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

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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

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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

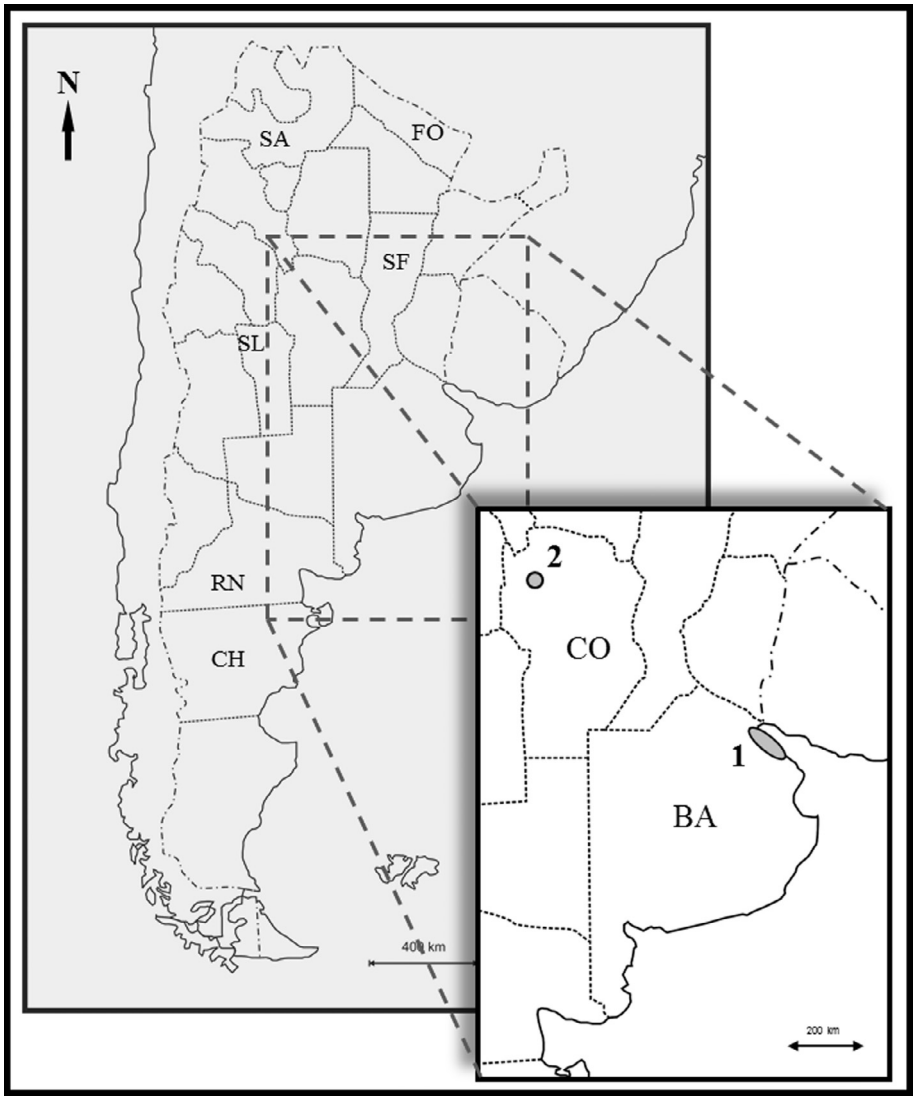

Fig. 1. Map of Argentina: areas in which *Tubulinosema*-infected bumblebees were sampled are highlighted by gray color. 1: Area between La Plata and Buenos Aires cities, northeastern Buenos Aires province (BA); 2: Cruz del Eje, northwestern Córdoba province (CO). Infections were not detected in provinces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