (Cantwell, 1970)

- sample bees at hive entrance (avoid bees under the age of 8 days)
- 60 bees should be collected (in order to detect 5% of diseased bees with 95% confidence)
- fixation in 4% formol, 70% EtOH or freezing (in order to prevent them from decomposing)
- separate abdomens and ground in 2-3 ml water
- 3 drops on slide, examination at x400 under bright-field or phase-contrast optics

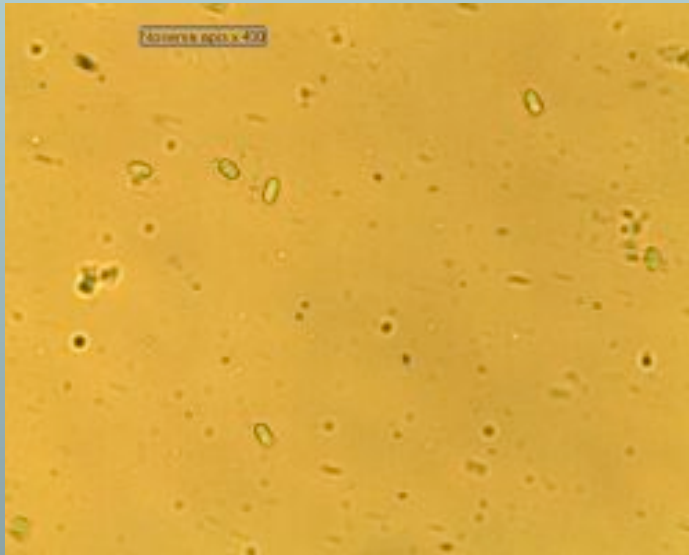
STAINED

- air-dried, EtOH-fixed smears of infected tissues
- Giemsa staining (10% in 0.02M phosphate buffer)

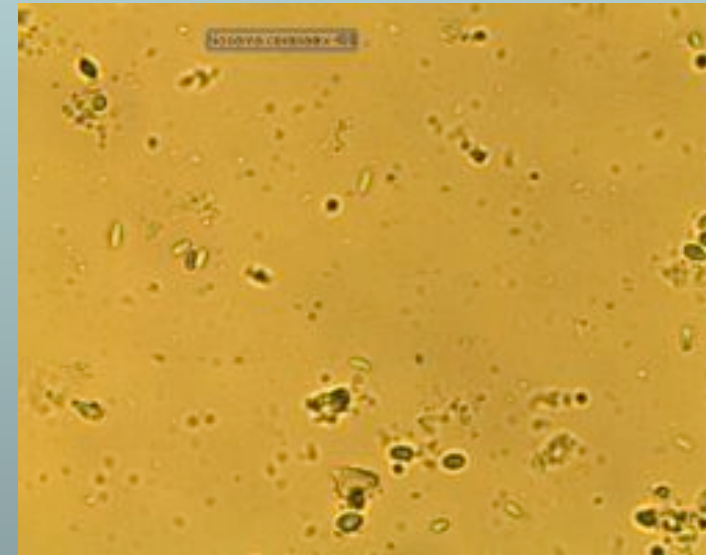
Diagnostics according to the OIA Manual

UNSTAINED

Nosema apis



Nosema ceranae



STAINED

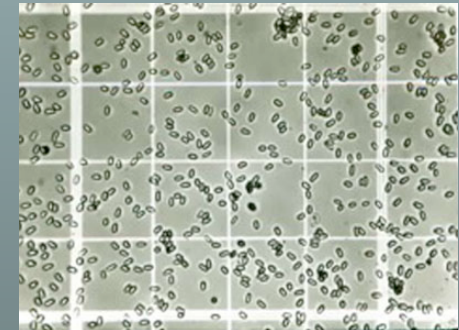
N. apis: unstained wall, indistinct blue interior without visible nuclei

insect cell, fungal spores and other protozoa: thinner wall, blue/purple cytoplasm, magenta coloured nuclei

Diagnostics according to the OIA Manual

Microscopy QUANTITATIVE

- 10 abdomens macerated in 5 ml water
- filtration through 2 layers of muslin
- water level equalised
- centrifugation, supernatants decanted
- tubes refilled to 10 ml level
- counting in a haemocytometer at 400x





Diagnosics according to the OIA Manual

Polymerase Chain Reaction

SAMPLE PREPARATION

- 10-20 abdomens macerated in 10 ml water
- filtration
- centrifugation, supernatants decanted
- induction of spore germination (NaCl+NaHCO₃)
- DNA extraction (routine procedure/comercial kits)

MULTIPLEX PCR

- primers used < [Martin-Hernandez et al. \(2007\)](#)

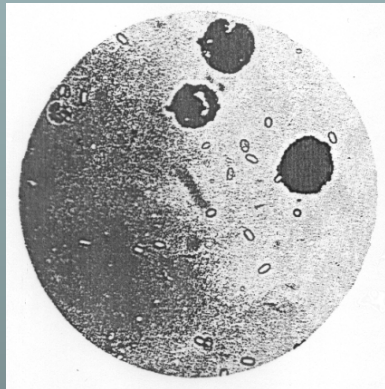
NAME	AMPLICON	SPECIFIC
218MITOC FOR	218-219	N. ceranae
218MITOC REV		
321APIS FOR	321	N. apis
321APIS REV		

Beyond OIE Manual

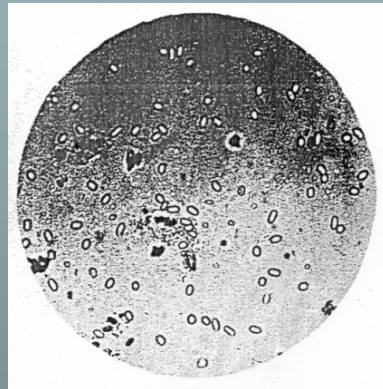
Microscopy

SEMI-QUANTITATIVE (Gross & Rutner, 1970)

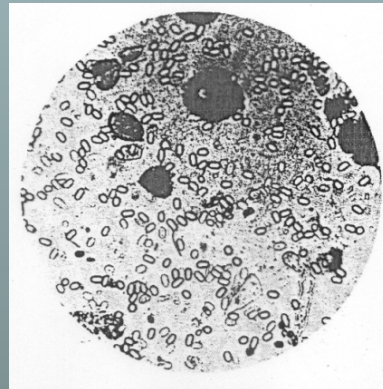
- 10-20 abdomens macerated in 10 ml water
- crushed midguts
- examination at 400x
- average infection level (30 fields of vision)



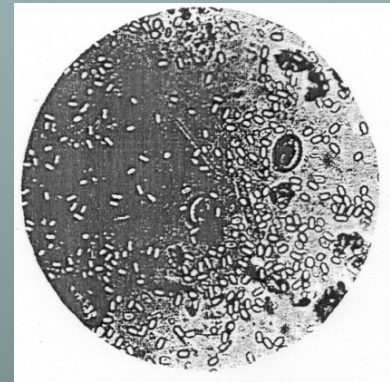
infection level I
+/- 10 spores



II
+/- 50 spores



III
+/- 100-150
spores



IV
> 150 spores

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Polymerase Chain Reaction FOR SEQUENCING

Higes et al. (2006) (*N. apis*-based)

primers **NOS FOR and NOS REV (240 bp)**

Higes et al. (2007) (*Enterocytozoon*-based)

primers **MICROCE-F and MICROCE-R (1171 bp)**
INTERFOR and INTER-REV

Huang et al. (2007) (divers origin)

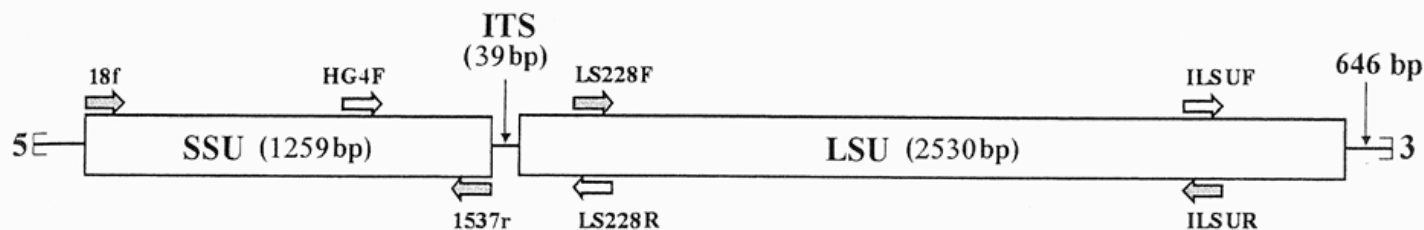


Figure 1. Schematic diagram of the *Nosema ceranae* isolate rRNA gene. The rRNA domains are indicated by boxes. The grey arrows represent primers used to amplify the major coding regions of rRNA while the white arrows represent primers for the ITS and the SSP-PCR.



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N. ceranae rRNA gene vs. Higes/OIE-primers:

>gi|94962170|gb|DQ486027.1|

CACCAGGTTGATTTCTGCCTGACGTAGACGCTATTCCTTAAGATTAACCCATGCATGTTTTTGACATTTGAAAAATGGACTGCTCAGTAATACTCACTTTATTTTATGTAAATTTTAATTAECTA
CGTTAAAGTGTAGATAAGATGTTTACAGTAAGAGTGAGACCTATCAGCTAGTTGTTAAGGTAATGGCTTAACAAGGCTGTGACGGGTAACGGTATTACTTTGTAATATCCCGGAGAAGGAGCCTG
AGAGACGGCTACTAAGTCTAAGGATTGCAGCAGGGGCGAACTTGACCTATGGATTTTATCTGAGGCAGTTATGGGAAGTAATATTATATTGTTTCATATTTTAAAAGTATATGAGGTGATTAAT
TGGAGGGCAAATCAAGTGCCAGCAGCCGCGGTAATACTTGTTCACAGAGTGTGTATGATGATTGATGCAGTCAAAAAGTCCGTAGTTTATTTATTTAAGAAGCAATATGAGGTGTACTGTATAGT
TGGGAGAAAGATGAAATGTGACGACCCCTGACTGGACGAACAGAAGCGAAAGCTGTACACTTGTATGTATTTTTTGAACAAGGACGTAAGCTGGAGGAGCGAAGATGATTAGATACCATTGTAGTT
CCAGCAGTAACTATGCCGACGATGTGATATGAAAATATTAATTTGTATTACATACTAGAAATTTGAGTTTTTTGGCTCTGGGGATAGTATGATCGCAGCATTGAAAATTAAGAATTTGACGGA
AGAATA

(H) TGCCGACGATGTGATATGAG → →
(O) CGGCGACGATGTGATATGAAAATATTA

CCACAAGGAGTGGATTGTGCGGCTTAATTTGACTCAACGCGAGGTAACCTTACCAATATTTTATTTATTTT ← GAGTACCGGTTTTTTGTTTGGAGAATGATAATAGTGGT ← ATGCCCGTTTTCAATG
G

GCCAAAAACAAACTCTTACTGGCCC (O)

GCAAAAGTTACC

ATGCTGTGAAGTTTTGATTAATTTCAACAAGACGTGAGACCCTTATTTTTTATTAAGACAGACACAATCAGTGTAGGAAGGAAAGGATTAAAACAGGTCGGTTATGCCCTCTGACATTTTGGGC
T

TACGACAC (H)

GCACGCGCAATACAATAGATATATAATCTTTTATGGGATAATATTTTGTAAAGAGATATTTGAACTTGAATTTGCTAGTAAATTTTATTAATAAGTAGAATGAATGTGTCCCTGTTCTTTGTACA
CACCGCCCGTTCGCTATCTAAGATGATATAATGTTGTGAAATAGTGAACACTACTTAAACAATATGTATTAGATCTGATATAAGTCGTAACATGGTTGCTGTTGGAGAACCATTAGCAGGATCATA
ATGATTTTTTAAAATTTATTTATTTTATTTTATTTTATTTTGGCCACACATGGGATCAATAGGATACCATAACGATGAAGGTCGTAATAGAATACGAAAGTATTTTAAATTTTACCGAATTA
TATTTAATAATATTGATTACCCTTTGAACCTAAGCATATCATAAAAGGAGGAAAAGAACTAAGTATTTCTTTAGTAGCGGCGAGTGAACAAGAAATAACCCCTGATTTGTAATCCTTAATT
GGAGATGTAATCAATTTTATTTTATTTTAAATATATCATAGAGAAATATTTATTTTCGTTATTTATAAGATGATAATCAAAATATTTGAGTAGGGCTGCTTGGTAGTGCAGTTTGAATAAAGGTA
GAATGAGATATCTAAGGTTAAATATAATGGTATACCGATAGCAAAATAAGTACTGCGAAGGATTTTGTGAAAATGTGTGTGTATAGCCTTATTTTAAAGGACCCGCTTGAACCCAGCAGGAGA
TTATATTTTATACCGAGATAATTTTTTATTTTAGCCGTTATTAATTTGTAAGTTATATTTAAGACCCGAAACACAGTGAACATACATGTTCTGGTTGAAGATAAGCAACAGTTTATTGGGAAGA
CCATAATCATTCTGACGTGCAAAATCGATGATTTAAGATGTGTATAGTGGCGAAAGACCAATCGAAGTGTGTGGTAGCTGGTTTACAGCGAAATGCTCTCAGGACAGCAGTCAATTTTTTTTTA
TATAGGACATAGATGTGGAACACTGTTATAATATTTATATTTATGAGAAATCTTAAATCTATGGAATTTTATAAAATTAATTTTATAAAATGTATCTATATTTTATGACTAGTGGGCACATGATT
GTAAGAATGATGTGCAAAAAGGGATGAACCTTATGAAACATTAATATTTCTATATAGTAGATATTTATACCCATAAATATGATGAAGACGTGGAGACAGCGCCGGCTGTTATGGAAGTAGAAAT
CCGCTAAGAAGCTGTTACAACGTATCTACCGAATGTATTTATGATAAAATGGAAGAAGATTACTACTTTTTATGAGATGGTTTCTGTTNTATTTTATTTATCAGGTAGCTGTGCAATTTGTTTG
TATTGAAGTATATATGTGAATATATAGAAAGAAACAAATGAGCCGATCTTGGAGGCAGTAACAATATATTTGTATAGATATTAGACTAGGGTTTTCAATTATTATTGAAGTGAATCGGTTGTAT
TTTATAAAAACAAAAAGATTAATAATCTTTATTTAACTATTTTAAAGCGACTTGATGATGACAGTATATTTATTAATCTAAGATAAATGAATAGATTATTTATTTTAACTATGGATTGTC
ATGATAAGAAGTAGTTATTTATATATACTTGATAAAAATATTTATAGTTTTAACCGTACCTATACCGCATCAGGAGTCTTTGTATCATAACAAATATTTTAAATTAAGTAAGTAAGGAATTCGG
CAAATTAGATCTGTAACCTTTGGGATAAAGATTGGCTCTAGCATGCTAGAACTTTTACTATGTAAGGAATCTGACTGTTTATTAAAAACATAGCTTTTTGTAAATACAAGAAGTGAATCTGCCCCA
GTGCATTTATTTGTTAAAGTTGTGTAAGCGAATGTAACCGGGGAGTAACTATGACTCTCTTAAGGTAGCCAAATGCCCTCGTCATTTAATTTGGTGACGCGCATGAATGGAGCAACGAGATTTCTTA
CTGCTCCCTACTTACAATTTTGTGAAACCACTATAACAAGGACCGGGCTGTTTATTTTATCAGCGGGGAAAGAACCTTGTGAGCTTGACTTTAGTATGTTCTAATAATTTTCGATATATTGTA
GAGAGGTGGGATATTTGTGAAACCCTAGTATGTTTGAATAATTTATTTATTTTATGAAATATATGGGGAGTTTGGCTTGGGCGGTACAGCTGTTAAAAGTAACACAGCTGTCCATAAGGTAG
ATGAATGATGGATGGTAACCATCAGTTTATTAAGGGAATGAATCTGCTTTACTTTATACATATGACTGTATTTAGTAGGGAAACCTTGGCCTAGCGATCCCAATTACATTCATTATGTATTGG
GTGATTGAAAAGTTACCACAGGGATAAATGGCTTGTAGCAGGCAAGCGATCATAGCGACTCTGCTTTTTGATCTTCGATGTCGCTCTCTCTGATCATCGTAGTGTATATGTTACGAAGTGTGG
ATTGTTTACCCCGTAATGAGGAACGTGAGATGGGTTTAGACCGTCTGTAGACAGGTTAGTTTTACCCTACTGTTTGTATTTTGTAGTGAACCTTTGATAGTACGAGAGGAACTCTAAGTGTGACC
ACTGGTAGTGGCATTAACTATTAAGTTATGTTGCTTAGCTACGTCATTTCCGTTAAGGCTGAAAGCCCTCTAAAGCCTGAAGCGTAGCTACAGGATATTTTTTGAAGTAACTACTACTCTTTTAAAGCA
GAAGTGAAGAAATGAAGTTGATGCGGCATTTTGAAGTTACTGCTTTTTTTGTTAATGATGAAGTAGTTATAAACACTAATATTCGATATATTTATGATGATAACTGCCCTCAGCTGAAAAGCGG
AAGTATAAAGAACTTACC GCCAGAAGAGACAATAATCTCTCATACAAAAGTTAAGGAGCCCTGCCAATGAGAGCTCCACAGTCCGCGGAGGGTGGGACTGGGGTTTTATAACTCCTGTTGGCGA
ATCTCATAAGTCTTACCAATACCTCCTGCCCTCCACAATGAGAGCGTCCACGTCGCGGAGGGTGGGACTGGGGTTTTATAACTCCCGGTGCGAATCTCATAAGTCTTACCCTACCTCTGGCTG
CTGGCATGGTAAGTAGGATCAGCGATATCCTTGGGGTGTAGTAGACCCATTGCCGATAAAAGAGTCCGTTACCCCTCGGGGAATCTTAAGTCTTTCTTCTAAGAAAGTAGGGCAAGCTATGCTCT

PCR FOR RAPID DIFFERENTIATION

Klee et al. (2007)

SSU rRNA

PCR-RFLP using *MspI*, *PacI* and *NdeI*

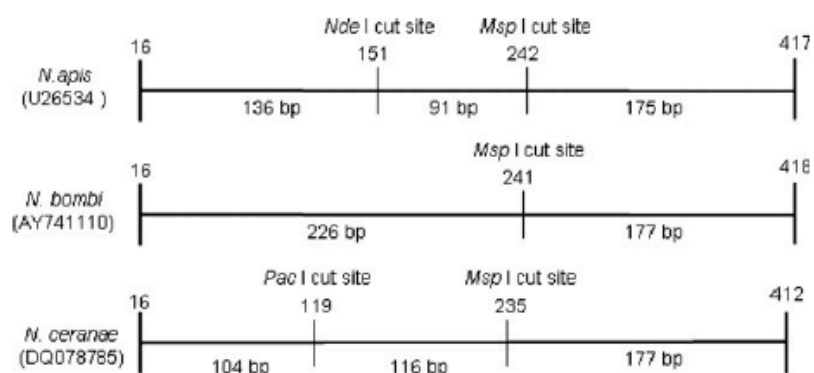


Fig. 2. Diagram showing the total length and size (in base pairs) of expected restriction fragments of *N. apis*, *N. bombi* and *N. ceranae* partial SSU rRNA PCR products amplified with the primer pair SSU-res-f1 and SSU-res-r1, and digested with *PacI*, *MspI* and *NdeI*. Base pair cut sites based 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*.

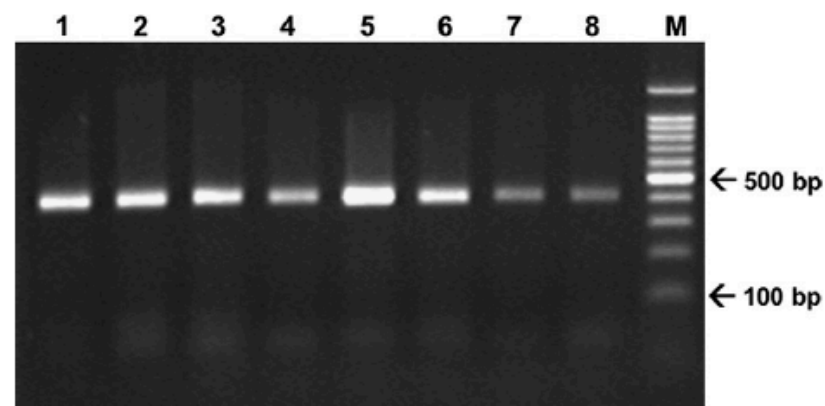


Fig. 3. Ethidium bromide stained agarose gel showing examples of PCR products for the fragment of SSU rRNA amplified with the primer pair SSU-res-f1 and SSU-res-r1. Lane 1, *N. ceranae* reference DNA extract; lane 2, *N. apis* reference DNA extract; lane 3, *N. bombi*; lane 4, Spain (Huelva); lane 5, 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.

1=*N. bombi*
 2=*N. apis*
 3=*N. ceranae*

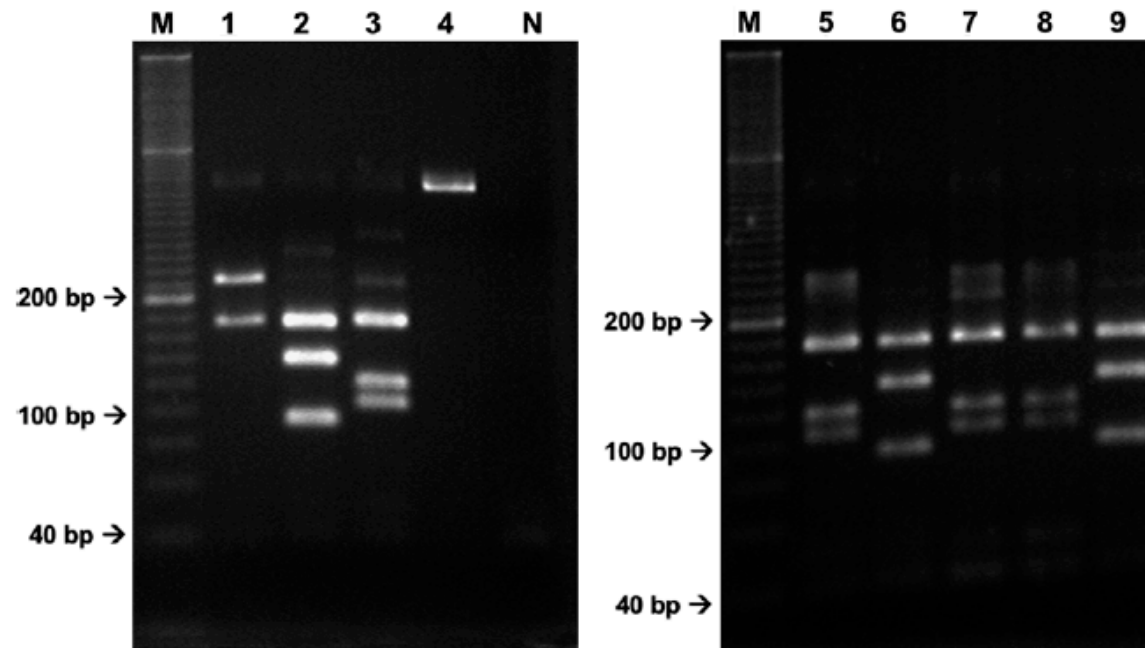
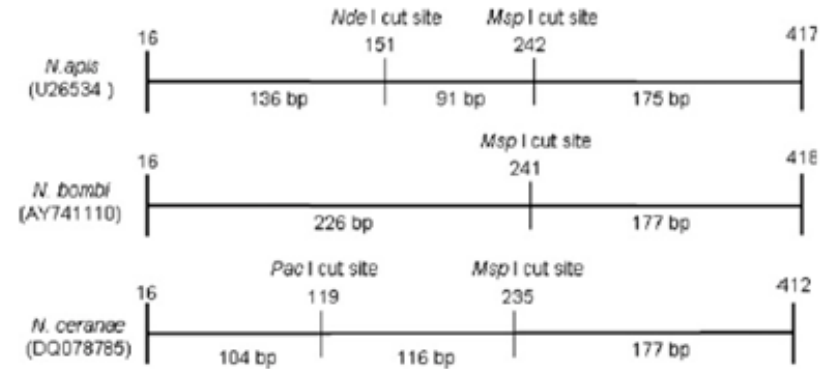


Fig. 4. Ethidium bromide stained agarose gel giving examples of RFLP analysis of partial SSU rRNA of *N. apis*, *N. ceranae* and *N. bombi* digested with the enzymes *MspI*, *PacI* and *NdeI*. Lane M, 20 bp ladder; lane 1, *N. bombi*; lane 2, *N. apis* reference DNA extract; lane 3, *N. ceranae* DNA 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.

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Chen et al. (2008)

- rRNA gene based
- 3 primer sets separately applied

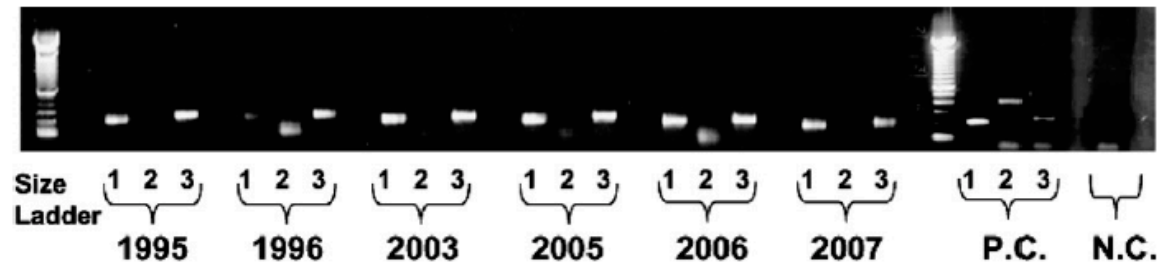


Fig. 1. PCR amplification of representative bee samples collected from 1995 to 2007 in the United States. For *x*-axis, No. 1 indicates that DNA was amplified with generic primers, *Nosema* F/R; No. 2 and 3 indicates DNA was amplified with specific primers, *N. apis* F/R and *N. ceranae* F/R, respectively. The primer pairs, *Nosema* F/R, *N. apis* F/R, and *N. ceranae* F/R generated PCR fragments of 208, 401, and 250 bp, respectively. DNA that was extracted from bees collected from Canada and identified to be positive for both *N. ceranae* and *N. apis* was used as a positive control (P.C.). Water was used as a negative control (N.C.). The PCR amplification bands are seen in samples amplified both with primer pairs *Nosema* F/F and *N. ceranae* F/R but in samples amplified with primer pair *N. apis* F/R.

SEARCH FOR POLYMORPHISM

Huang et al. (2008)

- comparison InterGenic Spacer region (IGS)
- comparison Internally Transcribed Spacer (ITS)
- no differences in sequences (except 1 point mutation < Martinique)

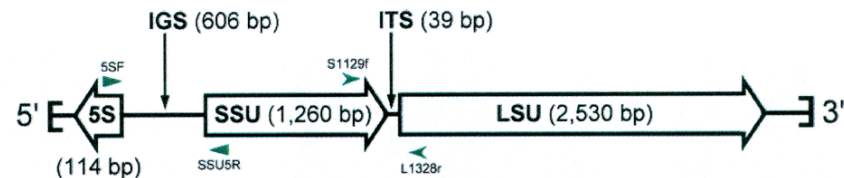


Fig. 1. Schematic diagram of the *Nosema ceranae* rRNA genes. The rRNA domains are indicated by hollow arrows and the direction of arrow indicates the direction of transcription. The small arrowheads represent primers used to amplify the IGS and ITS regions.

		*	20	*	4C		
<i>Nosema bombi</i> DQ472177	:	TCA	----	GGAAGTATAA	----	GTTTGTGGTATGTC	: 28
<i>Nosema apis</i> U97150	:	CGA	----	AGAATTGCAATTTT	----	TAGAATTAGTTTATA	: 33
<i>Nosema apis</i> Spain	:	CGA	----	AGAATTCAATTTT	----	TAGAATTAGTTTATA	: 33
<i>Nosema</i> sp. France	:	CAAG	----	AATATTATT	----	TTT	: 28
<i>Nosema ceranae</i> Martinique Island	:	TGATTTT	AAAAATTATTTATTT	CGTATTATTATTTTAT			: 39
<i>Nosema ceranae</i> France	:	TGATTTT	AAAAATTATTTATTT	CAATTATTATTATTTTAT			: 39
<i>Nosema ceranae</i> Spain	:	TGATTTT	AAAAATTATTTATTT	CAATTATTATTATTTTAT			: 39
<i>Nosema ceranae</i> Taiwan	:	TGATTTT	AAAAATTATTTATTT	CAATTATTATTATTTTAT			: 39
<i>Nosema ceranae</i> Taiwan Domested	:	TGATTTT	AAAAATTATTTATTT	CAATTATTATTATTTTAT			: 39
<i>Nosema ceranae</i> Taiwan <i>A. cerana</i>	:	TGATTTT	AAAAATTATTTATTT	CAATTATTATTATTTTAT			: 39
		gA	a	aAtTat	ttt	ta	atTa tTttat

Fig. 3. Alignment of the ITS from *N. cerana* isolates, *N. apis*, and *N. bombi*. The isolate, *Nosema ceranae* Taiwan domestic, was collected from the apiary which kept *A. cerana* and *A. mellifera* in the same environment. The isolate, *Nosema ceranae* Taiwan *A. cerana*, was collected from the wild colony of *A. cerana*. Other isolates were all collected from *A. mellifera* colonies.

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Sagastume et al. (2010)(continue)

- processus of homologue recombination suggested
(but putatively asexual microsporidia)

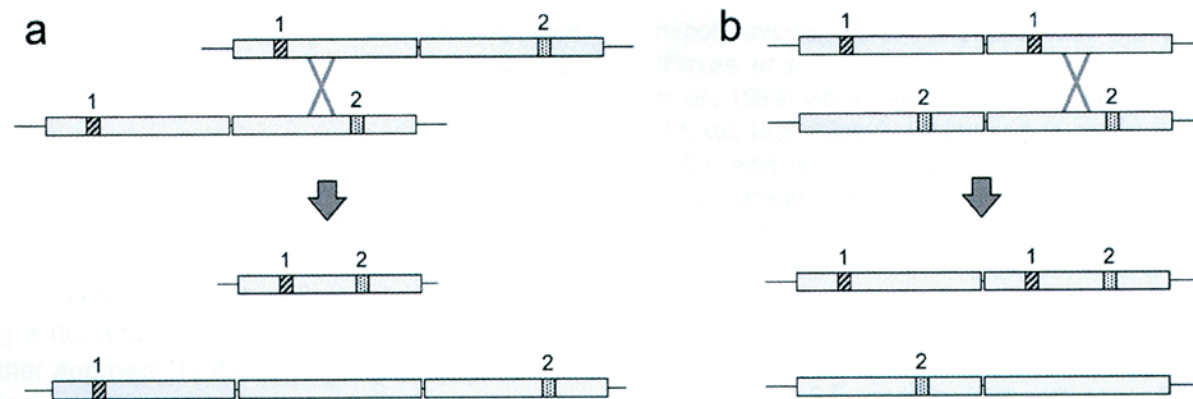


Fig. 3. Origin of new sequence variants by recombination.

A. Sister chromatid exchange generates new combinations of alleles that are already present in different repeats of a same genome.
B. Homologue recombination requires a diploid stage where two genomes of different origin meet in the same cell.



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REAL-TIME PCR FOR QUANTIFICATION Bourgeois et al. (2010)

Table 1
Primer and probe specifications for real-time PCR amplification of *Nosema apis* and *N. ceranae*.

Species	Primers	Product size (bp)	Location
<i>Nosema apis</i> genbank: U97150	For: GCCCTCCATAATAAGAGTGTCAC Rev: ATCTTCATCCCAAGAGCATTGC Probe: ACTTACCATGCCAGCAGCCAGAAGA	142	4364
<i>Nosema ceranae</i> genbank: DQ486027	For: AAGAGTGAGACCTATCAGCTAGTTG Rev: CCGTCTCTCAGGCTCTTCTC Probe: ACCGTTACCCGTCACAGCCTTGTT	104	218

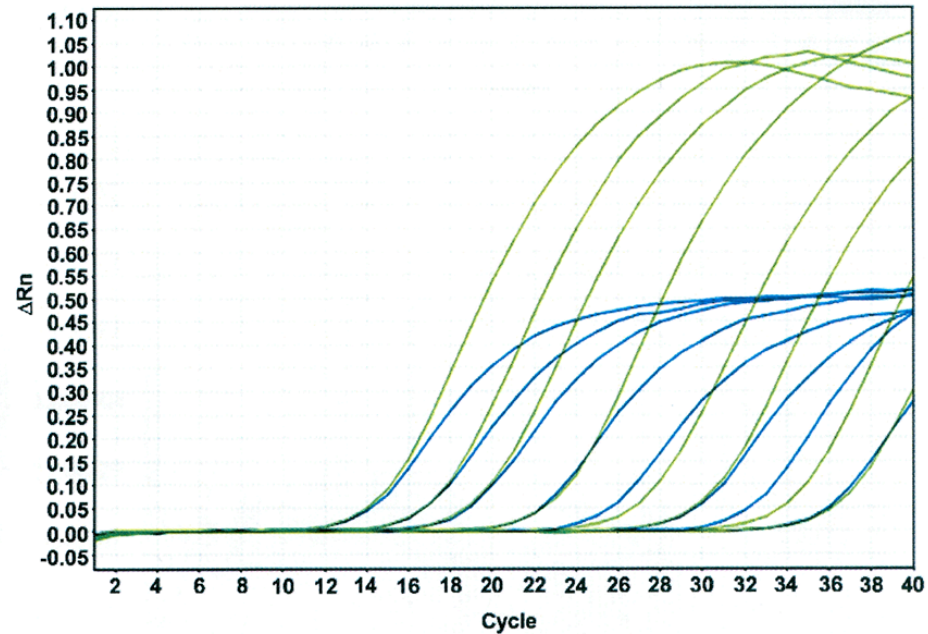
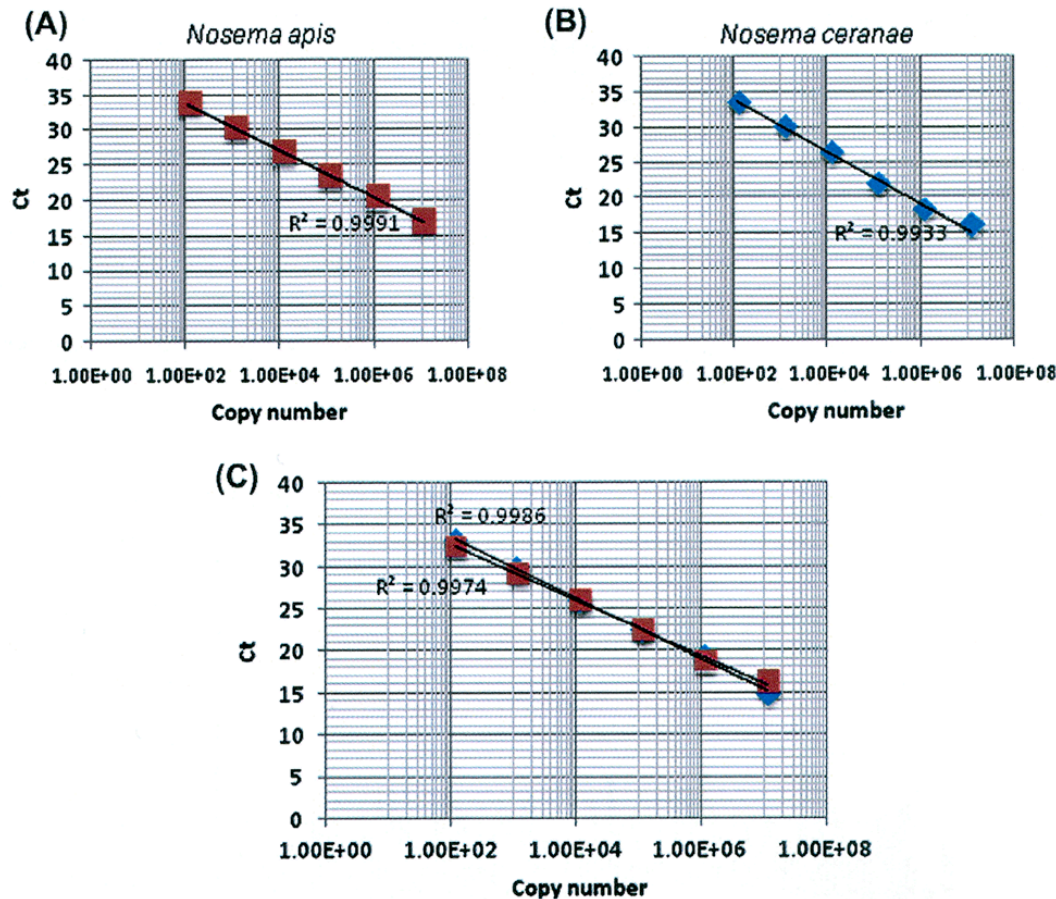


Fig. 2. Real-time PCR fluorescence curves for eight *Nosema apis* and *N. ceranae* standards co-amplified. Standard template was added in 10-fold serial dilutions from 500 pg template to 50 ag template DNA, representing 1.18×10^8 to 11.8 copies of the target fragments.

Bourgeois et al. (2010)(continue)



Due to the protocol used for DNA extraction, conversion factors from copies/ μ L to spore equivalents/bee were calculated for both individual and pooled samples as follows:

Individual samples:

$$\frac{\text{Num. Individual } \textit{Nosema}}{\text{bee}} = \frac{\left(\frac{a \text{ } \textit{Nosema} \text{ copies}}{\mu\text{L PCR}}\right) \left(\frac{25 \mu\text{L}}{0.2 \text{ bee}}\right)}{10 \text{ copies per genome}}$$

where a = copy number from real-time PCR, and 0.2 bee represents the 200 μ L aliquot taken from the 1 mL homogenate.

Pooled samples:

$$\frac{\text{Num. Individual } \textit{Nosema}}{\text{bee}} = \frac{\left(\frac{a \text{ } \textit{Nosema} \text{ copies}}{\mu\text{L PCR}}\right) \left(\frac{25 \mu\text{L}}{(0.04) (b \text{ bees})}\right)}{10 \text{ copies per genome}}$$

where a = copy number from real-time PCR, b = number of bees pooled in the DNA extraction.

Fig. 1. Standard curves for real-time PCR assay developed for detection of *Nosema apis* and *N. ceranae*. (A) Standard curve based on six standards for *N. apis*. (B) Standard curve based on six standards for *N. ceranae*. (C) Standard curves for *N. apis* and *N. ceranae* co-amplified in the same reaction (i.e., multiplexed). C_t is the cycle at which fluorescence was above background levels.

Conclusions

- *N. ceranae* jumped recently from the Asian to the European honeybee (discovered 2x independently)
- impact is region dependent a may be related to its freezing-°T sensitivity
- traditional microscopic techniques cannot differentiate *N. apis* and *N. ceranae*
- there are several molecular tools that can
- haplotyping based on rRNA gene seems no releable marker for differentiation
- quantification of spore-load is also possible by real-time PCR