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Ascosphaera apis, the entomopathogenic fungus affecting larvae of native bees (*Xylocopa augusti*): First report in South America



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ABSTRACT

Background: Nowadays several invertebrate pollinators of crops and wild plants are in decline as result of multiple and, sometimes, unknown factors; among them, the modern agricultural practices, pests and diseases are postulated as the most important factors. Bees of the genus *Xylocopa* are considered effective pollinators of passion fruit crops in tropical regions, as well as important pollinators in wild plants, but these bees are attacked by several pathogens that affect different stages in their life cycle. The fungal species of the genus *Ascosphaera* are commonly associated with social and solitary bee larvae causing chalkbrood disease.

Aims: The aim of the present study was to demonstrate the presence of *Ascosphaera apis* affecting larvae of *Xylocopa augusti* in South America.

Methods: For this purpose, *A. apis* was isolated from affected larvae in YGPSA medium. Final identification was run out by three techniques: (1) Microscopic examination of the hyphae and sizes of the fruiting bodies; (2) Mating test, and specific sexual compatibility test, and (3) PCR detection, using specific primers.

Results: This study demonstrates for the first time the presence of *A. apis* affecting larvae of *X. augusti* in South America.

Conclusions: The evidence of *A. apis* affecting the larvae of *X. augusti*, and the fact that the sharing of pathogens between different bee species has been underestimated, suggests the need for further epidemiological studies in order to determine not only the prevalence of this pathogen among wild pollinators, but also its relationship to the sudden collapse of honey bee colonies in this region.

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Infeción por *Ascosphaera apis* en larvas de abejas carpinteras (*Xylocopa augusti*): primer reporte en América del Sur

RESUMEN

Antecedentes: En la actualidad, las poblaciones de una gran cantidad de insectos polinizadores están disminuyendo debido a múltiples factores, no siempre conocidos, como las prácticas agrícolas modernas, las plagas y las nuevas enfermedades. Las abejas del género *Xylocopa* se consideran unos importantes polinizadores de los cultivos de maracuyá en el trópico, así como de gran cantidad de plantas silvestres. Estos polinizadores silvestres se ven afectados por diversos agentes patógenos en diferentes etapas de su ciclo de vida. En particular, varias especies del hongo entomopatógeno *Ascosphaera* están comúnmente asociadas a las larvas de abejas sociales y solitarias, en las que causan una infección conocida como cría yesificada.

Palabras clave:

Abeja carpintera

Sudamérica

Ascosphaera apis

Hongos entomopatógenos

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Objetivos: El objetivo del presente estudio fue demostrar la presencia de *Ascosphaera apis* en larvas de *Xylocopa augusti* en Sudamérica.

Métodos: Se aisló *A. apis* de las larvas afectadas en medio YGPSA. La identificación final fue realizada utilizando tres técnicas: 1) examen microscópico del tamaño de las hifas y los cuerpos de fructificación; 2) test de apareamiento y test específico de compatibilidad sexual, y 3) detección mediante PCR, utilizando iniciadores específicos.

Resultados: En este estudio se ha registrado por primera vez en Sudamérica la presencia de *A. apis* en larvas enfermas de la abeja nativa *X. augusti*.

Conclusiones: La presencia de *A. apis* en larvas enfermas de *X. augusti*, sumada al hecho de que se ha subestimado la presencia de los mismos patógenos en diferentes especies de abejas, evidencia la necesidad de realizar más estudios epidemiológicos para determinar no solo la prevalencia de este patógeno entre polinizadores silvestres, sino también su relación con el repentino colapso de las colonias de abejas de la miel en esta región.

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Among the insect pollinators, solitary and social bees provide most of pollination in both managed and natural ecosystems; among them, honeybees (*Apis mellifera* L.) dominate crop pollination worldwide; however, local native bee species also play their part.¹ In this way, pollination service depends on both domesticated and wild pollinator populations, which might be affected by a range of recent and projected environmental changes.¹ Within these changes, introduction of managed pollinators for crop pollination and honey production can have an impact on native bee species²² through competition for resources or direct interaction. The genus *Xylocopa* (Apidae: Xylocopini) comprises approximately 470 described species worldwide.¹⁴ These bees are commonly known as carpenter bees owing to their behavior of building nests in dead wood or hollow internodes of bamboo stems.¹⁰ In nature, the nest has an internal branched structure with a single entrance connected to a system of tunnels. The internal structure can also be unbranched when it is raised in an artificial nest, as internodes of canes. The female builds a linear series of cells throughout the tunnels, each provisioned with a mixture of pollen and nectar (“bee bread”), then places the egg above the mass and, finally, closes the cell with a partition made of a mixture of sawdust and saliva. The brood is reared in individual and isolated cells until adult emergence. Species of *Xylocopa* are attacked by several parasites and predators during their life cycle.^{3,12,13,19}

The *Ascosphaera* genus includes 28 species capable of infecting bees; they have a worldwide distribution. These fungi are frequently associated to both social⁷ and solitary bees, although most of the species (25 out of 28) were originally described from solitary bees.^{8,20} Although species of *Ascosphaera* are pathogens with a preference for bees belonging to the family Megachilidae, several species were also cited affecting other solitary bees.²⁰ *Ascosphaera apis* is, particularly, an opportunistic pathogen of honeybees, although it has been also found in larvae of solitary bees such as *Nomia melanderi*¹⁶ and *Xylocopa californica*.⁶

The aim of the present study was to demonstrate *A. apis* affecting larvae of *Xylocopa augusti* in South America. In Argentina, *A. apis* was detected only in honey bee larvae causing chalkbrood disease.^{15,17}

The study was carried out at the Unidad de Vivero Forestal Universidad Nacional de La Plata (34°54'39" S, 57°55'37" W, 18 m.a.s.l), Argentina, from October 2013 to January 2014. During this period, ten dead larvae of *X. augusti* were collected from artificial nests used for biological studies. The larvae exhibited typical signs of *A. apis* infection such as fungal growth outside the larval integument, covered by white fibrous mycelium. Other signs founded in the larvae were dark and white areas visible through the cuticle that were light brown colored.

Collected larvae were stored at –20°C until they were processed. For the isolation of *A. apis* from larvae, the method of Jensen et al.¹¹ was used with modifications. Briefly, each sample

was sterilized with a 10% commercial bleach (sodium hypochlorite) solution for 10 min, and rinsed twice in sterile distilled water for 10 min each. Then, larvae were cut into small pieces with a sterile scalpel, placed on YGPSA plates² and incubated at 25 °C in microaerophilic conditions (10% CO₂ in air) in an anaerobic jar. After 48 h plates were removed from the jar and incubated aerobically at 25 °C. The fungal colonies developed after 5–7 days. The final identification was run out by three techniques: (1) Microscopic examination of the hyphae and sizes of the fruiting bodies; (2) Mating test, a specific sexual compatibility test use to determine *A. apis*. For this purpose we used two reference strains of our laboratory, CCM 95 (mating type +) and CCM 96 (mating type –), and (3) PCR detection, using specific primers previously reported by Garrido-Bailón et al.⁵ that amplify a fragment of 137 pb from the 5.8S rRNA of *A. apis*.

Even though the ten *X. augusti* studied larvae were detected dead inside their brood cells (Fig. 1a), only six larvae developed white colonies that suddenly turned to the characteristic olive-brown to black due to spore cysts of *A. apis*. Since all the samples rendered heterothallic isolates, a two-fold series dilution was done in order to obtain a single isolate. Once each single isolate was obtained, the mating test was run out. We could see a black zone in all cases, which indicated successful reproduction (Fig. 1b).

The microscopic examination of the black zone showed septate hyphae with the usually pronounced dichotomous branching. All the isolates presented reproductive structures, and the average sizes of the structures for 50 replications were within the expected for *A. apis*.¹⁸ For instance, spore cysts diameter was 86.7 ± 10.6 µm, spore-balls diameter was 14.8 ± 3.1 µm, and the length and width of the ellipsoidal, hyaline one celled ascospores were 3.2 ± 0.12 × 1.6 ± 0.08 µm (Fig. 1c). These average sizes of the reproductive structures were similar to the result reported for Reynaldi et al.,¹⁵ who isolated *A. apis* from a survey of honey of Argentina.

PCR results were positive for all the isolates, being observed the expected amplicon of 137 pb. Moreover, BLAST result confirmed the identity of the PCR-amplified sequences showing a high level of homology (99–100%) with others sequences of 5.8S ribosomal RNA gene of *A. apis* previously reported in GenBank.

The characteristic morphology of the hyphae, the sizes of the fruiting bodies, the positive mating test and PCR reactions allowed us to conclude that the studied isolates were *A. apis*.

The larvae studied may have probably been infected by spores present in the pollen carried by the female when the larval provision was prepared. Considering the isolated development of the larva of *Xylocopa* inside the brood cell, it is possible that spores are introduced by females that have foraged on flowers contaminated by spores left by honey bees. Like with other solitary bees, the brood cells of *Xylocopa* provide a stable and undisturbed

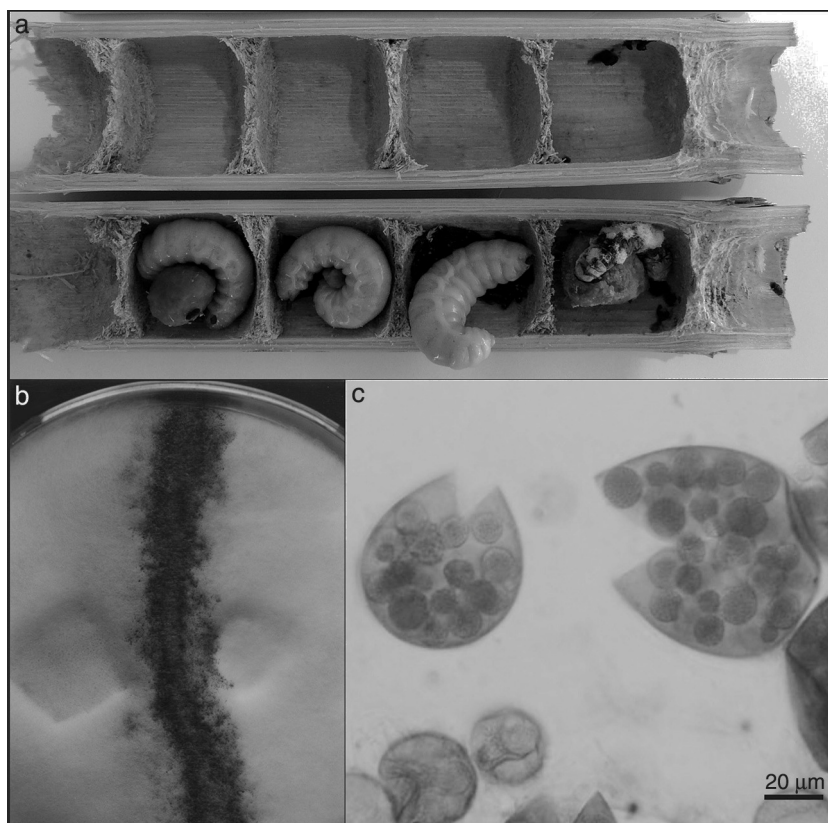


Fig. 1. (a) Internal structure of nests of *X. augusti*. (b) Positive mating test. (c) Characteristically *A. apis* fruiting bodies, for instance spherical spore cysts which contain numerous spore balls composed of hyaline spores.

micro-environment that appears suitable for the growth of these fungi. Evison et al.⁴ suggest that *Ascospaera* fungus is very common in the environment of pollinators, and that vectoring by non-host pollinators may have an important role in its movement around the environment.

We detect, for the first time, *A. apis* affecting larvae of *X. augusti* in South America. The chance of finding a honeybee pathogen affecting other hosts was already seen by Evison et al.⁴ who proposed that the interaction between host species in the movement of parasites may be significant. Moreover, they suggested that the shared use of flowers by multiple pollinator species, as well as the robbery of food storages in some cases, has the potential to make the transmission or vectoring of parasites between taxa relatively frequent. This situation calls our attention from an epidemiological point of view. Perhaps, the studies of microorganisms that affect other pollinators, such as *X. augusti*, allow us to mark some geographical areas with high levels of pathogens as *A. apis*.

In summary, this study describes the first report of *A. apis* affecting larvae of *X. augusti* in nature in South America. Even though little is known about the potential for inter and intra-specific transfer of pathogens in bee communities, there is evidence that the extent and role of host shifts and shared pathogens have been underestimated.^{9,21} This reason suggests the need for further epidemiological studies in order to determine not only the prevalence of this pathogen among wild pollinators, but also their relationship to the sudden collapse of honey bee colonies in this region.

Conflict of interest

The authors have no conflict of interest to declare.

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