Department of Physiology
Laboratory of Zoophysiology

# "Nosemosis: old story, new issue"

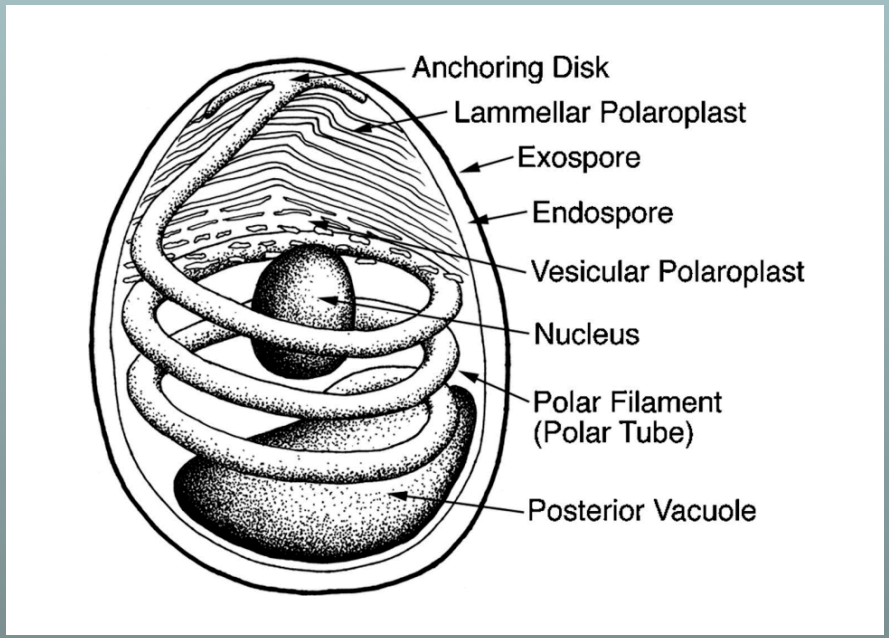
prof. dr. Dirk C. de Graaf

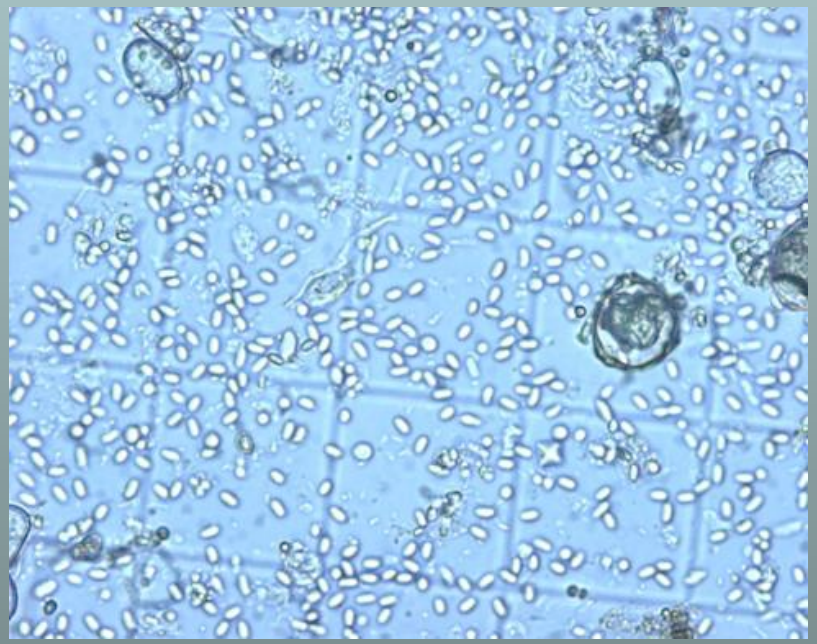
# *overview*

- **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*

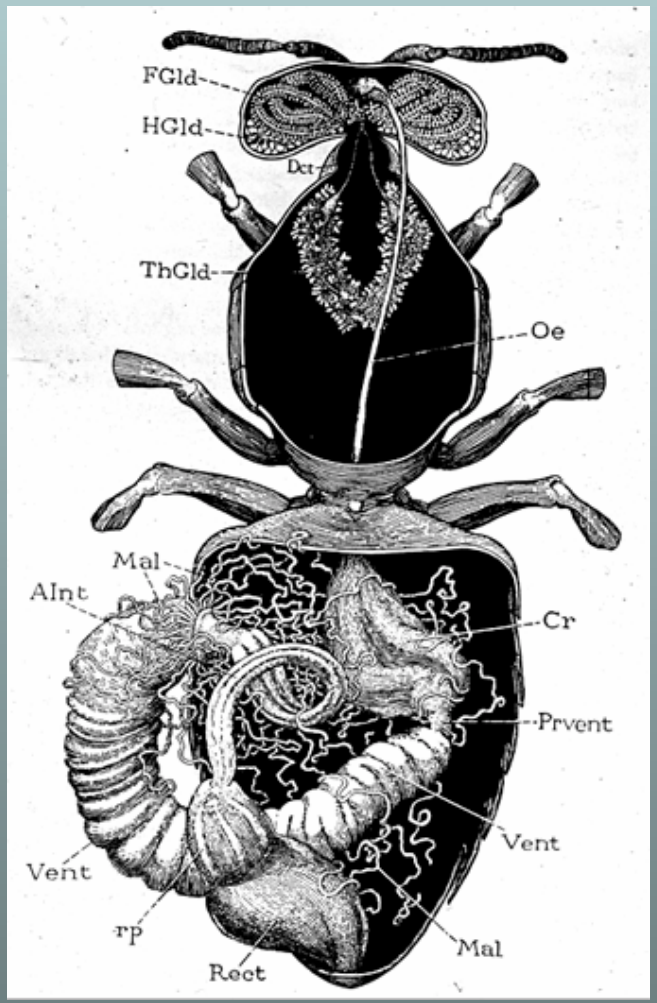
- microsporidia (fungi)
- spores: characteristic internal structure
- first desciption in bees: Dönhoff & Leukart (1857)
- classification by Enoch Zander in 1909



# The old story: *Nosema apis*

## - tissue specific: midgut




JOURNAL OF INVERTEBRATE PATHOLOGY 58, 277–278 (1991)

### Tissue Specificity of *Nosema apis*

*Nosema apis* (Microsporida, 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 1 hr postexposure 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 proceed 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 $10^6$ 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

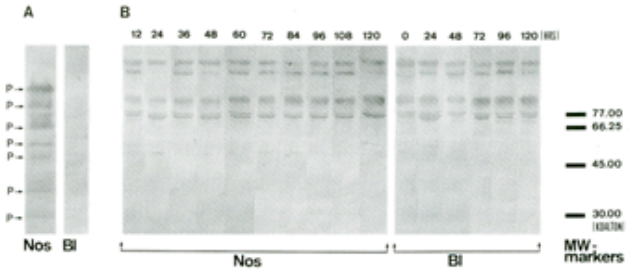



FIG. 1. Immunoperoxydase staining with anti-*Nosema* serum after SDS–PAGE (9% acrylamide). (A) Immunoblotting pattern from heavy *Nosema*-infected midguts (Nos) showing parasitic components (p). Healthy midguts (Bl, blank) are unstained. (B) Immunoblotting pattern of hemolymph from *Nosema*-infected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

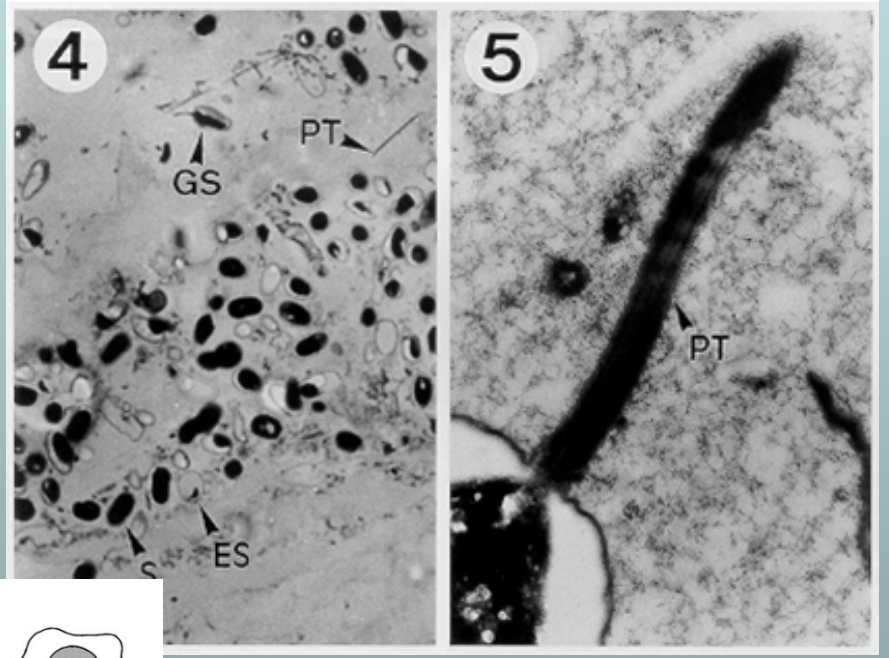


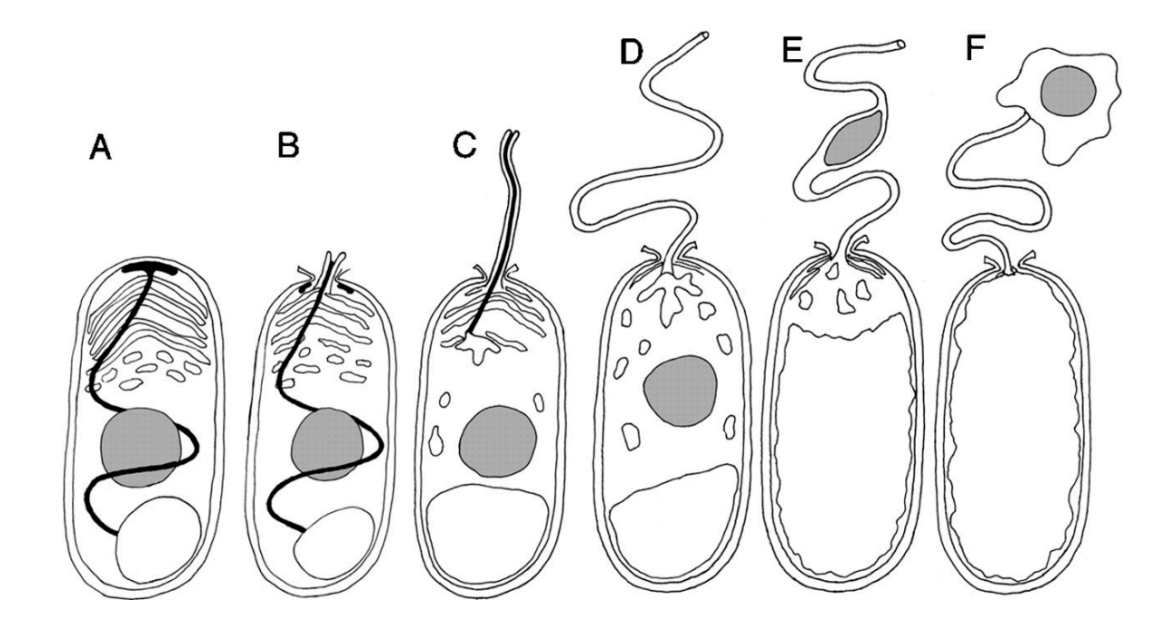
0022-2011/91 $1.50

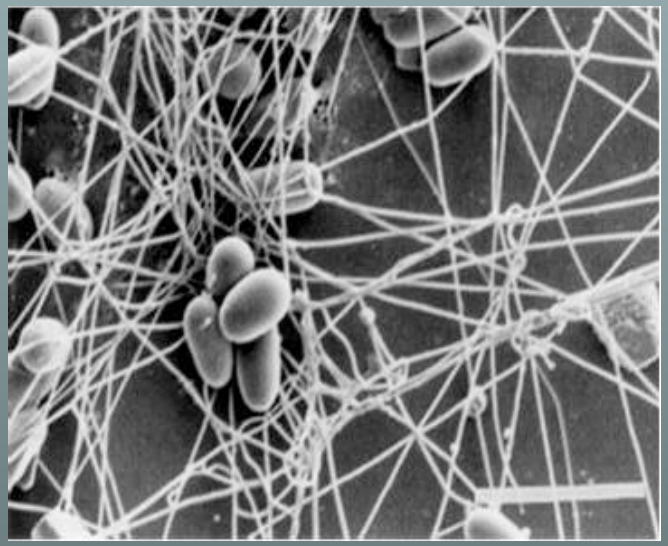
# The old story: *Nosema apis*

- hatching of spores
- invasion of host cells

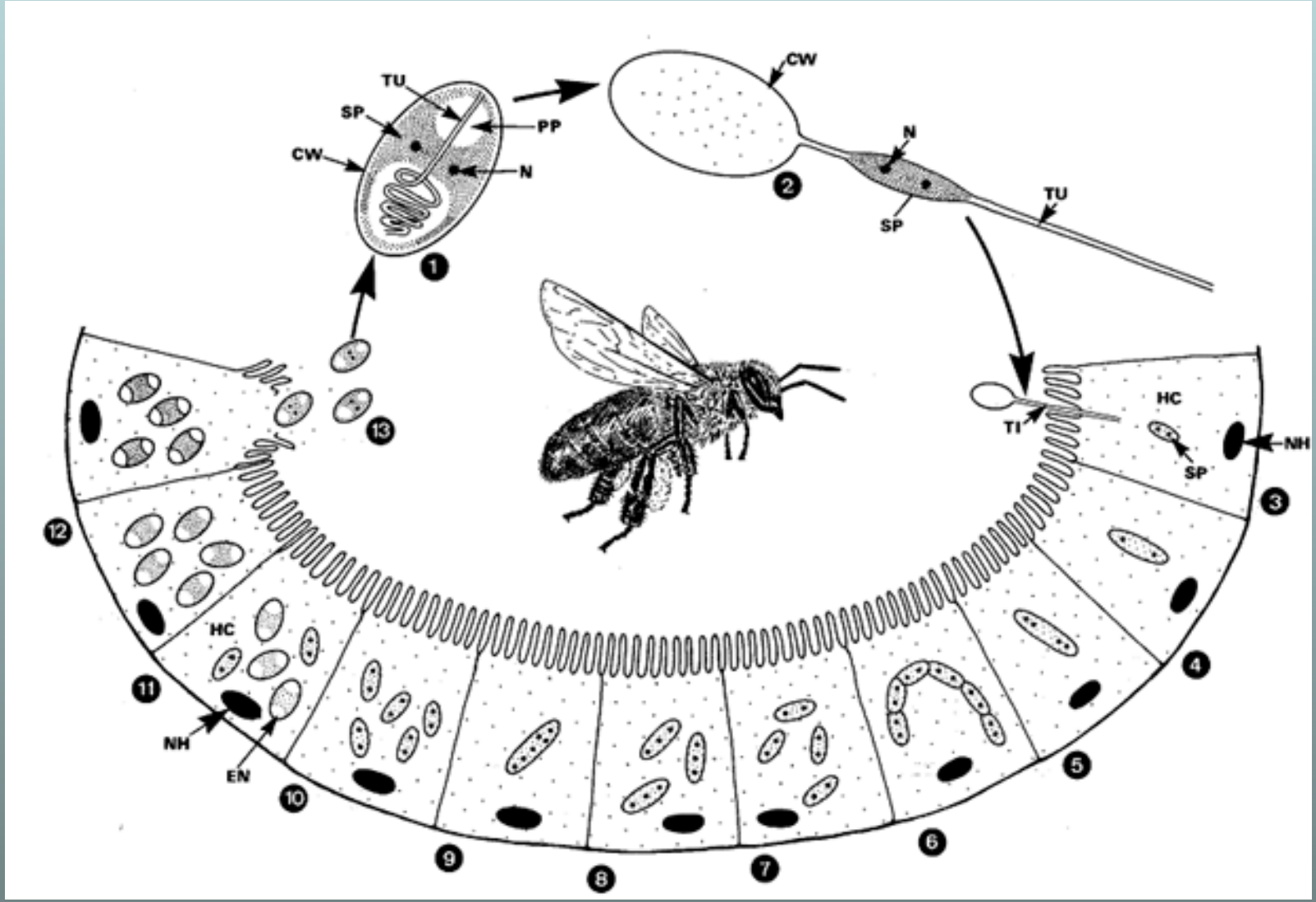
> polar tube
> polaroplast
> posterior vacuole
> sporoplasm



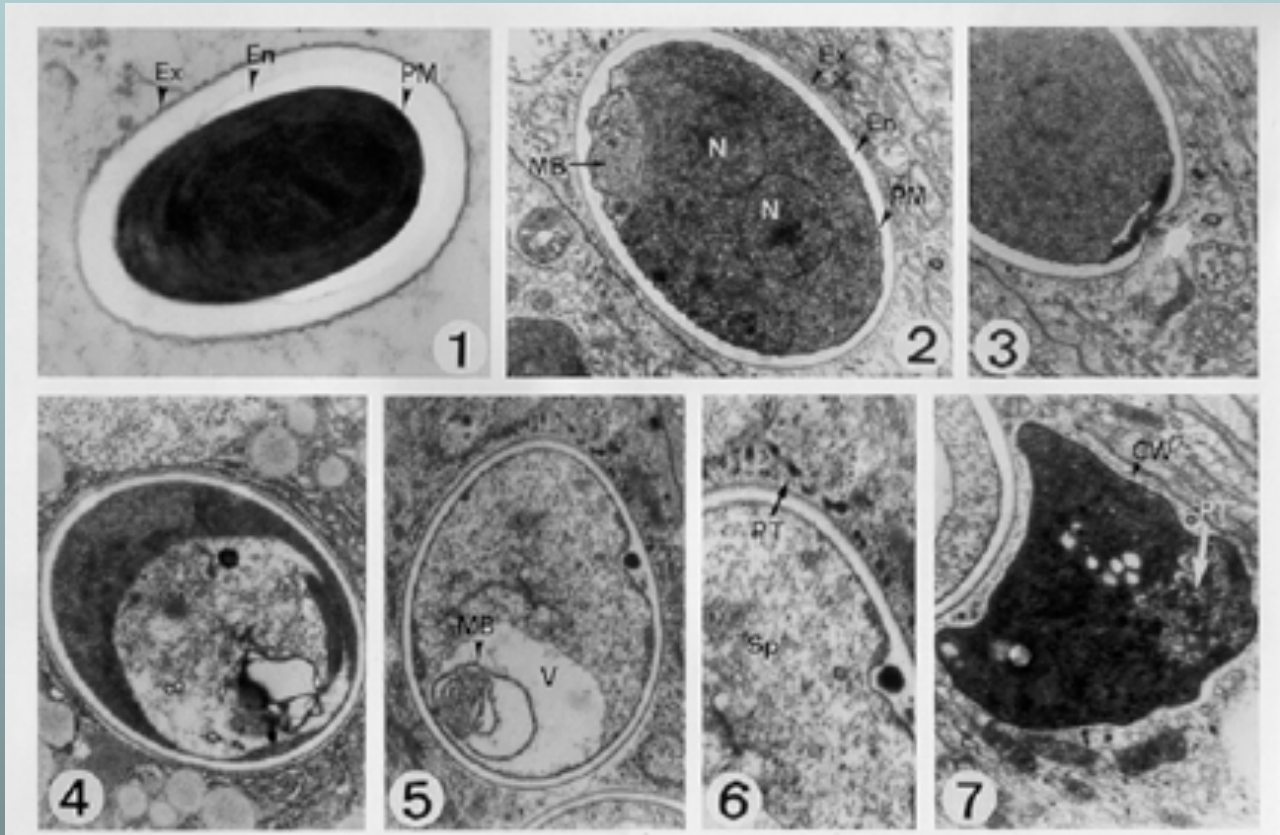



# The old story: *Nosema apis*



# The old story: *Nosema apis*

- thick and thin-walled spores




FIGS. 1–7. Abbreviations used: CW, cell wall; En, endospore; Ex, exospore; MB, membranous 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×).

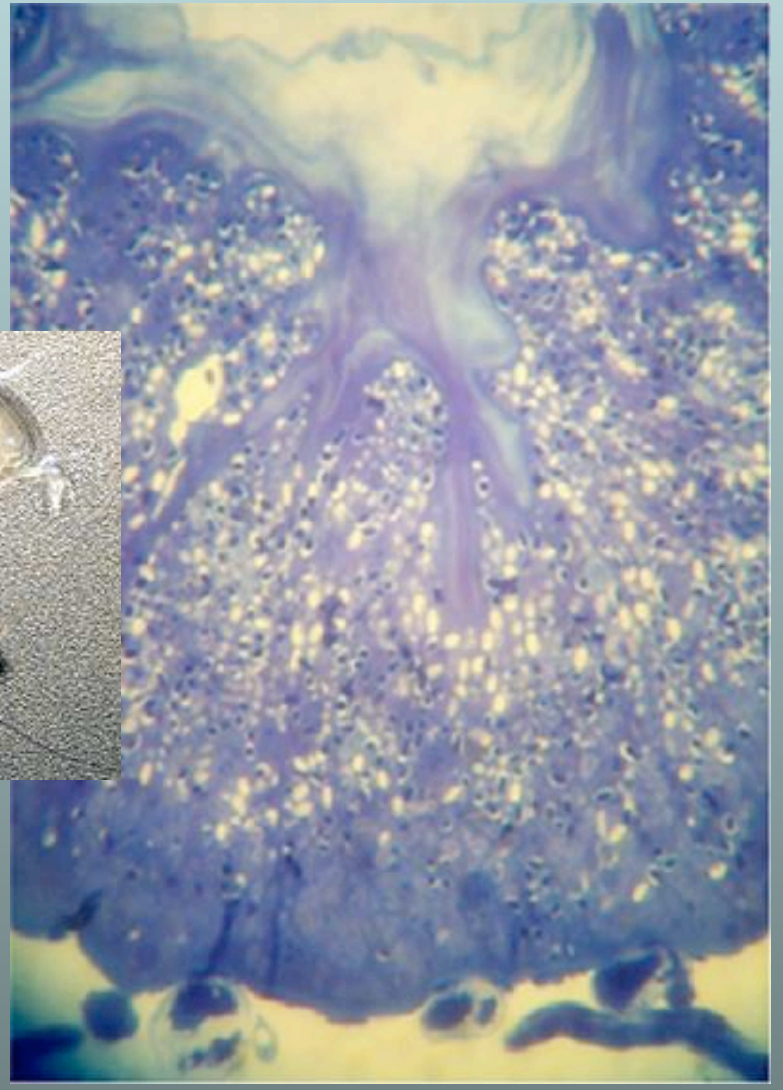
FIGS. 2–6. Intercellular germination, 48 hr pi. Fig. 2, spore of the early population at the start of germination (18,000×); Fig. 3, in one of the following sections the same spore has an invaginated spore wall (19,000×); Fig. 4, partially germinated spore (22,800×); Fig. 5, emptied spore (19,600×); Fig. 6, detail of Fig. 5 showing the extruded polar tube (53,000×).

FIG. 7. Sporoblast, 48 hr pi (58,000×).

# The old story: *Nosema apis*

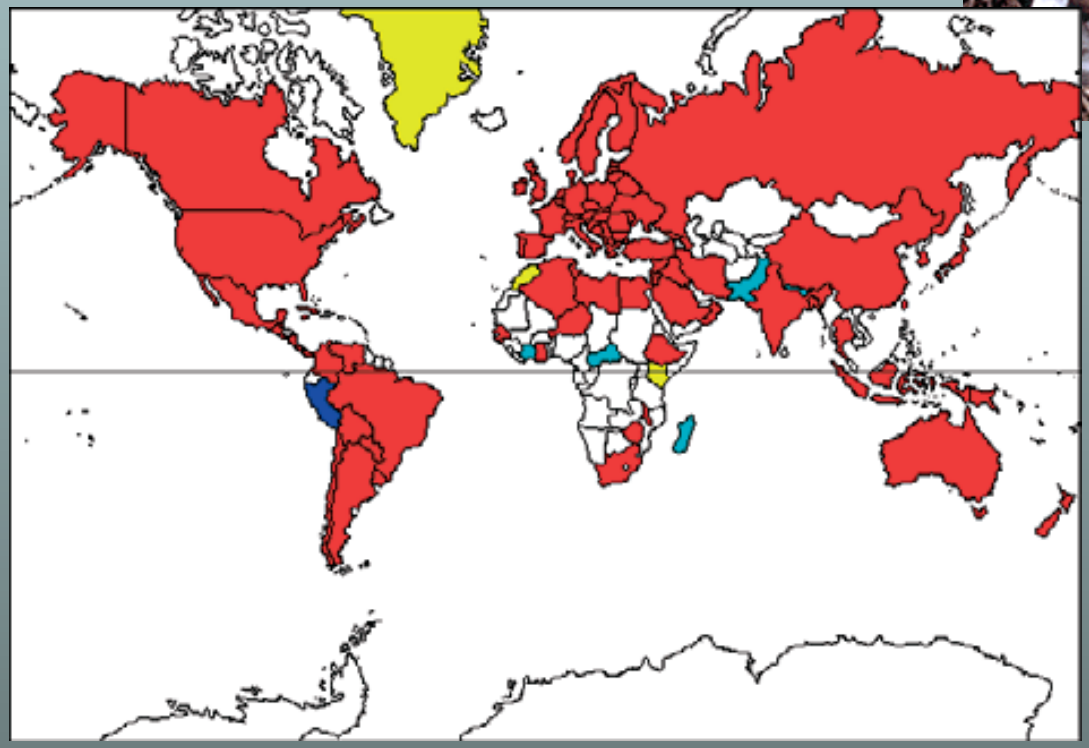
**- diseased midgut**

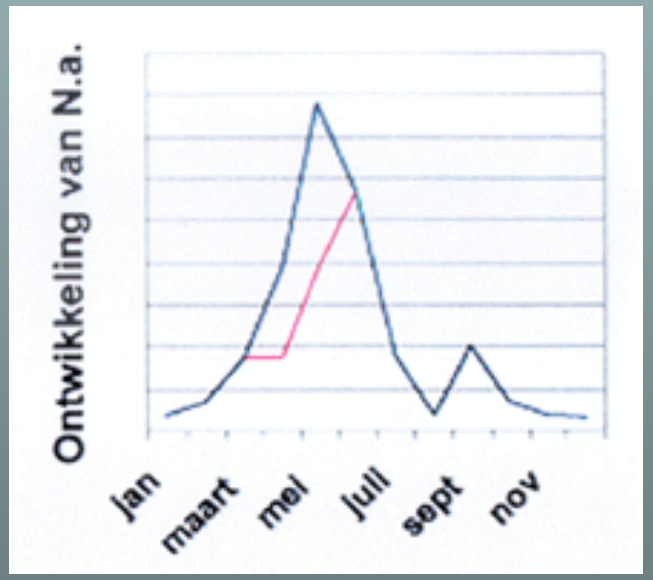
**> microscopic section**
**> whole intestinal tract**



# 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)**

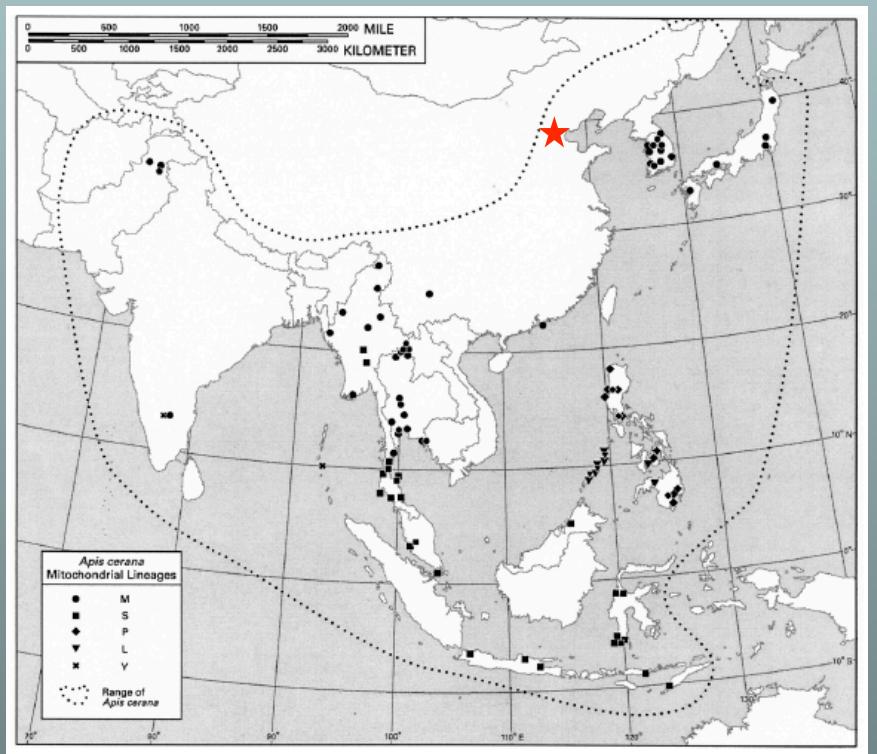



**Figure 1.** Approximate range of *Apis cerana*, showing major mitochondrial lineages (after de la Rua et al., 2000; Hepburn et al., 2001; Smith and Hagen, 1996, 1999; Smith et al., 2000). Circle = Mainland Asia, square = Sundaland, diamond = Oceanic Philippine islands, inverted triangle = Palawan, cross = "yellow plains bees".

# *N. ceranae*, parasite of Asian honeybee

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

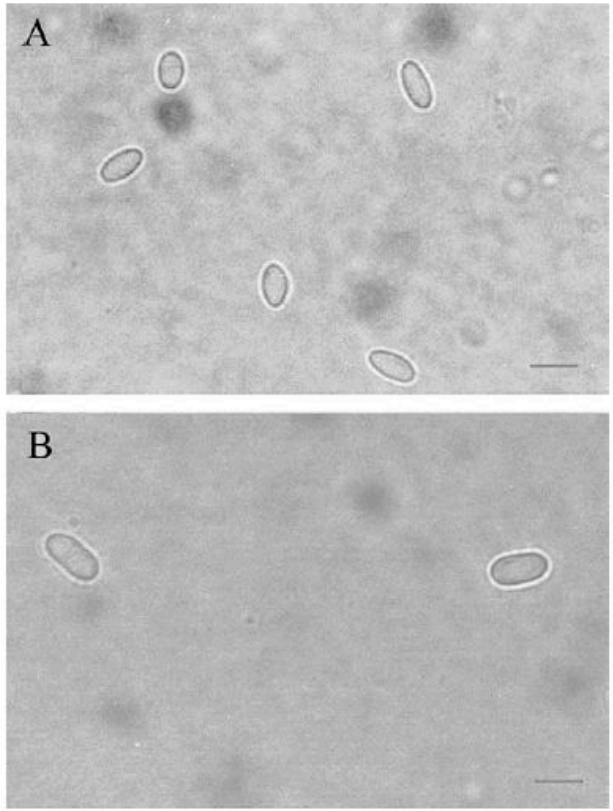



Fig. 1. Spores of *N. ceranae* (A) are distinctly smaller than spores of *N. apis* (B). Nevertheless, they can be hard to distinguish by light microscopy, in particular where mixed infections occur. Bars = 5 µm. (From Fries et al. 2006a).

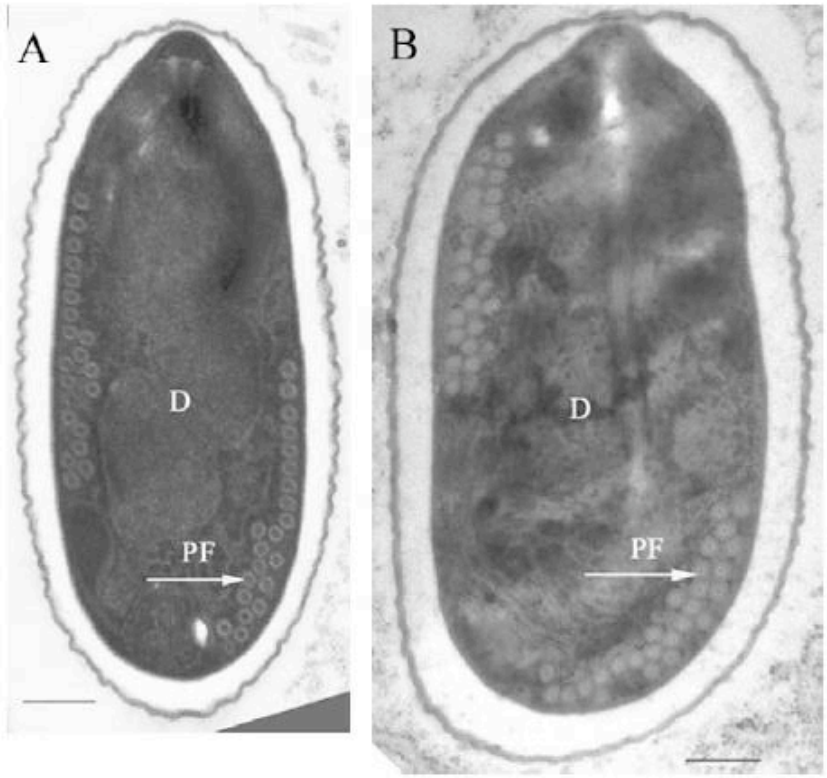
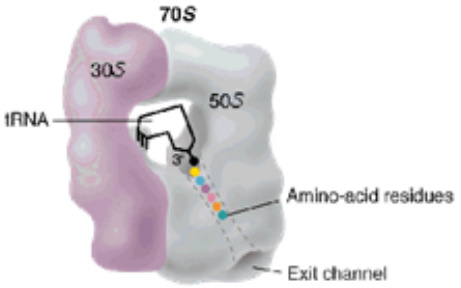



Fig. 2. The internal structures of the diplokaryotic (D) *N. ceranae* (A) and *N. apis* (B) are similar. Notably, *N. ceranae* spores contain fewer polar filamant (PF) coils compared to *N. apis*. Bars = 0.5 µm. (from Fries et al. 2006a).

# *N. ceranae*, parasite of Asian honeybee

**- ribosomes/rRNA**

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



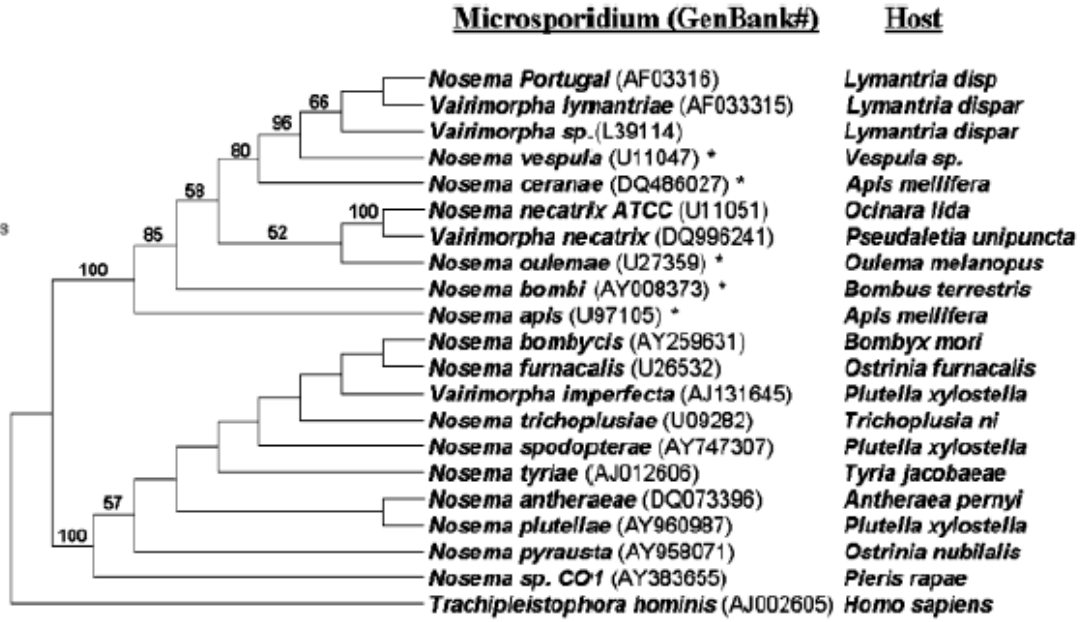




Fig. 10. Phylogenetic tree of microsporidia. Phylogenetic tree of microsporidia infecting insects based on the sequences of the small subunit rRNA gene and constructed by maximum parsimony analysis under a heuristic search. *Trachipleistophora hominis*-infecting *Homo sapiens* was used as an outgroup. 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

Mariano Higes [a,*], Raquel Martín [a], Aránzazu Meana [b]

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# A new issue: *Nosema ceranae*

- rRNA sequenties
- ultastructural characteristics

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Original article

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

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# 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)

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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 ± standard deviation) in infected and control groups

| | Infected groups | | | | Control |
|---|---|---|---|---|---|
| | 3 h | 3 d | 6 d | 7 d | |
| Parasite ratio (%) | 0 | 4.4 ± 1.2 | 66.4 ± 6 | 81.5 ± 14.8 | 0 |
| Mortality (%) | 0 | 0 | 66.7 ± 5.6 | 94.1± 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

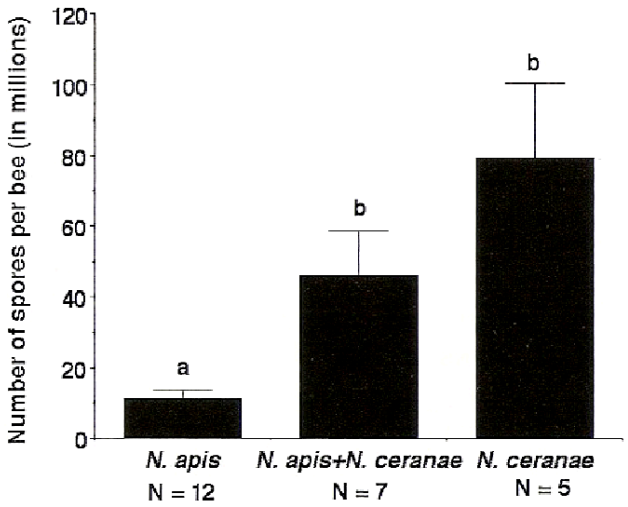


**Figure 3.** Numbers of spores per bee ($\times 10^6$) ± SE for N = 24 *A. mellifera* colonies from Southern Finland, infected with either *N. apis*, a mixed infection of *N. apis*/*N. ceranae* or *N. ceranae* between 1986 and 2006 and sampled in spring. Spore identification was based on RFLPs. Different 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)

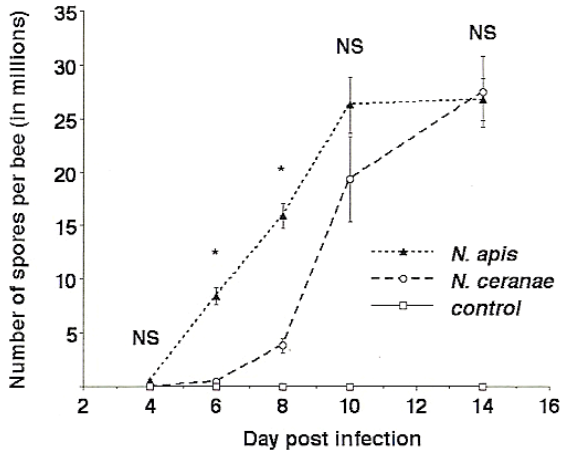


**Figure 4.** Number of spores per bee ($\times 10^6$) ± SE post infection for *A. mellifera* experimentally infected with either *N. apis* (filled triangles and dotted line), *N. ceranae* (open circles and dashed line) or uninfected (control, open squares and solid line). Each point represents the mean 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.

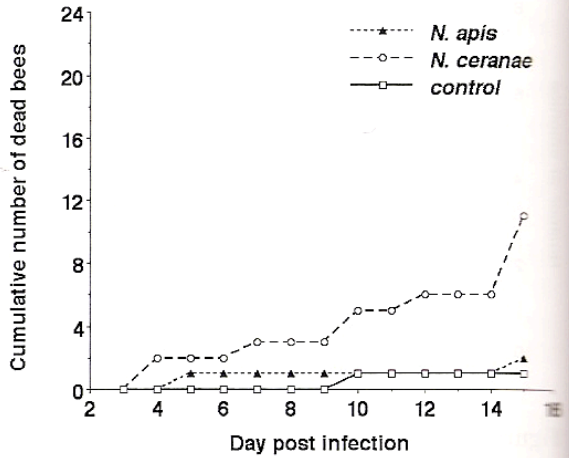


**Figure 5.** Mortality post infection of 25 *A. mellifera* workers experimentally infected with either *N. apis* (filled triangles and dotted line), *N. ceranae* (open circles and dashed line) or uninfected (control, open squares and solid line).

# A new issue: *Nosema ceranae*

## Forsgren & Fries (2010):

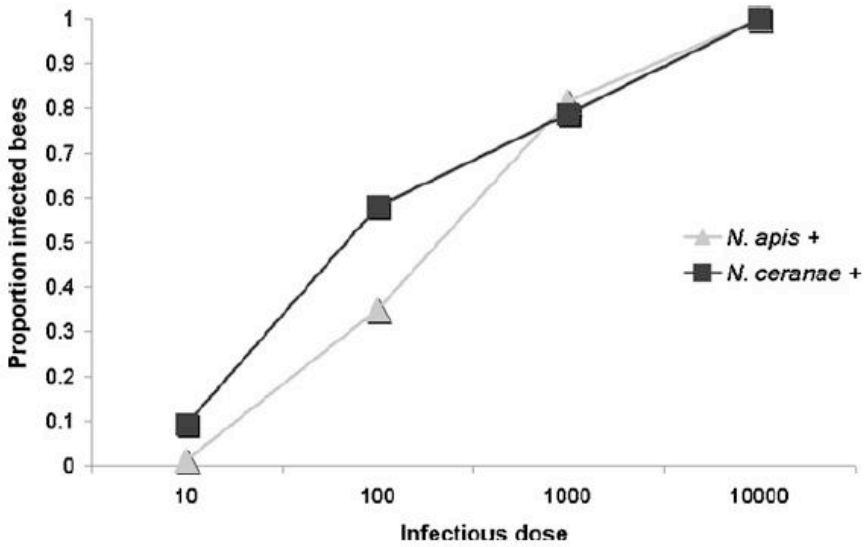


**Fig. 1.** The proportion of infected bees 14 days post-infection. Bees fed 10, $10^2$, $10^3$ and $10^4$ spores of *N. apis* or *N. ceranae*. Results presented as a mean based on three cages with 30 bees ($n = 90$) for each treatment.

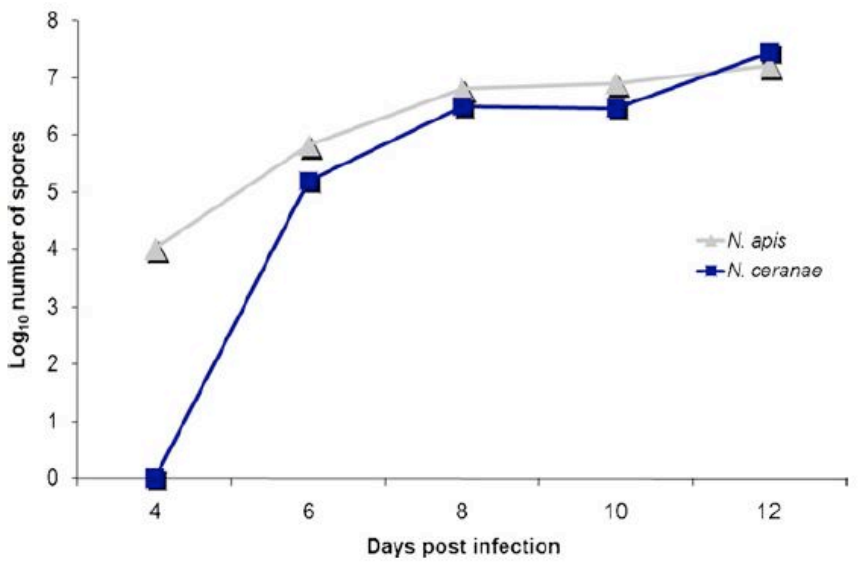


**Fig. 2.** The course of infection for *N. apis* and *N. ceranae*. Bees were individually infected with 10 000 spores of the respective parasite. At day 12 post-infection, the infection is probably fully developed with a similar number of spores produced by both species.

# 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 |

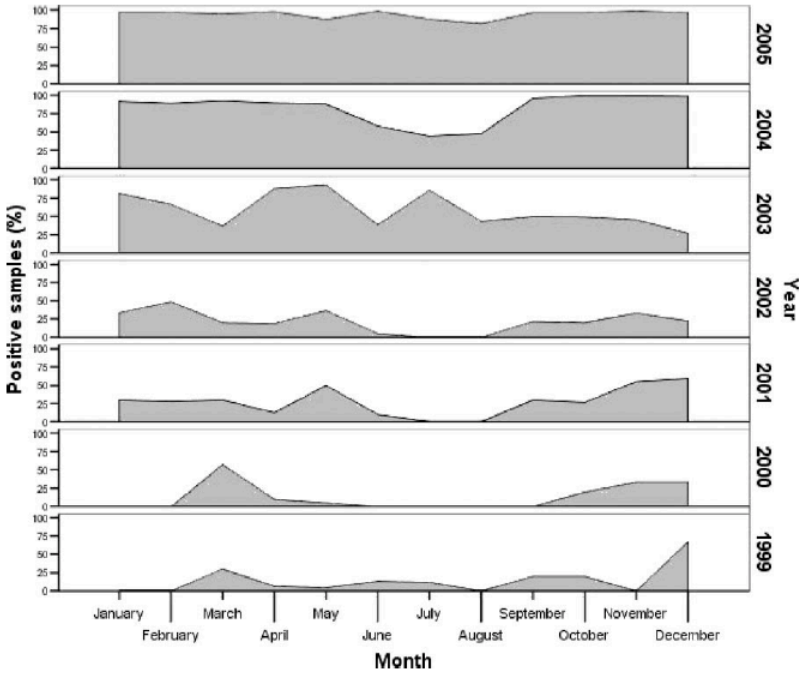


FIG. 2. Monthly distribution of *Nosema*-positive samples in every year from 1999 to 2005, obtained by comparing the number of samples positive for *Nosema* spores with the total number of samples received in the Laboratory of Bee Pathology.

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* |
|---|---|---|---|---|---|---|
| *N. apis* + *N. ceranae* | 10 | 1 | 11 | 5.82 | 3.20–10.59 | 0.0000 |
| *N. ceranae* | 44 | 8 | 52 | 5.42 | 3.03–9.68 | 0.0000 |
| *N. apis* | 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): Metagonomic 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 ($n$ = 30) | Non-CCD ($n$ = 21) | Total ($n$ = 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 |
| *N. apis* | 27 (90%) | 10 (47.6%) | 37 (72.5%) | 73.0 | 90.0 | 52.4 |
| *N. ceranae* | 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 survived colonies | | | No. of collapsed colonies | | | *P*-value ($chi^2$) |
|---|---|---|---|---|---|---|---|---|
| | | 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 |
| *Nosema* 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 | *P*-value |
|---|---|---|---|---|
| Type of beehive | 4313 | 3889<br>wood / styrofoam<br>(2594) / (1295) | 424<br>wood / styrofoam<br>(282) / (142) | 0.94 ($chi^2$) |
| Starting condition[1] | 4293 | 3876<br>O N C<br>(2731) (724) (421) | 417<br>O N C<br>(317) (65) (35) | 0.78 ($chi^2$) |
| Queen age years (n) | 4021 | 3639<br>0 1 2 3 4<br>(2002) (1238) (192) (5) (2) | 382<br>0 1 2 3 4<br>(156) (181) (45) (0) (0) | 0.0052 ($chi^2$) |
| Colony strength in October (frames with bees ± sd) | 4313 | 3889<br>12.3 ± 5.1 | 424<br>10.0 ± 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):

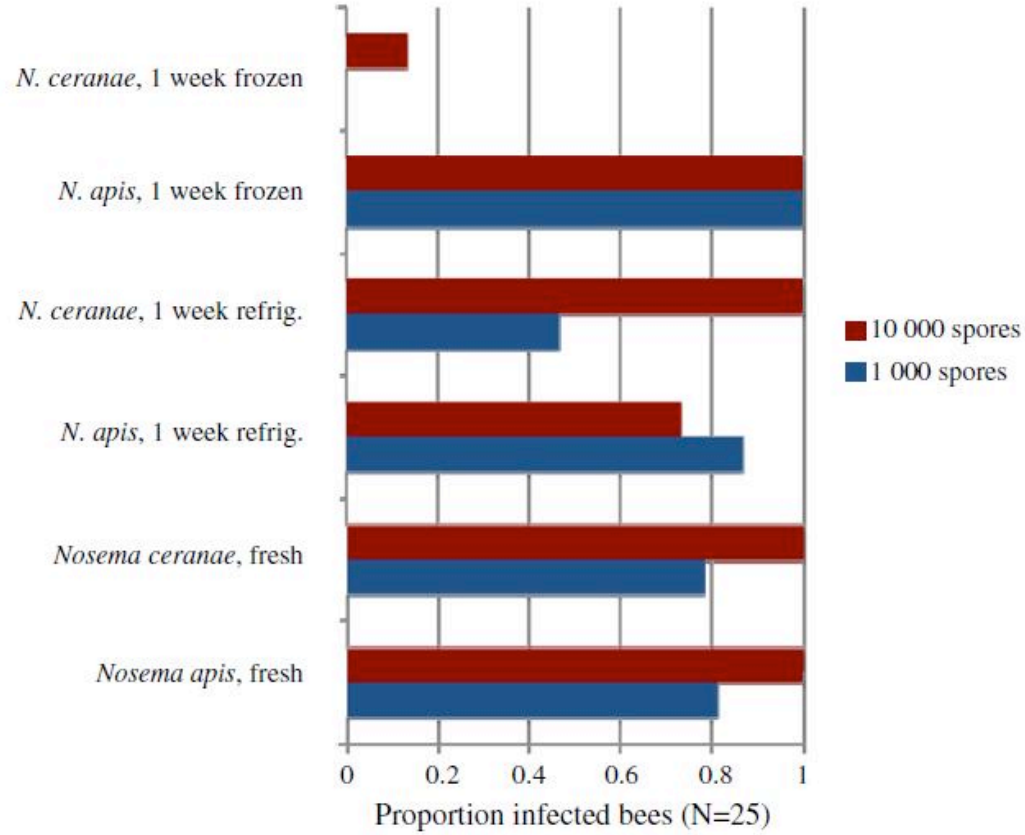


**Fig. 3.** The effect of freezing on *N. ceranae* spore viability is dramatic. Each bar represents 25 bees individually fed with 10 µl sugar suspension containing 1000 or 10,000 spores of either *N. ceranae* or *N. apis*. Spores were fed from fresh infections (fresh) or (using the same suspension) after one week in a refrigerator at +8 °C (refrig.) or in a deep freezer at −18 °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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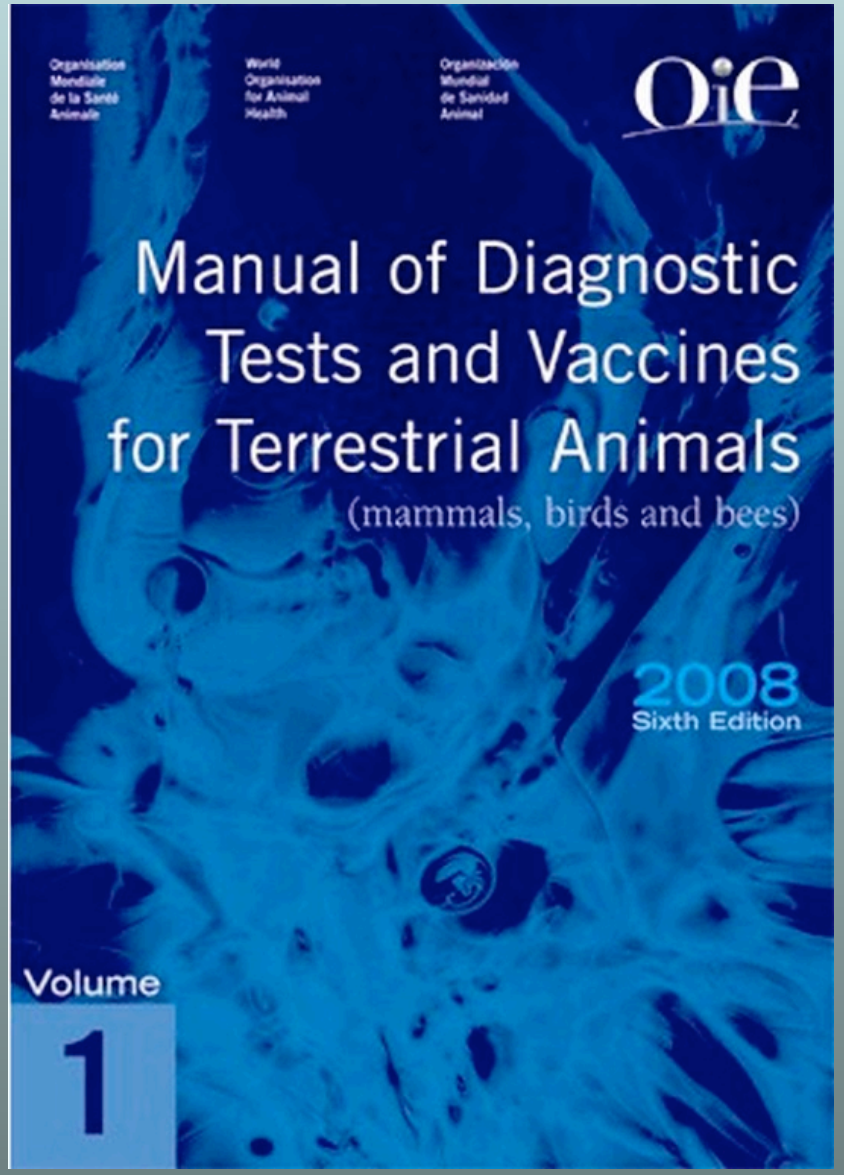
# Diagnostics according to the OIA Manual

**Office International des Epizooties**

**World Organisation for Animal Health**

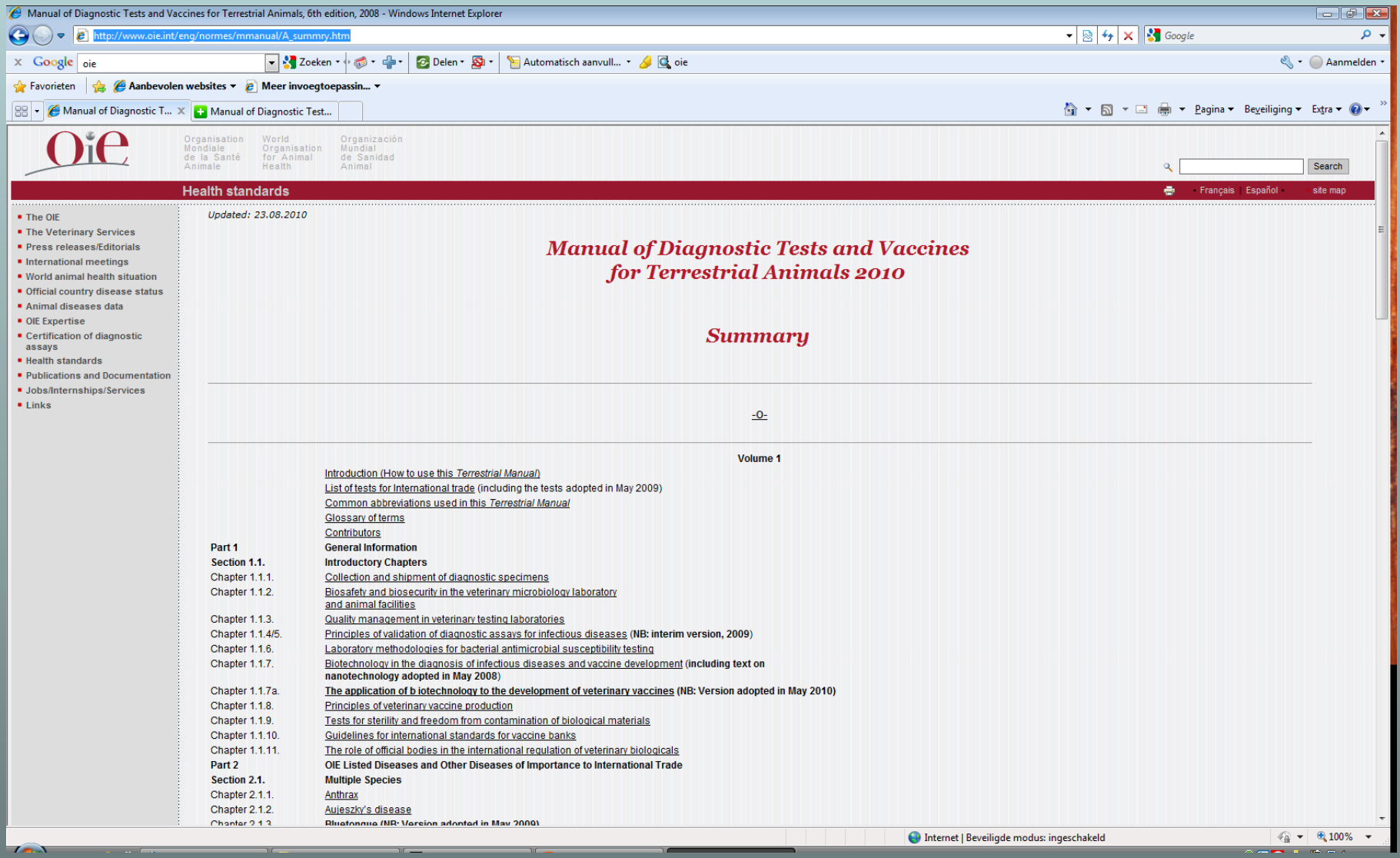
**= intergovernmental organisation responsible for improving animal health worldwide**

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



# Diagnostics according to the OIA Manual

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



# 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

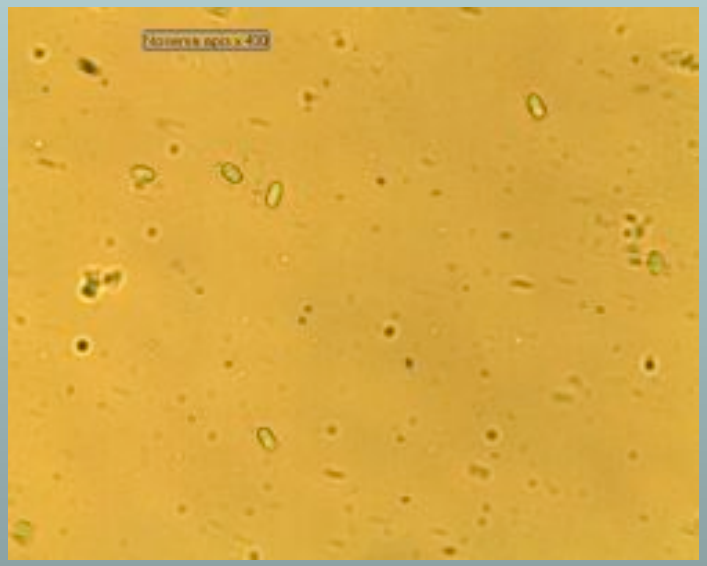
### STAINED

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

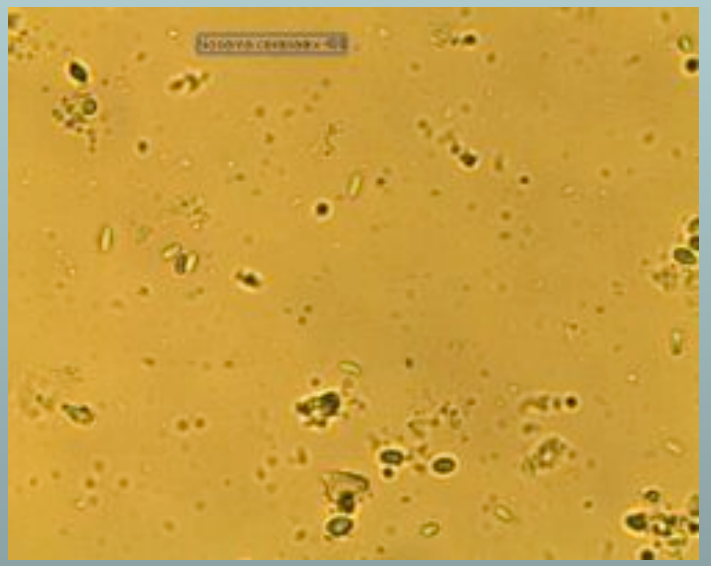
# Diagnostics according to the OIA Manual

## UNSTAINED

***Nosema apis***



***Nosema ceranae***



## STAINED

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

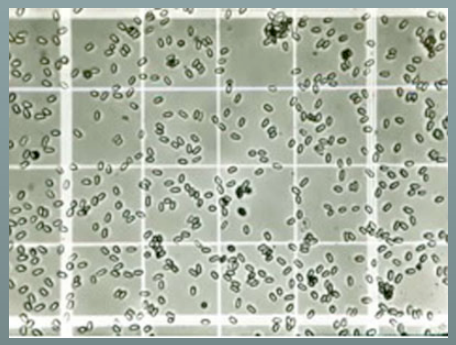
**insect cell, fungal spores and other protozoa: thinner wall, blue/purple cytoplasm, magenta coloured nuclei**

# 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



# Diagnostics 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)

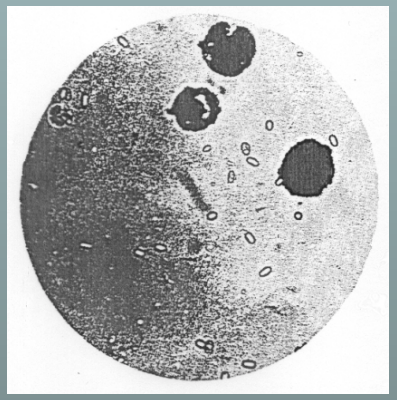
## MULTIPLEX PCR

- primers used < Martin-Hernandez et al. (2007)

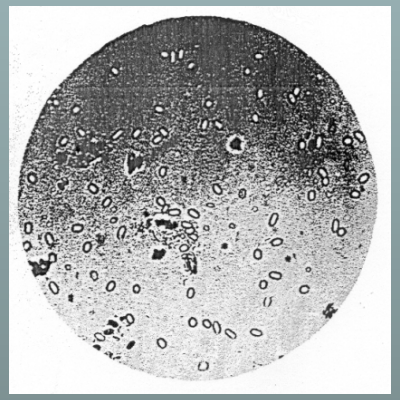
| NAME | AMPLICON | SPECIFIC |
|---|---|---|
| 218MITOC FOR | 218-219 | N. ceranae |
| 218MITOC REV | | |
| 321APIS FOR | 321 | N. apis |
| 321APIS REV | | |

# Beyond OIE Manual

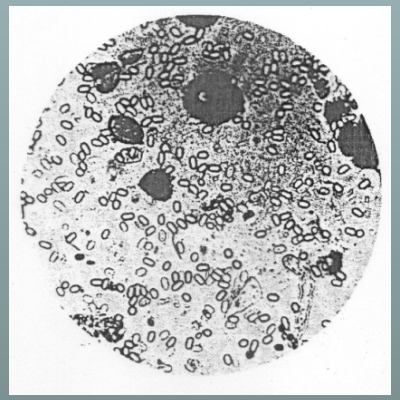
**Microscopy**
**SEMI-QUANTITATIVE (Gross & Rutner, 1970)**
- 10-20 abdomens macerated in 10 ml water
- crushed midguts
- examination at 400x
- average infection level (30 fields of vision)



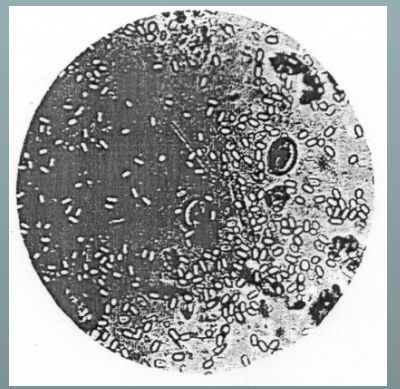
infection level I
+/- 10 spores



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)

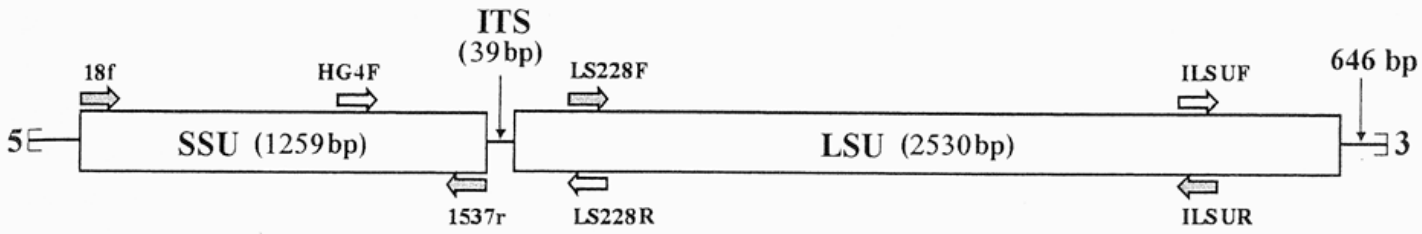
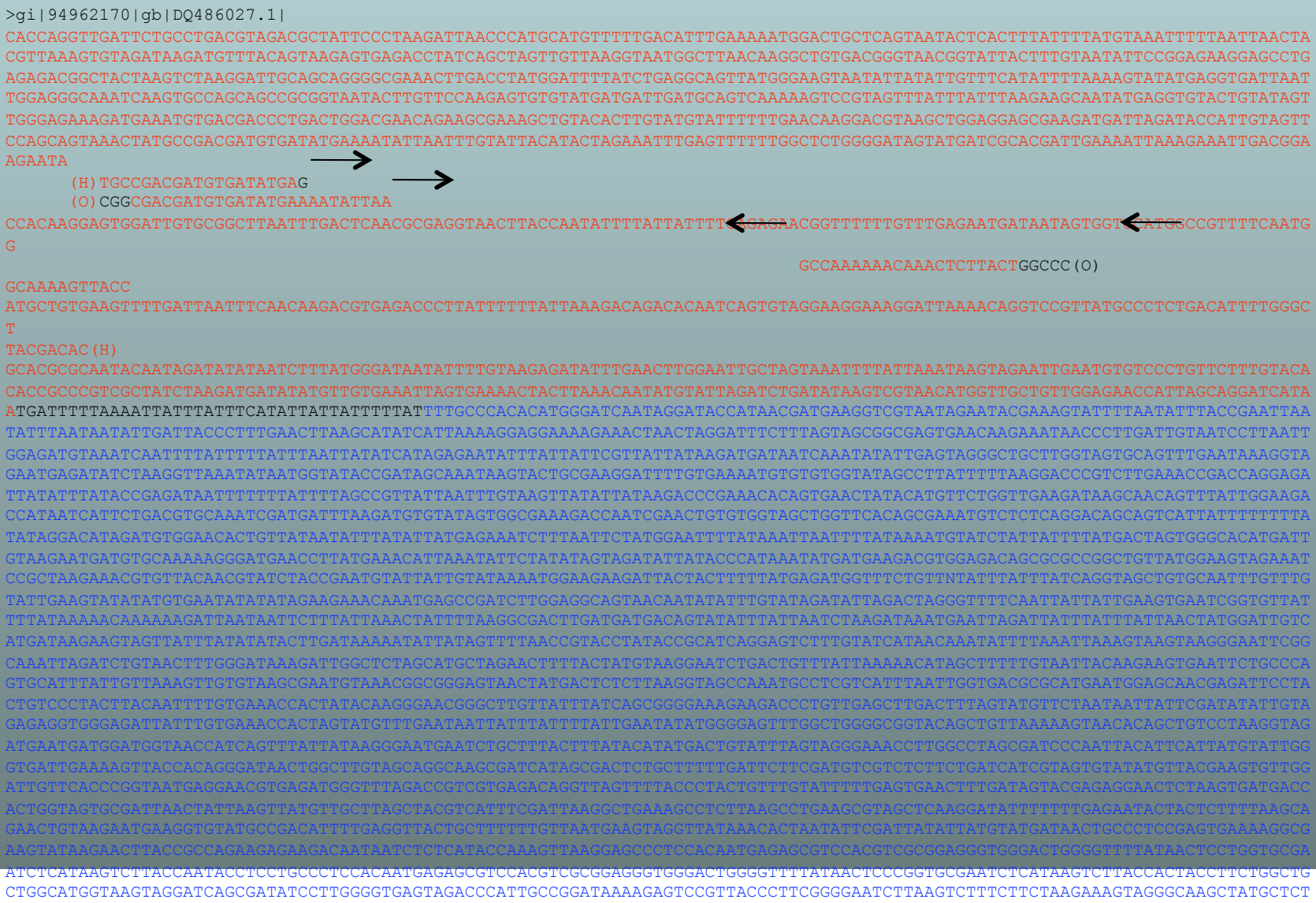



**Figure 1.** Schematic diagram of the *Nosema ceranae* isolate rRNA gene. The rRNA domains are indicated by boxes. The grey arrows represent primers used to amplify the major coding regions of rRNA while the white arrows represent primers for the ITS and the SSP-PCR.

# Beyond OIE Manual

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



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## PCR FOR RAPID DIFFERENTIATION

### Klee et al. (2007)

**SSU rRNA**

**PCR-RFLP using *Msp*I, *Pac*I and *Nde*I**

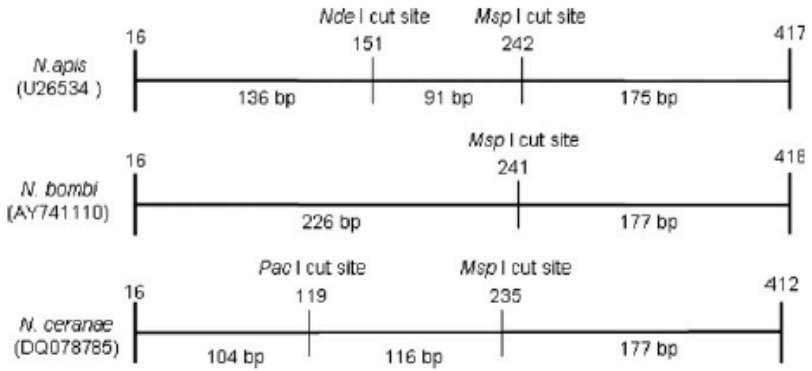


Fig. 2. Diagram showing the total length and size (in base pairs) of expected restriction fragments of *N. apis*, *N. bombi* and *N. ceranae* partial SSU rRNA PCR products amplified with the primer pair SSU-res-f1 and SSU-res-r1, and digested with *Pac*I, *Msp*I and *Nde*I. Base pair cut sites based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

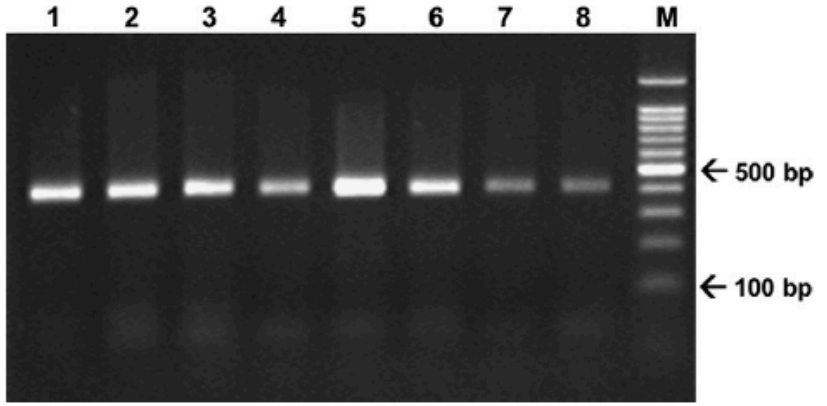


Fig. 3. Ethidium bromide stained agarose gel showing examples of PCR products for the fragment of SSU rRNA amplified with the primer pair SSU-res-f1 and SSU-res-r1. Lane 1, *N. ceranae* reference DNA extract; lane 2, *N. apis* reference DNA extract; lane 3, *N. bombi*; lane 4, Spain (Huelva); lane 5,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

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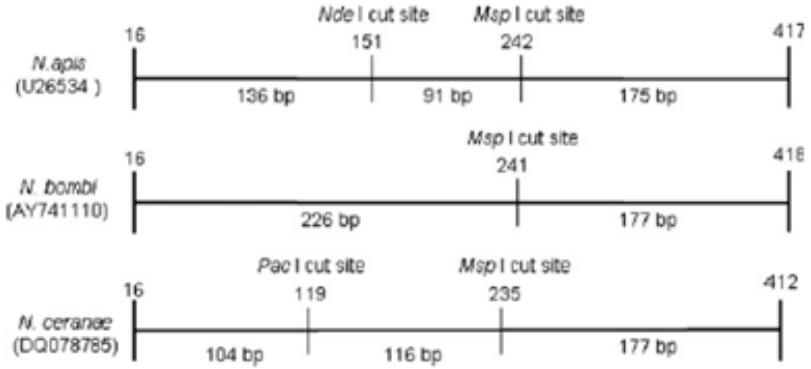
**1=*N. bombi***
**2=*N. apis***
**3=*N. ceranae***



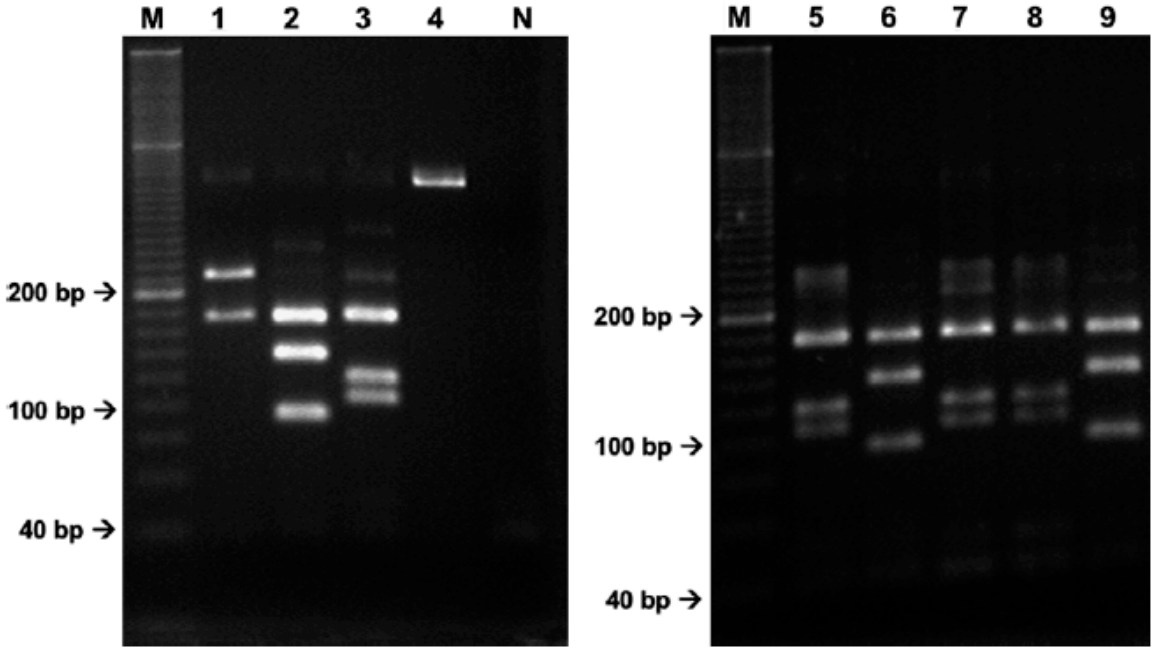




Fig. 4. Ethidium bromide stained agarose gel giving examples of RFLP analysis of partial SSU rRNA of *N. apis*, *N. ceranae* and *N. bombi* digested with the enzymes *Msp*I, *Pac*I and *Nde*I. Lane M, 20 bp ladder; lane 1, *N. bombi*; lane 2, *N. apis* reference DNA extract; lane 3, *N. ceranae* DNA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

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## Chen et al. (2008)

- rRNA gene based
- 3 primer sets separately applied

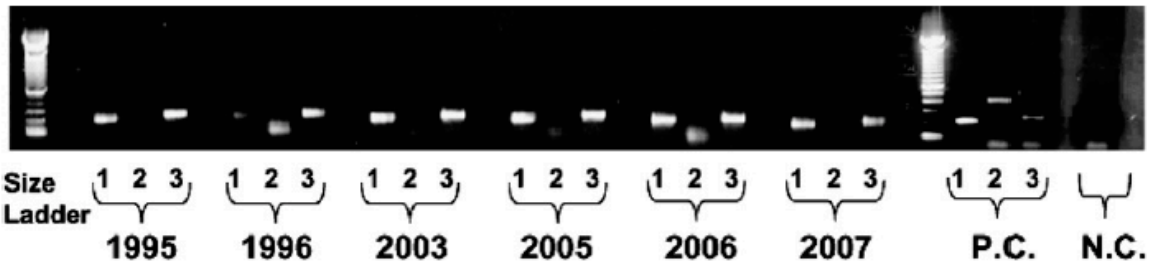



Fig. 1. PCR amplification of representative bee samples collected from 1995 to 2007 in the United States. For *x*-axis, No. 1 indicates that DNA was amplified with generic primers, *Nosema* F/R; No. 2 and 3 indicates DNA was amplified with specific primers, *N. apis* F/R and *N. ceranae* F/R, respectively. 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*.

# Beyond OIE Manual

## 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)

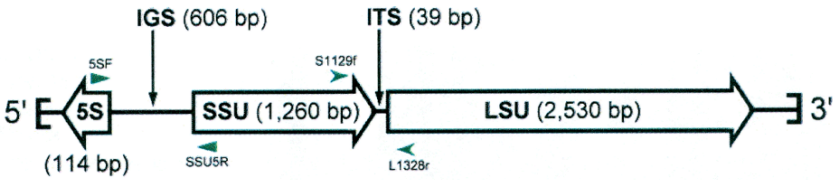



Fig. 1. Schematic diagram of the *Nosema ceranae* rRNA genes. The rRNA domains are indicated by hollow arrows and the direction of arrow indicates the direction of transcription. The small arrowheads represent primers used to amplify the IGS and ITS regions.

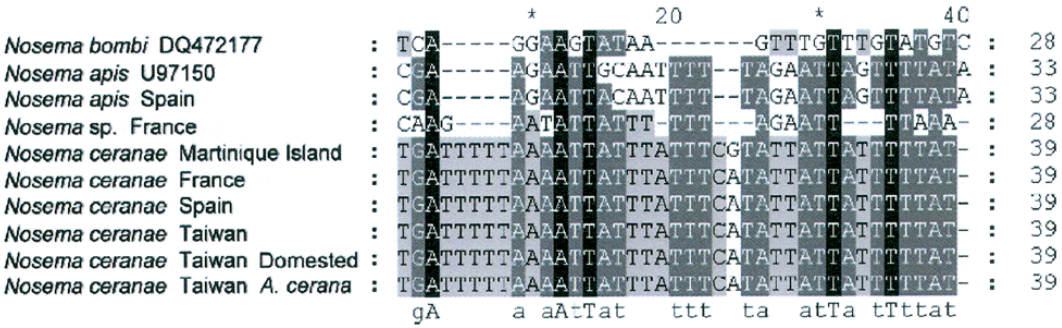



Fig. 3. Alignment of the ITS from *N. cerana* isolates, *N. apis*, and *N. bombi*. The isolate, *Nosema ceranae* Taiwan domested, was collected from the apiary which kept *A. cerana* and *A. mellifera* in the same environment. The isolate, *Nosema ceranae* Taiwan *A. cerana*, was collected from the wild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

# Beyond OIE Manual

## SEARCH FOR POLYMORPHISM
## Sagastume et al. (2010)

- genotyping based on IGS and SSU
- high diversity of sequences: 79 haplotypes
- '2 sequences from one isolate as different as any pair of sequences from a different sample'
-'identical haplotypes found in different regions'
=> no reliable marker for differentiation

**Table 4.** Insertion (1)/deletion (0) composition of the different haplotypes, extracted from Table 2 (*n*: number of haplotypes).

| | *Indel* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *A* | *B* | *C* | *D* | *E* | *F* | *G* | *H* | | | | | | | | | | | | | | | | | | | | | | | |
| | 42N | TTGTAT | AA | AT | TATGTA | AT | GATT | TG | *n* | *Haplotypes* | | | | | | | | | | | | | | | | | | | | | |
| sq01 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 5 | 7 | 11 | 36 | 37 | 38 | | | | | | | | | | | | | | | | | |
| sq02 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 21 | | | | | | | | | | | | | | | | | | | | | |
| sq03 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 5 | 3 | 20 | 34 | 65 | 60 | | | | | | | | | | | | | | | | | |
| sq04 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 19 | 1 | 2 | 6 | 8 | 9 | 10 | 14 | 15 | 19 | 22 | 26 | 27 | 31 | 39 | 43 | 45 | 55 | 59 | 66 | | | |
| sq05 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 3 | 17 | 28 | 68 | | | | | | | | | | | | | | | | | | | |
| sq06 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 13 | 16 | 23 | 30 | 32 | 41 | 42 | 46 | 52 | 53 | 57 | 64 | 77 | 78 | | | | | | | | | |
| sq07 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 79 | | | | | | | | | | | | | | | | | | | | | |
| sq08 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 51 | | | | | | | | | | | | | | | | | | | | | |
| sq09 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 5 | 4 | 18 | 29 | 33 | 49 | | | | | | | | | | | | | | | | | |
| sq10 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 2 | 35 | 63 | | | | | | | | | | | | | | | | | | | | |
| sq11 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 5 | 70 | 71 | 73 | 74 | 48 | | | | | | | | | | | | | | | | | |
| sq12 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 3 | 40 | 44 | 69 | | | | | | | | | | | | | | | | | | | |
| sq13 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 12 | 5 | 12 | 13 | 24 | 47 | 50 | 54 | 56 | 61 | 62 | 72 | 76 | | | | | | | | | | |
| sq14 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 58 | | | | | | | | | | | | | | | | | | | | | |
| sq15 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 2 | 25 | 75 | | | | | | | | | | | | | | | | | | | | |
| sq16 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 67 | | | | | | | | | | | | | | | | | | | | | |

# Beyond OIE Manual

## Sagastume et al. (2010)(continue)

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

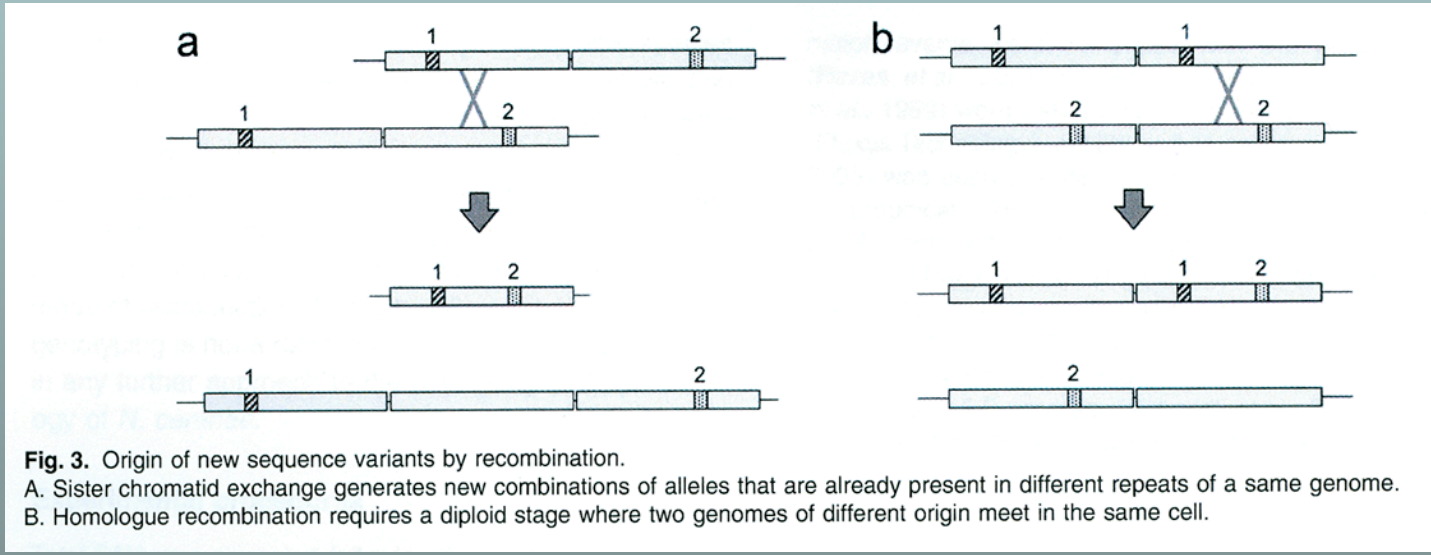



**Fig. 3.** Origin of new sequence variants by recombination.
A. Sister chromatid exchange generates new combinations of alleles that are already present in different repeats of a same genome.
B. Homologue recombination requires a diploid stage where two genomes of different origin meet in the same cell.

# Beyond OIE Manual

## 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 |
|---|---|---|---|
| *Nosema apis* genbank: U97150 | For: GCCCTCCATAATAAGAGTGTCCAC Rev: ATCTCTCATCCCAAGAGCATTGC Probe: ACTTACCATGCCAGCAGCCAGAAGA | 142 | 4364 |
| *Nosema ceranae* genbank: DQ486027 | For: AAGAGTGAGACCTATCAGCTAGTTG Rev: CCGTCTCTCAGGCTCCTTCTC Probe: ACCGTTACCCGTCACAGCCTTGTT | 104 | 218 |

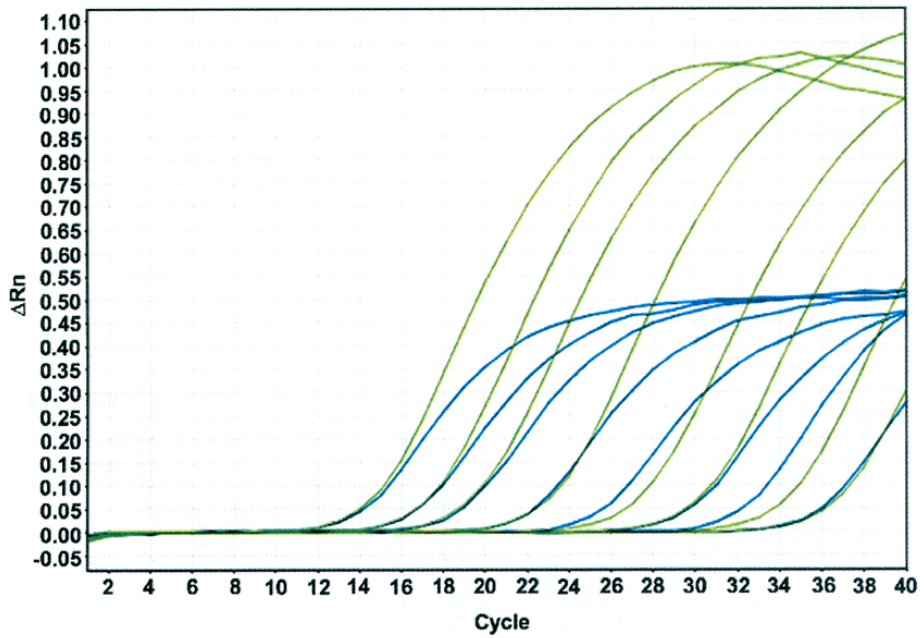
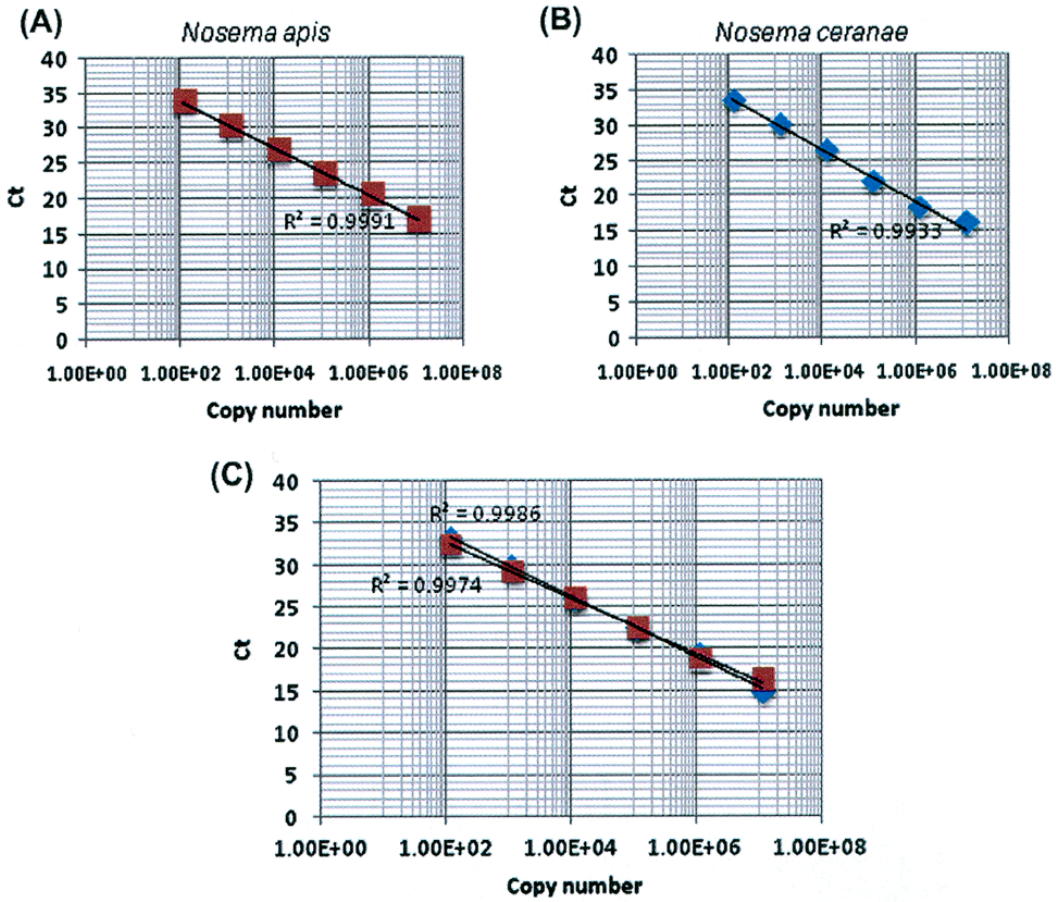



**Fig. 2.** Real-time PCR fluorescence curves for eight *Nosema apis* and *N. ceranae* standards co-amplified. Standard template was added in 10-fold serial dilutions from 500 pg template to 50 ag template DNA, representing $1.18 \times 10^8$ to 11.8 copies of the target fragments.

# Beyond OIE Manual

## Bourgeois et al. (2010)(continue)




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

Individual samples:

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

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

Pooled samples:

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

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