Accepted Manuscript

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PII: S1878-6146(11)00225-X

DOI: 10.1016/j.funbio.2011.11.002

Reference: FUNBIO 244

To appear in: *Mycological Research*

Received Date: 17 December 2010

Revised Date: 4 November 2011

Accepted Date: 7 November 2011

Please cite this article as: Scorsetti, A.C., Jensen, A.B., Lastra, C.L., Humber, R.A., First report of *Pandora neoaphidis* resting spore formation *in vivo* in aphid hosts, *Mycological Research* (2011), doi: 10.1016/j.funbio.2011.11.002

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 percent in the SSU rRNA sequence analysis and with bootstrap values above 99 percent the 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.

Key words: Entomopathogenic fungi, Entomophthorales, *Pandora neoaphidis*, resting spores, winter survival.

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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 world-wide distribution, and is well known from Europe, Asia, Africa, South America and Australia (Glare and Milner 1991; Wilding and Brady 1984; Hatting et al. 2000; Scorsetti et al. 2007). *P. 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 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 days after incubation at 5ºC 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 and 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 *E. muscae* species complex could each complete their entire life cycle in a single dipteran host species. Comparing sequences of the ribosomal repeat (SSU and 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. 18ºC.

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 cm3). 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 20º 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).

P. neoaphidis-infected aphids were stored at 4 ºC for one month in 60 mm Petri dishes with a moistened filter paper, and then examined using a stereoscopic microscope every 72 hours 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) and observed on an Olympus CH3 microscope. Fungal preparations were observed on an Olympus BX51 microscope and photographed using an Olympus DP71 camera.

Semi-permanent 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 4ºC in darkness until either extraction of the DNA or fixation for transmission electron microscopy.

Electron microscopy

For transmission electron microscopy, thick-walled spores from a mechanically disrupted infected aphid were fixed for 2 hours in 3% glutaraldehyde, post-fixed for 1 hour 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) transmission electron microscope.

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 x G. The supernatants were discarded and 100 µl MilliQ water was added to each preparation. Five µl 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 µl 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 *P. nouryi* ARSEF 362, *P. nouryi* ARSEF 366 and *P. bullata* ARSEF 116, following the manufacturer's instructions. The three *in vitro* cultures were obtained from the ARSEF the USDA-ARS Collection of Entomopathogenic Fungal Cultures 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 and DePriest 1996) were used to amplify the nuclear small subunit ribosomal RNA gene (SSU rRNA) 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 Internal Transcribed Spacer (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 x 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 and 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 and ITS4 (White et al., 1990), Nu-5.8S-5' (Jensen and 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 six (n=4) or nine (n=2) days 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 *L. 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 transmission electron microscopy (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. 1 a,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 color 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. 1 c,d).

We obtained a sequence of the SSU rRNA gene from resting spores of one aphid. The closest BLAST correspondences (those with 99 to 100% nucleotide identify) of the 1800 bp sequence from these resting spores were with eight different *P. 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 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 percent and 100 percent in the NJ analysis (data not shown).

The size of the amplified ITS region varied among the different species included in this analysis from ca. 1000 to 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.*

neoapidis 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 percent nucleotide identity with all the 18 deposited *P. neoaphidis* sequences. *P. kondoiensis* followed with only 87 percent 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 percent (100 percent 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. 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 and 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 and 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 entomphthoralean 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 and Willink 1973), and it is important to note that this site has a temperate climate with an average annual temperature of 18º C and winter temperatures generally within the range of 3–8º C. Zhou and Feng (2010) demonstrated that lower temperatures and longer daylight may result in more resting spore production by *P. 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 and 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 favor 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.

Acknowledgments

This study was partially supported by the National Research Council of Argentina (CONICET) (PIP 0049) and University of La Plata (UNLP). We also thank two anonymous reviewers for helpful comments on the manuscript.

References

Bałazy S, 1993. Entomophthorales. *Flora of Poland (Flora Polska) Fungi (Mycota), vol. 24*, Polish Academy of Science W. Szafer Institute of Botany, Krakow.

Baverstock J, Baverstock KE, Clark SJ, Pell JK, 2008a. Transmission of *Pandora neoaphidis* in the presence of co-occurring arthropods. *Journal of Invertebrate Pathology* **98**: 356-359.

Baverstock J, Clark SJ, Pell JK. 2008b. Effect of seasonal abiotic conditions and field margin habitat on the activity of *Pandora neoaphidis* inoculum on soil. *Journal of Invertebrate Pathology* **97**: 282-290.

Brobyn P, Wilding N, Clark S, 1985. The persistence of infectivity of conidia of the aphid pathogen *Erynia neoaphidis* on leaves in the field. *Annual Applied Biology* **107**: 365-376.

Cabrera AL, Willink A, 1973. *Biogeografía de América Latina*. Secretaría General de la Organización de los Estados Americanos, Washington, D.C.

Feng MG, Nowierski RM, Klein RE, Scharen AL, Sands DC, 1992. Spherical hyphal bodies of *Pandora neoaphidis* (Remaudière & Hennebert) Humber (Zygomycetes: Entomophthorales) on *Acyrthosiphon pisum* (Harris) (Homoptera: Aphididae): A potential overwintering form. *Pan-Pacific Entomology* **68**: 100-104.

Gargas A, DePriest PT, 1996. A nomenclature for fungal PCR primers with examples from intron-containing SSU rDNA. *Mycologia* **88**: 745–748.

Glare TR, Milner RJ, 1991. Ecology of entomopathogenic fungi. In: Arora DK, Ajello L, Mukerji KG (eds.), *Handbook of Applied Mycology*, vol 2: *Humans, Animals, and Insects*. Marcel Dekker, New York, pp. 547-612.

Hajek AE, 1997. Ecology of terrestrial fungal entomopathogens. *Advances in Microbiological Letters* **15**: 193-249.

Hatting JL, Poprawski TJ, Miller RM, 2000. Prevalences of fungal pathogens and other natural enemies of cereal aphids (Homoptera: Aphididae) in wheat under dryland and irrigated conditions in South Africa. *BioControl* **45:** 179–199.

Humber RA, 1989. Synopsis of a revised classification for the Entomophthorales (Zygomycotina). *Mycotaxon* **34:** 441–460.

Humber RA, 1997. Fungi: Identification. In: Lacey L, (ed.), *Manual of Techniques in Insect Pathology*. Academic Press, San Diego, pp. 153-185.

Jensen AB, Eilenberg J, 2001. Genetic variation within the insect-pathogenic genus *Entomophthora*, focusing on the *E. muscae* complex, using PCR-RFLP of the ITS II and the LSU rDNA. *Mycological Research* **105**: 307–312.

Jensen AB, Eilenberg J, Lopez Lastra C, 2009. Differential divergences of obligately insect-pathogenic *Entomophthora* species from fly and aphid hosts. *FEMS Microbiology Letters* **300**: 180–187.

Keller S, 1987. Arthropod-pathogenic Entomophthorales of Switzerland. I. *Conidiobolus*, *Entomophaga* and *Entomophthora*. *Sydowia* **40**: 122–167.

Keller S, 1991. Arthropod-pathogenic Entomophthorales of Switzerland. II. *Erynia*, *Eryniopsis*, *Neozygites*, *Zoophthora*, and *Tarichium*. *Sydowia* **43**: 39–122.

Morgan LW, 1994. Survival, germination responses and infectivity of conidia of *Erynia neoaphidis* (Zygomycetes: Entomophthorales). PhD thesis, University of Wales, Cardiff, UK, 187 pp.

Nielsen C, 2002. Interactions between aphids and entomophthoralean fungi. Characterisation, epizootiology and potential for microbial control. Ph.D. Thesis. Department of Ecology. The Royal Veterinary and Agricultural University, Copenhagen, Denmark.

Nielsen C, Hajek AE, Humber RA, Bresciani J, Eilenberg J, 2003. Soil as an environment for survival of aphid-pathogenic Entomophthorales. *Biological Control* **28**: 92-100.

Nielsen C, Jensen AB, Eilenberg J, 2007. Survival of entomophthoralean fungi infecting aphids and higher flies during unfavourable conditions and implications for conservation biological control. In: Ekesi S, Maniania NK (eds.), *Use of Entomopathogenic Fungi in Biological Pest Management*. Research Signpost, Trivandrum, India, pp. 13-38.

Pell JK, Eilenberg J, Hajek AE, Steinkraus DC, 2001. Biology, ecology and pest management potential of Entomophthorales. In: Butt TM, Jackson CW, Magan N (eds.), *Fungi as Biocontrol Agents: Progress, Problems and Potential,* CABI Publications, Wallingford, Oxon, UK, pp. 71-153.

Schofield G, Pell JK, Harrington R, 1995. Overwintering of the entomophthoralean fungus *Erynia neoaphidis* on foliage under and laboratory conditions. In: Program and Abstracts, Ann. Mtg. Society for Invertebrate Pathology, Ithaca, New York, 16-21 July 1995, p. 54.

Scorsetti AC, Humber RA, Garcia JJ, Lopez Lastra CC, 2007. Natural occurrence of entomopathogenic fungi (Zygomycetes: Entomophthorales) of aphid (Hemiptera: Aphididae) pests of horticultural crops in Argentina. *BioControl* **52:** 641–655.

Scorsetti AC, Macia A, Steinkraus DC, Lopez Lastra CC, 2010. Prevalence of *Pandora neoaphidis* (Zygomycetes: Entomophthorales) infecting *Nasonovia ribisnigri* (Hemiptera: Aphididae) on lettuce crops in Argentina. *Biological Control* **52**: 46-50.

Steinkraus DC, Kramer JP, 1989. Development of resting spores of *Erynia aquatica* (Zygomycetes: Entomopthoraceae) in *Aedes aegypti* (Diptera: Culicidae). *Environmental Entomology* **18**: 1147-1152.

Tamura K, Dudley J, Nei M, Kumar S, 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**:1596-1599.

Thomsen L, Jensen AB, 2002. Application of nested-PCR technique on resting spores from the *Entomophthora muscae* complex: implications for analyses of host-pathogen population interactions. *Mycologia* **94**: 794-802.

Uziel A, Kenneth RG, 1986. *In vitro* resting-spore formation in *Erynia neoaphidis*. In: Samson RA, Vlak JM, Peters D (eds.), *Fundamental and Applied Aspects of Invertebrate Pathology*, Proc. Intern. Colloq. Insect Pathol., Veldhoven, The Netherlands, p. 230.

Weiser J, Batko A, 1966. A new parasite of *Culex pipiens* L., *Entomophthora destruens* sp. nov. (*Phycomycetes, Entomophthoraceae*). *Folia Parasitologica (Praha)* 13, 144-149.

White TJ, Bruns TD, Lee SB, Taylor JW, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JS, White TJ (eds), *PCR Protocols: A Guide to Methods and Applications*, Academic Press, San Diego, pp. 315–321.

Wilding N, 1970. The effect of temperature on the infectivity and incubation periods of the fungi *Entomophthora aphidis* and *E. thaxteriana* for the pea aphid *Acyrthosiphon pisum*. In: Proc. 4th Internat. Colloq. Insect Pathology, College Park, Maryland, p. 84-88.

Wilding N, Brady BL, 1984. Descriptions of pathogenic fungi and bacteria. Set 82, Nos. 812, 814, 815, 817, 820, Commonwealth Mycological Institute, UK.

Zhou X, Feng MG, 2010. Biotic and abiotic regulation of resting spore formation in vivo of obligate aphid pathogen *Pandora nouryi*: Modeling analysis and biological implication. *Journal of Invertebrate Pathology* **103**: 83-88.

Figure captions:

Figure 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.

Figure 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 = 0.6405 and retention index = 0.8105. 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.

Figure 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 = 0.8613 and retention index = 0.9693. 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 10 nucleotide changes.

Table captions:

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

Table(s)

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 [a]	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

Figure 1

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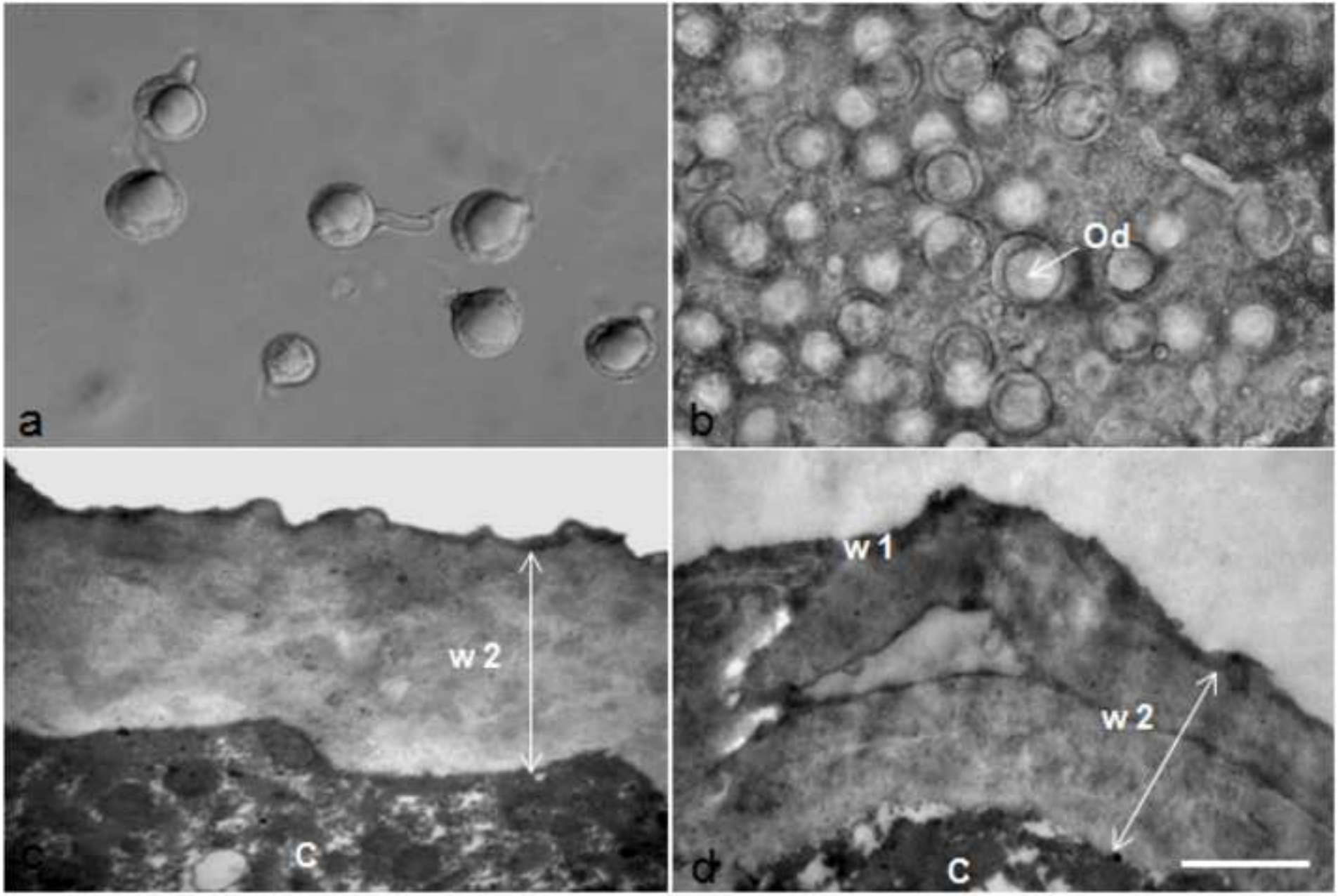

Figure 2

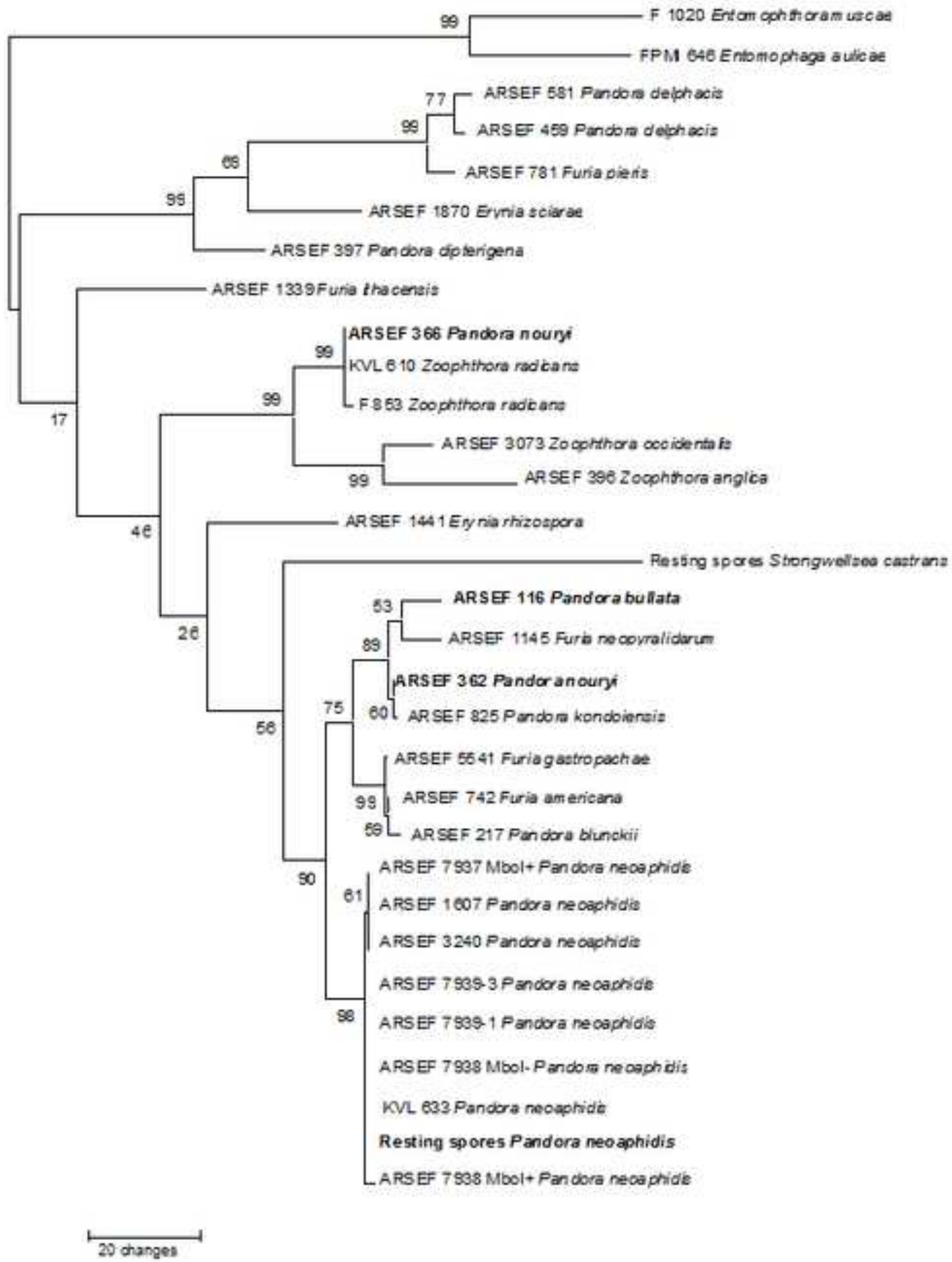

Figure 3

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