Diagnostics and genetic variation of an invasive microsporidium (*Nosema ceranae*) in honey bees (*Apis mellifera*)

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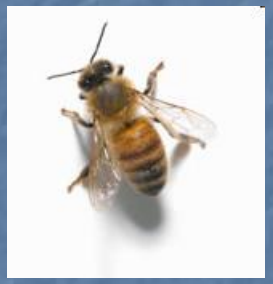
CHANGING LIVES IMPROVING LIFE

Importance of honey bees


Pollination

Agriculture



Honey bee

Foods


Health/medicine


Industry


Economy

Honey bees are social, beneficial, economic insects

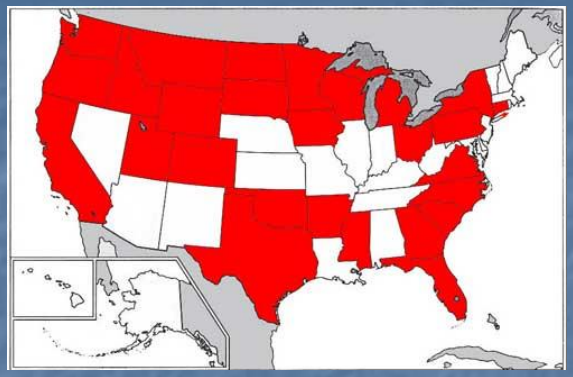
Are bees essential for pollination ?

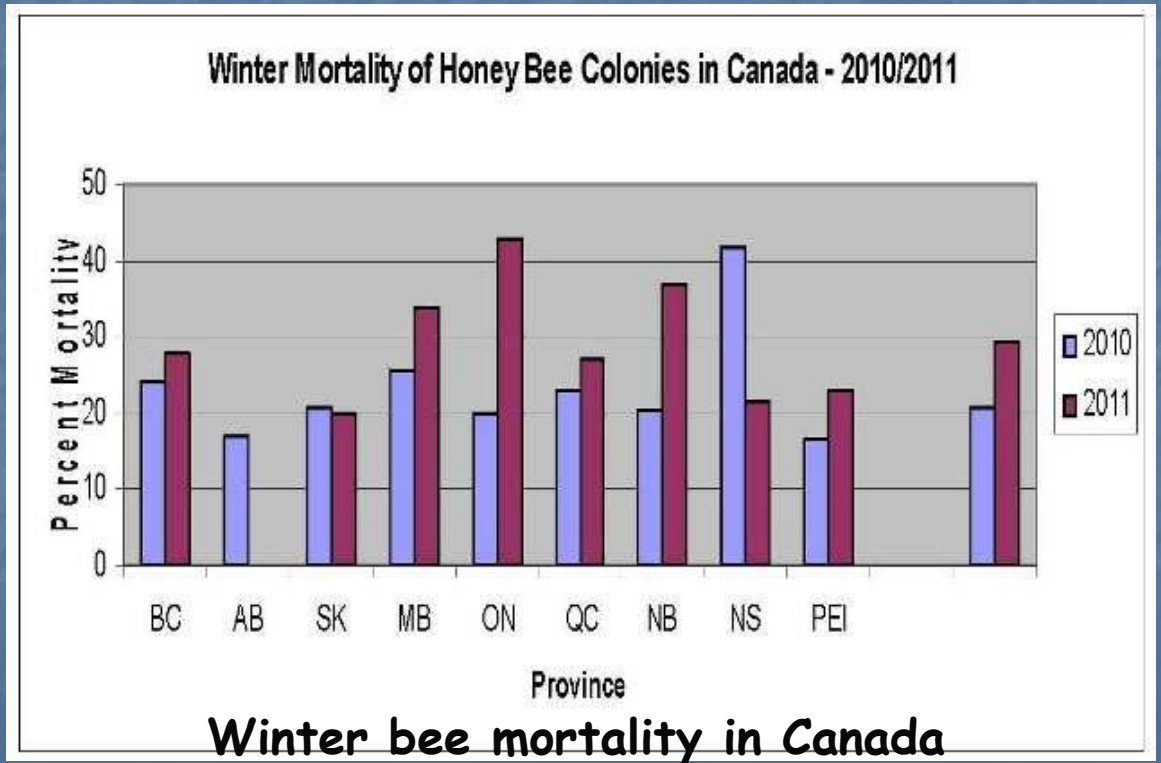
- Honey bees are responsible for pollinating 1/3 of our food crops

Adequately pollinated cucumber: healthy, higher yield, higher market value

Inadequately pollinated cucumber: distorted, lower yield, lower market value

Bee colony losses





Winter bee mortality in Canada

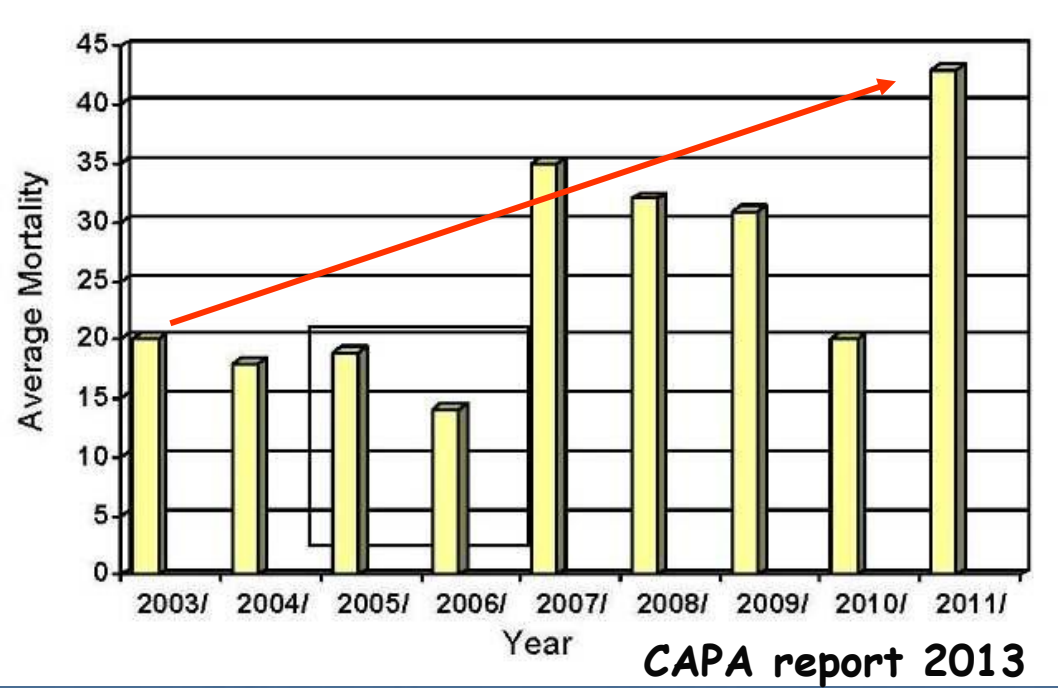
Honey bee colony mortality in Canada as well other countries has been increasing

Bee colony losses in Ontario

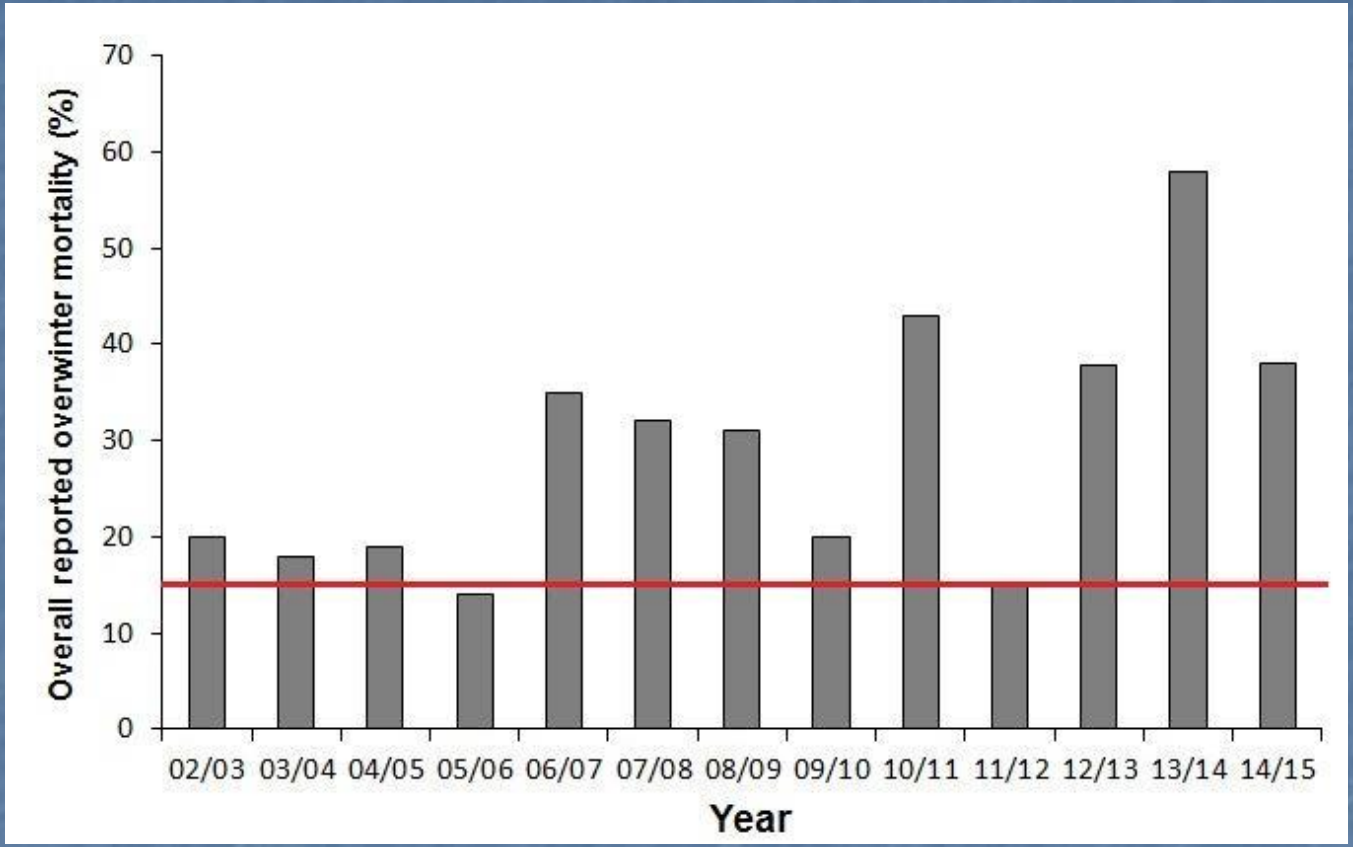
Colony mortality above 30% during the last six winters. Three times the expected loss

>25,000 colonies; > 3 million pounds of honey; thousands of tons of crops

Estimated losses in colonies, honey yields, crops (for lack of pollination): more than 50 million dollars annually



Bee colony mortality in Canada: CAPA report 2015



What has caused these high colony mortality rates ?

Nobody really knows !

Main suspects of colony mortality

- Parasitic mites
- Viruses (IAPV and others)
- Stress (malnutrition, transportation)
- Pesticides (internal and external)
- Inadequate management practices (leading to weak colonies)
- Climate change
- Microsporidia (*Nosema apis* and/or *Nosema ceranae*)

Nosema disease of honey bees

Cause: Microsporidians (*Nosema apis* and/or *N. ceranae*)

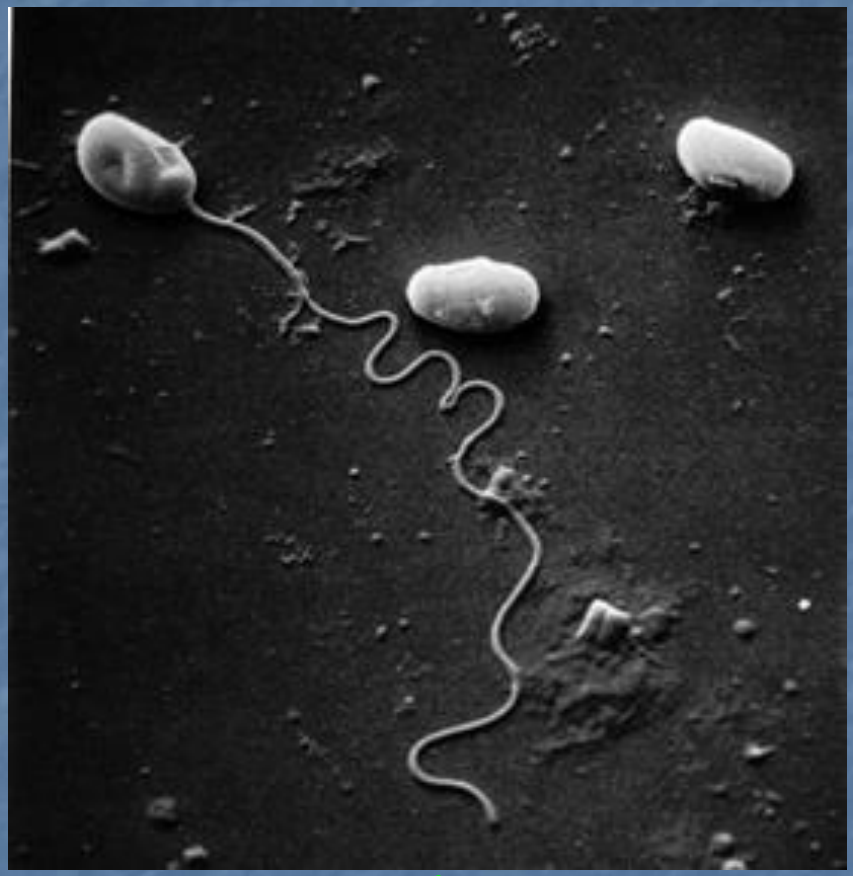
What is microsporidia?

Fungi;

Obligate intracellular parasites;

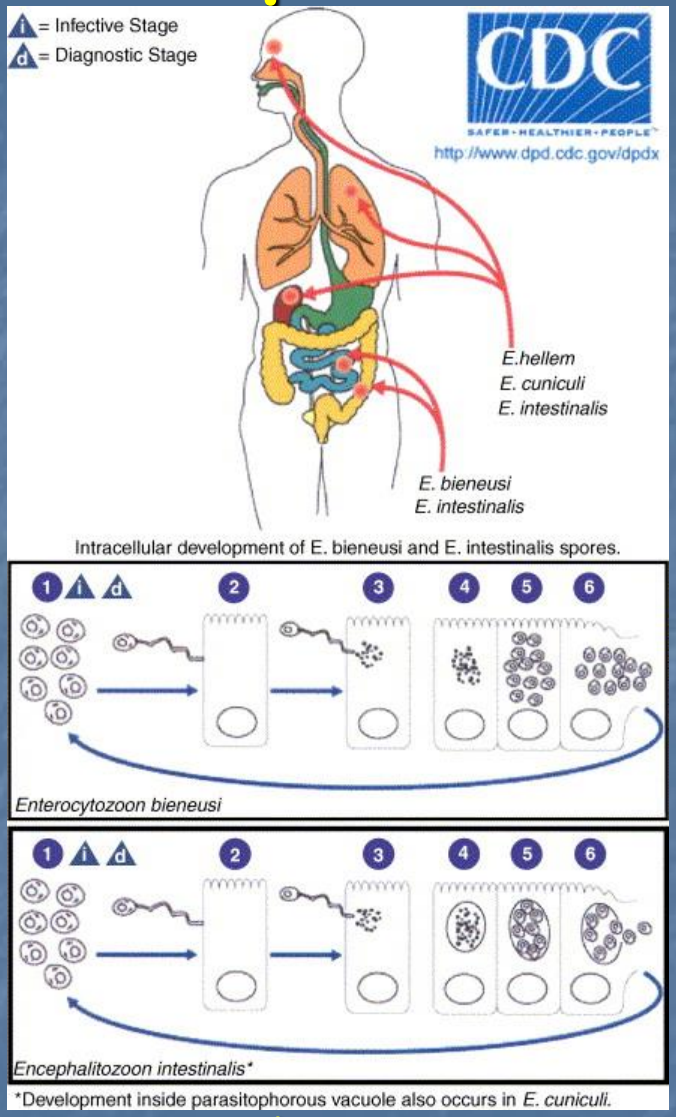
Infect a variety of animals, specially insects;

Transmitted between hosts as spores.



Microsporidia ejecting the polar filament

Importance of microsporidia (*Nosema*)



Human being

Encephalitozoon intestinalis,
E. bieneusi

Bees

Nosema apis
N. ceranae

Rabbit

Encephalitozoon cuniculi
Or, *Nosema cuniculi*

Nosema disease (Nosemosis) of honey bees

Cause: Microsporidians (*N. ceranae* and/or *N. apis*)

Fecal staining on the outside and entrance of the hive

Nosema Dysentery symptom

History of Nosema disease in bees

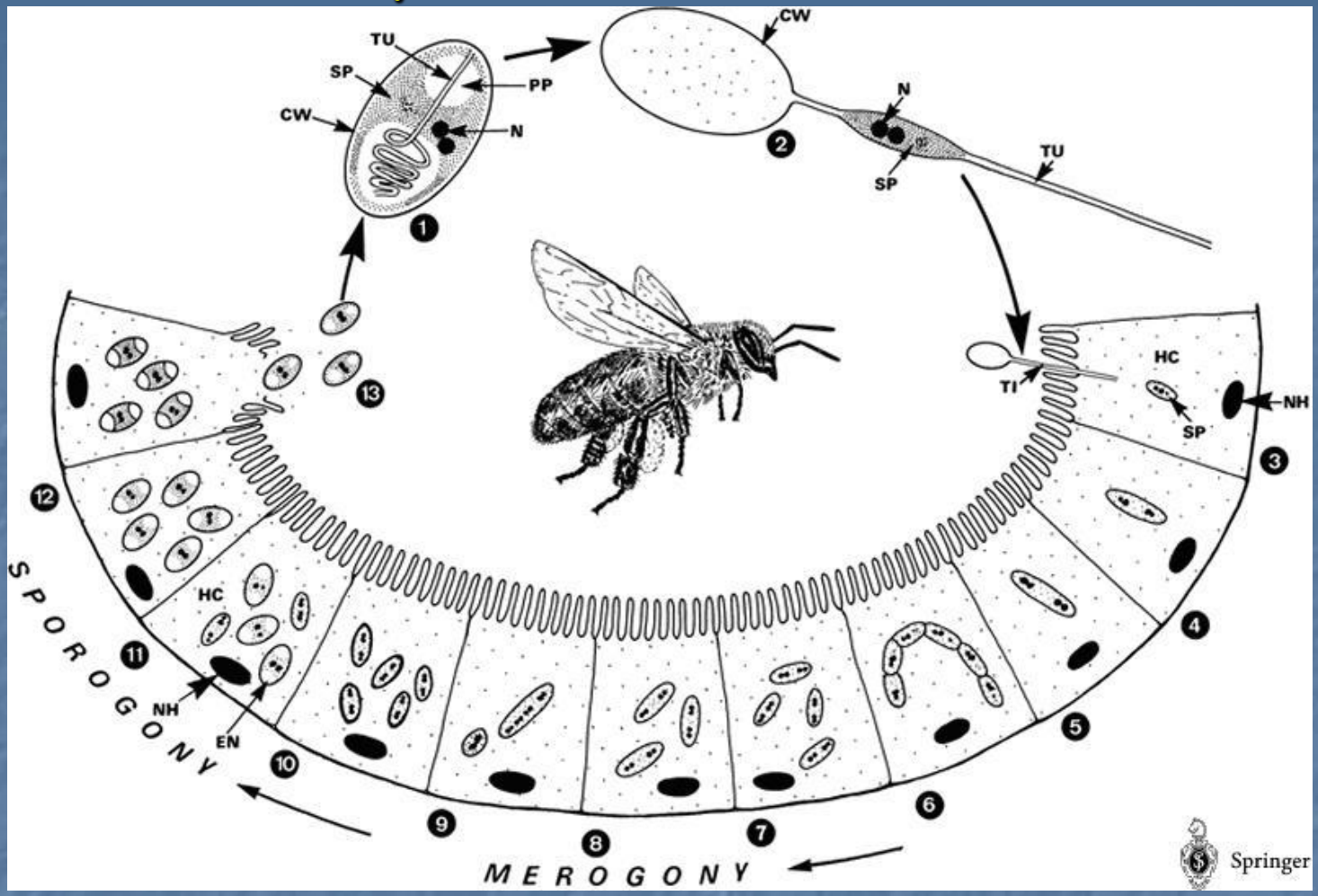
The first report of *N. apis* was in Europe (Germany) in the Western honey bees, *Apis mellifera* (Zander, 1909).

Almost a century after that *N. ceranae* was reported in East Asia (China) in the Asian honey bees, *Apis cerana* (Fries *et al.*, 1996).

Originally it was thought that *N. ceranae* was restricted to Asian honey bees (*Apis cerana*).

N. ceranae was reported in European bees (*Apis mellifera*) since 2006 by many research groups in all over the world.

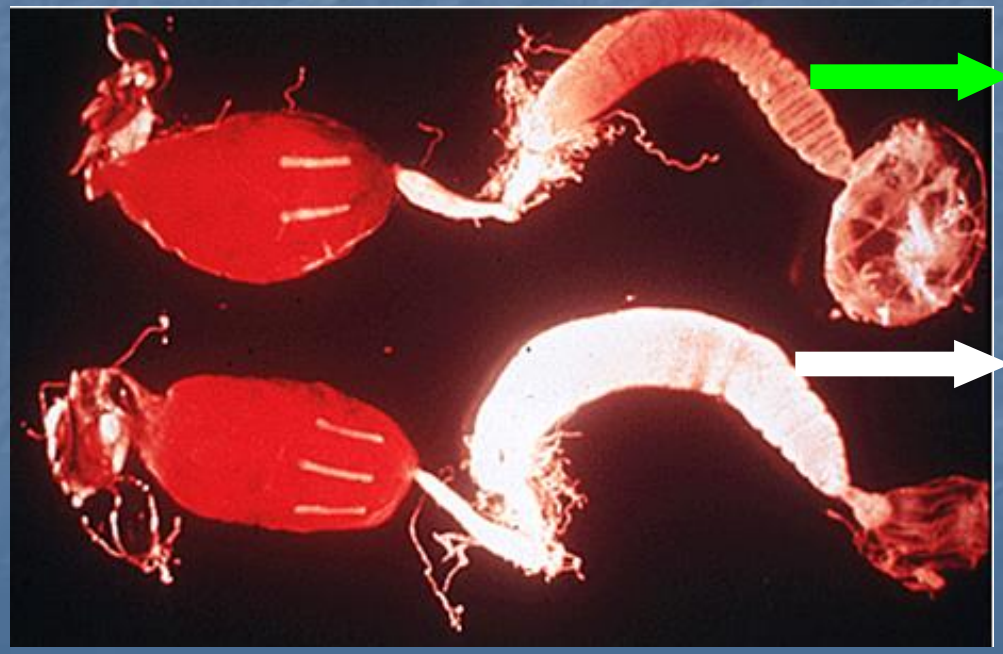
Life cycle of Nosema disease



Life cycle of *Nosema apis*. The spore injects its contents into a gut epithelial cell, multiplies, and eventually causes the cell to burst and release the new spores back into the gut. *Nosema* can also reproduce "vegetatively" cell to cell.

Symptoms of Nosema disease in bee guts

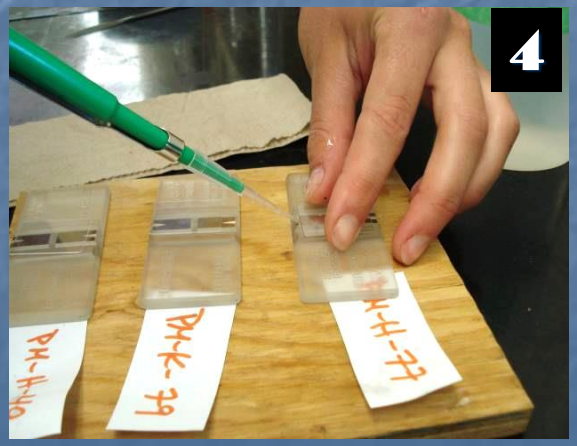
Infectious disease of the digestive tract of honey bees caused by parasites of the genus *Nosema*

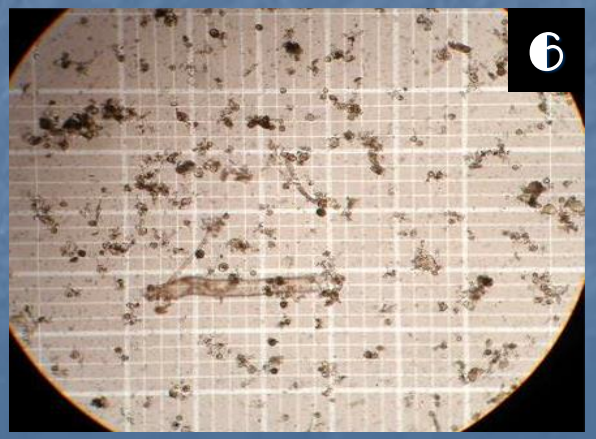


Healthy gut:
amber, translucent, constrictions present

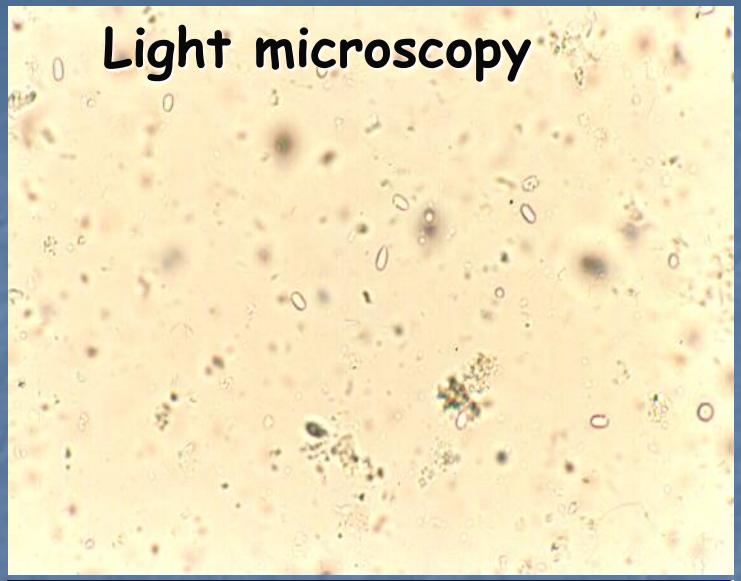
Infected gut:
chalky white, swollen, constrictions unclear

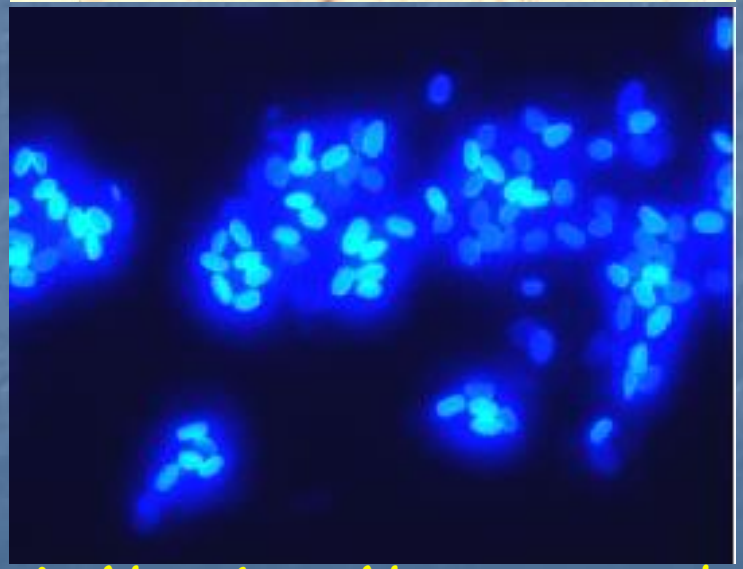
Detection procedures of *Nosema* spores under the microscope

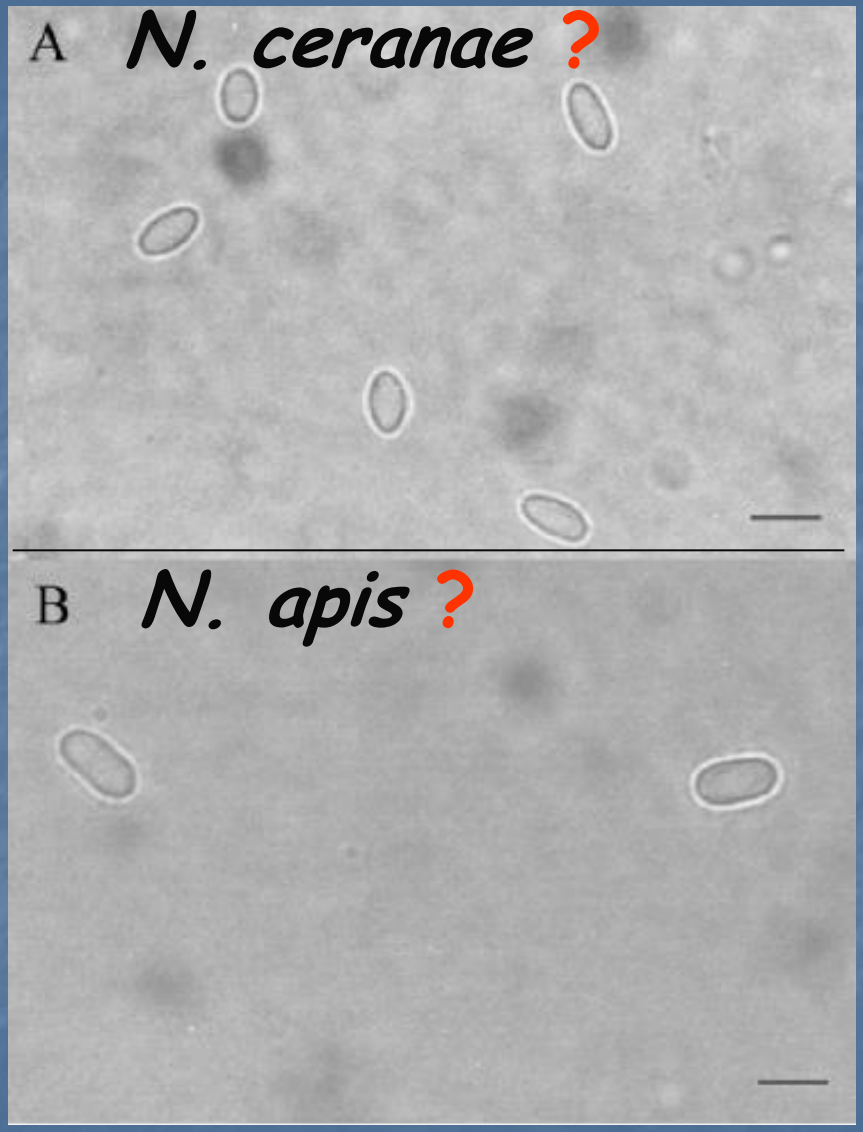




Nosema spp. under microscope







Is it *N. apis* or *N. ceranae* or both species? Detection and quantification of spores is only possible with infection levels of 50,000 spores/bee or more.

Challenges !

- Recently *N. ceranae* was found in Canada and it is replacing *N. apis*

- Good/reliable lab. methods to diagnose and quantify *N. ceranae* and/or *N. apis* infections are needed

- Analyze the genetic diversity of *N. ceranae*

Objectives

- Develop a simple and reliable multiplex PCR (polymerase chain reaction) method to diagnose and quantify Nosema disease (*N. ceranae* and/or *N. apis*) in honey bees

- Determine genetic variation in *N. ceranae* samples collected from different regions of the world using genetic markers and sequencing techniques

DNA extraction method

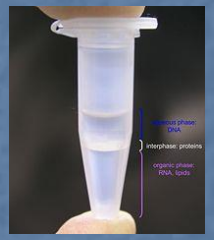
Kit method (Roche Diagnosis)	Honey Bee Research Centre (HBRC) method
Commercial Kit for DNA extraction	DNA extracted with CTAB buffer developed
10-20 bees per sample	Single bee per sample
Extraction of spores & filtration needed	Total abdomen used (no spore extraction needed)
Germination buffer needed	No germination buffer needed
Incubation period needed	No incubation period needed

Honey bee DNA extraction and PCR technique

DNA extraction (HBRC)

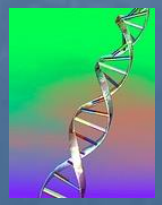
Dissection of bees (separate abdomen from thorax)

Macerate with CTAB buffer and heat



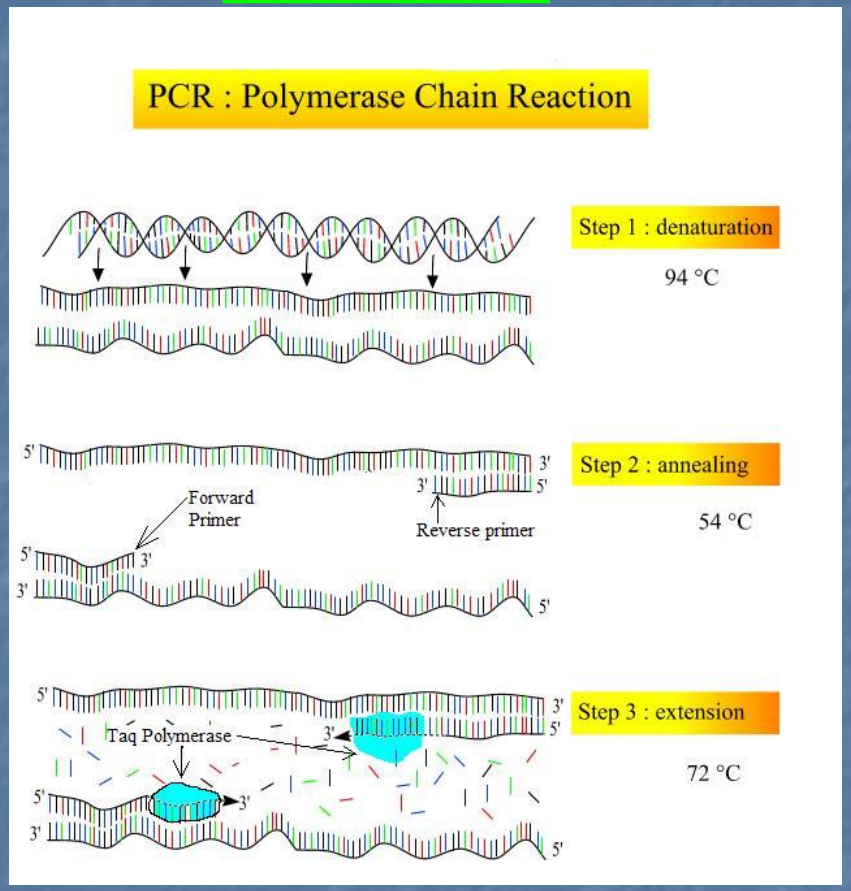
Phenol/chloroform washing steps

Precipitation with ETOH and centrifuge



DNA

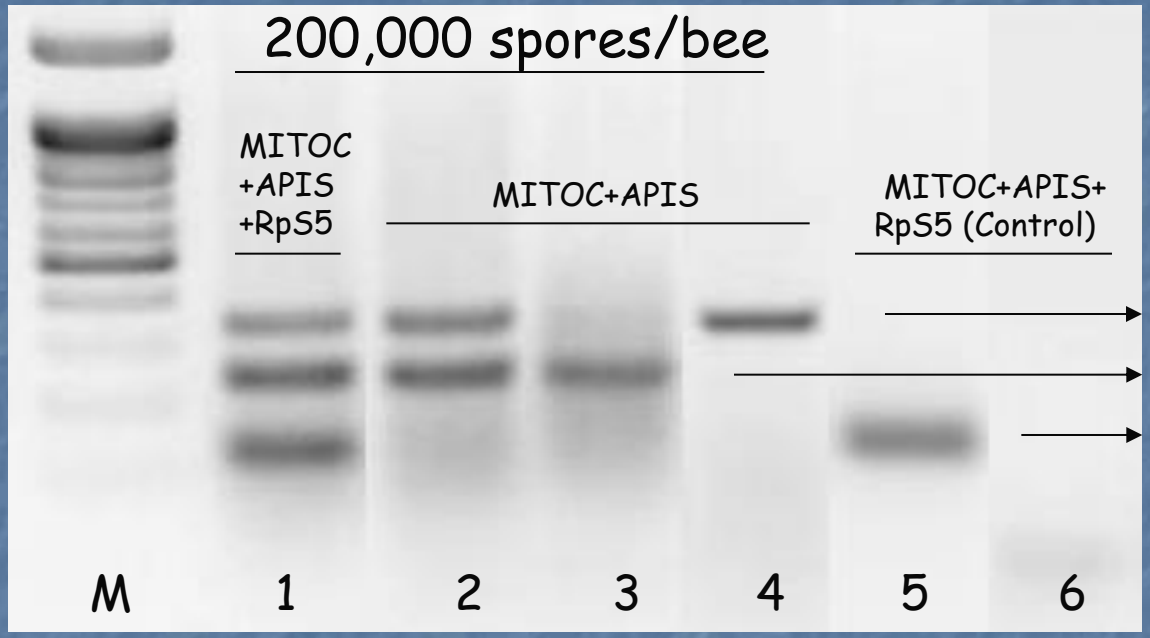
PCR reaction



Run the gel (Electrophoresis)

Observe the band under UV

Diagnosis of *N. ceranae* or *N. apis* separately and both together in the same sample

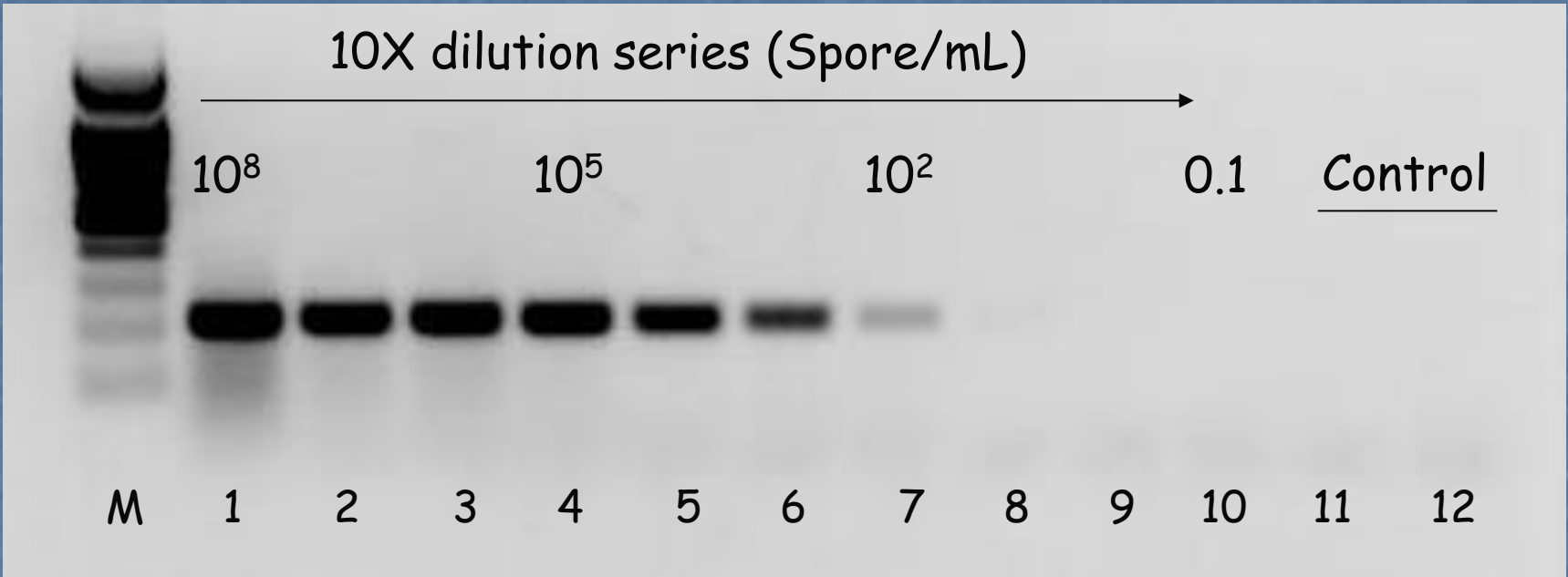


By observing the number of bands (with specific product length) we can diagnose *N. ceranae* or *N. apis* separately and both spp. together which is not possible under the light microscope

Sensitivity of the HBRC method

What is the lowest number of spores in a sample that can be detected with the HBRC method ?

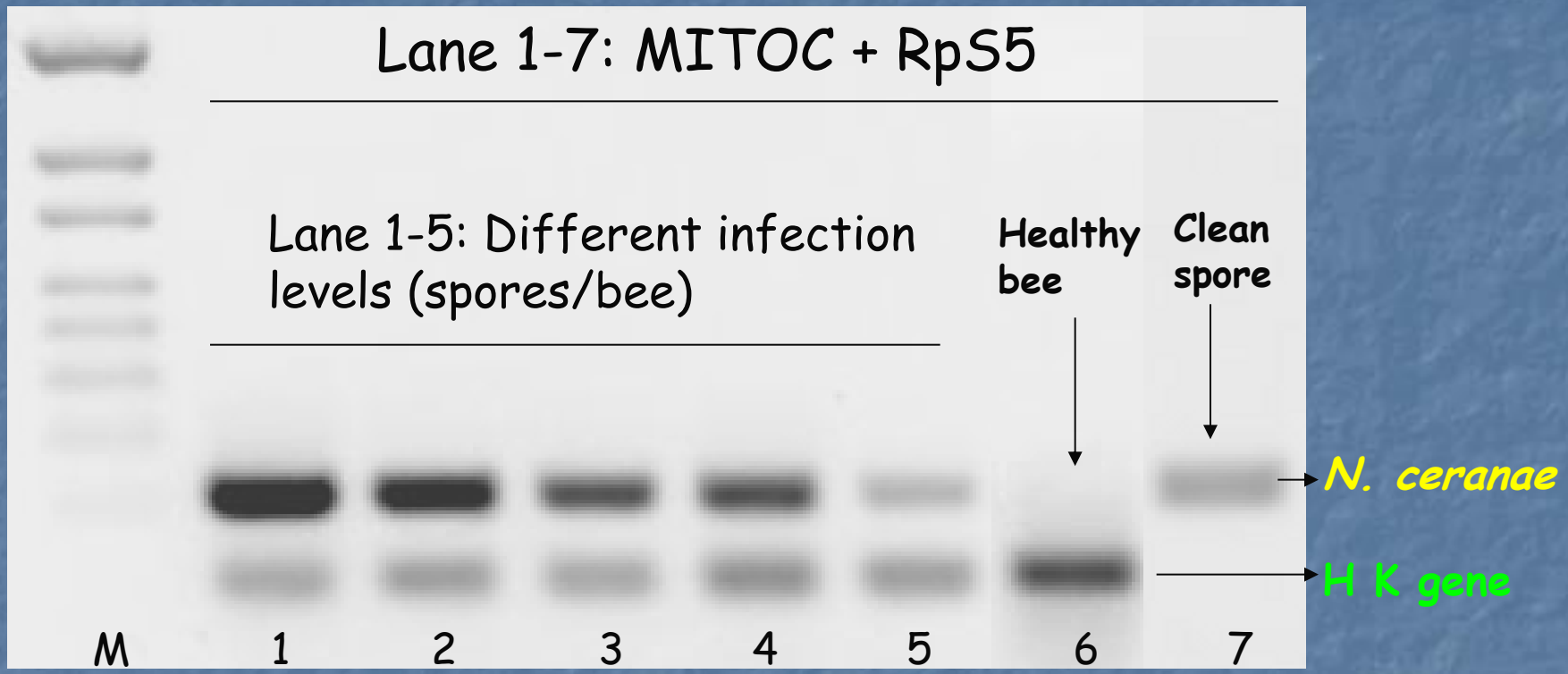
Detection of *Nosema ceranae* from a series of spore dilutions using the HBRC-PCR technique



-HBRC method detected 100 spores/bee

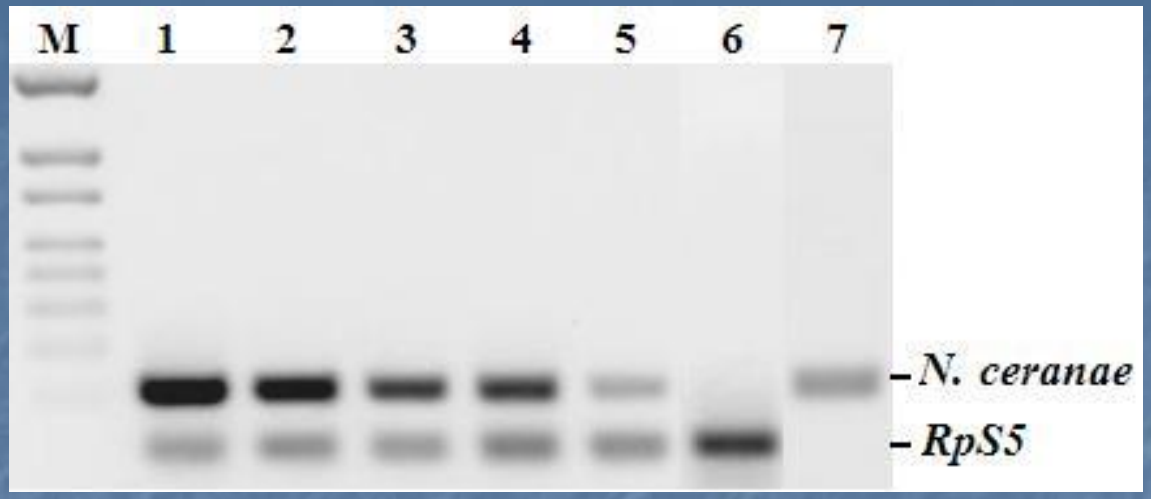
-Microscope method requires 50,000 spores/bee or more

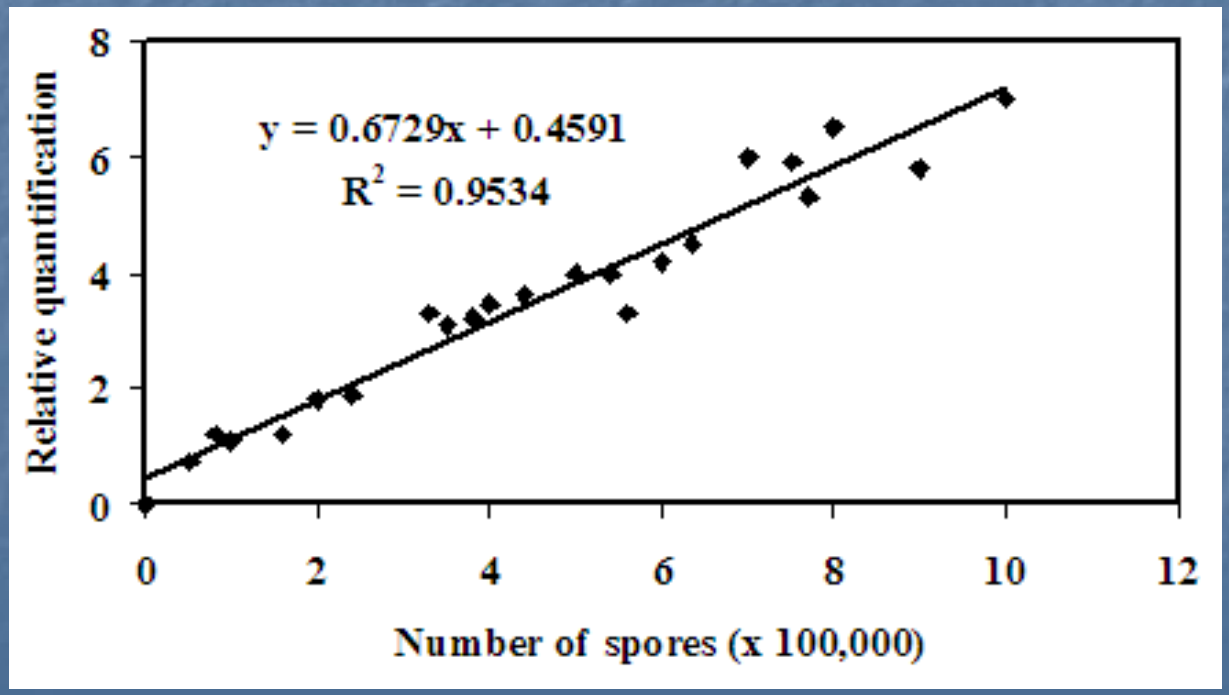
Quantification of *Nosema* infection levels using the HBRC method



Ratio of the expression level of target gene over HK gene was positively correlated with infection levels

Relationship between no. of spores and gel band intensity





Relative expression of target gene increased positively with number of spores

Analyze genetic variation in *N. ceranae*

Genetic markers and sequencing techniques

- Simple Sequence Repeats (SSR) or microsatellites
- Translation elongation factor 1 - alpha
- Single Nucleotide Polymorphism (SNP)
- Random amplified polymorphic DNA (RAPD)
- Gene Sequencing

An invasive population of a species in general have decreased genetic diversity in comparison to its native population (Besnard *et al.*, 2007).

Thus, *N. ceranae* could have a more genetic variability in its native range in eastern Asia, both in *A. cerana* and *A. mellifera*, compared to its invasive range in the rest of the world.

Microsatellites or Simple Sequence Repeats (SSR)

What are Microsatellites

Also known as STR, SSR, VNTR

STR: Short Tandem Repeats

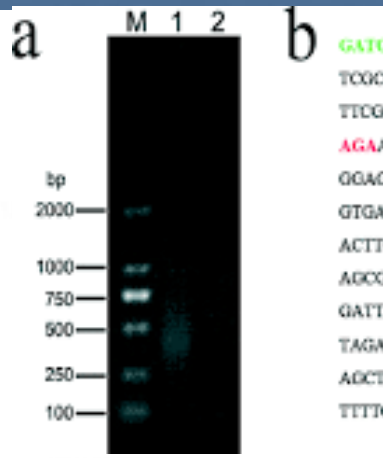
SSR: Simple Sequence Repeats

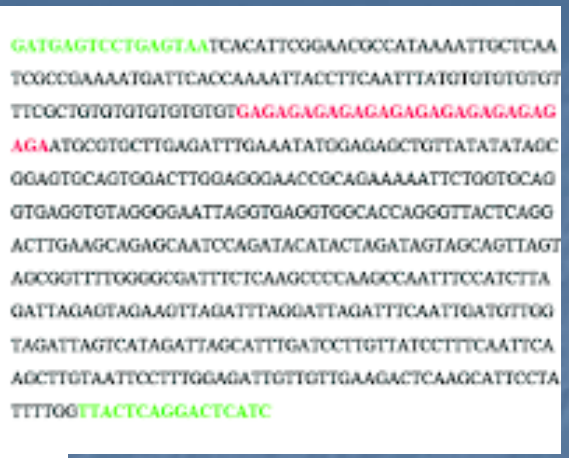
VNTR: Variable Number Tandem Repeats

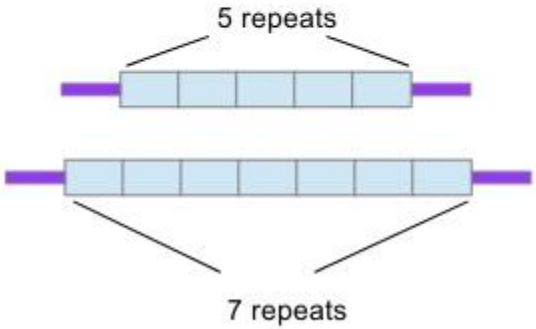
Tandemly repeated DNA sequences with the repeat/size of 1 – 6 bases repeated several times.

Highly polymorphic; can be analyzed with the help of PCR.

Individual alleles at a locus differ in number of tandem repeats of unit sequence owing to gain or loss of one

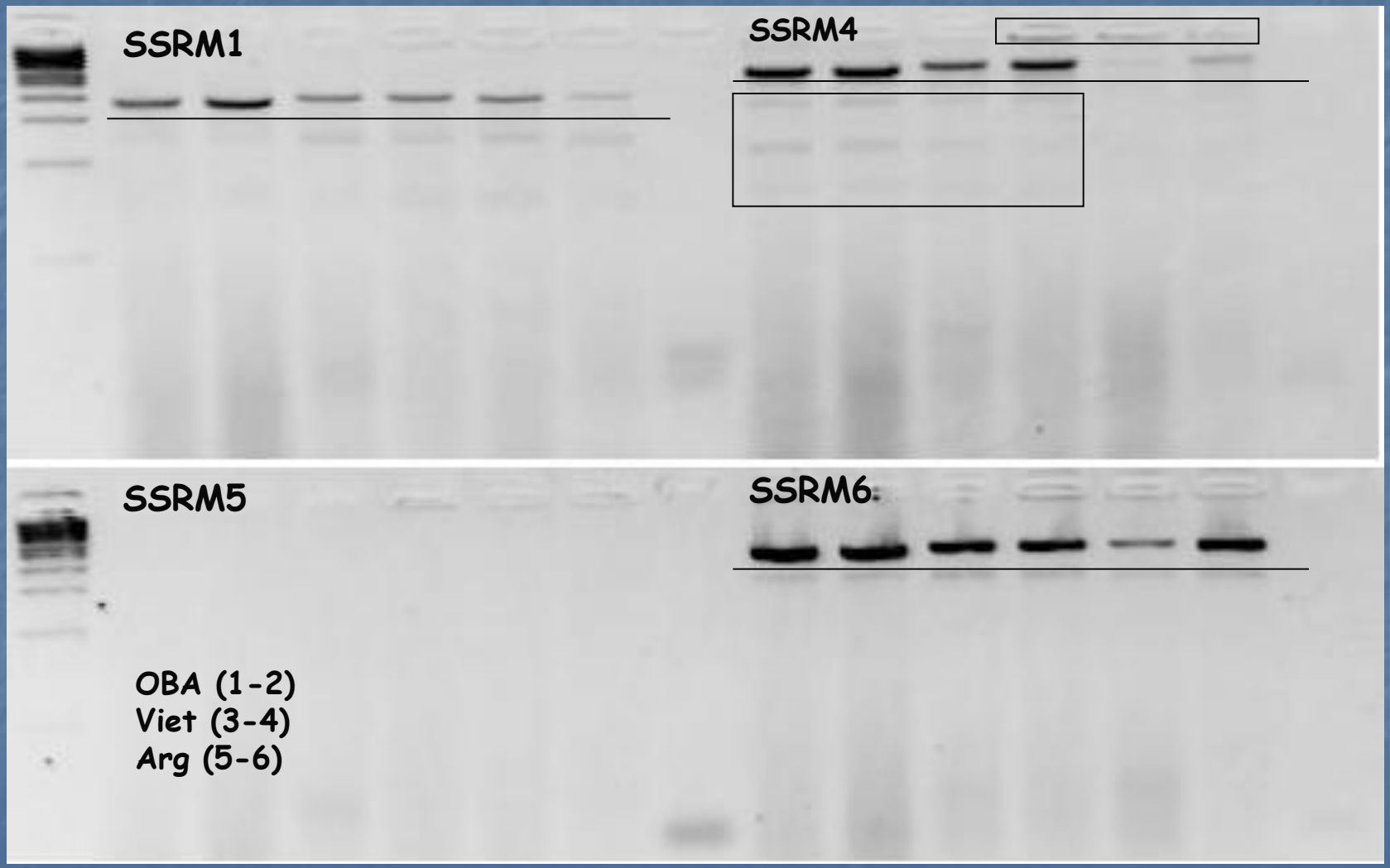






A microsatellite is a tract of repetitive DNA in which certain DNA motifs (ranging in length from 2-5 base pairs) are repeated, typically 5-50 times. Microsatellites occur at thousands of locations within an organism's genome; additionally, they have a higher mutation rate than other areas of DNA leading to high genetic diversity.

Detection of genetic diversity within *Nosema ceranae* using SSR primers (Simple Sequence Repeats)

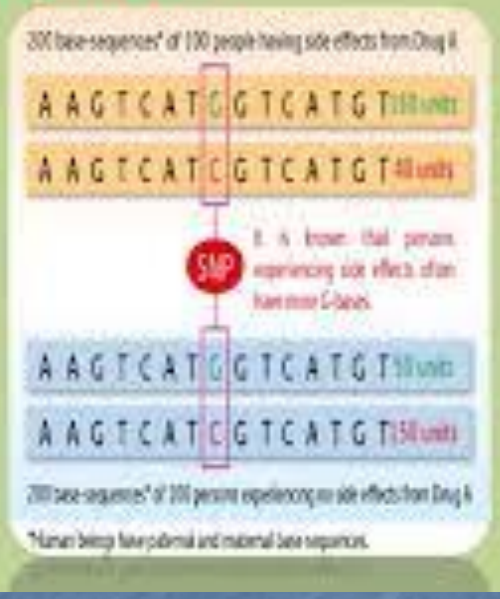


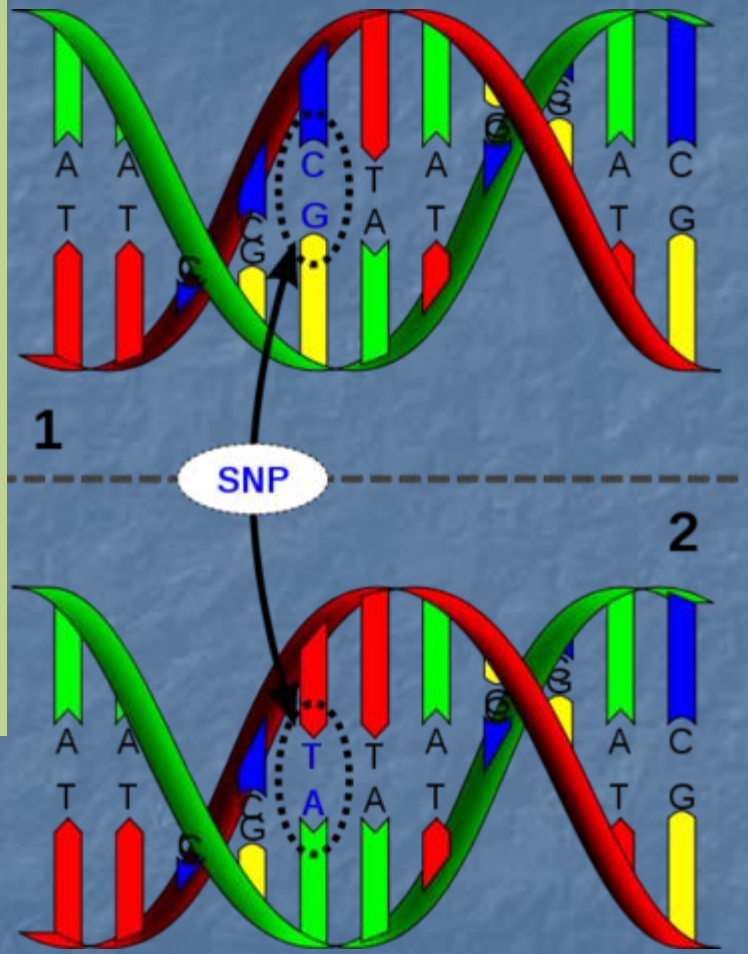
PCR, DNA purification, sequencing, alignments, phylogenetic tree

Single Nucleotide Polymorphism (SNP)

Single Nucleotide Polymorphism

- The most common sequence variations are single base changes – SNP
- < 1% of SNPs occur in coding regions and alter the genetic product → Disease
- Marker that is co-inherited with a disease causing gene due to physical proximity





*A single-nucleotide polymorphism (SNP, pronounced *snip*) is a DNA sequence variation occurring when a single nucleotide adenine (A), thymine (T), cytosine (C), or guanine (G) in the genome (or other shared sequence) differs between members of a species or paired chromosomes in an individual.*

Nucleotide sequence alignment of the translation elongation factor 1- alpha of *Nosema ceranae* infected honey bees

```
VI8       ATGTTAAGAAATATTGCTGTAACTAGATCTATAGAAAAATACAGAGAATC 50
VI10      ATGTTAAGAAATATTGTTGATACTAGAACTATAGAAAAATACAGAGAATC 50
GrCom     ATGTTAAGAAATATTGTTGATACTAGAACTATAGAAAAATACAGAGAATC 50
VI7       ATGTTAAGAAATATTGTTGATACTAGAACTATAGAAAAATACAGAGAATC 50
VI11      ATGTTAAGAAATATTGTTGATACTAGAACTATAGAAAAATACAGAGAATC 50
VI9       ATGTTAAGAAATATTGTTGATACTAGAACTATAGAAAAATACAGAGAATC 50
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Conclusions

1. The HBRC method was simple, more reliable, and cheaper than current methods
2. It is possible to detect *N. ceranae* or *N. apis* separately or together with the HBRC method which is not possible under the microscope
3. 100 spores/bee was detectable with the HBRL method when 50,000 spores/bee are needed under the microscope
4. First time it is shown that the quantification of *Nosema* infection levels is possible with a simple PCR technique
5. SSR primers and SNPs found as good molecular markers to determine genetic diversity within *Nosema ceranae* populations

Application of the HBRC method

- This method could be useful in epidemiological evaluations for regulatory purposes or disease control

- This method could be handy for researchers to study in depth *N. apis* and/or *N. ceranae* because it diagnoses single-bee samples

- SSR primers and translation elongation factor 1 – alpha could be useful to detect and track *N. ceranae* genotypes for parasite control, determine the sources of infestation and regulatory purpose when outbreaks occur

Honey bee research group at the Uni. of Guelph

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Our main goals

Healthy bees ➡ Healthy food ➡ Healthy society