Journal of Invertebrate Pathology 126 (2015) 31–42

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Tubulinosema pampeana sp. n. (Microsporidia, Tubulinosematidae), a pathogen of the South American bumble bee *Bombus atratus*

Santiago Plischuk [a,*], Neil D. Sanscrainte [b], James J. Becnel [b], Alden S. Estep [b,c], Carlos E. Lange [a,d]

[a] Centro de Estudios Parasitológicos y de Vectores – CEPAVE (CCT La Plata CONICET – UNLP), La Plata, Buenos Aires, Argentina
[b] Center for Medical, Agricultural and Veterinary Entomology – CMAVE (USDA, ARS), Gainesville, FL, USA
[c] Navy Entomology Center of Excellence, Naval Air Station, Jacksonville, FL, USA
[d] Comisión de Investigaciones Científicas (CIC), provincia de Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 3 September 2014
Revised 13 January 2015
Accepted 15 January 2015
Available online 28 January 2015

Keywords:
Argentina
Bee health
Cyst-like bodies
Nosema
Pampas region
Pollinator

ABSTRACT

An undescribed microsporidium was detected and isolated from the South American bumble bee *Bombus atratus* collected in the Pampas region of Argentina. Infection intensity in workers averaged 8.2×10^7 spores/bee. The main site of infection was adipose tissue where hypertrophy of adipocytes resulted in cyst-like body formation. Mature spores were ovoid and monomorphic. They measured 4.00 μm × 2.37 μm (fresh) or 3.98 μm × 1.88 μm (fixed). All stages were diplokariotic and developed in direct contact with host cytoplasm. Isofilar polar filament was arranged in 16 coils in one or, posteriorly, two layers. Coiling angle was variable, between perpendicular and almost parallel to major spore axis. Late meronts and sporogonial stages were surrounded by vesicles of approximately 60 nm in diameter. Based on both new and already designed primers, a 1827 bp (SSUrRNA, ITS, LSUrRNA) sequence was obtained. Data analyses suggest that this microsporidium is a new species of the genus *Tubulinosema*. The name *Tubulinosema pampeana* sp. n. is proposed.

1. Introduction

Bumble bees of the genus *Bombus*, social insects with three castes (queens, workers, males) and an annual life cycle, have a remarkable importance as pollinators (Goulson, 2010). Ubiquitous *Bombus atratus* is one of eight native species known to occur in Argentina (Abrahamovich et al., 2007). Palaearctic, non-native *B. terrestris* and *B. ruderatus* have recently invaded the country from the West into northern Patagonia after introductions in Chile (Schmid-Hempel et al., 2014). Despite their central role as pollinators, knowledge about the sanitary condition of bumble bees in South America has only begun to emerge in recent years (Plischuk, 2013). During surveys on pathogen diversity in *Bombus* from Argentina, an undescribed microsporidium was detected and isolated from *B. atratus*. Based on morphological and molecular grounds we describe this new microsporidium as *Tubulinosema pampeana* sp. n.

2. Material and methods

2.1. Sampling and processing

Between September 2009 and April 2013, 1959 adult bumble bees belonging to five species [native *B. atratus* ($n = 1381$), *B. bellicosus* ($n = 82$), *B. opifex* ($n = 16$), and exotic *B. terrestris* ($n = 472$) and *B. ruderatus* ($n = 8$)] were individually collected with cylindrical acetate sheet vials (20 cm long, 5 cm diameter) with removable screen ends (Plischuk and Lange, 2009) or with entomological nets while foraging throughout the Argentine provinces of Buenos Aires, Chubut, Córdoba, Formosa, Salta, San Luis, Santa Fe, and Río Negro. Bumble bees were identified following Torretta et al. (2006), Abrahamovich et al. (2007). After identification, insects were either immediately processed following classic dissection techniques (Larsson, 2007) under stereoscopic microscopy (10×, 40×) and tissues and organs were scrutinized for pathogens, or stored (70% ethanol or frozen at −32 °C) for later examination.

2.2. Light microscopy

Samples of different tissues were extracted using fine-point tweezers and observed as fresh smears with saline solution (Poinar and Thomas, 1984) under phase-contrast compound

* Corresponding author at: Centro de Estudios Parasitológicos y de Vectores – CEPAVE, Boulevard 120 e/60 y 64, La Plata 1900, Buenos Aires, Argentina. Tel.: +54 0221 423 2140; fax: +54 0221 423 2327.

http://dx.doi.org/10.1016/j.jip.2015.01.006
0022-2011/

microscopy (400×, 1000×). Samples of infected tissues were used to prepare Giemsa-stained smears following protocols described by Becnel (2012) for microsporidia. Measurements were obtained from both Giemsa and fresh mounted preparations using SPOT Advanced Plus Imaging Software v4.7 (Diagnostic Instruments, Inc.). In order to estimate spore loads *per* individual host, 53 infected *B. atratus* (10 queens, 20 workers, 23 males) were individually homogenized in double distilled water and an Improved Neubauer haemocytometer was utilized for spore counting (Undeen and Vávra, 1997).

2.3. Transmission electronic microscopy (TEM)

Small pieces of infected, frozen tissues were placed into 2.5% glutaraldehyde, allowed to return to room temperature and fixed for 2.5 h, washed in 0.1 M Cacodylate buffer (pH 7.2–7.3) three times, and postfixed in 1% OsO_4 (pH 7.5) at room temperature. After three washes in double distilled water, dehydration was performed by transferring the material through an ascending ethanol series into absolute ethanol. Tissue pieces were embedded in Epon–Araldite following protocols described by Becnel (2012). Ultrathin sections were obtained using diamond knives, mounted on copper grids (200 μm mesh), and stained with uranyl acetate followed by lead citrate. Sections were examined and photographed in a Hitachi H600 electron microscope at an accelerating voltage of 75 kV.

2.4. Molecular studies

Three spore suspensions of approximately 10^5 spores/ml (Klee et al., 2006) from three individual *B. atratus* were purified by filtering and centrifugation. Genomic lysis buffer (G-Biosciences) was added to each suspension, and incubated at 99 °C for 15 min to rupture microsporidia cell walls. Then, *Extraction from Solid Tissue* protocol from the OmniPrep genomic DNA extraction kit (G-Biosciences) was followed. Amplification of the partial DNA sequence of the small subunit ribosomal RNA gene (SSU), complete DNA sequence of the internal transcription spacer region (ITS), and partial DNA sequence of the large subunit ribosomal RNA gene (LSU) was performed using universal primers *18f* (CACCAGGTT

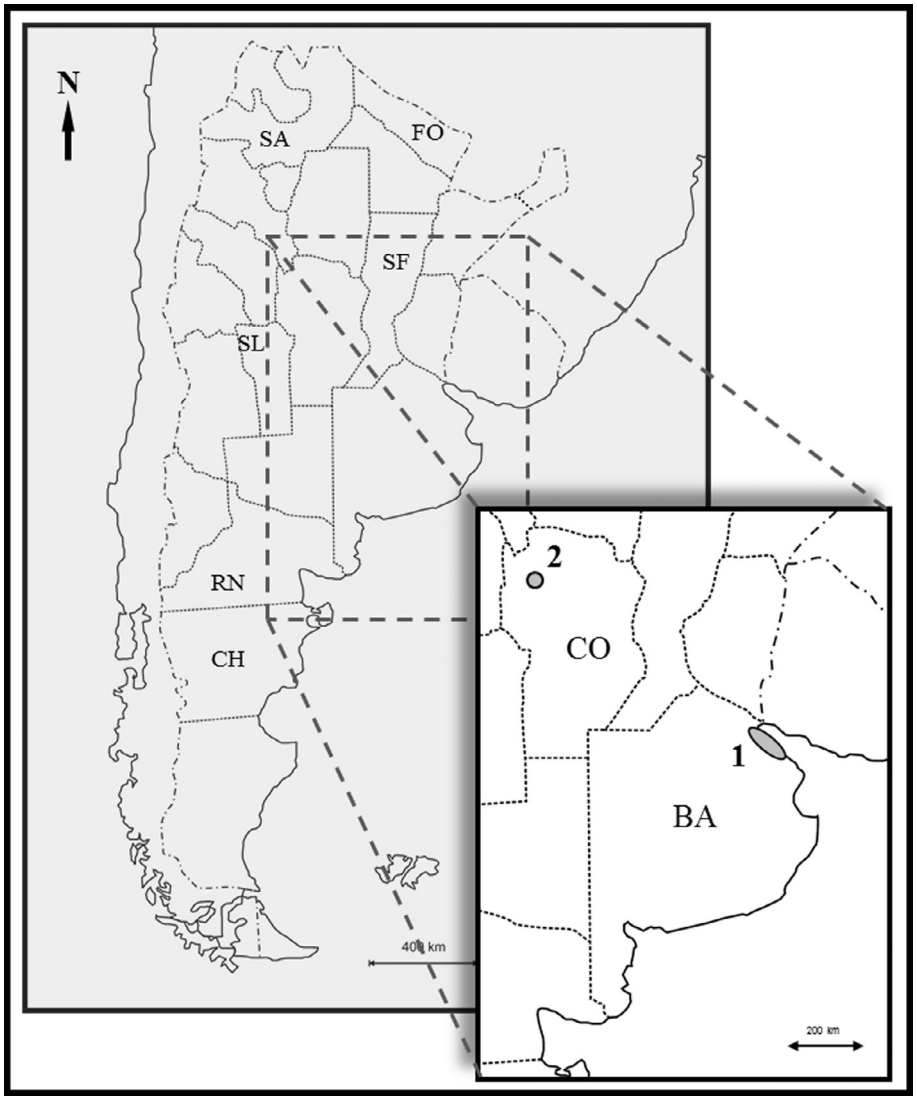

Fig. 1. Map of Argentina: areas in which *Tubulinosema*-infected bumblebees were sampled are highlighted by gray color. 1: Area between La Plata and Buenos Aires cities, northeastern Buenos Aires province (BA); 2: Cruz del Eje, northwestern Córdoba province (CO). Infections were not detected in provinces of Chubut (CH), Formosa (FO), Salta (SA), San Luis (SL), Santa Fe (SF), and Río Negro (RN).

Table 1
Prevalence of *Tubulinosema pampeana* sp. n. in *Bombus atratus* sampled at the northeastern Buenos Aires province area, Argentina. Percentage (%) values obtained by phase contrast microscopy are shown in brackets. Each season lasts from early spring to late fall.

Season	Samples		Queens		Workers		Males	
	Total	Infected	Total	Infected	Total	Infected	Total	Infected
2009–2010	378	45 (11.9)	62	3 (4.8)	218	19 (8.7)	98	23 (23.5)
2010–2011	727	11 (1.5)	33	3 (9.1)	678	6 (0.9)	16	2 (12.5)
2011–2012	73	5 (6.8)	23	5 (21.7)	22	0	28	0
2012–2013	39	4 (10.3)	10	2 (20.0)	22	0	7	2 (28.6)
Total	1217	65 (5.3)	128	13 (10.2)	940	25 (2.7)	149	27 (18.1)

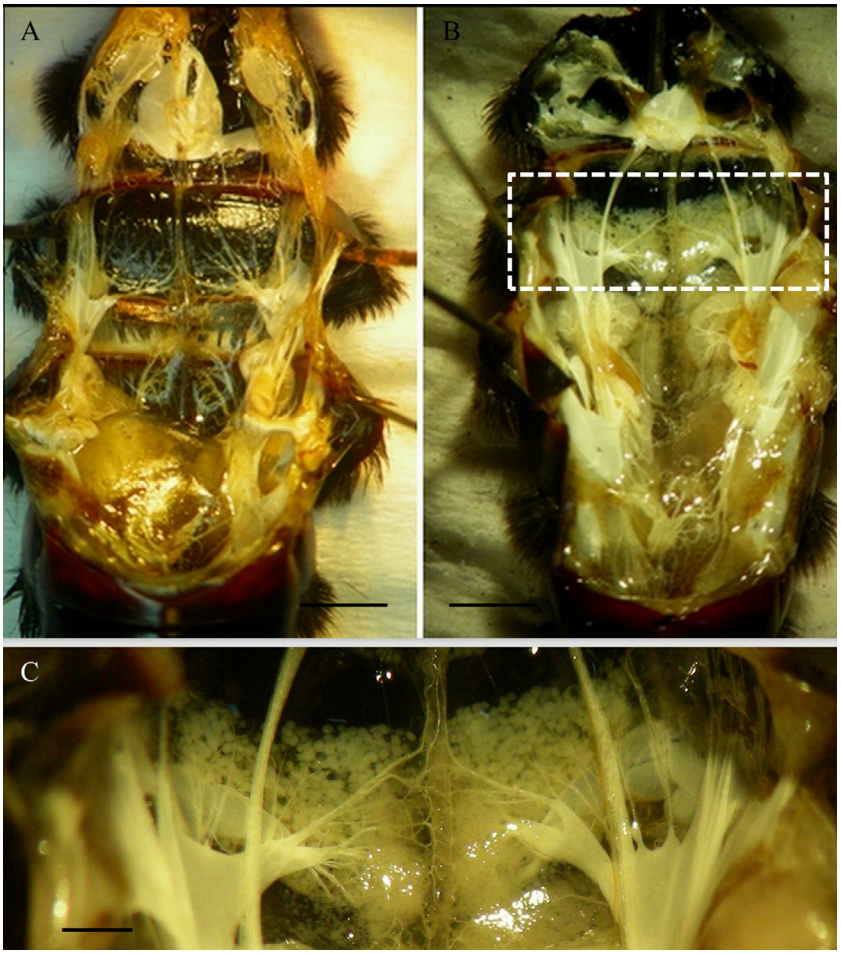

Fig. 2. Ventrally dissected queens of *Bombus atratus* infected with *Tubulinosema pampeana* sp. n. (A) Metasoma of uninfected individual; (B) Metasoma of infected individual; (C) Higher magnification of the marked area in B, showing the cyst-like bodies. [Stereomicroscopy; Bars = 5 mm (A–B) and 1 mm (C)].

GATTCTGCC), *1492r* (GGTTACCTTGTTACGACTT) and *LSU 580r* (GGTCCGTGTTTCAAGACGG) (Vossbrinck et al., 1993) plus the *Tubulinosema* SSU specific primers *782f* (TAAAGAAATTGGCGG AAGGA) and *844r* (TCCTTCCGCCAATTTCTTTA) designed by the authors. PCR was performed at 0.1 mM dNTPs, 0.4 μM forward and reverse primers, 1.5 mM $MgCl_2$, and 0.5 units of Taq polymerase (Invitrogen). Samples were heated at 95 °C for 3 min followed by 35 cycles of 95 °C for 15 s, 50 °C for 1 min, and 72 °C for 90 s followed by a final extension step of 72 °C for 10 min. Amplicons were separated by loading PCR product on ethidium bromide-stained 1% agarose gels and running at 91V. Gel images were observed and photographed using a Gel Logic 212 Pro imaging system. DNA was extracted from gels and purified using a QIAquick Gel Extraction Kit (QIAGEN) and sequenced by Macrogen, USA (Rockville, MD). Partial DNA sequence of the 3′ end SSU RNA gene, complete DNA sequence of ITS, and partial DNA sequence of the 5′ end of LSU RNA gene were assembled and a consensus sequence was submitted to GenBank.

The same methodology was applied to three individual *Hippodamia convergens* lady beetles from a colony known to be infected with *T. hippodamiae* (kindly provided by Dr. Susan Bjørnson, Saint Mary's University, Hailfax, NS) in order to obtain the complete ITS sequence and part of the 5′ end of LSU gene as only a SSU fragment for this species was available on GenBank (JQ082890.1).

For relationship analysis, other microsporidia partial sequences were retrieved from the GenBank database: *T. acridophagus* (JQ247017.1), *T. kingi* (L28966.1 and DQ019419.1), *T. hippodamiae* (JQ082890.1), *T. ratisbonensis* (AY695845.1), *Tubulinosema* sp.

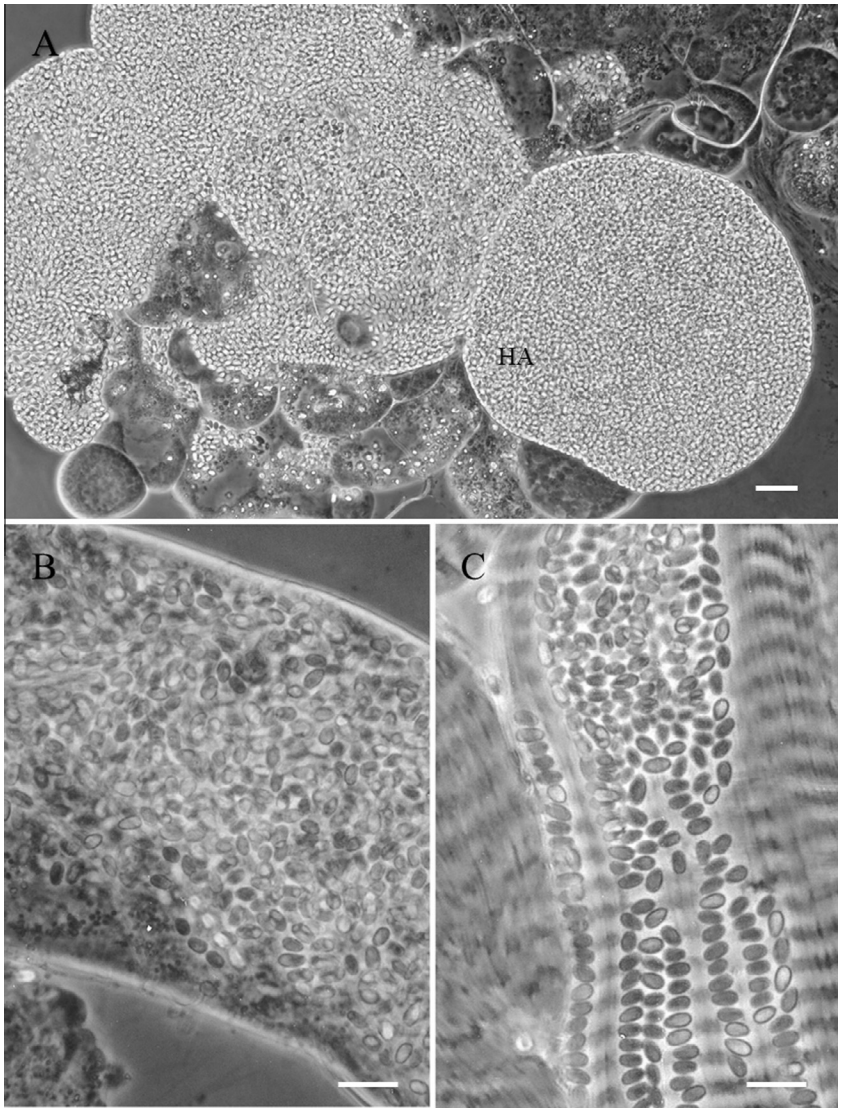

Fig. 3. Infection sites of *Tubulinosema pampeana* sp. n. in *Bombus atratus*. (A) Adipose cells; (B) Malpighian tubule; (C) Muscle. [Phase contrast microscopy; Bars = 20 μm (A) and 10 μm (B–C)]. HA = Hypertrophied adipocyte.

YT-2013 (described later as *T. loxostegi*; Malysh et al., 2013) (JQ906779.1), *Kneallhazia solenopsae* (AY312502.1), *Anncaliia algerae* (AY230191.1), *A. meligethi* (AY894423.1), *Bryonosema plumatellae* (AF84691.1), and *Pseudonosema cristatellae* (AF84694.1). Sequences from *Vairimorpha* sp. (HQ891818.1), *Nosema bombycis* (AY259631.1), and *N. spodopterae* (AY747307.1) were included in the analysis as out-groups. Comparisons of the SSU and combined SSU, ITS, and LSU were run separately. Full length alignments were performed in MAFFTv7.205 (Katoh and Standley, 2013) for the combined SSU, ITS, and LSU regions using default parameters. For the SSU-specific alignments, the initial longer alignment of the three contiguous regions was trimmed to just the SSU region. The *T. acridophagus* sequence was added and realigned using the same program and trimmed after alignment to result in a common 1500 bp alignment. Trees were built using the PhyML3.0 (Guindon et al., 2010) algorithm implemented through the SEAview4.0 (Gouy et al., 2010) interface. Model selection was performed in MEGA6.0 (Tamura et al., 2013) and the GTR + G substitution model was specified in PhyML3.0. The resulting tree was subjected to 100 rounds of bootstrapping to provide branch support values. Trees were displayed and annotated using FigTREEv1.4.0 (Rambaut, 2012). Distance between species was estimated using the Kimura two parameter algorithm instituted in MEGA6.0, for each member of the *Tubulinosema* group as well as more distant microsporidia, *A. algerae*, *B. plumatellae*, and *N. bombycis*. Calculations of percent similarity were performed by loading the alignment into the identity and similarity calculator at www.bioinformatics.org/sms2/ident_sim.html.

3. Results

3.1. Samplings and light microscopy

The microsporidium was found infecting 65 *B. atratus* individuals of all castes along a narrow area of northeastern Buenos Aires province that stretches from La Plata (35°00′35″S; 57°54′46″W) to Buenos Aires city (34°38′00″S; 58°26′40″W) (*ca.* 70 km long), plus one queen from Cruz del Eje, northwestern Córdoba province (30°43′40″S, 64°47′59″W) (Fig. 1). Prevalence per sampling season in the northeastern Buenos Aires area ranged from 1.5% (n = 727) to 11.9% (n = 378), averaging 5.3% (n = 1217).

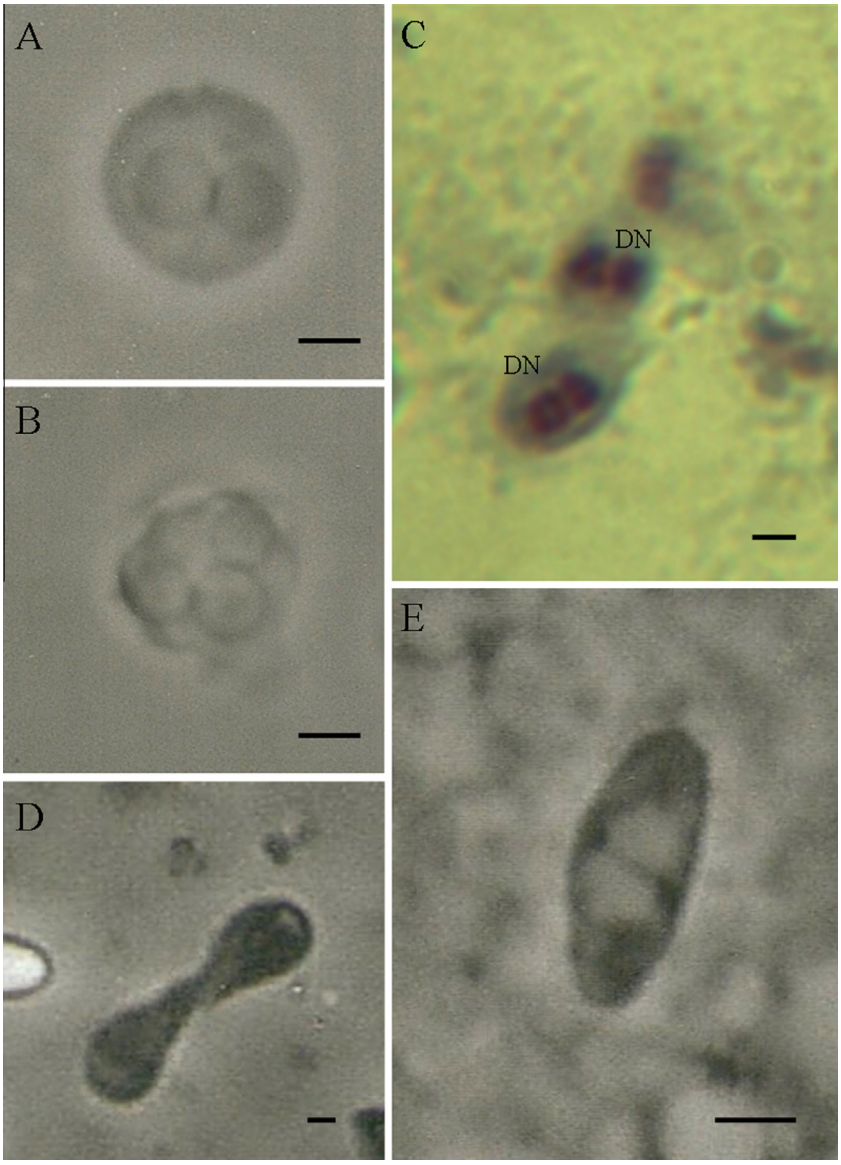

Fig. 4. Development stages of *Tubulinosema pampeana* sp. n. in the adipose tissue of *Bombus atratus*. (A) Binucleate meronts; (B) Tetranucleate meront; (C) Binucleate meront; (D) Disporoblastic division of a sporont; (E) Binucleate sporont [A, B, D, & E: Phase contrast microscopy. C: Giemsa stained, light microscopy; Bars = 1 μm]. DN = Diplokaryotic nuclei.

Males have shown highest percentage values along three of the four collecting seasons (Table 1).

Upon dissection, the microsporidium was always detected in adipose tissue. The great majority of infected bumble bees (91%) showed a characteristic alteration of this tissue mainly consisting of white lesions, general apparent enlargement, and a multiple cyst-like appearance (Fig. 2). At phase-contrast microscope, conspicuous cyst-like bodies were observed, which were usually even more evident in queens. These bodies looked as enlarged, distended fat cells up to four times larger than uninfected ones. Adipocytes showing different levels of infection progression or intensity and apparently healthy adipocytes were commonly seen. In addition to the adipose tissue, infections in Malpighian tubules and muscle cells were relatively common (24% and 15%, respectively) (Fig. 3). Neural tissue, digestive tract, and connective tissue were also infected, but less frequently. Infections were not found in gonads.

Infection intensities and spore sizes are expressed below as mean values ± SE followed by range. Mean spore load was $1.19 \pm 0.48 \times 10^9$ spores/bee in queens (3.09×10^7 – 5.28×10^9; $n = 10$), $8.23 \pm 1.65 \times 10^7$ spores/bee in workers (2.10×10^6 – 2.17×10^8; $n = 20$), and $7.67 \pm 1.69 \times 10^7$ spores/bee in males (4.00×10^6 – 3.40×10^8; $n = 23$).

In both fresh mounted and Giemsa-stained preparations, binucleate and tetranucleate stages presumed to be meronts with nuclei in diplokaryotic arrangement were observed (Fig. 4A–C, E). Disporoblastic division was frequently seen (Fig. 4D). Giemsa-stained spores measured 3.98 ± 0.03 (3.60–4.80) μm × 1.88 ± 0.05 (1.60–3.20) μm ($n = 50$). Observation of fresh-mounted preparations showed that mature spores had an ovoid shape, measuring 4.00 ± 0.05 (3.20–5.20) μm in length × 2.37 ± 0.04 (1.60–3.20) μm in width ($n = 50$). Extruded polar filament length averaged approximately 80 μm ($n = 3$) (Fig. 5).

3.2. Transmission electron microscopy (TEM)

Observation of ultrathin sections showed some sporonts, as well as numerous sporoblasts and mature spores. The few observed meronts were apparently not properly fixed.

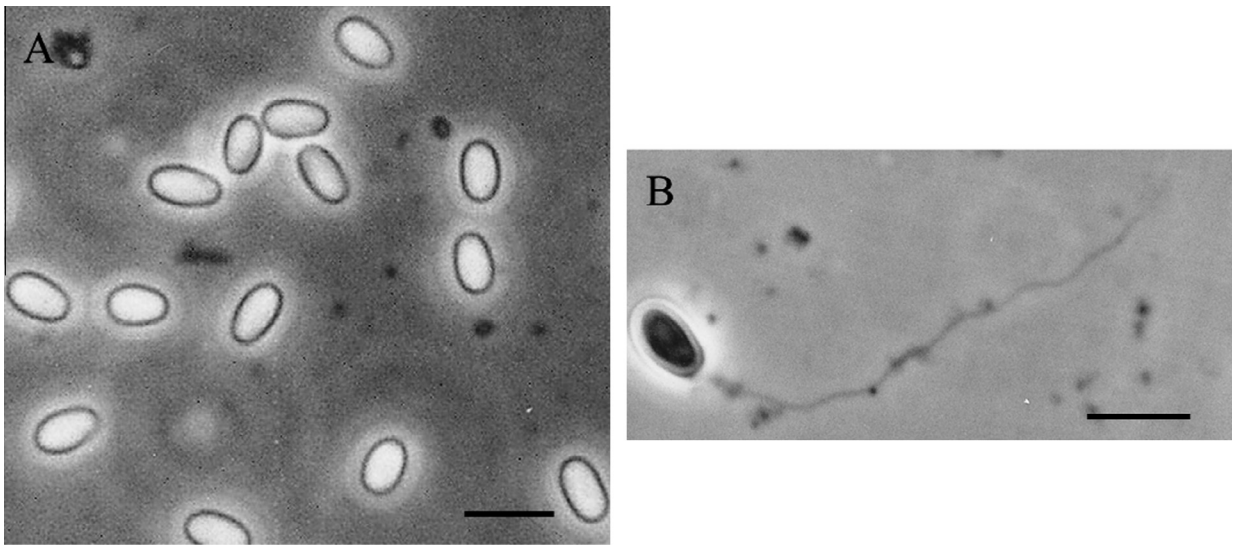

Fig. 5. Spores of *Tubulinosema pampeana* sp. n. in the adipose tissue of *Bombus atratus*. (A) Mature spores; (B) Spore with just extruded polar filament [Phase contrast microscopy; Bars = 5 μm].

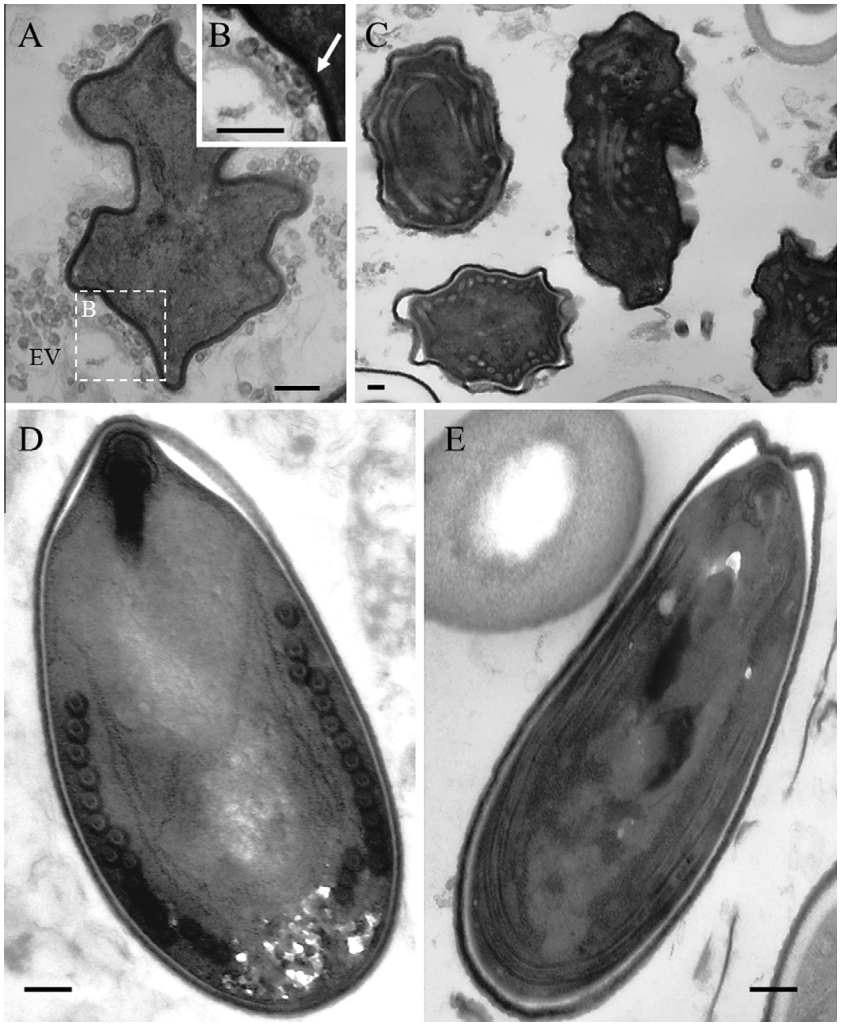

Fig. 6. Sporogony of *Tubulinosema pampeana* sp. n. in the adipose tissue of *Bombus atratus*; (A) Sporont surrounded by vesicles (cross sectioned microtubuli); (B) Detail of tubular elements attached to sporont wall (arrow); (C) Sporoblasts at different stages of wall formation and polar filament development; (D) Immature spore with coiling angle of polar filament coils perpendicular to the longitudinal axis; (E) Immature spore with coiling angle almost parallel to the longitudinal axis; [TEM; Bars = 200 nm]. EV = External vesicles.

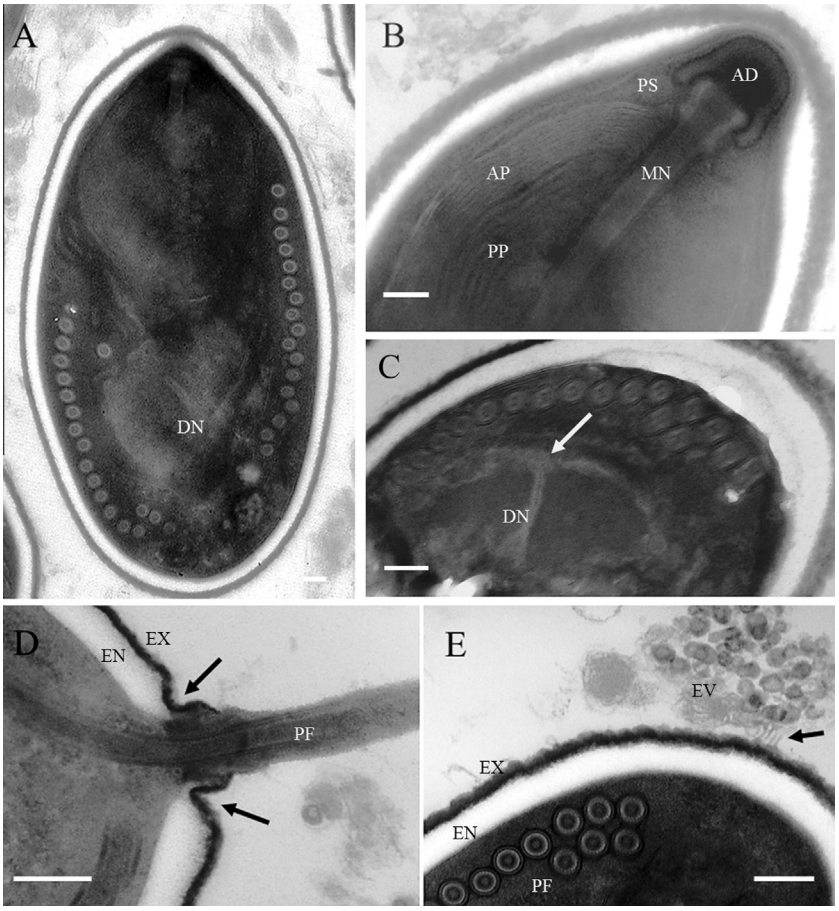

Fig. 7. Spores of *Tubulinosema pampeana* sp. n. in adipose tissue of *Bombus atratus*. (A) Mature spore; (B) Polar sac – anchoring disk complex, and polaroplast; (C) Diplokaryotic arrangement of nuclei (arrow). (D) Collar-like structure at the site of attachment of extruded polar filament (arrows); (E) Tubular structures in close proximity and possible interaction with mature spore wall (arrow). [TEM; Bars = 200 nm]. AD = Anchoring disk; AP = Anterior polaroplast; DN = Diplokaryotic nuclei; EN = Endospore; EV = External vesicles; EX = Exospore; MN = Manubrium; PF = Polar filament; PP = Posterior polaroplast; PS = Polar Sac.

Table 2
Comparison of *Tubulinosema* species and several more distantly related microsporidia based on the small subunit ribosomal RNA gene (SSU) by nucleotide identity [first row], and by Kimura 2 parameter distance analysis [second row]. *A. algerae* = *Anncaliia algerae*; *B. plumatellae* = *Bryonosema plumatellae*; *N. bombycis* = *Nosema bombycis*.

T. pampeana sp. n.	*T. kingi*	*T. ratisbonensis*	*T. acridophagus*	*T. hippodamiae*	*T. loxostegi*	*A. algerae*	*B. plumatellae*	*N. bombycis*
Percent identity	97.36%	98.21%	98.28%	95.85%	98.36%	75.69%	67.46%	55.20%
Kimura two parameter distance	0.0053 ± 0.0022	0.0018 ± 0.0012	0.0009 ± 0.0008	0.0072 ± 0.0026	0.0009 ± 0.0009	0.3229 ± 0.0233	0.4636 ± 0.0353	0.8454 ± 0.0630

Measurements are expressed below as mean ± SE (followed by range if indicated). Sporoblasts, irregular in shape, were normally found surrounded by small vesicles averaging 58.89 ± 1.82 nm (n = 20) in diameter, corresponding to sectioned external microtubuli (Franzen et al., 2005a) (Fig. 6A–C). Spores showed an isofilar polar filament with 14–18 coils (16 on average) with the last 2–3 coils located inwardly (Fig. 7A, E). Mean diameter of the polar filament cross-section was 103.4 ± 1.8 nm (90.9–119.7; n = 15). The coiling angle was variable from typically perpendicular to the longer spore axis to almost parallel to it (Fig. 6D–E). Mature spores measured 3.17 ± 0.08 (2.43–3.80) µm × 1.76 ± 0.05 (1.38–2.14) µm (n = 18). All spores contained diplokaryotic nuclei, a lamellar polaroplast, and a typical anchoring disk – polar sac complex (Vávra and Larsson, 1999) (Fig. 7A–C). Average thickness of the mature spore wall was 151.25 ± 8.47 nm (112.24–209.52; n = 12). The endospore measured 105.38 ± 7.34 nm (81.63–171.43) while the exospore 45.87 ± 3.18 nm (30.61–71.43) on average. A thinning of the former near the anchoring disk was observed. As in fresh preparations, several spores with extruded polar filaments were found on thin sections. Those spores were usually surrounded by a slight wall subsidence forming a collar-like structure (Fig. 7D). In some mature spores the presence of small external vesicles and small tubules apparently associated with the wall were also detected (Fig. 7E).

3.3. Molecular studies

Using 16S SSU rRNA universal microsporidia primers *18f* and *1492r*, an amplicon ~1370 bp was initially obtained. The identity of the novel sequence with *Tubulinosema* spp. orthologs was 95.85% or greater (Table 2, row 1). Pairwise phylogenetic distances between the novel species and other species of the genus *Tubulinosema* varied from 0.0009 (if paired with *T. loxostegi*) to 0.007 (with *T. hippodamiae*). In comparison, phylogenetic distance

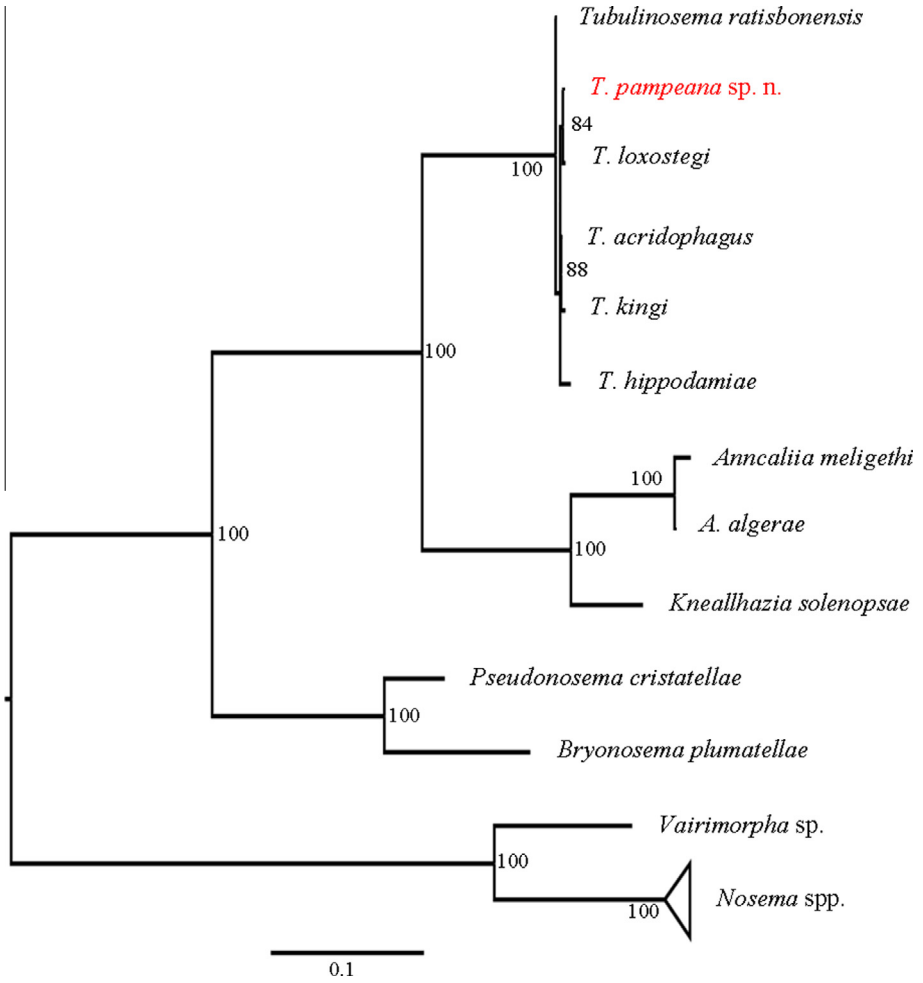

Fig. 8. Maximum likelihood phylogeny of selected ribosomal small subunit sequences, showing separation of *Tubulinosema pampeana* sp. n. from other species of the genus. Accessions of sequences used are listed in the methods section. Tree was constructed using MAFFT for alignment, then exported into MEGA6.0 and trimmed to include only the SSU region of all sequences. Substitution model selection was performed in MEGA6.0. The trimmed alignment was used for tree building with PhyML 3.0 (substitution model GTR + G) as implemented through SEAview4.0. One hundred replications of bootstrapping were performed. FigTREEv1.4.0 was used for tree display and annotation. Three *Nosema* species (shown collapsed) and one *Vairimorpha species* were used as an outgroup.

between the currently described *Tubulinosema* and *Nosema bombi* was as high as 0.8 (Table 2, row 2). Maximum likelihood analysis of the SSU regions agreed with the distance and identity calculations and placed this novel *Tubulinosema* most closely with *T. loxostegi* with high bootstrap values.

The *Tubulinosema* consensus sequence obtained from three different *B. atratus* individuals included partial SSU, complete ITS, and partial LSU sequence that was 1827 bp with a GC content of 41.16% and was deposited to GenBank under accession #KM883008. In the same way we also obtained DNA sequence of the ITS and the 5′-end of the LSU rRNA gene of *T. hippodamiae* (GenBank accession #KM883009). Phylogenetic relationships were obtained as described in the methods (Section 2.4) and the resultant tree demonstrated clustering of our undescribed species and *T. loxostegi* (Fig. 9), which confirms the relationships identified by analysis of the SSU alone.[1] Overall topology of the two trees was similar and relationships to more distant clades were conserved, whether analysis included just the SSU (Fig. 8) or the combined SSU–ITS–LSU region (Fig. 9).

[1] We noted that the available sequence used for *T. acridophagus* was truncated and the lower bootstrap number might be indicative of this abbreviated sequence.

4. Discussion

Up to the present, few microsporidia species have been detected in association with bumble bees. *N. bombi* was described early in the 20th century (Fantham and Porter, 1914) and it is known to have more than 20 natural hosts, all of them native to the northern hemisphere (Cordes et al., 2012; Paxton, 2008). In South America *N. bombi* has been isolated for the first time only recently from non-native *B. terrestris* in Chile (Schmid-Hempel et al., 2014). *Nosema ceranae* was described associated to the Asian honey bee *Apis cerana* (Fries et al., 1996). After its description, *N. ceranae* had been only detected parasitizing other *Apis* species (Botías et al., 2009) until Plischuk et al. (2009) found infections in three native *Bombus* from Argentina (*B. atratus*, *B. bellicosus*, and *B. morio*). Subsequent surveys have identified *N. ceranae* from bumble bees in Uruguay, China, and United Kingdom (Arbulo et al., 2011; Graystock et al., 2013; Li et al., 2012). Accidental infections by species such as *N. thomsoni*, *N. portugal* or *Vairimorpha* sp. commonly associated with lepidopterans have also been reported in bumble bees (Li et al., 2012; Schmid-Hempel et al., 2014). Our data suggest that the microsporidium found in *B. atratus* of Argentina is different from the other species reported in these insects (Plischuk et al., 2012).

The following ultrastructural characteristics of the novel species match the diagnostic features of the genus *Tubulinosema*: oval or

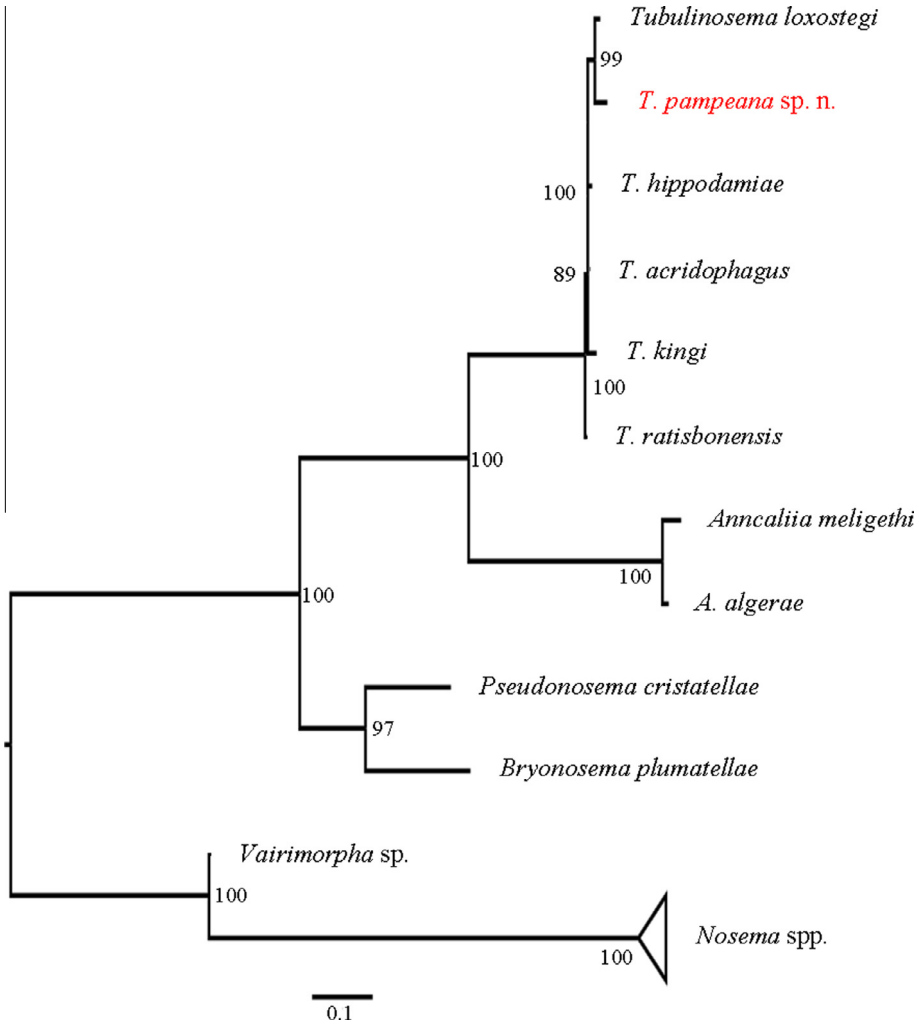

Fig. 9. Maximum likelihood phylogeny of combined ribosomal small subunit sequences, internally transcribed spacer, and large subunit sequences showing separation of *Tubulinosema pampeana* sp. n. from other species of the genus. Accessions of sequences used are listed in the methods section. Tree was constructed using MAFFT for alignment, then exported into MEGA6.0 and used to select the best fit model of nucleotide substitution. The trimmed alignment was used for tree building with PhyML 3.0 (substitution model GTR) as implemented through SEAview4.0. One hundred replications of bootstrapping were performed. FigTREEv1.4.0 was used for tree display and annotation. Three *Nosema* species (shown collapsed) and one *Vairimorpha species* were used as an outgroup.

slightly pyriform spores, thick endospore wall getting thinner over the anchoring disk, and the presence of tubular elements associated with envelopes of sporogonial stages (Franzen et al., 2005a; Issi et al., 2008). This genus was erected by Franzen et al. (2005a) and includes six other species (*T. acridophagus*, *T. hippodamiae*, *T. kingi*, *T. loxostegi*, *T. maroccanus*, and *T. ratisbonensis*) associated with insects in the Orders Coleoptera, Diptera, Lepidoptera, and Orthoptera. Members of family Tubulinosematidae appear to possess opportunistic properties that would allow them to grow and develop in mammal cells. Three species (*Anncaliia vesicularum*, *A. algerae*, *T. acridophagus*) have been detected in humans (Franzen et al., 2005b; Meissner et al., 2012; Solter et al., 2012), whereas a complete lifecycle of *T. ratisbonensis* has also been successfully achieved by Franzen et al. (2005b) in Vero cells.

A salient feature of this new species seems to be the ability to induce the formation of cyst-like bodies in the adipose tissue, the presence of which was confirmed in almost all cases. A few exceptions coincided with low spore loads, suggesting relatively early infections. This kind of pathology was not observed in other cell types, perhaps due to the adipocytes' plasmalemma propensity to distend. Henry (1967) reported "tumorlike growths" for *Tubulinosema* (*Nosema*) *acridophagus* in grasshoppers. However, the typical tissue pigmentation described in these insects was not seen in *B. atratus* and, in addition to adipose, other tissue types are apparently modified by *T. acridophagus*. Is it unlikely that structures observed in grasshoppers and bumble bees are homologous, and information about *T. loxostegi* "spore masses" in the fat bodies of *Loxostege sticticalis* (Malysh et al., 2013) is insufficient to make comparisons. An alternative possibility is that this new species could induce the formation of xenomas, which are defined as a symbiotic complex formed by hypertrophying host cells and multiplying intracellular parasites, and sometimes seen in some microsporidia (Onstad et al., 2006). However, these types of complex are limited by a basement membrane plus a wall (Becnel and Andreadis, 2014), structures that could not be observed in this study.

Another noticeable feature was the high spore loads recorded, particularly in queens but also in workers and males. Curiously, captured bumble bees did not show evident problems for foraging or flying, even those with heavy infections. Hence, high loads but low virulence could allow the parasite greater chances of success in its transmission and dispersal (Gandon et al., 2001).

The main ultrastructural difference between the species found in Argentina and the already described *Tubulinosema* species is

Table 3
Comparison between *Tubulinosema pampeana* sp. n. and the two known microsporidia of *Bombus* spp., *Nosema bombi* and *Nosema ceranae*.

	Nosema bombi	*Nosema ceranae*	*T. pampeana* sp. n.
Distribution	Almost cosmopolitan	Almost cosmopolitan	Pampas region, Argentina
Type host	*Bombus agrorum* (= *B. pascuorum*)	*Apis cerana*	*Bombus atratus*
Other hosts	>20 *Bombus* spp. *A. mellifera* (experimental)	4 *Apis* spp. 14 *Bombus* spp.	Unknown
Main target organ/tissue[a]	Malpighian tubules	Midgut	Adipose tissue
Other infected organs/tissues[a]	Midgut, muscles, adipose tissue, nerve tissue	Adipose tissue	Malpighian tubules, muscles, midgut, nerve tissue
Spore shape	Ovocylindrical to elongated	Oval (often slightly bent)	Ovoid
Mean spore size (fresh) (μm)	2.73–6.24 × 1.69–3.50[b]	4.70 × 2.70	4.00 × 2.37
Mean spore size (Giemsa stained) (μm)	4.53 × 2.79[c]	3.60 × 1.70	3.98 × 1.88
Number of polar filament coils	12–24	20–23	14–18
Polar filament coiling angle	55°–65°	55°–60°	~20°–85°
External microtubular structures	Not observed	Not observed	Yes
Mean intensity in *Bombus* workers (spores/insect)	~5×10^5	~8×10^6	~8×10^7
Gross pathology[a]	Enlarged Malpighian tubules	Whitish and shrunken midgut	Cyst-like bodies in adipose tissue
References	Fantham and Porter (1914) McIvor and Malone (1995) Larsson (2007) Otti and Schmid-Hempel (2007) Sokolova et al. (2010) Li et al. (2012) Schmid-Hempel et al. (2014)	Botías et al. (2009) Chen et al. (2009) Graystock et al. (2013) Li et al. (2012) Plischuk et al. (2009)	This study

[a] Only information about *Bombus* spp. is included.
[b] Highly variable. See Larsson (2007).
[c] According to McIvor and Malone (1995).

perhaps the polar filament. It reaches 16 coils on average, and shows a strongly variable angle of coiling, features not observed previously in any of the other species of the genus. It also showed a thinner spore wall (~151 nm) than *T. hippodamiae* (~320 nm), *T. kingi* (~213–237 nm), and *T. maroccanus* (~200 nm), and thicker than those of *T. loxostegi* (~145 nm) and *T. acridophagus* (~81–121 nm). Data are not available for *T. ratisbonensis* (see Malysh et al., 2013 for a comparison table). Major differences with *N. bombi* and *N. ceranae* are the spore morphology, the high infection intensities, and specific pathology, *i.e.* formation of cyst-like bodies in adipose tissue (Table 3).

SSU-inferred phylogenetic analysis demonstrated close relatedness of all *Tubulinosema* spp. Low pairwise phylogenetic distances between the novel microsporidium and the species of the genus question whether the former represents a new species or it can be attributed to one of the previously described species. We therefore decided to perform analysis of a larger region including the ITS and the partial LSU to differentiate these closely related species (Vossbrinck et al., 2014). When comparison was based on larger sequences with combined SSU–ITS–LSU fragments, the novel microsporidium still grouped closest to *T. loxostegi*, thereby confirming the SSU alone phylogeny with only slightly better branch supports (Figs. 8 and 9).

Although the association of the described species within the genus *Tubulinosema* is well supported by molecular data, there are no submitted DNA sequences of *T. maroccanus*, or a larger sequence of *T. acridophagus* to make a reliable, integrative comparison into the genus. Since previous studies have found that the genetic variability among their species could be very low (Malysh et al., 2013), until SSU-ITS-LSU fragments of all *Tubulinosema* are sequenced and compared, systematic relationships between them should be regarded as tentative. Also considering that *T. maroccanus*, *T. kingi* and *T. acridophagus* were initially described as belonging to genus *Nosema* (Henry, 1967; Kramer, 1964; Krilova and Nurzhanov, 1987), it would be reasonable to expect new future amendments on other already described species, altering even more the little known phylogeny of the family Tubulinosematidae.

In summary, both structural (*e.g.*: shape and size of mature spore, diplokaryotic nuclei, presence of external vesicles during merogony, sporogony, and sometimes surrounding mature spores, number of coils and variable angle of polar filament) and molecular characteristics of this microsporidium would justify the erection of a new species. Authors propose the name of *Tubulinosema pampeana* sp. n., bringing to seven the number of known species for the genus.

5. Taxonomic summary

Tubulinosema pampeana sp. n., Plischuk, Sanscrainte, Becnel, Estep & Lange.

Merogony: Diplokaryotic meronts undergo binary fission.

Sporogony: Diplokaryotic and disporoblastic. Late meronts and sporogonial stages surrounded by vesicles (tubuli) (diameter = 58.89 ± 1.82 nm) in the host cell cytoplasm.

Interface: All stages develop in direct contact with the host cell cytoplasm.

Spores: Ovoid, diplokaryotic, lamellar polaroplast, isofilar polar filament (diameter = 103.4 (90.9–119.7) nm) arranged in 16 (14–18) coils in one or, posteriorly, two layers. Angle variable from perpendicular to almost parallel to major spore axis. Fresh spores sized 4.00 (3.20–5.20) μm × 2.37 (1.60–3.20) μm. Fixed and stained spores (Giemsa) sized 3.98 (3.60–4.80) μm × 1.88 (1.60–3.20) μm. Fixed and stained spores (TEM) sized 3.17 (2.43–3.80) μm × 1.76 (1.38–2.14) μm. Spore wall [151.25 (112.24–209.52) nm] divided in an exospore [45.87 (30.61–71.43) nm] and an endospore [105.38 (81.63–171.43) nm, narrower at anchoring disc level].

Type host: *Bombus atratus* Franklin (Hymenoptera: Apidae).

Type locality: La Plata, Buenos Aires province, Argentina (34°54′39″S; 57°55′37″W).

Detection areas: Northeastern Buenos Aires province, Argentina; Cruz del Eje, Northwestern Córdoba province, Argentina.

Site of infection: Adipose tissue (inducing adipocytes hypertrophy) [Main]. Malpighian tubules, muscle tissue, midgut, nerve tissue, connective tissue [Secondaries].

Transmission: Unknown.

Etymology: Specific epithet "*pampeana*" refers to the Pampas region, where the pathogen was mainly detected.

Deposition of types: Holotype in the International Protozoan Type slide collection, Smithsonian Institution, Washington DC, USA. Paratypes in collections of CEPAVE, La Plata, Argentina, and CMAVE, Gainesville (FL), USA.

Acknowledgments

Authors are grateful to Susan Bjørnson, Cory Stanley-Stahr, Mariano Higes, Leellen Solter and two anonymous reviewers. SP also acknowledges IDEA WILD. This study was supported by USDA – ARS, CICPBA, CONICET (Fin. Res. 4754/12), and FONCyT (PICT 2012-0851 and PICT 2012-0199).

References

Abrahamovich, A.H., Díaz, N.B., Lucía, M., 2007. Identificación de las "abejas sociales" del género *Bombus* (Hymenoptera, Apidae) presentes en la Argentina: clave pictórica, diagnosis, distribución geográfica y asociaciones florales. Rev. Fac. Agron. La Plata 106, 165–176.

Arbulo, N., Salvarrey, S., Santos, E., Antúnez, K., Martín-Hernández, R., Higes, M., Invernizzi, C., 2011. Nosema ceranae detection in bumblebees *Bombus atratus* and *B. bellicosus*, from Uruguay. In: Proceed. 42nd Apimondia Cong., Buenos Aires, Argentina.

Becnel, J.J., 2012. Complementary techniques: preparations of entomopathogens and diseased specimens for more detailed study using microscopy. In: Lacey, L.A. (Ed.), Manual of Techniques in Invertebrate Pathology. Academic Press, New York, pp. 337–353.

Becnel, J.J., Andreadis, T.G., 2014. Microsporidia in insects. In: Weiss, L.M., Becnel, J.J. (Eds.), Microsporidia. Pathogens of Opportunity. Wiley-Blackwell, Ames, Iowa, pp. 521–570.

Botías, C., Martín-Hernández, R., Garrido-Bailón, E., Higes, M., Anderson, D.L., 2009. Nosema ceranae is able to infect different Apis species. In: Proceed. 41st Apimondia Cong., Montpellier, France.

Chen, Y.P., Evans, J.D., Murphy, C., Gutell, R., Zuker, M., Gundensen-Rindal, D., Pettis, J.S., 2009. Morphological, molecular and phylogenetic characterization of *Nosema ceranae*, a Microsporidian parasite isolated for the European honey bee *Apis mellifera*. J. Eukaryot. Microbiol. 56, 142–147. http://dx.doi.org/10.1111/j.1550-7408.2008.00374.x.

Cordes, N., Huang, W.F., Strange, J.P., Cameron, S.A., Griswold, T.L., Lozier, J.D., Solter, L.F., 2012. Interspecific geographic distribution and variation of the pathogens *Nosema bombi* and *Crithidia* species in United States bumble bee populations. J. Invertebr. Pathol. 109, 209–216. http://dx.doi.org/10.1016/j.jip.2011.11.005.

Fantham, H.B., Porter, A., 1914. The morphology, biology and economic importance of *Nosema bombi* n. sp. parasitic in various bumblebees. Ann. Trop. Med. Parasitol. 8, 623–638.

Franzen, C., Fischer, S., Schroeder, J., Schölmerich, J., Schneuwly, S., 2005a. Morphological and molecular investigations of *Tubulinosema ratisbonensis* gen. nov., sp. nov. (Microsporidia: Tubulinosematidae fam. nov.), a parasite infecting a laboratory colony of *Drosophila melanogaster* (Diptera: Drosophilidae). J. Eukaryot. Microbiol. 52 (2), 141–152. http://dx.doi.org/10.1111/j.1550-7408.2005.04-3324.x.

Franzen, C., Fischer, S., Schroeder, J., Bleiss, W., Schneuwly, S., Schölmerich, J., Salzberger, B., 2005b. In vitro cultivation of an insect microsporidian *Tubulinosema ratisbonensis* in mammalian cells. J. Eukaryot. Microbiol. 52 (4), 349–355. http://dx.doi.org/10.1111/j.1550-7408.2005.00043x.

Fries, I., Feng, F., da Silva, A., Slemenda, S.B., Pieniazek, N.J., 1996. *Nosema ceranae* n. sp. (Microspora, Nosematidae), morphological and molecular characterization of a microsporidian parasite of the Asian honey bee *Apis cerana*. Eur. J. Protistol. 32, 356–365. http://dx.doi.org/10.1016/S0932-4739(96)80059-9.

Gandon, S., Jansen, V.A.A., van Baalen, M., 2001. Host life history and the evolution of parasite virulence. Evolution 55 (5), 1056–1062. http://dx.doi.org/10.1111/j.0014-3820.2001.tb00622.x.

Goulson, D., 2010. Bumblebees. Behaviour, Ecology and Conservation, second ed. Oxford University Press, New York.

Gouy, M., Guindon, S., Gascuel, O., 2010. Seaview version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Mol. Biol. Evol. 27, 221–224. http://dx.doi.org/10.1093/molbev/msp259.

Graystock, P., Yates, K., Darvill, B., Goulson, D., Hughes, W.O.H., 2013. Emerging dangers: deadly effects of an emergent parasite in a new pollinator host. J. Invertebr. Pathol. 114, 114–119. http://dx.doi.org/10.1016/j.jip.2013.06.005.

Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W., Gascuel, O., 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst. Biol. 59 (3), 307–321. http://dx.doi.org/10.1093/sysbio/syq010.

Henry, J.E., 1967. *Nosema acridophagus* sp. n., a Microsporidian isolated from grasshoppers. J. Invertebr. Pathol. 9, 331–341. http://dx.doi.org/10.1016/0022-2011(67)90067-5.

Issi, I.V., Tokarev, Y.S., Seliverstova, E.V., Nassonova, E.S., 2008. Specified ultrastructural data on *Tubulinosema maroccanus* Comb. Nov. (*Nosema maroccanus* Krilova et Nurzhanov, 1987) (Microsporidia) from the Moroccan locust *Dociostaurus maroccanus* (Orthoptera). Acta Protozool. 47, 125–133.

Katoh, K., Standley, D.M., 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30, 772–780. http://dx.doi.org/10.1093/molbev/mst010.

Klee, J., Tay, W.T., Paxton, R.J., 2006. Specific and sensitive detection of *Nosema bombi* (Microsporidia: Nosematidae) in bumble bees (*Bombus* spp.; Hymenoptera: Apidae) by PCR of partial rRNA gene sequences. J. Invertebr. Pathol. 91, 98–104. http://dx.doi.org/10.1016/j.jip.2005.10.012.

Kramer, J., 1964. *Nosema kingi* sp. n., a microsporidium from *Drosophila willistoni* Sturtevant and its infectivity for other Muscoides. J. Insect Pathol. 6, 491–499.

Krilova, S.V., Nurzhanov, A.A., 1987. *Nosema* sp. n. (Nosematidae) – microsporidium from Marokkan locust *Dociostaurus maroccanus* Thunb. (Orthoptera). Bull. All-Union Inst. Plant Protection, St. Petersburg 68, pp. 10–14.

Larsson, J.I.R., 2007. Cytological variation and pathogenicity of the bumble bee parasite *Nosema bombi* (Microspora, Nosematidae). J. Invertebr. Pathol. 94, 1–11. http://dx.doi.org/10.1016/j.jip.2006.07.006.

Li, J., Chen, W., Wu, J., Peng, W., An, J., Schmid-Hempel, P., Schmid-Hempel, R., 2012. Diversity of *Nosema* associated with bumblebees (*Bombus* spp.) from China. Int. J. Parasitol. 42, 49–61. http://dx.doi.org/10.1016/j.ijpara.2011.10.005.

Malysh, J.M., Tokarev, Y.S., Sitnicova, N.V., Martemyanov, V.V., Frolov, A.N., Issi, I.V., 2013. *Tubulinosema loxostegi* sp. n. (Microsporidia: Tubulinosematidae) from the Beet Webworm *Loxostege sticticalis* L. (Lepidoptera: Crambidae) in Western Siberia. Acta Protozool. 52, 299–308. http://dx.doi.org/10.4467/16890027AP.13.027.1319.

McIvor, C.A., Malone, L.A., 1995. *Nosema bombi*, a microsporidian pathogen of the bumble bee *Bombus terrestris* (L.). New Zeal. J. Zool. 22, 25–31. http://dx.doi.org/10.1080/03014223.1995.9518020.

Meissner, E.G., Bennett, J.E., Qvarnstrom, Y., da Silva, A., Chu, E.Y., Tsokos, M., Gea-Banacloche, J., 2012. Disseminated microsporidiosis in an immunosuppressed patient. Emerg. Infect. Dis. 18, 1155–1158. http://dx.doi.org/10.3201/eid1807.120047.

Onstad, D.W., Fuxa, J.R., Humber, R.A., Oestergaard, J., Shapiro-Ilan, D.I., Gouli, V.V., Lacey, L.A., 2006. An Abridged Glossary of Terms Used in Invertebrate Pathology. Society for Invertebrate Pathology. <http://www.sipweb.org/glossary>.

Otti, O., Schmid-Hempel, P., 2007. *Nosema bombi*: a pollinator parasite with detrimental fitness effects. J. Invertebr. Pathol. 96, 118–124. http://dx.doi.org/10.1016/j.jip.2007.03.016.

Paxton, R.J., 2008. The distribution and abundance of microsporidia in bumble bees of Europe. In: Biodiversity, Impact and Control of Microsporidia in Bumble Bee (Bombus spp.) Pollinators: Technical Report on Nosema bombi. The "Pollinator Parasites" Project Group. European Union, 5th Framework Programme (Quality of Life and Management of Living Resources), pp. 81–92.

Plischuk, S., 2013. Protistas entomopatógenos asociados a apoideos (Hymenoptera: Apoidea) polinizadores de la región Pampeana. Ecosistemas 22 (1), 89–91. http://dx.doi.org/10.7818/ECOS.2013.22-1.20.

Plischuk, S., Lange, C.E., 2009. Invasive *Bombus terrestris* (Hymenoptera: Apidae) parasitized by a flagellate (Euglenozoa: Kinetoplastea) and a neogregarine (Apicomplexa: Neogregarinorida). J. Invertebr. Pathol. 102, 263–265. http://dx.doi.org/10.1016/j.jip.2009.08.005.

Plischuk, S., Martín-Hernández, R., Prieto, L., Lucía, M., Meana, A., Abrahamovich, A.H., Lange, C.E., Higes, M., 2009. South American native bumblebees (Hymenoptera: Apidae) infected by *Nosema ceranae* (Microsporidia), an emerging pathogen of honey bees (*Apis mellifera*). Environ. Microbiol. Rep. 1, 131–135. http://dx.doi.org/10.1111/j.1758-2229.2009.00018.x.

Plischuk, S., Higes, M., Lange, C.E., 2012. Microsporidia from honey bees and bumble bees in southern South America. In: Proceed. Internat. Cong. Invertebr. Pathol. & Microbial Control, Buenos Aires, Argentina.

Poinar Jr., G.O., Thomas, G.M., 1984. Laboratory Guide to Insect Pathogens and Parasites. Plenum Press, New York.

Rambaut, A., 2012. FigTree v1.4: Tree Figure Drawing Tool. <http://tree.bio.ed.ac.uk/software/figtree/> (accessed 20.06.14).

Schmid-Hempel, R., Eckhart, M., Goulson, D., Heinzmann, D., Lange, C., Plischuk, S., Ruz Escudero, L., Salathé, R., Scriven, J.J., Schmid-Hempel, P., 2014. The invasion of southern South America by imported bumblebees and associated parasites. J. Animal Ecol. 83 (4), 823–837. http://dx.doi.org/10.1111/1365-2656.12185.

Sokolova, Y.Y., Sokolov, I., Carlton, C., 2010. Identification of *Nosema bombi* Fantham and Porter 1914 (Microsporidia) in *Bombus impatiens* and *Bombus sandersoni* from Great Smoky Mountains National Park (USA). J. Invertebr. Pathol. 103, 71–73. http://dx.doi.org/10.1016/j.jip.2009.10.002.

Solter, L., Becnel, J., Oi, D., 2012. Microsporidian entomopathogens. In: Kaya, H.K., Vega, F.E. (Eds.), Insect Pathology. Acad. Press, New York, pp. 221–263.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30 (12), 2725–2729. http://dx.doi.org/10.1093/molbev/mst197.

Torretta, J.P., Medan, D., Abrahamovich, A.H., 2006. First record of the invasive bumblebee *Bombus terrestris* (L.) (Hymenoptera: Apidae) in Argentina. Trans. Am. Entomol. Soc. 132, 285–289.

Undeen, H.H., Vávra, J., 1997. Research methods for entomopathogenic Protozoa. In: Lacey, L. (Ed.), Manual of Techniques in Insect Pathology. Acad. Press, New York, pp. 117–151.

Vávra, J., Larsson, J.I.R., 1999. Structure of the microsporidia. In: Wittner, M., Weiss, L.M. (Eds.), The Microsporidia and Microsporidiosis. American Society for Microbiology, Washington, D.C.

Vossbrinck, C.R., Baker, M.D., Didier, E.S., Debrunner-Vossbrinck, B.A., Shadduck, J.A., 1993. Ribosomal DNA sequences of *Encephalitozoon hellem* and *Encephalitozoon cuniculi*: species identification and phylogenetic construction. J. Euk. Microbiol. 40 (3), 354–362. http://dx.doi.org/10.1111/j.1550-7408.1993.tb04928.x.

Vossbrinck, C.R., Debrunner-Vossbrinck, B.A., Weiss, L.M., 2014. Phylogeny of the microsporidia. In: Weiss, L.M., Becnel, J.J. (Eds.), Microsporidia: Pathogens of Opportunity. Wiley-Blackwell, Hoboken, New Jersey, pp. 203–220.