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Nosema neumanni n. sp. (Microsporidia, Nosematidae), a new microsporidian parasite of honeybees, *Apis mellifera* in Uganda

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Abstract

The microsporidium *Nosema neumanni* n. sp., a new parasite of the honeybee *Apis mellifera* is described based on its ultrastructural and molecular characteristics. Structures resembling microsporidian spores were found by microscopic examination of honeybees from Uganda. Molecular confirmation failed when PCR primers specific for *Nosema apis* and *Nosema ceranae* were used, but was successful with primers covering the whole family of Nosematidae. We performed transmission electron microscopy and found typical microsporidian spores which were smaller (length: 2.36 ± 0.14 μm and width: 1.78 ± 0.06 μm; $n = 6$) and had fewer polar filament coils (10–12) when compared to those of known species infecting honeybees. The entire 16S SSU rRNA region was amplified, cloned and sequenced and was found to be unique with the highest resemblance (97% identity) to *N. apis*. The incidence of *N. neumanni* n. sp. in Ugandan honeybees was found to be much higher than of the two other *Nosema* species.

Keywords: African honeybee; Microsporidia; Nosematidae; Nosemosis; 16 SSU rRNA; Ultra-structure

Introduction

The microsporidia are a diverse group of intracellular parasites that infect both invertebrates and vertebrates (Franzen and Müller 1999). Until now, only two species of microsporidia are known to infect the honeybee, *Apis mellifera* worldwide. These are *Nosema apis* which was first reported over 100 years ago and the relatively recent *Nosema ceranae* (Higes et al. 2006; Huang et al. 2007; Traver et al. 2012). Both *N. apis* and *N. ceranae* are obligate intracellular parasites of the midgut of honeybees (de Graaf et al. 1994; Higes et al. 2006). They have been reported in all continents where beekeeping with *A. mellifera* occurs (e.g. in Africa by Fries et al. (2003), Higes et al. (2009) and Muli et al. (2014); in Europe by Higes et al. (2006) and Paxton et al. (2007); in North America by Chen et al. (2008); in South America by Calderón et al. (2008); in Asia by Chen et al. 2009). The two

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species of *Nosema* cannot clearly be distinguished under the light microscope. However, under the electron microscope, *N. ceranae* has 20–23 polar filament coils while *N. apis* has more than 30 polar filament coils (Fries et al. 1996).

Infection of honeybees by *N. apis* and *N. ceranae* has negative impacts that vary with the geographical location and the *Nosema* species involved. Damage to colonies can include suppression of the honeybee immune system (Antúnez et al. 2009), shortening of worker bee lifespan (Mayack and Naug 2009), decline in colony strength and productivity (Botías et al. 2013), queen supersedure (Alaux et al. 2011), increased winter losses and colony collapse (Higes et al. 2008). These impacts demonstrate *Nosema* spp. as threats to honeybees and hence the need to monitor.

Beekeeping is an important economic activity in the rural areas of Uganda providing a supplementary source of income for rural households (Chemurot et al. 2013; Kajobe et al. 2009; UEPB 2005). However, limited information is available on honeybee pests and pathogens in the country that can be used to plan for their management. In order to provide baseline data and gain some insights into microsporidian parasite infections in Ugandan honeybees, this study was conducted to identify the *Nosema* spp. present. Structures resembling microsporidian spores but slightly smaller in size when compared to the spores of known *Nosema* parasites of honeybees, were found by microscopic examination. Eventually, ultra-structural and molecular characterization enabled us to describe a new microsporidian parasite of the genus *Nosema* in honeybees.

Material and Methods

Study area and sample collection

This study was conducted in the eastern and western highlands agro-ecological zones (AEZ) of Uganda. Samples of worker honeybees were collected from the edges inside the beehives. A total of 175 colonies were sampled during the dry season and 195 during the wet season (December 2014–September 2015) from the eastern and western highlands AEZs of Uganda. Honeybee samples were preserved in 95% ethanol and refrigerated as soon as possible until transported to Ghent University, Laboratory of Molecular Entomology and Bee Pathology for analyses. Detailed description of the field data and sample collection are provided elsewhere (Chemurot et al. 2016).

Detecting and counting *Nosema* spores

To detect and evaluate *Nosema* spp. infection in the colonies, samples of bees (n = 10 bees per sample) were soaked in 10 ml PBS in 15 ml tubes and electronically agitated in a cold room (4 °C) for one day to wash off the ethanol. The PBS was changed the following day and the sample was agitated in the cold room for another day to rehydrate the honeybees. Then, the honeybees were transferred into 5 ml tubes containing 5 ml PBS, about 0.25 ml zirconia beads and 5 metal beads. The honeybees were crushed in 5 rounds lasting 5 min each in the Bullet Blender Storm 5® /VISUM IDPBW at maximum speed. A volume of 20 μl of the sample was pipetted onto a haemocytometer (Bürker) and examined at 400× magnification for spore counting.

Nosema spp. confirmation by RT-PCR

Nosema spp. confirmation was performed using RNA-based molecular methods as RNA extractions were already available from these samples for other purposes (i.e., virus analyses; data not shown here), which was technically feasible because the microsporidian SSU rRNA gene lacks introns (Vossbrinck et al. 1987).

Total RNA was extracted from 10 bees per sample using QIAamp® viral RNA kit (Qiagen) following the manufacturer's guidelines and descriptions by Ravoet et al. (2013) and Amakpe et al. (2015). Briefly, 560 μl of buffer AVL containing carrier RNA was pipetted into 1.5 ml Eppendorf tubes. Then, 140 μl of the homogenized honeybee sample was added and briefly vortexed and centrifuged. The samples were lysed 10 min at room temperature. After adding 560 μl of absolute ethanol, the mixture was briefly vortexed and centrifuged. The mixture was then pipetted into QIAamp columns and centrifuged at 8000 rpm for 1 min. The columns were washed with 500 μl of buffer AW1 and AW2 and centrifuged according to the manufacturer's protocol. Finally, the columns were transferred into 1.5 ml Eppendorf tubes and 50 μl of AVE elution buffer was carefully added and left to stand at room temperature for 1 min. This was centrifuged at 8000 rpm for 1 min and the eluted RNA was kept at −80 °C.

At first, the singleplex RT-PCR method of Carletto et al. (2013) was used to confirm the *Nosema* spp. present as it is currently the most sensitive method to discern *N. apis* and *N. ceranae*. First, cDNA was synthesized using random primers in the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit starting with 5 μl RNA in a volume of 12 μl. This mixture was denatured for 5 min at 65 °C and placed on ice. Then 4 μl of 5X reaction buffer, 1 μl of RiboLock RNase inhibitor (20 u/μl), 2 μl of 10 mM dNTP mix and 1 μl of Reverse transcriptase (200 u/μl) were added to make a total volume of 20 μl. The mixture was mixed gently and centrifuged before incubating for 5 min at 25 °C and 60 min at 42 °C. The reaction was finally terminated by heating at 70 °C for 5 min and the product used directly for PCR.

The PCR primers QNoUF2 (5′-GGA TTG TGC GGC TTA ATT TGA-3′) and QNo.AR: (5′-CCT CAG ATC ATA TCC TCG CAG-3′) were used to amplify a 77 bp fragment of *N. apis* 16S rRNA. On the other hand, for *N. ceranae*, the same forward primer QNoUF2 (5′-GGA TTG TGC GGC TTA ATT TGA-3′) was used but the reverse primer was replaced with QNoCR (5′-ACC ACT ATT ATC ATT CTC

AAA C-3′) targeting a 97 bp fragment of 16S rRNA. For each PCR reaction, 2.5 μl of 10X buffer, 1 μl of 10 mM dNTP mix, 1.25 μl forward primer (100 μM), 1.25 μl reverse primer (100 μM), 0.25 μl of HotStar Taq polymerase (5 u/μl) (Qiagen) and 13.75 μl of water were prepared (mixed thoroughly) and pipetted into PCR tubes. Then 5 μl of cDNA was added and the mixture centrifuged briefly before being placed in the thermocycler. The following program for amplifying *Nosema* spp. cDNA was used: 5 min at 94 °C, denaturation for 30 s at 94 °C, annealing 30 s at 60 °C, extension for 30 s at 72 °C (35 cycles) and final extension for 3 min at 72 °C. The amplified PCR products were electrophoresed for 60 min at 100 volts through 1.5% agarose TBE gel in standard TBE buffer, stained with ethidium bromide, and visualized using UV illumination.

Several samples with visible spores under microscopy gave negative PCR results for *N. apis* and *N. ceranae*. Therefore, a set of Nosematidae family specific primers designed to amplify a 240 bp region of the 16S rRNA gene (Nos-F: 5′-TAT GCC GAC GAT GTG ATA TG-3′ and Nos-R: 5′-CAC AGC ATC CAT TGA AAA CG-3′) corresponding to nucleotides 644 to 883 (Higes et al. 2006; Fernández et al. 2012) was used. For each sample, the reaction consisted of 2.5 μl of 10X buffer, 1 μl of 10 mM dNTP mix, 1.25 μl forward primer (Nos-F; 100 μM), 1.25 μl reverse primer (Nos-R; 100 μM), 0.25 μl of Taq polymerase (5u/μl), 13.75 μl of water and 5 μl of cDNA. The parameters of amplification were set as follows: 5 min at 95 °C, denaturation for 30 s at 94 °C, annealing 30 s at 52 °C, extension for 30 s at 72 °C (35 cycles) and final extension for 10 min at 72 °C. Amplified PCR products were electrophoresed for 45 min at 120 volts through 2% agarose TBE gel in standard TBE buffer and stained/visualized as here above. Amplicons of three selected samples amplified using Hotstar Hifi Taq (Qiagen) were sequenced. The sequences obtained were edited and alignments performed using Clustal-W and compared with those in the GenBank using BLAST.

Transmission electron microscopy (TEM) of spores

One sample of the honeybee homogenate (collected from Kasese district) with observable microsporidia-like spores under the light microscope but which turned negative for both *N. apis* and *N. ceranae* was prepared for TEM. Spores were fixed in 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M Na cacodylate buffer, pH 7.2 and centrifuged at 1500 rpm. Low melting point-agarose was used to keep the cells concentrated for further processing. Spores were fixed for 4 h at RT followed by fixation O/N at 4 °C after replacing with fresh fixative. After washing in buffer, they were post-fixed in 1% OsO_4 with 1.5% $K_3Fe(CN)_6$ in 0.1 M Na cacodylate buffer at room temperature for 1 h. After washing, cells were subsequently dehydrated through a graded ethanol series, including a bulk staining with 1% uranyl acetate at the 50% ethanol step followed by embedding in Spurr's resin. Semi-thin sections were first cut at 0.5 μm and stained with toluidine blue. Ultrathin sections of a gold interference color were cut using an ultra-microtome (Leica EM UC6), followed by a post-staining in a Leica EM AC20 for 40 min in uranyl acetate at 20 °C and for 10 min in lead stain also at 20 °C. Sections were collected on Formvar-coated copper slot grids. Grids were viewed with a JEM 1400plus transmission electron microscope (JEOL, Tokyo, Japan) operating at 60 kV.

Sequencing of the 16S SSU rRNA and diagnostic tool development

A set of primers (Nos_ssu_18F: 5′-CACCAG GTT GAT TCT GCC-3′ and Nos_ssu_1537r: 5′-TTA TGA TCC TGC TAA TGG TTC-3′) designed to amplify the entire 16S rRNA gene of Nosematidae (Dong et al. 2010) was used on the cDNA of a sample (also used for TEM) with visible spores under microscopy but which gave negative results for *N. apis* and *N. ceranae* specific PCR assay. The amplicon was generated using the same reaction conditions as described above using Hotstar Hifi Taq polymerase. The thermocycler program was set as follows: 2 min at 95 °C, 30 s at 94 °C, 1 min at 50 °C, 2 min at 68 °C for 35 cycles and final extension 10 min at 68 °C. The amplicon was purified using the GenJET Gel extraction kit (Thermo Fisher Scientific) following the manufacturers' protocol with slight modifications (elution with 25 μl elution buffer instead of 50 μl). The purified PCR product was cloned into a plasmid using the TOPO® TA cloning® Kit (Invitrogen) for sequencing following the manufacturers protocol. The GenJet plasmid miniprep kit was used to extract the plasmids and sent for commercial sequencing. The sequences obtained from both strands were edited and alignments performed using Clustal-W and compared with those in the GenBank using BLAST.

The entire 16S rRNA gene of the new species was found to be 97% similar to *N. apis* (U97150.1). In order to develop a species-specific PCR, a section of the sequence that was different from *N. apis* was selected and primers were developed with the forward corresponding to QNoUF2 (5′-GGA TTG TGC GGC TTA ATT TGA-3′) and the reverse primer (Nos_new_R1: 5′-CCT CAA ATA GAA TCA TCG CCG G-3′ and Nos_new_R2: 5′-CAC TAG AAG TGT CAG TCC TAC-3′). After testing, only the first reverse primer was specific for the new *Nosema* species. The thermocycler was set as follows: 15 min at 95 °C; 30 s at 94 °C; 30 s at 60 °C; 30 s at 72 °C: 35 cycles and final extension for 3 min at 72 °C. Electrophoresis on a 1.5 agarose TBE gel and staining/visualization was as here above. All samples (370) collected from Uganda were screened using the developed primers to establish the incidence of the new species.

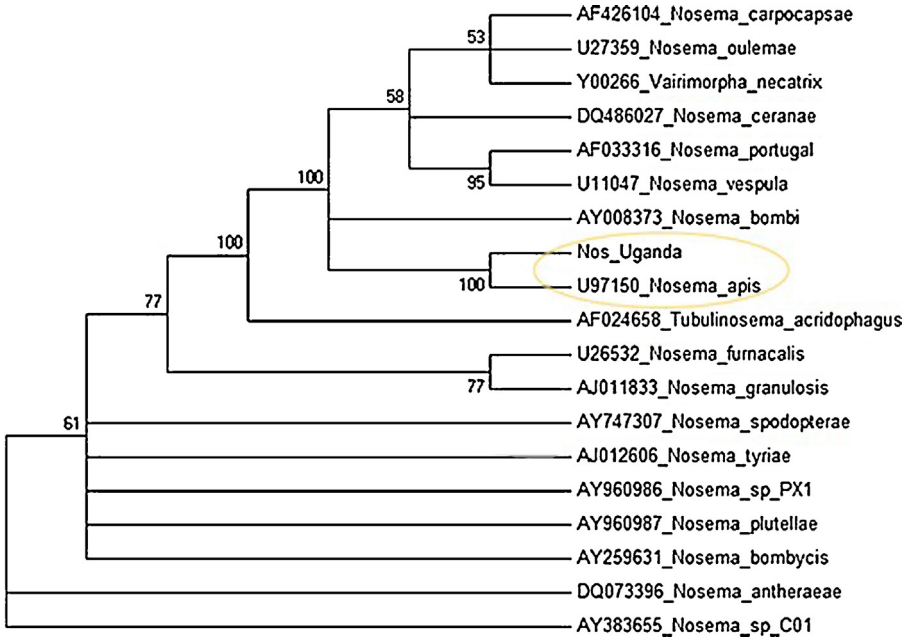

Fig. 1. Phylogenetic tree based on SSU rRNA sequences of *N. neumanni* n. sp. and other related microsporidia. The phylogenetic tree was constructed using the Maximum likelihood method. The bootstrap values are indicated at the nodes. The GenBank accession number for each sequence is given adjacent to the corresponding species name. The new *Nosema* spp., *N. neumanni* n. sp. (indicated in the tree as Nos_Uganda) and *N. apis* to which it is more closely related are encircled together in an orange oval.

Phylogenetic tree using SSU rRNA gene sequences

The SSU rRNA gene sequences of 19 microsporidia were aligned and edited using the Clustal-W Program. Phylogenetic trees based on the resultant alignments were constructed using the Maximum Likelihood method of MEGA7 software (Kumar et al. 2016). In this method, initial trees were obtained automatically by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. All positions containing gaps and missing data were eliminated leaving a total of 1030 positions in the final dataset.

Results

Discovery of a new *Nosema* spp.

The two known *Nosema* spp. that infect honeybees were detected in the study sites. However, several samples with visible spores under the light microscope gave negative PCR results for both *N. apis* and *N. ceranae.* Further PCR analyses of these samples with Nosematidae family primers gave positive results. Amplicons (240 bp) of the 3 samples that were sequenced were 95% identical to *N. apis* (FJ789798.1) with 9 nucleotide differences. Further analyses showed a new species here-in referred to as *Nosema neumanni* n. sp. to honor the bee researcher Prof. Dr. Peter Neumann, presently affiliated to the University of Bern, Switzerland. Prof. Dr. Peter Neumann was founder and coordinator (now president) of COLOSS (Prevention of honey bee COlony LOSSes). COLOSS started as a COST-Action of the European Commission, but has now evolved into an international, non-profit association headquartered in Bern, Switzerland that is dedicated to improving the well-being of bees at a global level. COLOSS currently consists of 935 members from 97 countries and continues to grow.

The electron micrographs showed that the spores of *N. neumanni* n. sp. are smaller in size (length: 2.36 ± 0.14 μm and width: 1.78 ± 0.06 μm; $n = 6$) and have fewer polar filament coils compared to those of *N. apis* and *N. ceranae* (Fig. 2). Specifically, a mature spore of *N. neumanni* n. sp. has a spore wall consisting of an electron dense exospore and an electron lucent endospore. Its polar filament tubule comprised of 10–12 coils, while the spore nucleus was diplokaryotic. All these features match the principle characteristics of the genus *Nosema*.

The SSU rRNA region of *N. neumanni* n. sp. consists of 1242 bp (MF882996) and the GC content is 38.9%. It is between 93 and 97% identical with corresponding regions of the rRNA of other *Nosema* species (*N. apis*, *Nosema bombi*, *Nosema oulemae*, *Nosema thomsoni*, *Nosema portugal*, *Nosema vespula*, *Nosema necatrix* and *N. ceranae*). The results also suggest that this isolate may be closely related to *N. apis*, with which it shares 97% identity of the SSU rRNA. The phylogenetic tree (Fig. 1) based on the SSU rRNA sequences confirms that *N. neumanni* n. sp. represents a true *Nosema* spp. that is most related to *N. apis* (U97150.1).

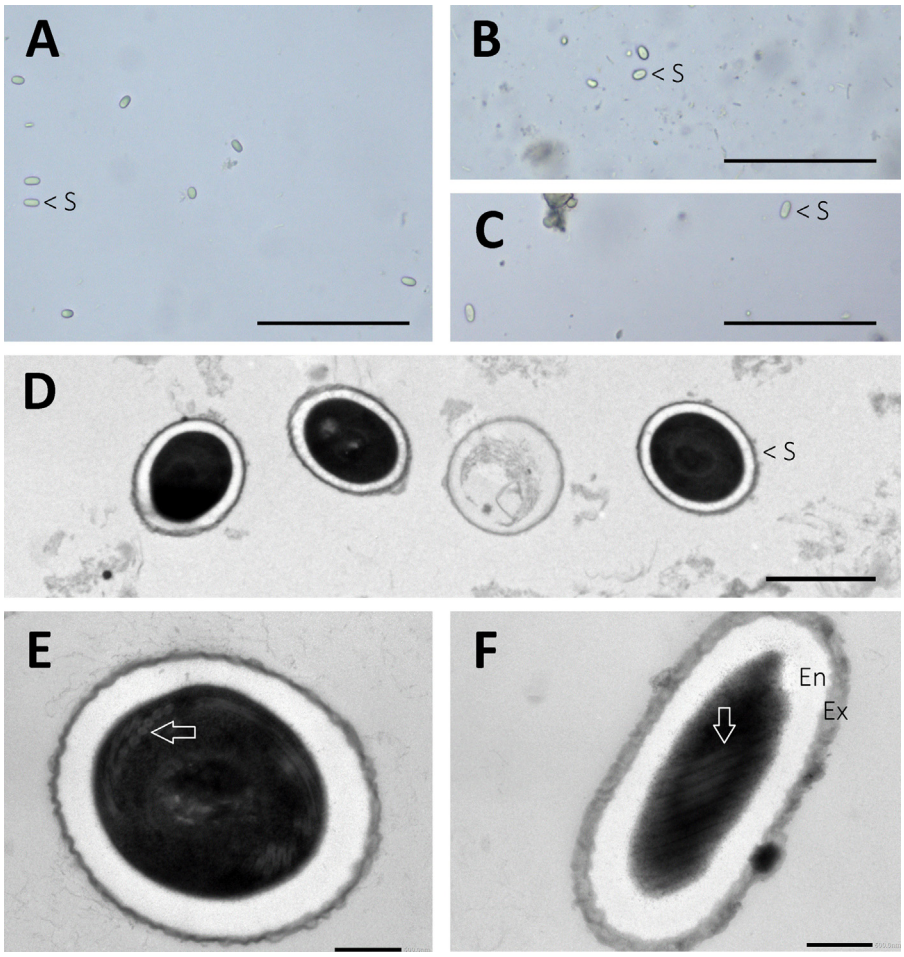

Fig. 2. In A: Light micrographs of spores (S) of *Nosema neumanni* n. sp. which seem slightly smaller than spores of *Nosema ceranae* (in B) and *Nosema apis* (in C). In D, E and F: electron micrographs of spores of *Nosema neumanni* n. sp. with electron dense exospore (Ex) and electron lucent endospore (En). Spore size measurements under transmission electron microscopy confirms that spore length (2.36 ± 0.14 μm) and width (1.78 ± 0.06 μm) is smaller than of the two already known *Nosema* spp. associated with honeybees. Moreover, *Nosema neumanni* n. sp. has fewer filament coils (10–12; white arrows). Scale bars: in A, B and C 50 μm; in D 2 μm and in E and F 500 nm.

Seasonal incidence of *Nosema* spp.

There was a varied incidence of *Nosema* species infections in colonies in the two AEZs of Uganda during the dry and wet seasons. *N. neumanni* n. sp. was more common when compared to *N. apis* and *N. ceranae*. Generally, the proportion of samples positive for *N. neumanni* n. sp. was higher in the dry than in the wet season (Fig. 3). Mean *Nosema* spore count in the eastern AEZ of Uganda was highest in the wet season (2800.63 ± 802.75 spores per bee) compared to the dry season (43.69 ± 24.21 spores per bee). While, in the western highlands AEZ of Uganda, the mean spore count was highest during the dry season (4247.61 ± 1578.65 spores per bee) compared to the wet season (2960.15 ± 1645.85 spores per bee). In both zones, the seasonal spore counts were statistically different (eastern: Mann–Whitney $U = 1269.5$, $Z = -7.478$, $P < 0.01$; western highlands: Mann–Whitney $U = 3389.0$, $Z = -3.942$, $P < 0.01$).

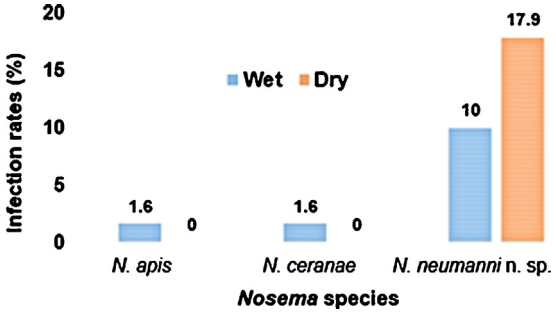

Fig. 3. Comparison of the seasonal incidence of different *Nosema* spp. in the wet and dry season at the study sites.

Discussion

Although microsporidia infect a broad range of vertebrate and invertebrate hosts including insects, fish and mammals, the genus *Nosema* are entomopathogenic (Huang et al. 2004).

This study is the first documentation of *Nosema* spp. in Ugandan honeybee colonies. Importantly, it shows the discovery of a new microsporidian, *Nosema neumanni* n. sp., detected in Ugandan colonies in addition to the known *N. apis* and *N. ceranae*. There are clear differences in the ultra-structural and molecular characteristics of the new *Nosema* species mentioned here when compared to the other described honeybee microsporidian parasites *N. apis* and *N. ceranae*. Spores of *N. neumanni* n. sp. are smaller under the TEM and the number of polar filament coils is between 10 and 12, compared to 20–23 for *N. ceranae* and more than 30 often seen in *N. apis* (Fries et al. 1996).

Microsporidia under the genus *Nosema* are characterized by production of spores with walls consisting of an electron dense exospore and an electron lucent endospore, polar filament coils, and diplokaryotic nuclei (Cali et al. 2011; Franzen and Müller 1999; Huang et al. 2007). Based on our molecular and TEM data, this study confirms that the microsporidian discovered in Ugandan honeybees truly represents a new species of genus *Nosema*. Previous studies on nosemosis in Africa could have overlooked *N. neumanni* n. sp. because of the relatively small size of its spores when compared to *N. apis* and *N. ceranae*. It seems also reasonable to believe that investigations only based on microscopic examinations might have misdiagnosed infections of the new species as *N. apis* or *N. ceranae* and thus should be revisited to ascertain the actual species involved. It is clear that worldwide epidemiological screening for this newly detected microsporidian parasite of honeybees is necessary to determine its true distribution.

Nosema parasite incidence levels of the new microsporidian were high compared to *N. apis* and *N. ceranae* although the spore counts were always low in Ugandan honeybee colonies. This suggests that the new microsporidian parasite is endemic in Ugandan honeybee colonies and the local honeybees are able to maintain infection at low levels. So far, we could not associate the presence of *N. neumanni* n. sp. with particular clinical manifestations. We plan to make observations on naturally infected colonies and to perform experimental infections of caged bees in order to better understand the implications of infections with this new *Nosema* species.

In conclusion, our study demonstrates that honeybees can be infected by a third distinct microsporidian parasite that we named *Nosema neumanni* n. sp. to honor Prof. Dr. Peter Neumann.

Taxonomic summary

Phylum Microsporidia Balbiani, 1882.

Genus *Nosema* Naegeli, 1857.

Spores ovocylindrical, straight, diplokaryotic, $2.36 \pm 0.14 \times 1.78 \pm 0.06\,\mu m$ in TEM sections and covered with a thin electron dense exospore and a thick electron lucent endospore. Isofilar polar filament with 10-12 coils.

Species *Nosema neumanni* n. sp.

The SSU rRNA region consists of 1242 bp (MF882996) and the GC content is 38.9%. It shares 97% identity with the SSU rRNA of its closest related species *Nosema apis*, also parasite of the honeybee.

Diagnosis: microscopic examination of crushed honeybees (or their abdomina) showing suspicious spores (size and shape) should be confirmed by PCR (or RT-PCR) reaction using QNoUF2 forward (5′–GGA TTG TGC GGC TTA ATT TGA–3′) and Nos_new_R1 reverse (5′-CCT CAA ATA GAA TCA TCG CCG G-3′) primers.

Tissue and host: midgut (based on spore counts of separated abdominal tissues) of the honeybee *Apis mellifera*.

Etymology: species name refers to the bee scientist Prof. Dr. Peter Neumann, presently affiliated to the University of Bern, Switzerland.

Type of habitat: apiary of the western agro-ecological zone, Kasese district, in Uganda.

Type material: Syntypes: Spurr's resin embedded homogenates of infected honeybees and frozen specimens coming from the same hive, in care of the Laboratory of Molecular Entomology and Bee pathology, Faculty of Sciences, Ghent University in Belgium. Illustration Fig. 2A, D, E and F, this paper.

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