

Biological and molecular features of *Nosema rachiplusiae* sp. n., a microsporidium isolated from the neotropical moth *Rachiplusia nu* (Guenée) (Lepidoptera: Noctuidae)

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Received: 20 October 2017 / Accepted: 19 February 2018 / Published online: 28 February 2018
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Abstract

Light, electron microscopy and DNA analyses were performed to characterize a microsporidium infecting *Rachiplusia nu* larvae from a laboratory rearing in Argentina. Diplokaryotic spores were oval and measured $3.61 \pm 0.29 \times 1.61 \pm 0.14 \mu\text{m}$ (fresh). The spore wall was composed of an electron-dense exospore and an electron-lucent endospore, ca. 30 nm and 100–120 nm thick, respectively. The polar filament was arranged in a single rank of 10–12 coils (typically 11). Microsporidian cells were found in the cytoplasm, next to the endoplasmic reticulum (especially the prespore stages) and generally surrounded by electron-lucent spaces. The infection was polyorganotropic; the fat body appeared as the most heavily invaded tissue, followed by tracheal matrix and epidermis. A molecular phylogeny based on the small (SSU) and large subunit (LSU) ribosomal RNA genes clearly placed the new isolate within the “*Nosema bombycis* clade”. Considering both SSU and LSU concatenated partial sequences, the microsporidium from *R. nu* showed 99.5% nucleotide similarity with *N. bombycis* and 99.8% with its closest relative, a microsporidium isolated from *Philosamia cynthia*. According to its genetic and biological features, the *R. nu* isolate is proposed as the new species *Nosema rachiplusiae* sp. n., expanding the limited knowledge on microsporidia associated to endemic South-American moths.

Keywords *Nosema* · *Rachiplusia nu* · Ultrastructure · Histopathology · Phylogeny

Introduction

Microsporidia are obligate parasites related to, but different from the “true” fungi, presently assigned to superphylum Opisthosporidia (Karpov et al. 2014). They are pathogens for a wide range of animal groups, including arthropods. Several economically important moth species have been recorded as their hosts. Although their use in integrated pest management programs is limited, microsporidia contribute to regulation of insect populations in the field and represent

a frequent problem in mass-rearing of Lepidoptera (Bjørnson and Oi 2014; Arneodo et al. 2016). Infections are often sub-lethal; typical symptoms include delayed larval development, melanization, failure to pupate, smaller pupal size and reduced fecundity in surviving adults. Up to now, most of the microsporidia described from Lepidoptera have been classified within the phylogenetically closely related genera *Vairimorpha* and *Nosema* (Wang et al. 2009; Liu et al. 2012, 2015; Solter et al. 2012). Though less common, other genera, such as *Endoreticulatus*, *Cystosporogenes*, and *Tubulinosema* were also reported (Malysh et al. 2013; Pilarska et al. 2015).

The “sunflower looper” *Rachiplusia nu* (Guenée, 1852) is a polyphagous noctuid pest endemic to southern South America. It causes particular damage to soybean, but also to sunflower, maize, alfalfa, tobacco, and a number of horticultural crops (Specht et al. 2006; Barriiduevo et al. 2012; Rimoldi et al. 2012). Despite its agronomic significance, and possibly due to its restricted geographic distribution, scientific literature concerning the biology and management of *R. nu* is scarce. Current control measures rely mainly on the use of

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broad-spectrum synthetic insecticides and, when available, Bt-transgenic crops (Macrae et al. 2005; Rimoldi et al. 2012; Blanco et al. 2016). Natural enemies (i.e., predators and parasitoids) also play a certain role in reducing *R. nu* populations in the agroecosystem (Araya et al. 1997; Luna and Sánchez 1999; González et al. 2009; Armendano and González 2011; Avalos et al. 2016). Except for baculoviruses (Rodríguez et al. 2012), the occurrence of entomopathogens affecting *R. nu* remains largely unsurveyed.

In 2015, a disease was observed in a laboratory colony of *R. nu* originated from wild specimens captured in Buenos Aires province (Argentina). Larvae exhibited sluggish movement, reduced feeding and growth, high mortality (especially during early instars) and unsuccessful pupation. Preliminary light microscopic examinations of insect cadavers revealed the presence of microsporidian-like spores. Hence, a histological, ultrastructural and molecular study was undertaken in order to accurately determine the identity of this *R. nu*-associated microorganism.

Materials and methods

Light and electron microscopy

Fresh smears were prepared from diseased *R. nu* larvae and observed with an Olympus BX51 optical microscope (Olympus Co., Tokyo, Japan), to assess the size and shape of the spores. These were photographed at $\times 100$ and measured (longest and broadest axis) using image analyzing software Image-Pro Plus (Media Cybernetics Inc., Bethesda, MD, USA). Symptomatic *R. nu* individuals were fixed in 2.5% glutaraldehyde for further scanning (SEM) and transmission (TEM) electron microscopy processing. In the case of SEM examinations, larvae were washed with phosphate buffer and dehydrated in acetone series (50, 80, and 90%). Specimens were directly placed on the plate and their teguments torn with a razor blade to expose the spores contained in the body cavity. Observations were made in a FEI Quanta—250 microscope (FEI Co., Eindhoven, The Netherlands) at 4000– \times 10000. For TEM preparations, portions of two heavily infected larvae (checked by optical microscopy) were postfixed with osmium tetroxide, embedded in epoxy resin and cut with an ultramicrotome. Prior to ultrastructural studies, semi-thin sections of ca. 0.5 μm were colored with toluidine blue to investigate tissue tropism at 40– \times 100 in a Nikon Eclipse 80i microscope equipped with a Nikon DS-U3 digital camera (Nikon Corporation, Tokyo, Japan). Then, ultra-thin sections of ca. 100 nm were stained with uranyl acetate and lead citrate, and examined in a JEOL 1200 EX II microscope (JEOL Ltd., Tokyo, Japan) at 10000– \times 30000 to describe the internal structure of the spores and to visualize other stages of the spore formation process.

DNA extraction, PCR, and sequencing

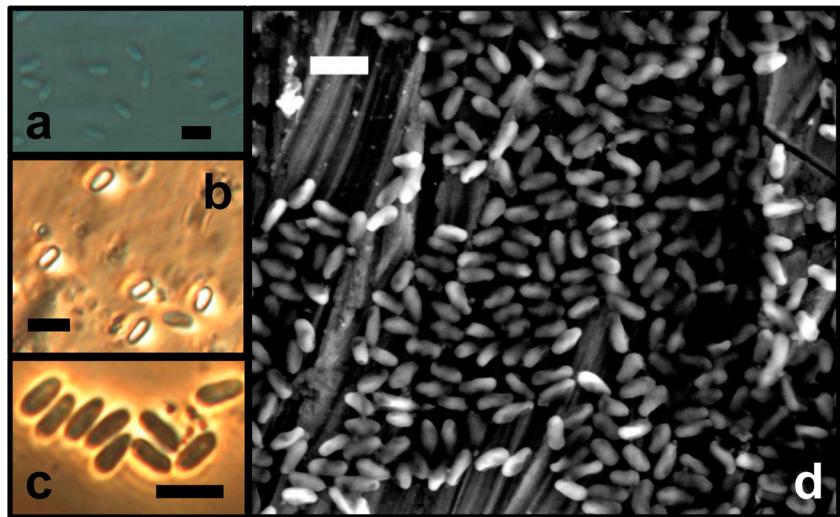
R. nu larvae suspected of microsporidial infection were individually placed in polypropylene microtubes and grounded in CTAB buffer using pestles. Zirconia/silica beads (0.1 mm in diameter) (Biospec Products, Bartlesville, USA) were added to the homogenates, which were vortexed for 20 min in order to break the microsporidian spores. Total DNA isolation continued as described elsewhere (Doyle and Doyle 1990). Partial amplification of the microsporidian large subunit (LSU) ribosomal RNA (rRNA) gene was carried out in an Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany) with primers LSF/ILSUR (Zhu et al. 2010). Part of the small subunit (SSU) rRNA gene was amplified with primers 18F/1492R (Weiss and Vossbrinck 1999). The internal transcribed spacer (ITS) and its flanking regions (3' end of the LSU and 5' end of the SSU) were amplified with primers ILSUF/S33R (Huang et al. 2004). Amplicons obtained from two different *R. nu* specimens were purified (ADN PuriPrep-GP Kit, Inbio, Tandil, Argentina) and directly sequenced (both strands) in an ABI PRISM 3500 XL genetic analyzer (Applied Biosystems, Foster City, CA) at the Instituto de Biotecnología – INTA (Buenos Aires, Argentina). After discarding ambiguous or insufficiently supported bases (less than 2 \times coverage each, with forward and reverse primers) from the 5' and 3' ends, sequences were submitted to GenBank and compared to those previously reported using BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Newly obtained and reference sequences were trimmed to a common length and concatenated (excluding the relatively more variable ITS) to conduct a Maximum-Likelihood phylogenetic analysis with MEGA7 software (Kumar et al. 2016).

Results

Morphological and histopathological traits

Under the optical microscope, fresh smears of symptomatic larvae showed large numbers of microsporidian spores (Fig. 1a–c). These had a uniform oval shape, and measured $3.61 \pm 0.29 \times 1.61 \pm 0.14 \mu\text{m}$ ($n = 60$). In accordance, SEM observations revealed their massive occurrence in the body cavity of diseased *R. nu* larvae (Fig. 1d). The average spore size, affected by the fixation and dehydration processes, was $3.11 \pm 0.25 \times 1.52 \pm 0.11 \mu\text{m}$ ($n = 52$). When examined by TEM, spores exhibited an electron-dense undulating exospore about 30 nm thick and a translucent endospore 100–120 nm thick. This wall was thinner at the anterior part of the spore. Spores were diplokaryotic and contained an isofilar polar filament usually coiled in 11 turns (range = 10–12, $n = 47$) arranged in single rows. The polar filament was $87.6 \pm 6.3 \text{ nm}$ in diameter ($n = 113$), and expanded considerably at its union

Fig. 1 Mature spores of *Nosema rachiplusiae* sp. n. **a** Fresh smears of infected larval tissues viewed by optical microscopy. **b, c** Idem as “a”, but in phase-contrast. **d** Spores in the body cavity of a *R. nu* larva by SEM. Scale bars 5 μ m



with the “mushroom-like” anchoring disc. Next to it, a lamellar polaroplast was visible (Fig. 2). In one sample, some cells appeared to be co-infected with reovirus-like particles (Fig. 2a). Studies are underway to clarify this issue. Early stages of spore formation were also found in the cytoplasm of the infected host cells, adjacent to the mature spores, in close association with the host endoplasmic reticulum. Sporoblasts and spores were increasingly surrounded by electron-lucid, rounded spaces, which often contained dissimilar developmental phases of the parasite, including the “star-like” stage observed in other *Nosema* isolates (Fig. 3). Semi-thin cross sections of infected *R. nu* larvae indicated that the fat body was the main site of microsporidia proliferation. However, the infection was markedly polyorganotropic: microsporidian cells were also abundant in the tracheal matrix and, to a lesser extent, in the epidermis. Comparatively fewer spores were

observed in the midguts. The muscles seemed to be spared from infection, at least in the analyzed sections (Fig. 4).

Genetic characterization

PCR amplifications with microsporidian-specific primers LSF/ILSUR (LSU), ILSUF/S33R (ITS and adjacent regions) and 18F/1492R (SSU) yielded products of the expected sizes (ca. 1.4, 0.5 and 1.4 bp, respectively). Positive results with ILSUF/S33R already suggested that the isolate under study follows the particular arrangement (LSU-ITS-SSU) found in the “true *Nosema* clade”. Partial sequences obtained with primers LSF/ILSUR, ILSUF/S33R and 18F/1492R were deposited at GenBank under accession numbers MG062733 (1261 bp), MG062734 (317 bp) and KY126433 (1108 bp), respectively. There were no differences among the nucleotide

Fig. 2 Ultrastructure of *Nosema rachiplusiae* sp. n. spores. **a** Mature spore showing single rows of isofilar polar filament (white arrowheads). Spore wall formed by an undulating exospore (black arrow) and a translucent endospore (asterisk). Unexpected reovirus-like particles are noticed (black arrowheads). **b** Detail of polar filament coils, in transversal section (white arrowheads). **c** Anterior part of the spore: anchoring disc (black arrow) and lamellar polaroplast (asterisk). Scale bars 200 nm

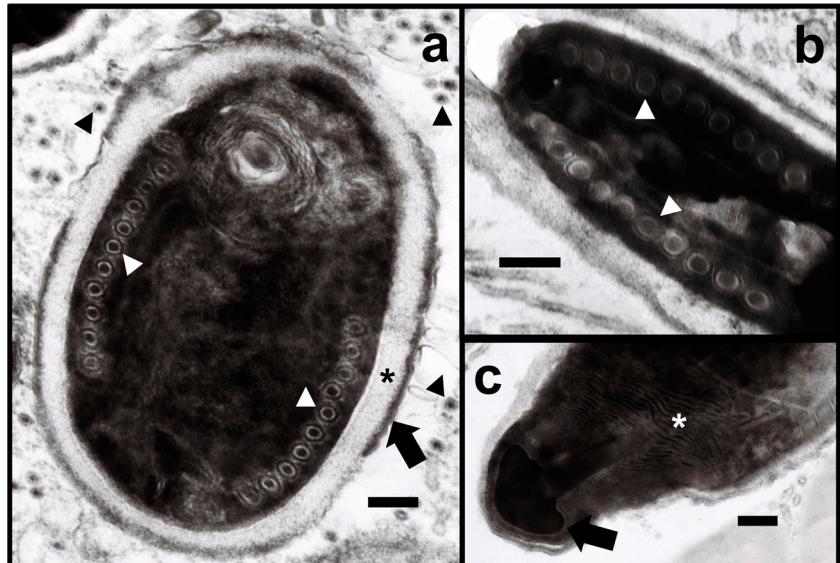


Fig. 3 Prespore stages of *Nosema rachiplusiae* sp. n. **a** Wall thickening (black arrow) of a microsporidian cell next to the host endoplasmic reticulum. **b** Sporoblast surrounded by an electron-lucent space (asterisk) which is, in turn, in close association with the endoplasmic reticulum. **c** Sporoblast at the “star-like” stage (arrow) and spore (arrowhead) sharing the same electron-lucent space (asterisk). **d** Late sporoblast/young spore encircled by a round electron-lucent space (asterisk). Scale bars 500 nm

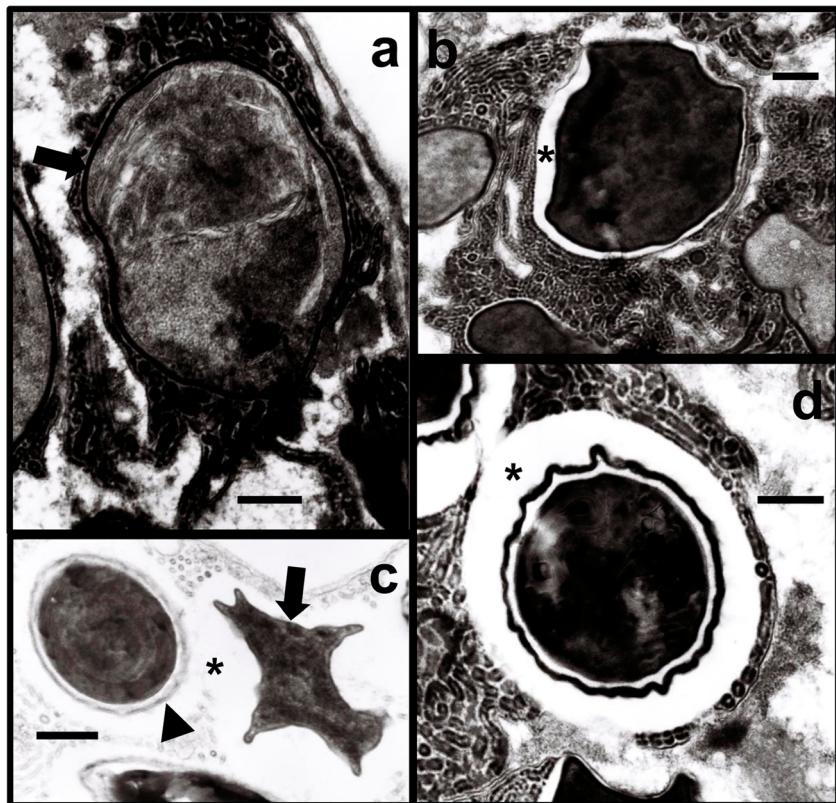
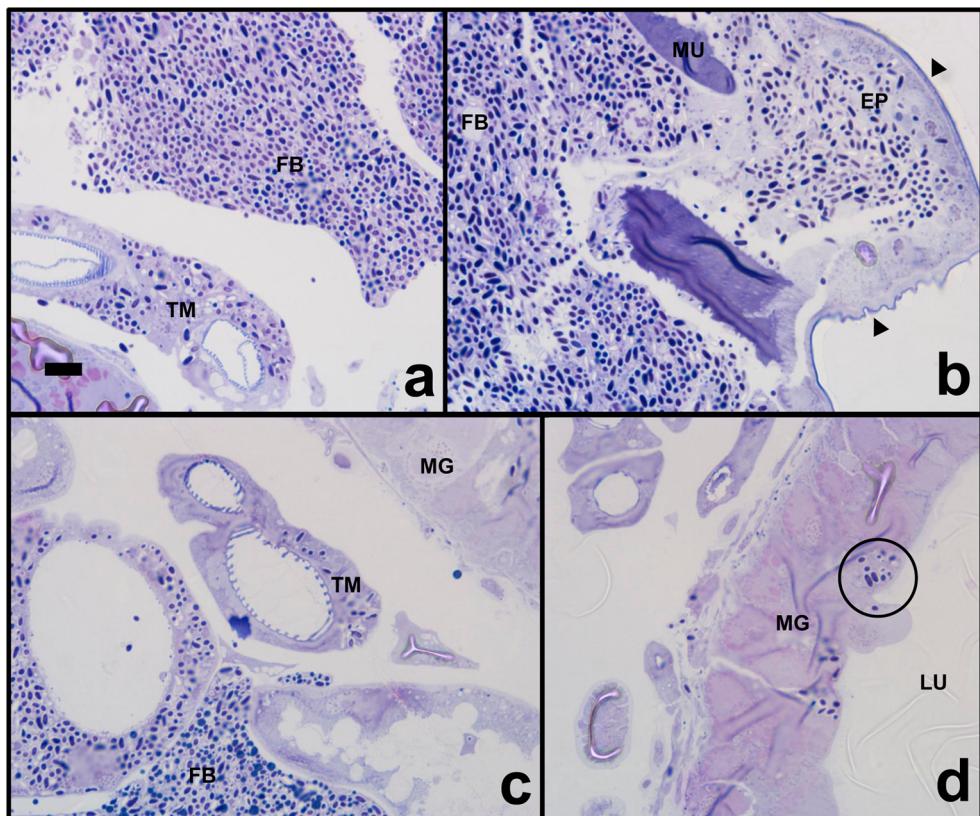


Fig. 4 Larval tissues of *R. nu* infected with *Nosema rachiplusiae* sp. n. **a** Fat bodies (FB) and tracheal matrix (TM). No sign of infection in the muscles (MU). **b** Fat bodies and epidermis (EP). The insect cuticle is marked by arrowheads. **c** Fat bodies, tracheal matrix, and midguts (MG). **d** Spores in the midguts, highlighted by a circle. LU, intestine lumen. Scale bar 10 μm (same magnification for all panels)



sequences determined from the two individually processed *R. nu* larvae. For most of the ITS portion of the ILSUF/S33R-primed product, however, direct sequencing failed to provide clean sequence data, possibly due to intra-sample variability. Phylogenetic analysis of concatenated partial LSU and SSU sequences (2612 informative positions in total) clearly placed the *R. nu*-associated microsporidium within the “true *Nosema* clade”, and more specifically, within the “*Nosema bombycis* clade” (99% bootstrap support). It formed a small subcluster (with a moderate bootstrap support of 57%) with *Nosema* sp. SC, a microsporidium isolated from *Philosamia cynthia* (Zhu et al. 2010) (Fig. 5). Both isolates shared 99.8% rRNA identity. With respect to *N. bombycis* (the type species of the genus), identity was 99.5% (taking together LSU + SSU, in all cases). If only considering SSU, for which more sequences are available at the public databases, the new *Nosema* isolate was identical to those infecting *P. cynthia* (GenBank accession number FJ767862), *Malacosoma americanum* (AY589503) and *Antheraea mylitta* (AB009977); and shared up to 99.8% identity with different isolates of *N. bombycis*. Based on molecular and biological characteristics, the microsporidium isolated from *R. nu* is proposed to be assigned to a novel species within the genus *Nosema*, namely, *N. rachiplusiae* sp. n.

Discussion

Microscopic observations and rRNA analyses have been extensively used to characterize *Nosema* spp. from many lepidopteran species belonging to different families. As the majority of the *Nosema* spp. recorded so far in lepidopteran hosts, *N. rachiplusiae* sp. n. shared high rRNA nucleotide identity with *N. bombycis* (a pathogen of the silkworm, *Bombyx mori*),

the first identified microsporidium (Nägeli 1857). Accordingly, the new species was phylogenetically grouped in the so-called “*N. bombycis* clade”. When the analysis was circumscribed to the SSU rRNA gene, three microsporidian partial sequences showed homology to that of *N. rachiplusiae* sp. n. infecting a noctuid species in Argentina. Two of them were obtained from moths in the Saturniidae family (*P. cynthia* and *A. mylitta*, collected in China and India, respectively), while the other corresponded to a North-American host (*M. americanum*) in the Lasiocampidae. As for other isolates in the “*N. bombycis* clade”, there was no obvious correlation between the phylogeny of the pathogen and the host taxonomy or geographic origin. Slight nucleotide differences between *N. rachiplusiae* sp. n. and other members of the clade were detected in the ITS and LSU sequences. Taking together SSU and LSU, the closest phylogenetic relative of *N. rachiplusiae* sp. n. was still *Nosema* sp. SC from *P. cynthia*.

Little genetic variability of the SSU and LSU rDNA emphasize the need to explore additional genomic regions and/or perform detailed ultrastructural and histopathological examinations to further differentiate closely related *Nosema* isolates. Indeed, their species classification is subject to constant revision (Tokarev et al. 2015; Kyei-Poku and Sokolova 2017). Here, *N. rachiplusiae* sp. n. displayed some distinctive biological attributes with respect to other accepted or proposed species in the “*N. bombycis* clade”. Compared to *Nosema* sp. SC, fresh spores of *N. rachiplusiae* sp. n. were substantially smaller: $3.3\text{--}3.9 \times 1.5\text{--}1.8 \mu\text{m}$ vs. $4\text{--}4.5 \times 2\text{--}2.5 \mu\text{m}$, as deduced from the light micrographs in Zhu et al. (2010). Also, 10–11 polar filament coils were reported in *Nosema* sp. SC (Xie 1989), whereas *N. rachiplusiae* sp. n. exhibited 10–12. All these *N. rachiplusiae* sp. n. features fell within the range informed for *N. bombycis* (reviewed by Kyei-Poku and

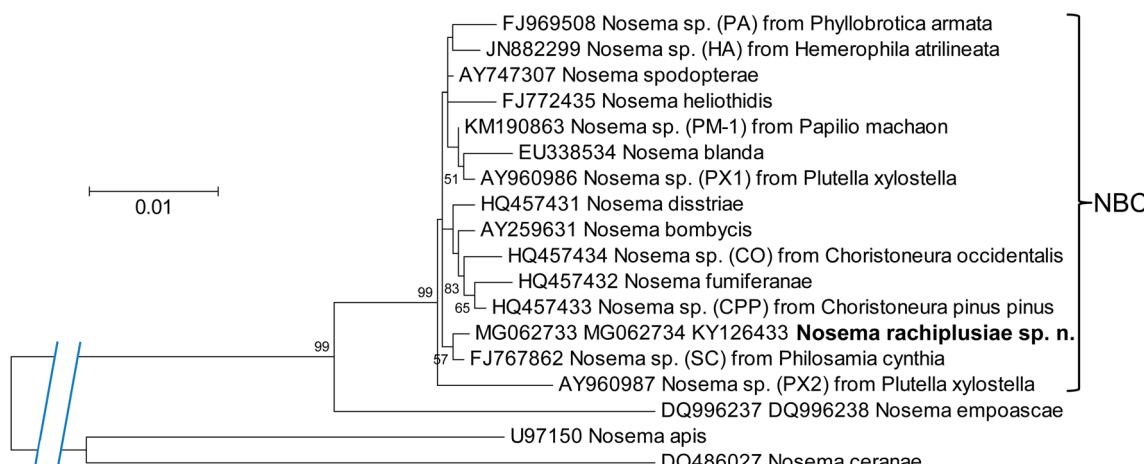


Fig. 5 Maximum-likelihood phylogenetic tree of concatenated LSU and SSU sequences of *Nosema rachiplusiae* sp. n. (in bold) and reference *Nosema* spp./isolates. Bootstrap supports over 50% based on 1000 replicates are indicated at each node. The Hasegawa-Kishino-Yano

model was used (Gamma distributed with invariant sites). Accession number/s given before the sp./isolate names. For incompletely characterized *Nosema* spp., host identities are provided next to the isolate names. NBC: *Nosema bombycis* clade. Scale bar 0.01 substitutions per site

Sokolova 2017). However, differences were detected between *N. bombycis* and *N. rachiplusiae* sp. n. regarding tissue tropism. While the guts have been mentioned as the major site of infection for *N. bombycis*, *N. rachiplusiae* sp. n. spores were mainly observed in the host fat bodies. High spore loads were also recorded in the tracheal matrix and less prominently in the epidermis, but the midguts (the presumed initial site of infection) were scarcely affected. Furthermore, unlike *B. mori* specimens infected with *N. bombycis* and related isolates (Kawarabata 2003), the muscles in the vicinity of other heavily colonized tissues were apparently free of spores. Although the possible occurrence of microsporidia in the muscles at a terminal infection stage (or in unexplored parts of the insect) cannot be excluded, it seems evident that this tissue is not a preferred site for parasite development. Unfortunately, no such histopathological studies are available for *Nosema* sp. SC in its natural host.

Microsporidian infections have been cited in more than 100 lepidopteran species (Kyei-Poku and Sokolova 2017). It is a fact that most *Nosema* isolates are only partially characterized. Incomplete genetic information and poor or missing biological data make it difficult to compare previously and newly reported *Nosema* spp. An unknown number of microsporidians are yet to be discovered, especially those infecting species of little economic significance. Moreover, non-specific microsporidian infections (Kawarabata 2003; Kyei-Poku and Sokolova 2017) may result in variable host-pathogen interactions. This question should be addressed through cross-infection assays between different *Nosema* isolates and insect hosts. Until these knowledge gaps are filled, the taxonomic status of many *Nosema* isolates will remain uncertain.

In conclusion, this paper reports the biological and molecular characterization of *N. rachiplusiae* sp. n., a microsporidium associated to *R. nu*, a moth native to Argentina, Chile, Uruguay, Paraguay, and southern Brazil. The information provided here should be taken into account during mass-rearing and bioecological studies concerning this important agricultural pest.

Taxonomic summary

Name: *Nosema rachiplusiae* sp. n. (Microsporidia: Nosematidae)

Host and locality: *Rachiplusia nu* (Lepidoptera: Noctuidae) from Buenos Aires province, Argentina

Subcellular localization and tissue tropism: Spores and prespore stages are found in the host cell cytoplasm, normally surrounded by electron-lucent spaces. The fat body is the most heavily infected organ. Tracheal matrix also display high spore loads, and epidermis is partially affected. Midguts are only slightly infected.

Spores: Oval, $3.61 \pm 0.29 \times 1.61 \pm 0.14 \mu\text{m}$ (fresh), diplokaryotic, with lamellar polaroplast. Wall formed by an electron-dense undulating exospore ca. 30 nm thick and a translucent endospore 100–120 nm thick. Isofilar polar filament (average diameter ca. 88 nm) arranged in single layers of 10–12 (mode 11) coils.

Etymology: The specific epithet “*rachiplusiae*” refers to the host genus.

Type material: Light and electron microscopy preparations, purified DNA and intact infected larvae (frozen) are preserved at the Instituto de Microbiología y Zoología Agrícola (IMyZA) – INTA, Hurlingham, Buenos Aires province, Argentina. Ribosomal DNA sequences were deposited at GenBank under accession n° MG062733 (partial LSU), MG062734 (partial ITS and adjacent regions) and KY126433 (partial SSU).

Acknowledgments The authors warmly thank Viviana Barrera (IMyZA-INTA), Fernando Delgado (Instituto de Patobiología-INTA) and the personnel of the Laboratorio de Microscopía (CICVyA-INTA) for sharing the microscopy facilities. Also to Débora Moreyra and Roberto Igarza (IMyZA-INTA) for their assistance in *R. nu* mass rearing.

Funding Information This work was funded by INTA and Agencia Nacional de Promoción Científica y Tecnológica (grant PICT-2016-1949), Argentina.

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