Long-term prevalence of the protists *Crithidia bombi* and *Apicystis bombi* and detection of the microsporidium *Nosema bombi* in invasive bumble bees

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Running title: Pathogens in invasive bumble bees

Summary

An initial survey in 2009 carried out at a site in northwestern Patagonia region, Argentina, revealed for the first time in South America the presence of the flagellate *Crithidia bombi* and the neogregarine *Apicystis bombi*, two pathogens associated with the Palaearctic invasive bumble bee *Bombus terrestris*. In order to determine the long-term persistence and dynamics of this microparasite complex, four additional collections at the same site (San Carlos de Bariloche) were conducted along the following seven years. Both protists were detected in all collections: prevalence was 2% - 21.6% for *C. bombi* and 1.2% - 14% for *A. bombi*. In addition, the microsporidium *Nosema bombi* was recorded for the first time in the country in the last two collections, at prevalences of 12.4% and 2.4% and unusually high infection intensities (Average = 6.56×10^7 spores *per* individual). Due to the exceptional dispersal ability of the exotic *B. terrestris*, these three multihost pathogens should be considered as potential threats to South American native bumble bees.

Introduction

 An initial study in 2009 on the entomopathogenic protists associated with the Palaearctic invasive bumble bee *Bombus terrestris* (Hymenoptera: Apidae) in Argentina had revealed the presence of two species, the enteric flagellate *Crithidia bombi* (Euglenozoa: Kinetoplastea) and the neogregarine of the fat body *Apicystis bombi* (Apicomplexa: Conoidasida), both never reported to occur in the eight native bumble bee species known for the country (Plischuk and Lange, 2009). Diagnosis of the pathogens at that time was based on morphological characters, host-pathogen relationships, and host species involved, but later work by molecular means confirmed the identity of both protists (Plischuk *et al.*, 2011; Schmid-Hempel *et al.*, 2014). In order to further the knowledge on the occurrence and diversity of pathogens associated to *B. terrestris* in Argentina, we performed four additional collections at the same site where the first findings were registered. This communication presents the obtained data and reports on the first record of the microsporidium *Nosema bombi* in Argentina.

Figure 1 placement

Fig. 1. Sampling site of *Bombus terrestris* in Argentina (♦). Black circle indicates the main site of release of *B. terrestris* (Quillota, Chile). Empty circle indicates site of release of *Bombus ruderatus* (Temuco, Chile). Shaded area shows the known distribution of *B. terrestris* in Argentina.

Sampling of adult specimens of *B. terrestris* was performed at a site located 8 km West of San Carlos de Bariloche, Río Negro province (41°07'33"S; 71°23'55"W). With the exception of the first collection (Season 1) which was conducted from October 2009 to April 2010 (n = 107), all other three were carried out in January as season 2 (2011; n = 269), season 3 (2015; n = 162), and season 4 (2016; n = 166). Bumble bees

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were captured one by one with cylindrical acetate sheet vials (20 cm long, 5 cm diameter) with removable ends while foraging on flowers of autochthonous *Alstroemeria aurea* Graham (Alstroemeriaceae) and exotics *Lavandula angustifolia* Mill. (Lamiaceae) and *Hypericum calycinum* L. (Hypericaceae). After collection, bumble bees were frozen (-32° C) until their identification according to Fernández and Sharkey (2006) and Abrahamovich *et al*. (2007), and later processing.

Figure 2 placement

Fig. 2. *Nosema bombi* in *Bombus terrestris* from San Carlos de Bariloche, Argentina. A: Spores. B: Parts of Malpighian tubules of *B. terrestris* heavily filled with spores packed together tightly (upper) and more loosely (lower). C: Immature oocyst of the neogregarine *Apicystis bombi* among spores of *N. bombi* from the only mixed infection found. [Bars: A-C: 5 µm; B: 25µm; Phase-Contrast Microscopy].

Processing was performed under a Nikon SMZ745T stereoscopic microscope (x10, x40), dissecting each bumble bee and extracting small pieces of different tissues and organs in order to prepare fresh smears with one-quarter-strength Ringer's solution (Poinar and Thomas, 1984). Detection of pathogens was done using phase-contrast microscopy (x400, x1000) (Lange and Lord, 2012; Solter *et al*., 2012). After detections, each infected individual was individually homogenized in 2 ml of double distilled water and infection intensity was quantified using an Improved Neubauer hemocytometer as described by Undeen and Vávra (1997).

To perform molecular analyses of microsporidia, 200 µl of the suspensions prepared for spore quantification were used for DNA extraction with the PureLink® Genomic DNA Mini Kit (Invitrogen), according to manufacturer´s instructions. DNA samples were analyzed by multiplex PCR for simultaneous detection of *Nosema apis*

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and *Nosema ceranae* using primers 321APIS and 218MITOC, as described by Martín-Hernández *et al*. (2007). Samples were also subjected to PCR to detect the presence of *Nosema bombi*, using primers BOMBICAR according to Plischuk *et al*. (2009). Amplified DNA was electrophoresed on a 0.8% agarose gel, stained with GelRed (Biotium) and visualized using a UV-Transiluminator. Positive samples were purified and sequenced at Macrogen Inc. (Seoul, South Korea). Obtained sequences were compared with data available in the GenBank (Altschul *et al*., 1997).

Results and Discussion

Two deliberate introductions of bumble bees for pollination services were carried out in Chile late in the twentieth century. The first one was in 1982-83 when close to 400 queens of *Bombus ruderatus* were imported from New Zealand and released East of Temuco (Arretz and MacFarlane, 1986; Schmid-Hempel *et al*., 2014) (Fig. 1). The second introduction, although exact information is not available, seems to have occurred in 1997-98 when an unknown number of individuals of *B. terrestris* were brought from Israel and possibly Belgium in several localities in central Chile, particularly Quillota (Schmid-Hempel *et al*., 2014) (Fig. 1). In 1996 and 2006 both *B. ruderatus* and *B. terrestris* were respectively detected in Argentine Patagonia on the eastern slope of the Andes (Roig-Alsina and Aizen, 1996; Torretta *et al*., 2006). It has been suggested that one or more invasion episodes have occurred (and likely still occur) across the lowest mountain passes during periods of mild weather (Torretta *et al*., 2006; Plischuk and Lange, 2009). Populations of *B. terrestris* have established not only along western Patagonia region but also reached the Atlantic coast (Fig. 1), showing ubiquity

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and a remarkable dispersion capacity (Schmid-Hempel *et al*., 2014; Geslin and Morales, 2015).

As shown in Figure 3, after several years from our first discoveries results from the four additional collections have demonstrated not only a clear presence of *C. bombi* and *A. bombi* in the invading host as judged by their long-term persistence over several seasons but also the occurrence of an additional pathogen not previously detected. Heavy spore loads [Average = 6.6 x 10^7 ± 0.8 spores *per* individual; Range = 1.3 x 10^7 - 1.5 x 10^8; n = 18] of a microsporidium were found primarily in the Malpighian tubules of some bumble bees although sometimes other organs and tissues were also invaded (gonads, nervous tissue, digestive tract). Infections started to show up in January 2015. Following molecular techniques as described in the Experimental Procedures section, the microsporidium was identified as *Nosema bombi* and its sequence deposited in GenBank under accession number KX279355. A mixed infection with *A. bombi* was observed in one worker.

In the initial survey of 2008-09 (hereafter "Season 0") prevalence was 21.6% for *C. bombi* and 3.6% for *A. bombi* (n = 111) (Plischuk and Lange, 2009). During the four seasons that were surveyed afterwards these two pathogens ranged from 2% to 21.6% and 1.2% to 14%, respectively. *Crithidia bombi* showed values lower than 6% during Seasons 1 (5.6%), 2 (2%) and 3 (3.1%) [no statistical differences between them], but in the last survey (Season 4) prevalence was 20.5%, almost what was observed in season 0 (see Odds ratios and *p*-values in Supp. Info). Prevalence of *A. bombi* increased during seasons 1 (12.1%) and 2 (14%), and then decreased in seasons 3 (11.1%) and 4 (1.2%). Except for the last season, prevalence differences were statistically low between them (see Odds ratios and *p*-values in Supp. Info). Although our sampling does not allowed for an epizootiologic analysis (no data are available for 2012-2014, and there is

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disparity in sampling periods for seasons 0 and 1 relative to seasons 2-4) the overall

trend of occurrence for both pathogens concurs in terms of prevalence with studies

reported elsewhere in the world for the same host (Çancaya and Kaftanoglu, 2006;

Allen *et al.*, 2007; Erler *et al.*, 2012; Goulson *et al.*, 2012; Schmid-Hempel *et al.*, 2014)

and demonstrates long-term persistence.

Figure 3 placement

Fig. 3. Seasonal prevalence of *Apicystis bombi*, *Crithidia bombi,* and *Nosema bombi* in *Bombus terrestris* from San Carlos de Bariloche, Argentina, in 2008-09 [* reported by Plischuk and Lange (2009)], 2009-10, 2011, 2015, and 2016.

In order to evaluate statistical differences on prevalence for each pathogen all pairwise comparisons were made between seasons. Differences were detected using bilateral Fisher's exact test adjusted by Bonferroni method to obtain an overall significance of 5% (R Core Team, 2015). Same letters indicate no-significant statistical differences between seasons.

Infection by *N. bombi* produces deleterious effects at both individual and colony

level, and virulence apparently vary depending on the species of *Bombus* affected

(Solter, 2014). This microsporidium has a wide host range (at least 47 *Bombus* spp.) and

Holarctic distribution (Solter, 2014), although it has also been isolated from non-native

B. terrestris in New Zealand (Hopkins, 1914; McIvor and Malone, 1995). Until the

findings in Argentine Patagonia here reported, detections of *N. bombi* in South America

were limited to few infected *B. terrestris* individuals collected in Chile during 2010/11

(Schmid-Hempel *et al.*, 2014). In Argentina, after examination of *ca*. 3,100 native

bumble bees collected by our group since 2006 (seven species at more than 70

localities; Plischuk *et al*., 2009, 2011, 2013, 2015; Plischuk and Lange, 2016, and unpublished data), two other microsporidia were detected [*Nosema ceranae* (Plischuk *et al*., 2009); *Tubulinosema pampeana* (Plischuk *et al*., 2015)], but *N. bombi* was never found. This microsporidium could have entered to Chile within *B. terrestris*, *B. ruderatus*, or both, and infected specimens have afterwards crossed the Andes in recent years to Argentina. The ubiquity of the invasive *B. terrestris* (Schmid-Hempel *et al*., 2014; Geslin and Morales, 2015), the ability of *N. bombi* to infect numerous *Bombus* species [*i.e*., multihost pathogen (Paxton, 2006; Solter, 2014)], and the strong propagule pressure given by the high spore loads (Rutrecht and Brown, 2008; Chu and Cameron, 2016) lead to presume that *N. bombi* would continue dispersing even further along with its host. A similar scenario was reported previously (Plischuk and Lange, 2009) when both *C. bombi* and *A. bombi* were detected for the first time in South America also in *B. terrestris*, with negative results in 441 other native individuals. Some years later, other studies revealed their presence in South American native species [*A. bombi* in *Bombus dahlbomii* (Arbetman *et al*., 2013); *A. bombi* and *C. bombi* in *Bombus atratus* (Gamboa *et al*., 2015)]. *Nosema bombi* should be therefore considered, jointly with both *C. bombi* and *A. bombi*, as another threat to South American native bumble bees. In this sense, according to Cameron *et al*. (2016) there might be a link (although still rather elusive) between declining bumble bee species in North America and *Nosema bombi* infection rates. It should be important to monitor the possible spread of *N. bombi* into wild *Bombus* in South America, to see if a similar pattern to the one in North America will be observed.

Acknowledgments

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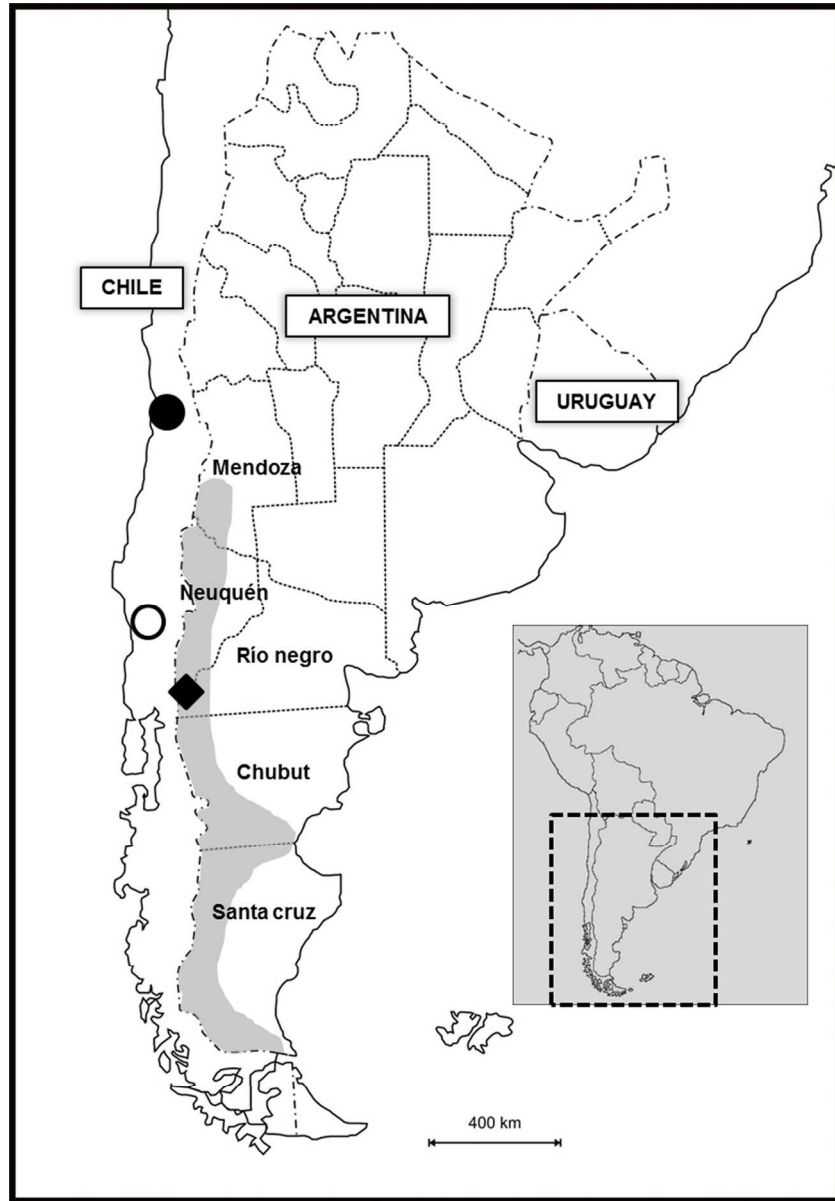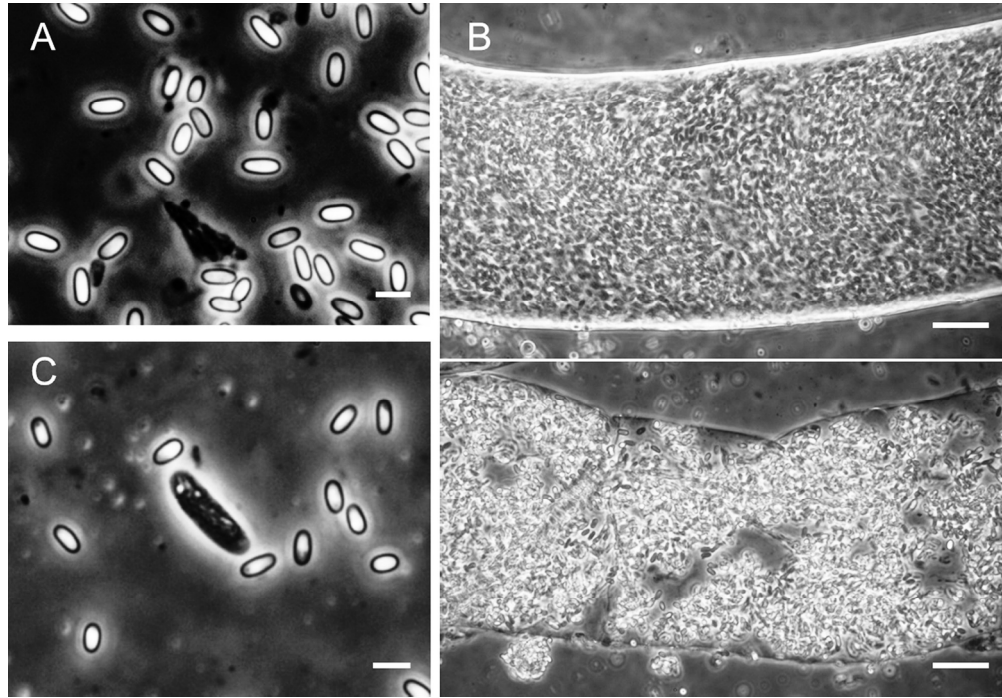
Sampling site of Bombus terrestris in Argentina (♦). Black circle indicates the main site of release of B. terrestris (Quillota, Chile). Empty circle indicates site of release of Bombus ruderatus (Temuco, Chile). Shaded area shows the known distribution of B. terrestris in Argentina
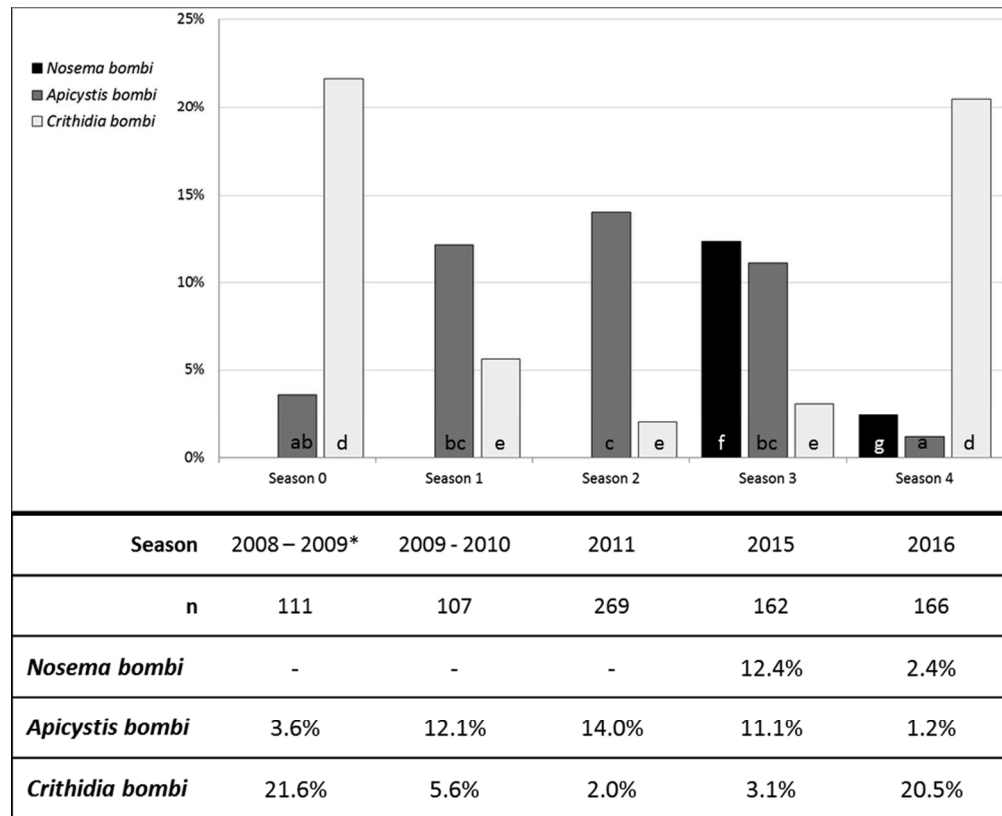Figure 1 placement
109x158mm (300 x 300 DPI)

Nosema bombi in Bombus terrestris from San Carlos de Bariloche, Argentina. A: Spores. B: Parts of Malpighian tubules of B. terrestris heavily filled with spores packed together tightly (upper) and more loosely (lower). C: Immature oocyst of the neogregarine Apicystis bombi among spores of N. bombi from the only mixed infection found. [Bars: A-C: 5 μm; B: 25μm; Phase-Contrast Microscopy]
Figure 2 placement
168x117mm (300 x 300 DPI)

Season	2008 – 2009*	2009 - 2010	2011	2015	2016
n	111	107	269	162	166
Nosema bombi	-	-	-	12.4%	2.4%
Apicystis bombi	3.6%	12.1%	14.0%	11.1%	1.2%
Crithidia bombi	21.6%	5.6%	2.0%	3.1%	20.5%

Seasonal prevalence of Apicystis bombi, Crithidia bombi, and Nosema bombi in Bombus terrestris from San Carlos de Bariloche, Argentina, on 2008-09 [* reported by Plischuk and Lange (2009)], 2009-10, 2011, 2015, and 2016
Figure 3 placement
109x89mm (300 x 300 DPI)