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First report of *Pandora neoaphidis* resting spore formation *in vivo* in aphid hosts

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ABSTRACT

The entomopathogenic fungus *Pandora neoaphidis* is a recognized pathogen of aphids, causes natural epizootics in aphid populations, and interacts and competes with aphid predators and parasitoids. Survival of entomophthoralean fungi in periods of unsuitable weather conditions or lack of appropriate host insects is accomplished mainly by thick-walled resting spores (zygospores or azygospores). However, resting spores are not known for some entomophthoralean species such as *P. neoaphidis*. Several hypotheses of *P. neoaphidis* winter survival can be found in the literature but so far these hypotheses do not include the presence of resting spores. Resting spores were found in an aphid population where *P. neoaphidis* was the only entomophthoralean fungus observed during surveys conducted in organic horticultural crops in greenhouses and open fields in Buenos Aires province, Argentina. This study sought to use molecular methods to confirm that these resting spores were, in fact, those of *P. neoaphidis* while further documenting and characterizing these resting spores that were produced *in vivo* in aphid hosts. The double-walled resting spores were characterized using light and transmission electron microscopy. The Argentinean resting spores clustered together with *P. neoaphidis* isolates with bootstrap values above 98 % in the small subunit ribosomal RNA (SSU rRNA) sequence analysis and with bootstrap values above 99 % the Internal Transcribed Spacer (ITS) II region sequence analysis. This study is the first gene-based confirmation from either infected hosts or cultures that *P. neoaphidis* is able to produce resting spores.

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Introduction

The entomopathogenic fungus *Pandora neoaphidis* (Remaudière & Hennebert) Humber (*Entomophthoromycotina*: *Entomophthorales*), is a recognized pathogen of aphids (*Hemiptera*: *Aphididae*). This fungus has a worldwide distribution, and is well known from Europe, Asia, Africa, South America, and Australia (Wilding & Brady 1984; Glare & Milner 1991; Hatting *et al.* 2000; Scorsetti *et al.* 2007). *Pandora neoaphidis* is aphid-specific, causes natural epizootics in aphid populations and interacts and competes with aphid predators and parasitoids (Baverstock *et al.* 2008a). Recently it has been demonstrated that *P. neoaphidis* causes natural epizootics in different seasons at different temperatures in South America and causes host

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mortality up to 56.6 %, thus confirming the capacity of this fungus to be an effective biological control agent (Scorsetti *et al.* 2010).

Survival of entomophthoralean fungi in periods of unsuitable weather conditions or lack of appropriate host insects is accomplished mainly by thick-walled resting spores (zygospores or azygospores). However, resting spores are not known for all entomophthoralean species. Most species that are only rarely collected and poorly known probably do, in fact, form resting spores as often and in the same manner as their better known relatives, but their resting spores remain undetected from their few known specimens (Nielsen *et al.* 2007). However, for so common, widespread, and much studied a species as *P. neoaphidis*, the absence of information about resting spores cannot be dismissed as either accidental or artifactual. It has long been believed that resting spore production by *P. neoaphidis* either does not happen or must be extraordinarily rare.

There are several hypotheses in the literature about winter survival of *P. neoaphidis*. One is that the fungus survives by continued infection of anholocyclic populations of aphids. This possibility is supported by the fact that the fungus is capable of infecting aphids at temperatures as low as 5 °C (Wilding 1970). Baverstock *et al.* (2008b) have suggested that *P. neoaphidis* could remain active throughout the year through a combination of continuous infection and as inoculum deposited on the soil. There is evidence reported about the capability of *P. neoaphidis* to survive for long periods outside the host in soil as conidia or as hyphal bodies (Feng *et al.* 1992). Schofield *et al.* (1995) showed that conidia remained infective for up to 32 d after incubation at 5 °C in 85 % RH. Nielsen *et al.* (2003) suggested that *P. neoaphidis* may overwinter in the soil as 'loricoconidia' (resistant structures formed by the marked thickening of a conidial wall; although they may function in a manner similar to resting spores, the morphological, and developmental differences between these spore types are discussed below).

In populations of aphids otherwise demonstrated sporulating infections of *P. neoaphidis*, Scorsetti *et al.* (2007) noted and illustrated a few resting spore-like structures from Argentinean aphid cadavers collected in the autumn (June 2003 and May 2004) but the taxonomic identification of these resting spores could not be confirmed by molecular or morphological techniques. The linkage between entomophthoralean resting spores and their conidial forms is not always straightforward but congruence of sequences for several genes from both resting spores and conidial states effectively confirm the linkages of resting spores with the corresponding conidial states that are the basis for entomophthoralean taxonomy. Thomsen & Jensen (2002) used a nested PCR techniques to link resting spores with the *Entomophthora* conidial stages affecting the same host species of flies and thus demonstrated that several differing genotypes within the *Entomophthora muscae* species complex could each complete their entire life cycle in a single dipteran host species. Comparing sequences of the ribosomal repeat (small subunit (SSU) and Internal Transcribed Spacer (ITS)) is however a stronger method that also allows blast search against the still growing collection of entomophthoralean sequences of these two regions in GenBank.

The objective of this study was to document the morphology of resting spores found in field-infected aphids where only *P. neoaphidis* infection was found in the aphid population and to use sequences of the ribosomal repeat (SSU and ITS) to confirm the *P. neoaphidis* identity of these resting spores.

Materials and methods

Field survey

Surveys were conducted in organic horticultural crops in greenhouses and open fields in Buenos Aires province, Argentina, according to Scorsetti *et al.* (2007). Sampling was performed weekly from April 2007 to April 2008. The climatic conditions in this region are temperate with average temperature of *ca* 18 °C.

Lettuce (*Lactuca sativa* L.) was the main crop that was sampled, but aphids were also collected from fields of tomato (*Lycopersicon esculentum* Mill.), cabbage (*Brassica oleracea* L.), pepper (*Capsicum annuum* L.), eggplant (*Solanum melongena* L.), Swiss chard (*Beta vulgaris* var. *cicla* L.), and artichoke (*Cynara scolymus* L.).

Laboratory

Field-collected dead and living aphids were placed individually in plastic cups with lids (150 cm^3). Dead aphids with or without externals signs of mycosis were collected with the piece of plant substrate onto which they were attached by rhizoids, and were then placed individually into sterilized plastic containers (100 cm^3) and transported to the laboratory for analysis of fungal infection.

Dead aphids showing no external signs of mycoses were put in 60 mm Petri dishes with a moistened filter paper and maintained at 20 °C for 24–72 h to allow the complete development of any fungi present in them. Healthy aphids were preserved in 70 % ethanol as reference specimens for further taxonomic identification.

The pathogen affecting the aphids collected during these studies was confirmed to be *Pandora neoaphidis* by using standard taxonomic literature (Keller 1987, 1991; Humber 1989; Bałazy 1993).

Pandora neoaphidis-infected aphids were stored at 4 °C for 1 m in 60 mm Petri dishes with a moistened filter paper, and then examined using a stereoscopic microscope every 72 h for the possible development of environmentally resistant resting structures.

Microscopic characteristics were described from aphids with resting spores mounted in aceto-orcein (1 % w v^{-1}) and observed on an Olympus CH3 microscope. Fungal preparations were observed on an Olympus BX51 microscope and photographed using an Olympus DP71 camera.

Semipermanent slides were made according to Humber (1997) and deposited as herbarium material in the Mycological Collection of the Institute of Botany Carlos Spegazzini (LPSC, La Plata, Buenos Aires, Argentina).

The aphids containing resting spores were preserved individually in Eppendorf microcentrifuge tubes containing 48 % ethanol and kept at 4 °C in darkness until either extraction of the DNA or fixation for transmission electron microscopy (TEM).

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Electron microscopy

For TEM, thick-walled spores from a mechanically disrupted infected aphid were fixed for 2 h in 3 % glutaraldehyde, post-fixed for 1 h in 1 % osmium tetroxide, dehydrated in a graded series of 30–100 % ethanol, cleared in propylene oxide, and embedded in Epon 812. Thin (60 nm) sections were cut with a diamond knife, stained with uranyl acetate and lead citrate, and examined using a JEM 1200EX II (Jeol) TEM.

DNA extraction, PCR amplification, and sequencing

Two of the infected aphids bearing resting spores were used for the molecular identification. Each of the aphids was transferred into new Eppendorf microcentrifuge tubes, the ethanol was allowed to evaporate, and the cadavers were mechanically disrupted with a sterile plastic pistil in 100 μl MiliQ water. The samples were washed twice by centrifugation for 2 min at 10 000×*g*. The supernatants were discarded and 100 μl MilliQ water was added to each preparation. Five microlitres of the washed spore suspension was added to a glass slides coated with Gel Slick Solution (BioWhitataker Molecular Application BMA; Rockland, ME, USA), and another coated slide was put on top of the spores. The resting spores were then disrupted mechanically by pressing the slides hard with the thumbs with simultaneous circular movements of the top slide. One microlitre of the crushed spores was used as template for the PCR amplification. The DNeasy® Plant Mini Kit (Qiagen GmbH, Hilden, Germany) was used to extract the DNA from *in vitro* cultures of *Pandora nouryi* ARSEF 362, *P. nouryi* ARSEF 366, and *Pandora bullata* ARSEF 116, following the manufacturer's instructions. Three in vitro cultures were obtained from the United States Department of Agriculture, Agricultural Research Service Collection of Entomopathogenic Fungal Cultures (ARSEF) and were grown in Graces liquid medium from where hyphal mass was collected and freeze dried upon DNA extraction.

Universal fungal primers nu-SSU-0021-5′ and nu-SSU-1780-3′ (Gargas & DePriest 1996) were used to amplify the nuclear SSU ribosomal RNA (rRNA) gene with an initial denaturation for 3 min at 94 °C, followed by 35 cycles with denaturation for 1 min at 94 °C, annealing for 1 min at 60 °C, extension for 1.5 min at 72 °C, and a final extension for 7 min at 72 °C. Universal fungal primers ITS1 and ITS4 (White *et al.* 1990) were used to amplify the ITS regions of ribosomal DNA with an initial denaturation for 3 min at 94 °C, followed by 35 cycles with denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C, extension for 1.5 min at 72 °C, and a final extension for 7 min at 72 °C. Fungal universal primers were used as a positive amplification control to assure availability of DNA from the full range of entomophthoralean species studied here.

The PCR reactions were carried out in 50 μl volumes each with 200 μM of each dNTP, 1 μM of each primer, 1× Phusion HF Buffer (with 1.5 mM $MgCl_2$), 0.5 unit Phusion DNA polymerase (Finnzymes; Espoo, Finland), and 1 μl template (DNA extractions from *in vitro* cultures or DNA from *in vivo* material). Prior to sequencing, the PCR products were purified with the illustra™ DNA and Gel Band Purification Kit (GE Healthcare; Buckinghamshire, UK). The purified PCR products were sent to Eurofins MWG Operon (Ebersberg, Germany) for sequencing in both directions. The following primers were used for sequencing of the SSU: nu-SSU-0021-5′ and nu-SSU-1780-3′ (Gargas & DePriest 1996) and nu-SSU-0553-3′, nu-SSU-0573-5′ and nu-SSU-1150-5′ (White *et al.* 1990) and for the ITS we used following primers for sequencing ITS1

Table 1 – List of the entomophthoralean species used in this study, strain code, and GenBank accession numbers.

Species	Strain code	SSU	ITS
Entomophaga aulicae	FPMI 646	EAU35394	
Entomophthora muscae	F 1020	D29948	
Erynia rhizospora	ARSEF 1441	AF368514	
Erynia sciarae	ARSEF 1870	AF368515	
Furia americana	ARSEF 742	EF392554	
Furia gastropachae	ARSEF 5541	EF392562	
Furia ithacensis	ARSEF 1339	AF351134	
Furia neopyralidarum	ARSEF 1145	AF368518	
Furia pieris	ARSEF 781	AF368519	
Pandora blunckii	ARSEF 217	AF368520	
Pandora bullata	ARSEF 116	HQ677592	HQ677588
Pandora delphacis	ARSEF 581	EF392551	
Pandora delphacis	ARSEF 459	AF368521	
Pandora dipterigena	ARSEF 397	AF368522	
Pandora kondoiensis	ARSEF 5707		AF543200
Pandora kondoiensis	ARSEF 5708		AF543201
Pandora kondoiensis	ARSEF 828		AF543199
Pandora kondoiensis	ARSEF 825	AF351133	
Pandora neoaphidis	ARSEF 3240	EF392560	
Pandora neoaphidis	ARSEF 1607	EU267188	
Pandora neoaphidis	ARSEF 1609		AF543210
Pandora neoaphidis	ARSEF 5374		AF543211
Pandora neoaphidis	ARSEF 7937 MboI+	EU267189	EU267189
Pandora neoaphidis	ARSEF 7938 MboI–	EU267191	EU267191
Pandora neoaphidis	ARSEF 7938 MboI+	EU267190	EU267190
Pandora neoaphidis	ARSEF 7939 -1	EU267192	EU267192
Pandora neoaphidis	ARSEF 7939 -3	EU267193	EU267193
Pandora neoaphidis	ARSEF 835		AF543209
Pandora neoaphidis	KVL 633	AF052405	
Pandora neoaphidis	NW 195		AF543204
Pandora neoaphidis	NW 283		AF543205
Pandora neoaphidis	NW 316		AF543206
Pandora neoaphidis	NW 327		AF543207
Pandora neoaphidis	NW 343		AF543202
Pandora neoaphidis	NW 415		AF543208
Pandora neoaphidis	Resting spores	HQ677591	HQ677587
Pandora nouryi	ARSEF 362	HQ677593	HQ677589
Pandora nouryi	ARSEF 366	HQ677594	HQ677590
Strongwellsea castrans	Resting spores	AF052406	
Zoophthora anglica	ARSEF 396	AF368524	
Zoophthora occidentalis	ARSEF 3073	AF368525	
Zoophthora radicans	KVL 610	AF052404	
Zoophthora radicans	ARSEF 1699		DQ864988
Zoophthora radicans	ARSEF 2411		EF151416
Zoophthora radicans	ARSEF 6003		EF137934
Zoophthora radicans	F 853	D61381	
Zoophthora radicans	NW 250		EF137938
Zoophthora radicans	NW 323		EF137936
Zoophthora radicans	NW 378		EF151414
Zoophthora radicans	NW 386		EF151412

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and ITS4 (White *et al.* 1990), Nu-5.8S-5′ (Jensen & Eilenberg 2001) and Nu-5.8S-3′ (Jensen *et al.* 2009).

In order to confirm the taxonomic affinity of the resting spores we blasted the sequences against the GenBank database. In addition the resting spore SSU rRNA sequence was aligned with published SSU rRNA sequences from other entomophthoralean species including three new SSU rRNA sequences – one from *P. bullata* and two from *P. nouryi* isolates – were obtained for the current study (Table 1).

The sequences were checked and aligned with *Bioedit* v7.0.8.0. Parsimony and neighbour-joining analyses with 1000 bootstrap replications to determine the support for internal branches were performed using MEGA 4 (Tamura *et al.* 2007). In the SSU rRNA analysis *Entomophthora muscae* and *Entomophaga aulicae* were used as outgroup taxa. In the ITS II analysis all the *Zoophthora radicans* isolates were used as outgroups.

Results

Among the 562 *Pandora*-infected aphids that were studied, six cadavers included structures with thick, double-layered walls that appeared to standard entomophthoralean resting spores. These six individuals were held at 4 °C for an additional 6 ($n = 4$) or 9 ($n = 2$) d to allow the full development and maturation of the spores. All cadavers with the putative resting spores were collected in mid-winter (August and September 2007) from *Lactuca sativa* plants growing in open fields, and all of these hosts were identified as *Nasonovia ribisnigris* (Mosley) (*Hemiptera*: *Aphididae*).

These six aphids were preserved in 48 % ethanol. Two of them were processed for TEM, another two were used for DNA extraction to determine the species identification by molecular analyses, and the remaining two are retained as voucher specimens deposited as herbarium material in the Mycological Collection of the Institute of Botany Carlos Spegazzini (LPSC 47460, La Plata, Buenos Aires, Argentina).

The resting spores in these aphids averaged 31.4 ± 2.4 μm diam (24.8–37.2 μm; $n = 300$) (Fig 1A and B), and were produced both internally and externally on the surface of the aphid cadaver. The macroscopic appearance of aphids filled with these structures was dry and 'sandy'. The resting spores were spherical, globose, with surface slightly covered by rounded depressions, varying from a pale yellow tint to deep amber (with the colour apparently confined to the walls). The spores appeared to be mature and dormant since there was a single consolidated oil droplet filling most of the volume of individual spores (Fig 1B).

Cross-sections of the resting spores observed with TEM show two layers of the spore wall, a thin electron-dense (dark) outer layer, and a much thicker electron-lucent (light) inner layer (Fig 1C and D).

We obtained a sequence of the SSU rRNA gene from resting spores of one aphid. The closest BLAST correspondences (those with 99–100 % nucleotide identify) of the 1800 bp sequence from these resting spores were with eight different *Pandora neoaphidis* sequences. The resting spore SSU rRNA sequence was aligned with other published entomophthoralean SSU rRNA sequences but we only used the first 1100 positions

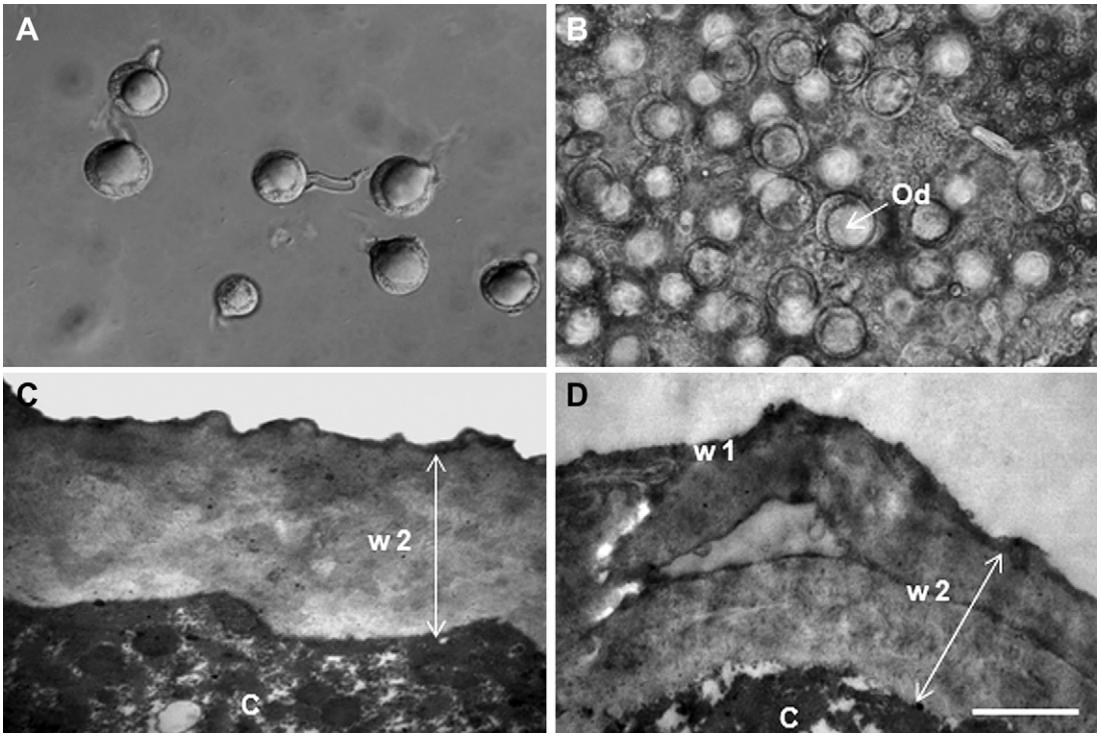

Fig 1 – Differential interference contrast micrograph (A), and phase contrast micrograph, (B) of *Pandora neoaphidis* mature resting spores, with a central oil droplet (Od) filling most of the spore's volume. Scale bar: 50 μm. Ultrastructure of double spore wall of the resting spore, immature (C), and mature spore (D). Thin outer layer (w1), wide inner layer (w2), c: Cytoplasm. X30000.

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for the sequence analyses (the SSU alignment used can be found as Supplement 1) since the SSU rRNA gene was only partially sequenced for several of the other published entomophthoralean species that we wanted to include.

The SSU rRNA sequence from the Argentinean resting spores clustered together with those from conidial material of *P. neoaphidis* (Fig 2) with bootstrap values above 98 % and 100 % in the NJ analysis (data not shown).

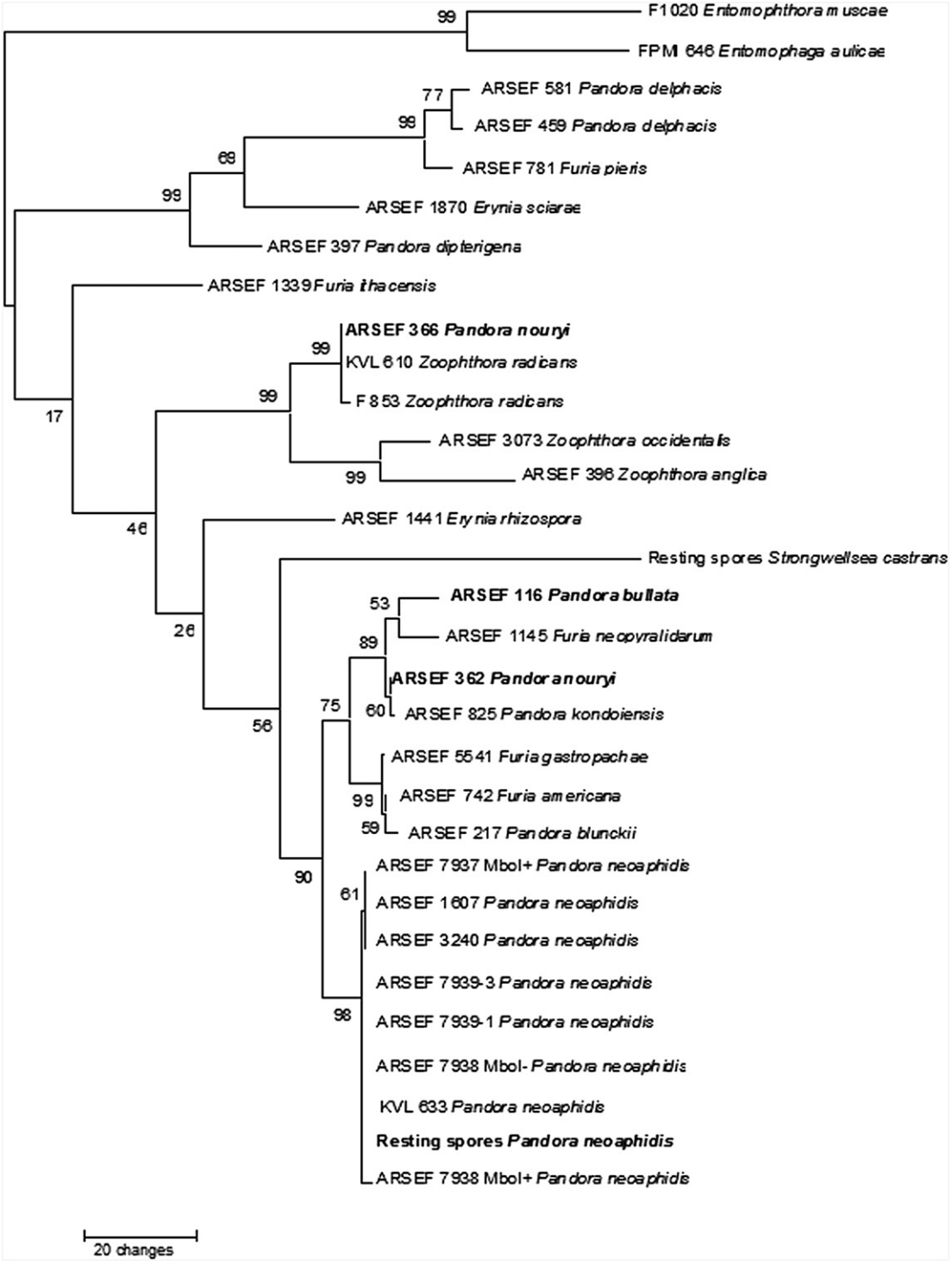

Fig 2 – Taxonomic relationships among members of *Entomophthoraceae* with uninucleate spores inferred from parsimonious analysis of 1080 positions of the nuclear SSU RNA gene. The tree is one out of 20 most parsimonious trees which require 562 steps with a consistency index = 0.640 and retention index = 0.810. Bootstrap percentages from 1000 replicates are shown above each of the major branches. GenBank accession numbers are given before each species name, for additional information see Table 1. The scale bar corresponds to 20 nucleotide changes.

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The size of the amplified ITS region varied among the different species included in this analysis from *ca* 1000–1500 bp (Table 1). Amplicons of the resting spores from both aphids had a length of 1080 bp, as did those from all the tested *P. neoaphidis* isolates. We obtained ITS sequences from the resting spores of both aphids, and these resting spore sequences were identical. When the sequences from the resting spores were blasted *P. neoaphidis* came out with 99 % nucleotide identity with all the 18 deposited *P. neoaphidis* sequences. *Pandora kondoiensis* followed with only 87 % nucleotide identity of the part that could be aligned. The variation of the whole ITS sequences within *Entomophthoraceae* and even within species that produce uninucleate conidia was too high for making unambiguous alignments. Most of the variation and also the ITS size differences were found in the ITS I region, and therefore only the ITS II region (including the 5.8s rRNA gene covering in all 585 positions including gaps) was used for the sequence analyses corresponding to 490–1030 bp of the GenBank sequence AF543202 of the *P. neoaphidis* isolate NW 343. Based on the SSU rRNA analysis we chose the *Zoophthora* clade as outgroup (the ITS II alignment used can be found as Supplement 2).

In the sequence analyses of the ITS II region the resting spore sequences clustered with those of *P. neoaphidis* (Fig 3) with bootstrap values above 99 % (100 % in the NJ analysis).

Discussion

The major finding of this study is that the resting spores of *Pandora neoaphidis*, the most important entomophthoralean pathogen of aphids worldwide, have been found from field-infected aphids that were collected in La Plata, Argentina.

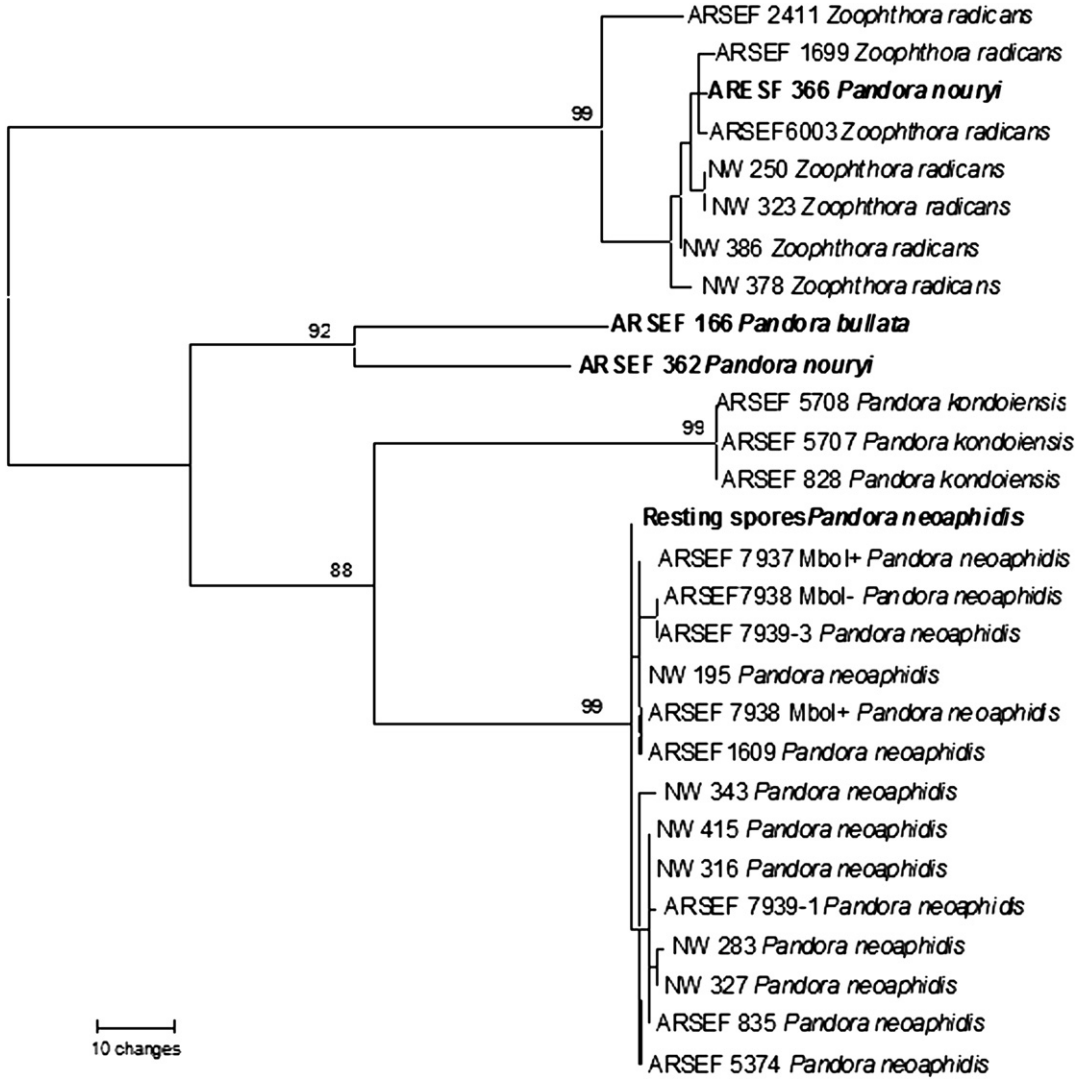

Fig 3 – Taxonomic relationships among members of *Entomophthoraceae* with uninucleate spores inferred from parsimonious analysis ITS II region including 588 positions of which 187 were informative. The tree is one out of 270 most parsimonious trees which require 270 steps with a consistency index = 0.861 and retention index = 0.969. Bootstrap percentages from 1000 replicates are shown above each of the major branches. GenBank accession numbers are given before each species name, for additional information see Table 1. The scale bar corresponds to ten nucleotide changes.

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Except for the report by Scorsetti *et al.* (2007) that is placed in its appropriate perspective by the findings reported here, no previous publication on the taxonomy or biology of this species has provided any significant information about the morphology – or even the existence – of resting spores for this nearly ubiquitous species.

In the literature, the studies of overwintering survival by *P. neoaphidis* focused on the conidial state (Brobyn *et al.* 1985; Morgan 1994; Nielsen *et al.* 2003). Despite several hypotheses about the morphological structures that might be responsible for the winter survival of this fungal species, there were hints in Scorsetti *et al.* (2007) that *P. neoaphidis* could produce resting spores in field-infected aphids, but there were not enough spores in those collections to allow gene-based confirmation of their identification as *P. neoaphidis*. Uziel & Kenneth (1986) suggested that a culture of *P. neoaphidis* produced what they believed to be resting spores in completely uncontrolled laboratory conditions, but the sizes of these structures (average diameter of 74.2 μm, range 52–119 μm) were substantially larger than any other resting spores reported within *Entomophthorales*, and the nature of these structures remains uncertain.

In the present study we never found either hyphal bodies or loricoconidia as environmentally resistant structures in *P. neoaphidis*-infected aphids as had been reported by other authors (Feng *et al.* 1992; Nielsen *et al.* 2003), but did find the presence of spores with the thick, double-layered walls that are characteristic of all entomophthoralean resting spores as the presumptive overwintering structures, and these thick-walled spores were collected only during the mid-winter. Apart from the molecular evidence presented here, the morpho-developmental evidence also confirms that these *P. neoaphidis* spores must be resting spores. Most of these spores formed inside the aphid bodies although some were superficial on the cadaver; their walls were distinctly double-layered; and their shapes were spherical rather than elongated (in no way similar to *P. neoaphidis* conidia). As defined by Weiser & Batko (1966), loricoconidia represent a single-layered thickening of a conidial wall that necessarily reflect the shapes of the conidia from which they form, and they can occur only where conidia are (or were) also present. The conidia of *P. neoaphidis* form exclusively on the surface of – and never inside – an infected host, and as in most entomophthoralean entomopathogens, no conidia were formed on the infected hosts where the resting spores formed.

The study site in Buenos Aires province is located in the Pampeana phytogeographic region of Argentina (Cabrera & Willink 1973), and it is important to note that this site has a temperate climate with an average annual temperature of 18 °C and winter temperatures generally within the range of 3–8 °C. Zhou & Feng (2010) demonstrated that lower temperatures and longer daylight may result in more resting spore production by *Pandora nouryi* in host cohorts at a given spore concentration. The positive effect of lower temperature on the resting spore formation agrees with most previous reports on the same phenomenon for other *Entomophthorales* (Steinkraus & Kramer 1989; Feng *et al.* 1992; Pell *et al.* 2001). Survival structures often also require periods of cold in order to be able to germinate (Hajek 1997). The exact requirements for dormancy and for initiation of germination and conidiation are poorly understood for almost all entomophthoraleans (Nielsen 2002), and they have remained completely unknown for *P. neoaphidis*.

This study of Argentinean aphids presents the first genomically verified evidence that *P. neoaphidis* is, indeed, able to produce resting spores. To learn more about the environmental (or other) conditions that allowed the production of this previously unobserved spore state should be a high priority. Progress on these issues will provide key insights about the field ecology of this fungus and the biological mechanisms controlling the initiations of epizootics of this aphid pathogen in Argentina and throughout the world. More studies are needed to locate further natural instances of these resting spores, to determine what conditions might favour the breaking of the (presumed) dormancy of fully mature spores, and whether there might be any means to manipulate the formation and germination of these resting spores to advance the practical application of *P. neoaphidis* as a potent biological control agent against some of the most significant insect pests of agricultural crops.

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Supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.funbio.2011.11.002.

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