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Natural occurrence of the entomopathogenic genus *Pandora* on spittlebug pests of crops and pastures in Argentina

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Abstract

The natural occurrence of entomophathogenic fungi infecting spittlebugs (Hemiptera: Cercopidae) considered serious pests to pasture grasses and crops in Argentina was investigated during summer-autumn (December to May) from 2013 to 2016. Adults and nymphs of spittlebugs were collected from *Sorghum halepense* and *Setaria parviflora* var. *parviflora* in San Miguel de Tucumán, Tucumán province. The entomopathogenic fungal species were characterized on the basis of morphological keys and molecular techniques. Microscopic characters were described from material mounted in lactophenol/aceto-orcein (1% w/v), and the amplification of the fungal SSU rDNA was carried out using the universal primers nu-SSU-0021-5′ and nu-SSU-1780-3′. Summarized information about occurrence of fungal infections on spittlebugs populations is provided. This study reports for the first time the occurrence of the genus *Pandora* infecting adults of the economically important spittlebugs *Deois (Deois) mourei*, *D. (D.) knoblauchii*, *Isozulia christenseni christenseni* and *Notozulia entreriana* from Argentina expanding the host range and geographical distribution of entomophthoralean fungi.

KEYWORDS
biological control, Cercopidae, Entomophthoraceae, Entomophthorales, froghoppers, Hemiptera

1 | INTRODUCTION

Spittlebugs (Hemiptera: Cercopidae) are a major threat to forage grass, milk and beef production in rangelands and pastures of the Neotropics (Valério, Lapointe, Kelemu, Fernandes, & Morales, 1996). These insects inflict heavy economic damage to pasture grasses and crops such as rice, sugarcane and corn and cause damage by mechanical feeding, by the physiological effects of saliva and by facilitating the entry of pathogenic microorganisms such as fungi and bacteria (Peck, 2001; Peck, Morales, & Castro, 2004). Spittlebugs of the genera *Aeneolamia*, *Mahanarva*, *Zulia*, *Deois*, *Notozulia* and *Prosapia* are considered to be serious pests in Central and South America where they cause up to 70% reduction in pasture yields within infested areas, particularly to the extensively planted genus *Brachiaria* (Trin.) Griseb. (Poaceae) (Lapointe & Sonoda, 2001; Leite, Machado Laerete, Goulart, Tavares, & Batista Filho, 2005; Sanz, 1997; Thompson, 2004). Additionally, Cercopidae have been identified as potential vectors of the bacteria *Xylella fastidiosa* to Brazilian grapevines (Ringenberg et al., 2014) and to Italian olives (Saponari et al., 2014). This family comprises approximately 1,450 species distributed principally in Neotropical regions (Carvalho & Webb, 2004; Fennah, 1953, 1968; Hamilton, 1977; de Remes Lenicov, Paradell, & Virla, 2004; Soulier-Perkins, 2013). Among the 24 species recorded for Argentina (Foieri & Marino de Remes Lenicov, 2015), the most economically important ones inhabiting the subtropical region of the country are *Deois (Deois) knoblauchii* (Berg), *D. (D.) mourei* (Berg) and *Notozulia entreriana* (Berg) (de Remes Lenicov et al., 2004). Holmann and Peck (2002) and Foieri, Marino de Remes Lenicov, and Virla (2016) pointed that the distribution of *D. (D.) mourei* and *N. entreriana* in the Americas is one of the widest known for spittlebugs (i.e. from southern USA to northern Argentina).

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Despite their importance, there is a significant lack of information about strategies to control these insects. The feeding behaviour of nymphs on roots below the soil surface makes it difficult to control them with chemical insecticides and brings up the need to look for alternatives. To date, few works in the Americas have demonstrated the importance of biological control for these pests, concentrating in regulating agents such as predators (Lastra, Gómez, & Castro, 2007; Mendonça, 2005), parasitoids (Triapitsyn, 2002; Valério & Oliveira, 2005), nematodes (Leite et al., 2005) and fungi (Dinardo-Miranda et al., 2004; García, Bustillo, Castro, & Arenas, 2012; Leite, Alves, Takada, Batista Filho, & Roberts, 2002; Matabanchoy Solarte, Bustillo Pardey, Castro Valderrama, Mesa Cobo, & Moreno Gil, 2012; Oliveira Campagnani et al., 2017; Oliveira Kassab et al., 2014). Unlike bacteria and viruses, fungi can infect insects not only through the gut, but also through spiracles and particularly through the surface of the integument. This opens up the possibility of fungi infecting sucking insects independently of their feeding activity (Ferron, 1978) and renders fungi one of the most promising candidates for biological control of spittlebugs. *Metarhizium anisopliae* (Metsch.) Sorokin (Ascomycota: Hypocreales) is the entomopathogenic fungus more commonly used against these pests in tropical and subtropical regions under field conditions (Dinardo-Miranda et al., 2004; Ferron, 1978; García et al., 2012; Leite et al., 2002; Matabanchoy Solarte et al., 2012; Oliveira Campagnani et al., 2017; Oliveira Kassab et al., 2014), and it is the only fungal species recorded to date for adults and nymphs spittlebugs from Argentina (Toledo, de Remes Lenicov, & López Lastra, 2008). Although fungi from the order Entomophthorales occasionally have been reported to cause epizootics in spittlebug pests of pasture and sugarcane in Brazil (Batista Filho, Leite, Takada, Lamas, & Ramiro, 1997; Leite et al., 2002), there are no records for this fungal order infecting any spittlebugs from Argentina.

The present work reports the presence of the entomopathogenic genus *Pandora* on spittlebugs from Argentina and provides both morphological and molecular characterization of the fungal species.

2 | MATERIALS AND METHODS

Adult and nymph spittlebugs were routinely collected every 15 days, during summer-autumn (December to May) from 2013 to 2016 in San Miguel de Tucumán, Tucumán province (26°47′S—65°18′W). Tucumán province is characterized by a subtropical climate with dry winters alternating with humid and rainy summers. The reasons for choosing this location and sampling season lie on previous studies carried out by our research group (Foieri & Marino de Remes Lenicov, 2015; Foieri et al., 2016) that indicated, on the one hand, that Tucumán presents one of the highest spittlebug species diversity in Argentina; and on the other hand, it has been shown that these insects increase their populations during rainy periods, remaining in a state of diapause egg during dry seasons. Specimens were collected mainly from *Sorghum halepense* (L.) Pers. and *Setaria parviflora* var. *parviflora* (Poir.) Kerguélen (Poaceae) where most frequently live in nature. Living adults and nymphs were collected using aspirators, nets or entomological forceps, placed in polyethylene–terephthalathe cylindrical cages (35 cm high, 18 cm diameter) and transported to the laboratory. Some insects were also collected, fixed in 70% ethanol and taken to the laboratory for specific identification using the taxonomic keys of Fennah (1953, 1968, 1985).

Adults were individually placed in glass assay tubes containing one leaf of *S. parviflora* var. *parviflora*. Assay tubes were sealed with dampened cotton, and insects were maintained under these conditions at 26 ± 1°C, 60%–80% relative humidity (RH) and of 14:10 h (light:dark) photoperiod. Nymphs were placed in breeding units, made with acrylic containers sealed with plastic lids, containing primary roots of *Cynodon dactylon* (L.) Pers. Nymphs were maintained under the above-mentioned conditions. Insects were checked daily, and the leaves and roots food were changed every 24 h up to 12 days. Dead insects with external signs of mycosis were removed. Attempts to isolate the fungus in pure culture were made using Sabouraud dextrose agar plus 1% yeast extract (SDAY 1%), a suitable media for fast mycelia growth commonly used in the isolation and culture of several species of Entomophthorales (Choi et al., 2016; Feng, Johnson, & Kish, 1990; Moubasher, Abdel-Rahman, Abdel-Mallek, & Hammam, 2010; Zhou, Su, & Liu, 2016). Isolates were made through the "descending conidia" and "ascending conidia" methods described by Eilenberg, Wilding, and Bresciani (1992) and Jensen, Thomsen, and Eilenberg (2006), respectively. Measurements of fungal structures were made to enable specific identifications. Fungal species were identified according to taxonomic keys and monographs of Humber (2012), Keller (2007) and Bałazy (1993). Microscopic characters were described from material mounted in lactophenol/aceto-orcein (1% w/v) and observed on a WILD M-20-21049 microscope. Fungal preparations were photographed using a Nikon Alphaphot-2 YS2 microscope fitted with a Nikon D40 digital camera. Infected insect hosts were photographed using a Wild M3 stereomicroscope fitted with a Sony Cyber-shot camera and were then deposited as herbarium material at Centro de Investigaciones de Fitopatología (CIDEFI), Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata.

Mycotized insects previously used for morphological identification were individually preserved in 96% ethanol for molecular identification. Total DNA (insect + fungus) was extracted individually from insect cadavers with a rotor–stator homogenizer (Glas-Col, Terre Haute, USA) using the DNeasy Plant Mini kit (Qiagen, Hilden, Germany) following the instructions provided by the manufacturer. Amplification of the SSU rDNA was carried out using the universal primers nu-SSU-0021-5′ (5′-CTGGTTGATTCTGCCAGT-3′) (Gargas & DePriest, 1996) and nu-SSU-1780-3′ (5′-AATGATCCTTCCGCAGGT-3′) (DePriest, 1993). The following amplification programme was used: denaturation at 95°C for 3 min, followed by 40 cycles with three-segment amplification (1 min at 95°C for denaturation, 30 s at 60°C for annealing and 2 min at 72°C for DNA chain elongation). The reaction mixture (20 μl) contained 200 μM of each dNTP, 1 μM of each primer, 1× Phusion HF buffer (containing 1.5 mM $MgCl_2$), 0.4 unit Phusion DNA polymerase (New England BioLabs, Ipswich, USA) and 1 μl of template DNA (insect + fungus). Amplicon sizes were checked by electrophoresis, and purified PCR products were sent to Macrogen Inc. (Seoul, Korea) for

sequencing in both directions. The sequences obtained were submitted to the National Center for Biotechnology Information (NCBI) GenBank database (www.ncbi.nlm.nih.gov) for gene annotation. Sequences were edited using the program BioEdit version 7.0.9.0 (Hall, 1999), and they were used to perform a phylogenetic analysis that included the sequences obtained in this study and those of some related species in the genera *Batkoa*, *Conidiobolus*, *Entomophaga*, *Furia*, *Pandora* and *Zoophthora* (Entomophthoromycota: Entomophthorales) available at GenBank. Sequences were aligned with the ClustalW tool of the program Mega5 (Tamura, Peterson, Peterson, Nei, & Kumar, 2011), assuming a penalty by opening gap and by extension of the gap of 15 and 6.66, respectively. The phylogenetic study was carried out using the analysis of maximum likelihood. The consensus tree was built using the latest 1,000 trees. Statistical support for the nodes was evaluated using 1,000 replicates.

3 | RESULTS

Two hundred and forty-eight insects (208 adults and 40 nymphs) were collected in the field. Adults were identified as *Deois (D.) mourei* (*n* = 89), *N. entreriana* (*n* = 77), *D. (D.) knoublauchii* (*n* = 28) and *Isozulia christenseni christenseni* (Lallemand) (*n* = 14). Nymphs were not taxonomically identified.

Insect infections were observed only in indoor experimental colonies, with no infected cadavers being detected in the field. The largest number of insects (*n* = 226) was collected between December 2014 and May 2015, in coincidence with fungal infections which were recorded principally on April and May 2015. Fungal infections were detected in 4.3% of the apparently healthy adults of *D. (D.) mourei* (one female and two males collected on 7 April 2015 and two females collected on 4 May 2015), *N. entreriana* (one female and one male collected on 7 April 2015) (Figure 1a–d), *I. c. christenseni* (one female collected on 4 May 2015) and *D. (D.) knoblauchii* (one male collected on 15 February 2016). Females accounted 2.4% of total insects collected whereas males represented 1.9%, with a 5:4 female: male ratio. No fungal infections were recorded among nymphs of any spittlebug species.

Pandora was the only fungal genus recorded infecting adults of spittlebug. Attempts to obtain pure cultures on SDAY 1% were not successful. Nevertheless, measurements and fungal descriptions were made from mycelia grown on one adult of each species of insect collected (*n* = 4). Mycelia were yellowish, covering the entire insect abdomen, with presence of some cystidia with thicknesses approximately equal to the diameter of conidiophores. No rhizoids were observed attaching any of the infected insects to the substrate. Conidiophores were digitately branched, 7.9–9.8 μm diameter (8.8 ± 0.2 μm) (four specimens, *n* = 40) (Figure 1e). Primary conidia were ovoid, elongate, bitunicate and uninucleate, 16.1–24.1 μm (20.7 ± 0.3 μm) × 10.7–16.1 μm (12.6 ± 0.3 μm), length/width ratio 1.5 μm (four specimens, *n* = 40) (Figure 1f). No secondary conidia or resting spores were observed. According to morphological characteristics and measurements, no differences were recorded among the structures observed from all mycotized insects. Thus, all insects were considered to be infected by the same fungal species which was principally identified by the shape and size of primary conidia. Table 1 compares conidia shape and measurement from the fungus of this study and other species of *Pandora*.

Specimens examined were preserved at Centro de Investigaciones de Fitopatología (CIDEFI), Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, as herbarium material under the accession number PD-DK1.

Although DNA was extracted from one adult of each *D. (D.) mourei*, *D. (D.) knoblauchii* and *N. entreriana*, a sequence of the fungal SSU rRNA gene could be obtained from only one of them; that is,

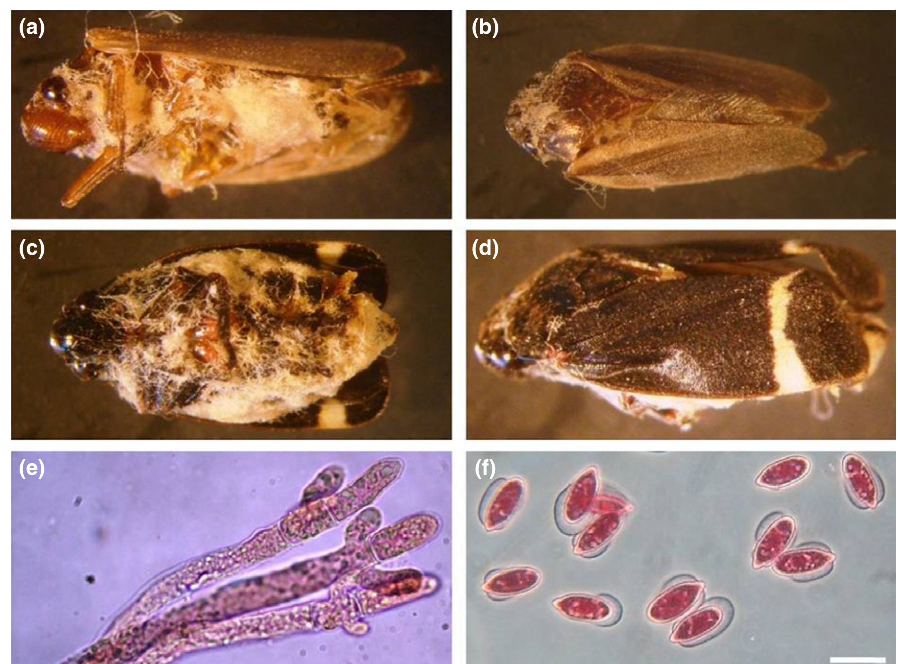

FIGURE 1 Adults of *Deois (Deois) mourei* (a,b) and *Notozulia entreriana* (c,d) infected with *Pandora* sp. (e,f) Branched conidiophores and primary conidia of *Pandora* sp. Scale bar: (a) 0.1 cm, (b) 0.2 cm, (c) 0.2 cm, (d) 0.1 cm, (e) 20.7 μm and (f) 20 μm

TABLE 1 Comparison of primary conidia measurements of *Pandora* closely related species

Fungal species	Primary conidia (µm)	Host	References
Pandora sp.	16.1–24.1 × 10.7–16.1	*D. (D.) mourei*, *D. (D.) knoblauchii* *I. c. christenseni* *N. entreriana* (Hemiptera: Cercopidae)	This study
P. blunckii (Bose & Mehta) Batko	15–20 × 7–11	*Plutella xylostella* L. (Lepidoptera: Plutellidae)	Humber (2012)
P. bullata (Thaxter & MacLeod ex Humber) Humber	23.2–34.8 × 11.5–16.3	*Chrysomya megacephala* Fabricius (Diptera: Calliphoridae)	Montalva, Collier, Luz, & Humber, (2016)
P. delphacis	23.8–26.6 × 9.9–11.9	*Spissistilus festinus* (Say) Hemiptera: Membracidae)	Miller and Harper (1987)
P. delphacis	22.9–23.3 × 12–12.4	*Empoasca fabae* (Harris) Hemiptera: Cicadellidae)	Nielsen et al. (2001)
P. noury (Remaudière & Hennebert) Humber	13.6–19.8 × 7.4–13.6	*Heterocaecilius* sp. (Psocodea: Pseudocaeciliidae)	Toledo, Humber, & López Lastra, (2008)

D. (D.) knoblauchii collected on 15 February 2016. The 1,283 bp sequence was annotated in GenBank as *Pandora* sp. under the accession number KY379099.

By means of the NCBI BLAST algorithm, it was found that the *Pandora* sp. SSU rRNA sequence was similar to those from species in the phylum Entomophthoromycota, class Entomophthoromycetes, order Entomophthorales. In the maximum likelihood tree resulting from the phylogenetic analysis, *Pandora* sp. was clustered in a monophyletic group supported by a bootstrap value of 89%, which includes representatives of the families Ancylistaceae (*Conidiobolus* species) and Entomophthoraceae (*Batkoa*, *Entomophaga*, *Furia*, *Pandora* and *Zoophthora* species) (Figure 2). However, the resulting sequence of the fungus described in this work was clustered together with *Pandora delphacis* (Hori) Humber ARSEF 581 in a same clade supported by a bootstrap value of 98%.

4 | DISCUSSION

This work provides the first record of the entomopathogenic genus *Pandora* in four of the most important spittlebugs species inhabit subtropical Argentina.

The largest number of insects was collected between December 2014 and May 2015, coinciding with a peak in fungal infections, recorded mainly for April and May 2015. Previous research has indicated that the development and survival of spittlebug species depend on climatic conditions, with populations proliferating mainly during hot, wet and rainy periods (Cosenza, 1989; Domingues & da Silva Santos, 1975; Pereira, Borges, Mendes, Toscano, & De Morales, 2011). Rain intensity and the duration of the rainy period are determining factors for the increment of spittlebug populations in the field. Accordingly, an unpublished survey (A. Foieri, personal communication) has recorded three well-defined generations of both *N. entreriana* and *D. (D.) mourei* populations, between November and May during the 2012–2013 and 2014–2015 periods, coinciding with the period of greatest precipitation and temperature. In contrast, spittlebug field populations fall down dramatically during the dry winter season (May to October). Both species probably survived the unfavourable conditions as diapause eggs, as suggested by Silveira Neto, Carvalho, and Paranhos (1968), Garcia, Botelho, and Parra (2006) and Auad et al. (2011).

Insect infections were observed only in indoor experimental colonies, with no infected cadavers detected in the field. It is known that the incidence of mycoses in insect populations is variable, depending on the host species, climate, microclimate and the season of the year (Remaudiére & Latgé, 1985). Rainfall and humidity are the two factors most often related with the appearance of entomopathogenic fungi (Leite et al., 2002); for instance, an average daily RH below 80% is unfavourable for many Entomophthorales fungi (Carruthers & Haynes, 1986; Le Ru & Iziquel, 1990; Oduor, Moraes, Van Der Geest, & Yaninek, 1996; Yu, Nordin, Brown, & Jackson, 1995). Weekly field data recorded during the 2012–2013 and 2014–2015 periods, indicated a daily average RH ranging from 37% to 95%, and <80% for most of the dates sampled. On the other hand, low-intensity rain is especially favourable for the development of fungal epizootics, while intense rains can reduce infection rates by washing conidia off dead insects and, causing these to fall to the ground, thus decreasing the density of inoculum (Leite et al., 2002). In Tucumán province, rains are usually very strong, with 90% of the annual precipitations concentrated in a 4-month time span. Recorded precipitation amounted to 979 and 1,072 mm, during the 2012–2013 and 2014–2016 sampling periods, respectively. The highest amount of rainfall was recorded in the months of December, January, February and March, representing between 71% and 84% of the total annual rainfall. Therefore, it is reasonable to assume that the frequency and intensity of rainfall,

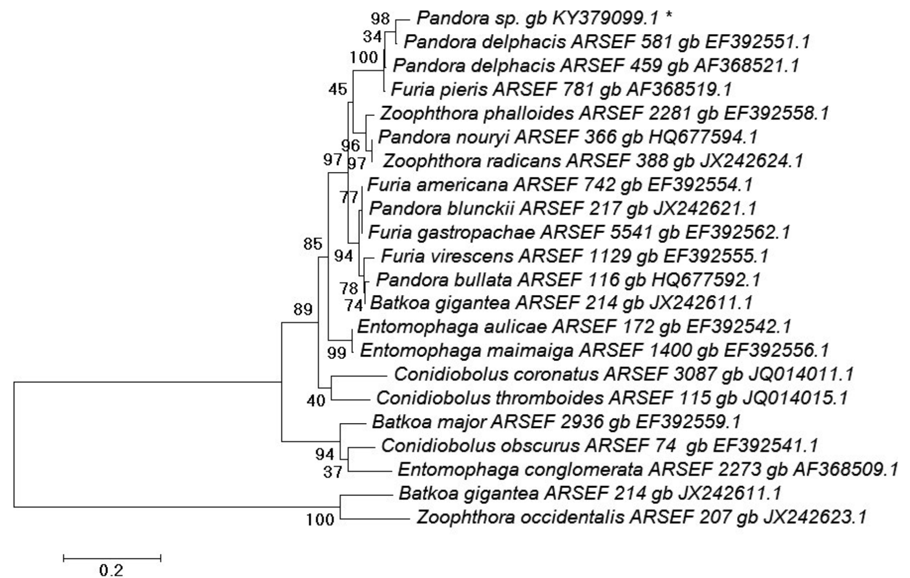

FIGURE 2 Phylogenetic tree generated by the maximum likelihood analysis of the SSU rDNA sequence of Argentinean *Pandora* sp. and related species. The sequence corresponding to this work is marked with an asterisk. Behind each reference isolate, its strain number and its access number to the GenBank are detailed. Bootstrap values are noted above of the internodes. The bar at the bottom indicates the number of substitutions per site

combined with a prevailing RH <80%, may account for the absence of epizootic fungi in field populations. However, the overcrowded conditions and the optimal temperature and RH values under which the insects were maintained in the laboratory enhanced the expression of fungal infections acquired in the field.

No fungal infections were recorded among nymphs of any spittlebug species. In that regard, it can be mentioned that nymphs are xylem-feeders and live on herbs, grasses or woody plants within their self-produced spittle masses. The spittle consists of the excretion of surplus water originating from the large amount of ingested xylem sap, which may be enriched with mucopolysaccharides and proteins from the Malpighian tubules (Marshall, 1966; Mello, Pimentel, Yamada, & Stroropoli-Neto, 1987). The spittle is thought to give shelter to the nymphs, protecting from predation, parasitism and providing a suitable microclimate (Whittaker, 1970). The absence of infected nymphs is in accordance with previously obtained results by Toledo, de Remes Lenicov, et al. (2008), who detected fungal infections produced by *M. anisopliae* in a 50:1 adult:nymph ratio.

The literature abounds with records about the occurrence of *Pandora* as a pathogen of hemipteran pests (Feng et al., 1990; Hatting, Humber, Poprawski, & Miller, 1999; Keller, 2006; Manfrino, Hatting, Humber, Salto, & López Lastra, 2014; Scorsetti, Maciá, Steinkraus, & López Lastra, 2010; Toledo, Giambelluca, de Remes Lenicov, & López Lastra, 2008); however, to date, one of the few reports of entomophthoralean fungus infecting Cercopidae from South America is those from Brazil recorded by Leite et al. (2002), who detected *Furia* sp. and *Batkoa* sp. infecting *Deois schach* (Fabricius) and *Mahanarva fimbriolata* (Stål), respectively.

Attempts to obtain pure cultures on SDAY 1% were not successful, despite this culture medium having been successfully used to cultivate the genus *Pandora* (Zhou et al., 2016). Consequently, efforts to isolate the fungus in pure cultures using other solid or liquid media will be the subject of future research. In addition, further attempts to culture the entomopathogenic fungus using living hosts (Hajek, Papierok, & Eilenberg, 2012) will be made in order to conduct laboratory pathogenicity tests against spittlebugs. Although these authors not recommend this method for massively producing Entomophthoralean fungi, it might be used for small-scale production purposes (enough for laboratory tests) to have an estimation about their performance in the field.

In the present study, fungus identification was primarily based on the shape and size of primary conidia. The conidial size of Argentinean fungus was found to be similar to those of *Pandora*, especially *P. delphacis*. Although this fungus has quite smaller primary conidia (16.1–24.1 × 10.7–16.1 μm) than *P. delphacis* characterized by Bałazy (1993) (29.2–35.6 × 13–17.5 μm) and Humber (2012) (30–35 × 12–18 μm), the measurements in this study were similar to those described by other authors from different hoppers, namely 23.8–26.6 × 9.9–11.9 μm from *Spissistilus festinus* (Say) (Hemiptera: Membracidae) (Miller & Harper, 1987) and 22.9–23.3 × 12–12.4 μm from *Empoasca fabae* (Harris) (Hemiptera: Cicadellidae) (Nielsen, Sommer, Eilenberg, Hansen, & Humber, 2001).

According to some morphological characteristics and phylogenetic analyses, the entomopathogenic fungus discussed here was placed in the family Entomophthoraceae and named as *Pandora* sp. This identification was based on the affinity of hosts and the phylogenetic analysis, where the Argentinean fungus is grouped together with *P. delphacis* ARSEF 581 with a bootstrap value of 98%. However, additional efforts to determine fungal identity at the species level using more than one gene and to be used in pathogenicity tests are needed in order to find new microbial agents useful to implement more specific biological control programmes against damaging spittlebug pests of agriculture.

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AUTHOR CONTRIBUTION

AF, NP and AT conceived research. AF conducted sampling, maintained the insects under laboratory conditions and identified taxonomically insect species. NP made DNA (fungus + insect) extraction, amplification and purification. AT made morphological characterization of fungal species, conducted phylogenetic analysis and wrote the manuscript. AT secured funding. All authors read and approved the manuscript.

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