Journal of Invertebrate Pathology 143 (2017) 108–114

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Pandora formicae, a specialist ant pathogenic fungus: New insights into biology and taxonomy

Joanna Małagocka *, Annette Bruun Jensen, Jørgen Eilenberg

Centre for Social Evolution, Department of Plant and Environmental Science, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark

ARTICLE INFO

Article history:
Received 11 February 2016
Revised 14 December 2016
Accepted 15 December 2016
Available online 18 December 2016

Keywords:
Formica polyctena
Pandora myrmecophaga
Field prevalence
Entomophthorales taxonomy
Resting spores

ABSTRACT

Among fungi from the order Entomophthorales (Entomophthoromycota), there are many specialized, obligatory insect-killing pathogens. *Pandora formicae* (Humber & Bałazy) Humber is a rare example of an entomophthoralean fungus adapted to exclusively infect social insects: wood ants from the genus *Formica*. There is limited information available on *P. formicae*; many important aspects of this host-pathogen system remain hitherto unknown, and the taxonomical status of the fungus is unclear. Our study fills out some main gaps in the life history of *P. formicae*, such as seasonal prevalence and overwintering strategy. Field studies of infection prevalence show a disease peak in late summer and early autumn. Typical thick-walled entomophthoralean resting spores of *P. formicae* are documented and described for the first time. The proportion of cadavers with resting spores increased from late summer throughout autumn, suggesting that these spores are the main overwintering fungal structures. In addition, the phylogenetic status of *Pandora formicae* is outlined. Finally, we review the available taxonomical literature and conclude that the name *P. formicae* should be used rather than the name *P. myrmecophaga* for ant-infecting fungi displaying described morphological features.

1. Introduction

Entomopathogenic fungi from the order Entomophthorales infect a variety of arthropod hosts; among these fungi, there is one known example of a fungus infecting social Hymenoptera, wood ants of the genus *Formica*. Infected ants display symptoms of the so-called summit disease before dying, i.e. they are manipulated by the fungal pathogen to climb vegetation, lock mandibles on its surface, and die in biting position above ground. The attachment is further reinforced by legs grasping the vegetation and rhizoids produced by the fungus. This phenomenon, known from other entomophthoralean fungi (Maitland, 1994; Carruthers et al., 1997) has been observed in ants by myrmecologists (Boer, 2008; Marikovsky, 1962; Turian and Wuest, 1977, 1969), parasitologists (Loos-Frank and Zimmermann, 1976) and by mycologists (Bałazy and Sokołowski, 1977). Despite the many observations of fungus-killed *Formica* ants, many basic features of *Pandora* remain unclear. It is of particular importance to elucidate how an entomophthoralean fungus interacts with a social insect host, because generally, specialized pathogens are rare in social insects, due to effective defenses against pathogens that had evolved in insect societies (Cremer et al., 2007). The majority of entomophthoralean fungi are obligatory pathogens, incapable of surviving outside host tissues in nature, that survive by horizontal passage from an infected host to an uninfected host when the hosts are available (Roy et al., 2006). In adverse conditions, when new hosts are unavailable, these fungi can survive by forming resting spores (or more rarely loricoconidia), thick-walled structures capable of persisting in the soil or host cadaver during the winter, and germinating into infective conidia in the spring (Bałazy, 1993; Nielsen et al., 2003; Scorsetti et al., 2012). Alternatively, the fungi can remain within the host, hibernating or dead, in the form of hyphal bodies (Keller, 1987). Recently, a third strategy for survival: slow conidial transmission in a group of hibernating hosts, was discovered (Eilenberg et al., 2013).

Colonies of *F. polyctena*, a dominant, territorial species, achieve impressive numbers of individuals that explore the surroundings of their nest (or interconnected nests) with an effective system of foraging trails connecting them to the aphid populations they tend, mostly on spruce trees (Buhl et al., 2009; Collingwood, 1979; Ellis and Robinson, 2014). Wood ant workers, even though they do not form discreet castes, can be divided into different task groups, with specialized foragers collecting aphid honeydew, collecting prey, scouts and nest workers (Domisch et al., 2009; Reznikova, 2011; Rosengren, 1977; Savolainen and Vepsalainen, 1989).

* Corresponding author.
E-mail address: jopi@plen.ku.dk (J. Małagocka).

http://dx.doi.org/10.1016/j.jip.2016.12.007
0022-2011/

Wood ant-infecting entomophthoralean fungi have been observed in large parts of Europe: Sweden, Denmark, the Netherlands, Switzerland, Germany, Russia, Romania, Poland, Czech Republic and Slovakia (Bałazy, 1993; Boer, 2008; Csata et al., 2013; Małagocka et al., 2015; Sosnowska et al., 2004). The known host range in these areas includes species of *Formica*: *F. rufa* L., *F. polyctena* Förster, *F. fusca* L. (original record by Turian and Wuest (1977) is interpreted as *F.* cf. *lemani* Bondroit by Boer (2008)), *F. pratensis* Retzius and *F. exsecta* Nylander. Infected *F. exsecta* ants have also been found in Finland (D. P. Hughes and A. B. Jensen, personal communication). However, these studies are observational (qualitative), without stratified prevalence sampling and with no records of winter survival structures.

In recent years, *P. formicae* infections of red wood ants *F. polyctena* have been observed in a stable ecosystem in a forest with dense population of ants (Małagocka et al., 2015). Ants killed by the fungal infection were found fixed to and biting grasses and twigs, mostly around wood ant nests and close to foraging trails (unpublished data), attached to vegetation with rhizoids. These cadavers subsequently produced a dense cushion of fungal conidiophores, actively shooting infectious conidia over ants passing below. Because entomophthoralean fungi need high relative humidity to produce conidia (Steinkraus, 2006), it is rather unlikely that they could develop inside a moisture-regulated, wood ant nest (Coenen-Stass et al., 1980). Therefore, we speculate that it is only in the nest surroundings that *P. formicae* can sporulate, and that the fungus is transmitted only among the outside workers. Because the cadavers were found mostly around the nest and foraging trails, we set to investigate if there was a difference in infection levels during the summer and autumn season between three cohorts; (1) ants active on the trail, (2) ants on the nest surface and (3) ants present in the area around the nest, but not on a trail.

The phylogenetic status of the fungus has not been studied so far, so even though conidia morphology and other morphological features place it within the genus *Pandora*, the specific position of the ant-infecting fungus is unknown. Also, one of the basic, unresolved questions is whether there is actually one or two different species from the genus *Pandora* Humber infecting *Formica* in Europe; *P. myrmecophaga* (Turian & Wuest) Keller and *P. formicae* (Humber & Bałazy) Humber are two valid names, but some authors argue that it is only one species (Bałazy, 1993; Keller, 1991).

The aim of our studies was to: (1) obtain data on seasonal trend in field prevalence of the fungus in ants sampled around the nest, on foraging trails or outside these areas, (2) document, if found, winter survival fungal structures, (3) clarify the phylogenetic and nomenclatural status of the fungus. In the current paper, findings about the seasonal prevalence and overwintering strategy will be shown and discussion of the nomenclatural status of the fungus will be presented.

2. Materials and methods

2.1. Prevalence assessment

Ants were sampled in 2011 (after initial observations and preliminary data gathered in 2009 and 2010), in Bidstrup Forest near Hvalsø in Middle Zealand, a managed forest with a mixture of spruce and beech stands. The climate in the area is, typically for Denmark, mild with warm and humid summer, with average temperatures in July and August around 20 °C during day and 10 °C during night, falling to ca 16 °C/8 °C in September and 12 °C/5 °C in October and rather mild cold season (Danish Meteorological Institute, www.dmi.dk). The studied ant colony consisted of two connected nests situated in a shady grass patch among spruce and pine trees, close to the forest road and a clearing (55.579242 N, 11.879287 E). There were four major foraging trails coming out of the nest.

To assess prevalence among foraging workers, the samplings were done five times throughout the 2011 season: on July 5th, August 1st, August 31st, October 10th, November 9th. One hundred individuals from each of three cohorts were sampled: (1) "trail" = foragers walking on foraging trails around 5 m from the nest, (2) "nest" = ants dwelling on nest surface, and (3) "scattered" = individual ants exploring a grass patch that was not on the foraging trail, 2–10 m from the nest. In result, three hundred ants were sampled each time, except for the November sampling, when only 50 individuals from the "trail" and "nest" cohorts were sampled (100 ants total). Overall, 1300 ants were sampled and incubated throughout the experiment. The cohorts from the foraging trail and from the nest surface were sampled by putting down a thin white lignin tissue – ants quickly latched on the tissue, and then the tissue was gently placed in a plastic bag with some moist moss and grass; this method minimized ant handling and stress. Ants scattered in the grass patch were put in a similar plastic bag, but sampled individually by gently picking up with insect tweezers. Each bag normally contained less than 25 individuals.

Upon arrival to the laboratory, bags with ants were immediately put in the refrigerator at 4 °C to avoid further disturbance to the ants and possible self-inflicted formic acid damage. When the ants calmed down, they were each placed in individual 30 ml plastic medicine cups with a layer of 2% water agar added for keeping moisture, with the intention of maximizing relative humidity to aid fungal growth, but avoid condensation. A drop of honey at the side of the cup was placed as food source. They were then kept for two weeks in a temperature-controlled room (20 °C ± 1), in natural photoperiod, and checked daily or every second day for mortality. Ants killed by *P. formicae* could easily be distinguished by a characteristic pattern of white bands growing through intersegmental membranes and other soft parts of the cuticle, and by a visible halo of discharged conidia in the cup.

Infection level is defined here as the percentage of cadavers that were killed by the fungus during incubation. The differences in infection levels between the sampled cohorts per sampling date were tested with a test of equal proportions in R statistical software (R Core Team, 2013).

2.2. Morphological observations

In the area surrounding the nests and close to the foraging trails, ants killed by *P. formicae* were frequently observed attached to grass blades, moss stems, short fallen spruce twigs and similar. These were sampled throughout the season, at different stages of fungal growth, for observation and morphological measurements of fungal structures. Ants killed by *P. formicae*, both sampled directly from the field and dead while incubated in medicine cups, were examined for morphological traits under a light microscope (Olympus AX70 Povis). Slides with fungal material were fixed with lactic acid, observed and measured, in phase contrast or differential interference contrast.

2.3. DNA extraction, PCR, sequencing and phylogenetic analysis

To obtain *P. formicae* DNA, whole infected ants were ground in liquid nitrogen with a mortar and pestle, and total DNA was extracted with DNeasy Plant Mini Kit (QIAGEN). Universal fungal primers ITS4 and ITS5 were used for amplification of ITS (internal transcribed spacer) region (White et al., 1990). Conditions for the PCR were as follows: initial denaturation for 3 min at 94 °C followed by 35 cycles of denaturation (30 s, 94 °C), annealing (30 s, 54 °C) and elongation (30 s, 72 °C) and finally 5 min of elongation in 72 °C. PCR products were visualized on a 1% agarose TAE gel

with EZvision One® (Amresco). Successfully amplified fragments were cleaned using Qiaquick® PCR purification kit (QIAGEN) and sent to Beckman Coulter Genomics (Essex, United Kingdom) for sequencing.

ITS sequences of two *P. formicae* isolates: JM02004 (from the study colony in Bidstrup) and JM04005 (sampled in a forest near Asserbo in North Zealand, ca 55 km from the study site) were used as seed for nucleotide BLAST, and sequences with highest similarity were used for phylogenetic tree construction. Sequence alignment was performed using MUSCLE (Edgar, 2004) with default settings and adjusted manually. Phylogenetic analysis of selected sequences was done in MEGA5 (Tamura et al., 2011); phylogenetic trees were constructed using Maximum Parsimony method, and branch support was estimated with bootstrap values (1000 replicates). The first out of three most parsimonious trees is shown. Overall, 13 sequences were analyzed. After eliminating positions with gaps and missing data, there were a total of 558 nucleotide positions in the final dataset.

3. Results

3.1. Morphological features of conidia and resting spores

All dead, infected specimens collected in the field were found attached to vegetation by a fascicle of thick rhizoids, clearly visible to the naked eye as shiny, transparent threads, terminating in discoid holdfasts (Fig. 1). Ants that died during incubation were also producing these structures. The fungus produced typical *Pandora*-like, ovoid, slightly tapering primary conidia, measuring on average 19.8 μm (±1.1) in length and 12.1 μm (±1.1) in width. The resting spores were smooth-walled, globose, and measuring on average 26.6 μm in diameter (±2.3) (Fig. 2). Their appearance under stereo microscope was shiny, orange[1] or yellowish in color, visibly different from the white, smooth cushion of conidiophores.

3.2. Seasonal prevalence of infection

In July, the infection was present, but prevalence was low compared to following months of the season, and no significant differences between the three cohorts were detected (Table 1). An increase of infection prevalence started later in the summer, with a peak in October. Infections were present also very late in the season, when there were still some ant workers active outside the nest. From August and until November, the highest prevalence was found in the ants sampled from the nest surface; also overall through the season, ants collected from the nest surface had significantly higher levels of infection than ants sampled on foraging trails (Table 1).

3.3. Winter survival: first record of resting spores

Among ants sampled live in the field and incubated in medicine cups, few infected specimens started producing resting spores in October (Fig. 3). These resting spores were found on the surface of ant cadavers, just where conidiophores and conidia are normally present. We did not observe resting spores inside of the dead ant; fungal infection leaves the exoskeleton empty. In November, the majority of infected ants produced these resistant structures during incubation in the laboratory, including 10 out of 11 infected ants from the cohort sampled around the nest. We did not see a case where only resting spores were produced; some conidia were always present in cadavers with resting spores.

[1] For interpretation of color in Fig. 2, the reader is referred to the web version of this article.

3.4. Sequencing and phylogenetic analysis

Sequencing resulted with fragments 852 and 856 base pair long, containing ITS1 (incomplete), 5.8S, ITS2, and LSU (incomplete) fragments of rDNA. They have been deposited in GenBank with accession numbers KY006579–KY006580; there was no difference in ITS sequence between these Danish strains. Sequence comparison and phylogenetic tree construction revealed that the fungus infecting ants is indeed placed within the genus *Pandora*; first of three most parsimonious trees is shown in Fig. 4. Sequences of *P. formicae* form a branch together with *P. bullata* and *P. nouryi*, which are pathogens of Diptera and Hemiptera, respectively.

4. Discussion

4.1. Seasonal prevalence and overwintering strategy – infected ants found close to the nest

Ants collected from the nest surface had the highest disease prevalence compared to ants collected from the trails or further away from the colony, which indicates that sick ants tend to stay close to the colony focal point. One explanation could be fungal manipulation; it has been shown for other entomophthoralean/host systems that fungi can manipulate their hosts to die in a place most favorable for disease transmission (Roy et al., 2006), and certainly summit behavior itself serves more effective conidia spread by increasing the range of conidial shower. One could speculate that not only the height above ground, but also locality of death are pathogen-manipulated. This is the case in distantly related fungal pathogens of ants, namely ascomycetes from the genus *Ophiocordyceps*: carpenter ants killed by *O. unilateralis* die in humid localities, attached to leaves quite far from their daily activities, where the conditions are optimal for fungal development (Andersen et al., 2009; Pontoppidan et al., 2009). Another possibility is that the ants actively choose location on the nest in relation to a phenomenon known as behavioral fever, an active increase in body temperature that can prevent disease development (Kalsbeek et al., 2001; Watson et al., 1993). Ant nest surface is typically warmer than surrounding areas (Frouz, 2000; Frouz and Finer, 2007), therefore it would be the optimal place to generate more body heat.

In addition, the highest proportion of infected ants producing overwintering resting spores later in autumn were found among ants sampled on the nest. It is therefore plausible to hypothesize that soil surrounding the nest acquires a considerable load of resting spores, which can initiate an infection cycle next year, like in the case of *Entomophaga maimaiga*-Gypsy moth system, where there is considerable amount of resting spores in the soil surrounding trees infected with moth larvae (Hajek et al., 1998). If *P. formicae* follows the same pattern as other entomophthoralean fungi, resting spores germinate when favorable conditions are again present and produce dischargeable infective conidia (Hajek and Humber, 1997). The dense traffic of workers going in and out of the nest for foraging increases the chances of the fungus to infect a new host. Also, the physical stability of the wood ant colony over years is favorable for *P. formicae*; the odds for a newly germinating resting spore to infect new host in the spring is potentially greater than in entomophthoralean species infecting more ephemeral hosts; persistent nest sites are one of the factors increasing disease risk in insect societies (Christe et al., 2002).

The discovery of resting spores produced in late autumn, mostly by infected ants found among the nest cohort, is the first documentation of resting spores in *P. formicae* and closes the gap in the knowledge about yearly cycle of the fungus. However, our experiments were not designed to study factors influencing resting spore

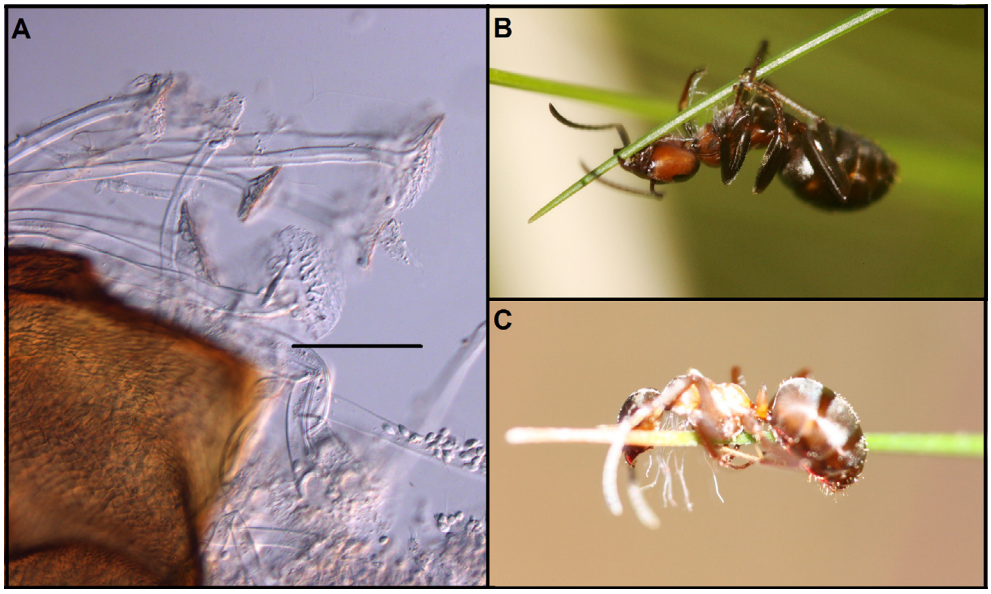

Fig. 1. Rhizoids produced by *P. formicae*. (A) Microscope slide showing discoid holdfasts (bar: 200 μm), (B and C) Ants killed by *P. formicae* and attached to grass straw with visible fascicle of rhizoids growing out from ventral side of the thorax.

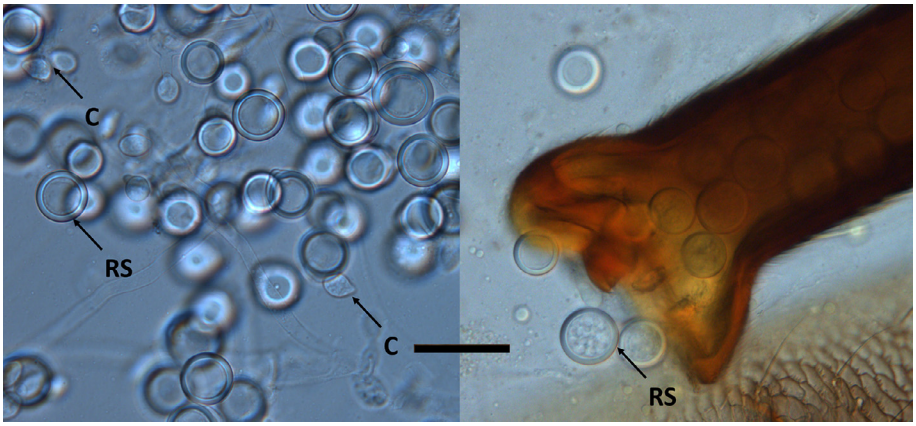

Fig. 2. Thick walled resting spores of *P. formicae*. Image on the left shows a mass of released mature resting spores (RS), with some conidia (C) also present. Image on the right shows resting spores (RS) in different degrees of maturity, released and inside of a detached ant leg. Scale bar = 50 μm.

Table 1
Prevalence of *P. formicae* infections by *F. polyctena* cohort. Each cohort consisted of 100 live sampled worker ants, incubated in the laboratory; 100 from the foraging trail, 100 from the nest surface and 100 ants scattered outside the dense traffic areas in the nest surroundings at each sampling date, except in November, where the cohort size was 50 and the scattered cohort was omitted. χ^2 statistic is given for a test of equal proportions among the three groups.

Sampling date	Trail (%)	Nest (%)	Scattered (%)	χ^2	p-value	df
5th July 2011	4	1	4	20,619	0.3567	2
1st August 2011	0	9	7	88,468	0.01199	2
31st August 2011	0	23	6	325,996	8.34E−05	2
10th October 2011	5	38	13	390,369	3.34E−06	2
9th November 2011[a]	6	22	NA	40,698	0.04366	1
2011 Season	4.70	18.20	7.50	497,088	1.61E−08	2

[a] Cohort size was 50, "scattered" cohort was omitted.

production; it was a discovery supplementing the study of yearly prevalence. We have never collected a cadaver with resting spores from the field, all observed cases come from laboratory incubation. It is known that both relative humidity and temperature can influence resting spore production in other entomophthoralean/host systems (Glare et al., 1989; Hajek and Shimazu, 1996). These two abiotic factors were kept constant during laboratory incubation. Only the photoperiod was natural in the setting, because the cups were kept in trays in temperature-controlled room, and not in a climate chamber. One possible explanation for the seasonal fluctuation in resting spore production is that the fungus developing within the host had already entered a specific developmental program, influenced by the environmental conditions prior to sampling day. Other possibility is that, in fact, the decrease in day

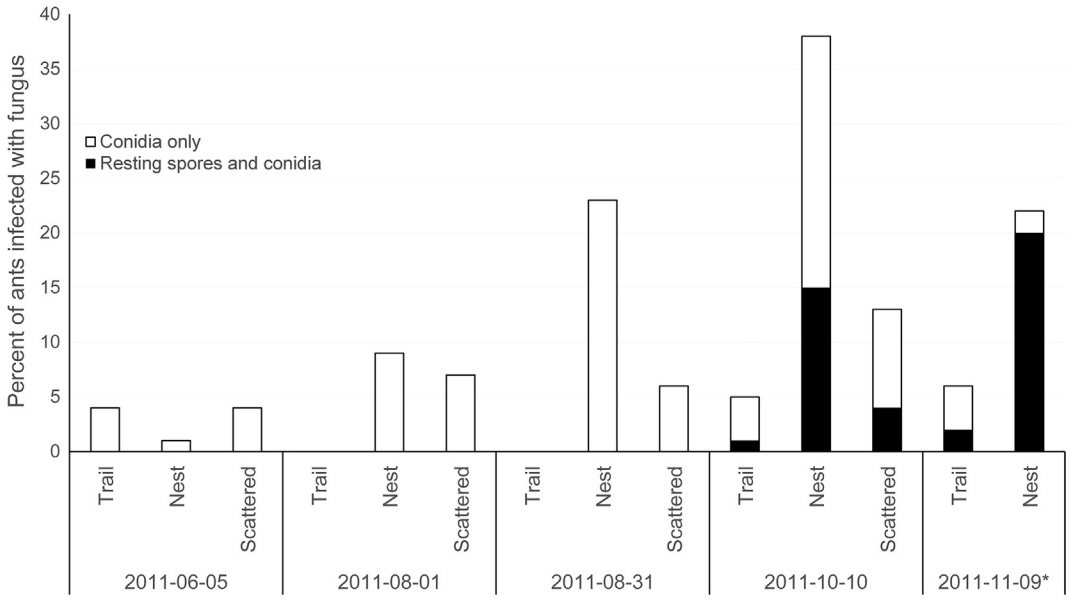

Fig. 3. Seasonal prevalence of *P. formicae* infections in *F. polyctena*. Percent of field-sampled live ants from three cohorts: nest, trail and scattered, killed by fungal infection during laboratory incubation, is shown. One hundred ants were sampled from each cohort ant each sampling date (300 ants per sampling date), except for November sampling (indicated with an asterisk), where only 50 ants from two cohorts, nest and trail, were sampled (100 total). White column area indicates cadavers producing conidia only, black indicates cadavers producing resting spores along with conidia.

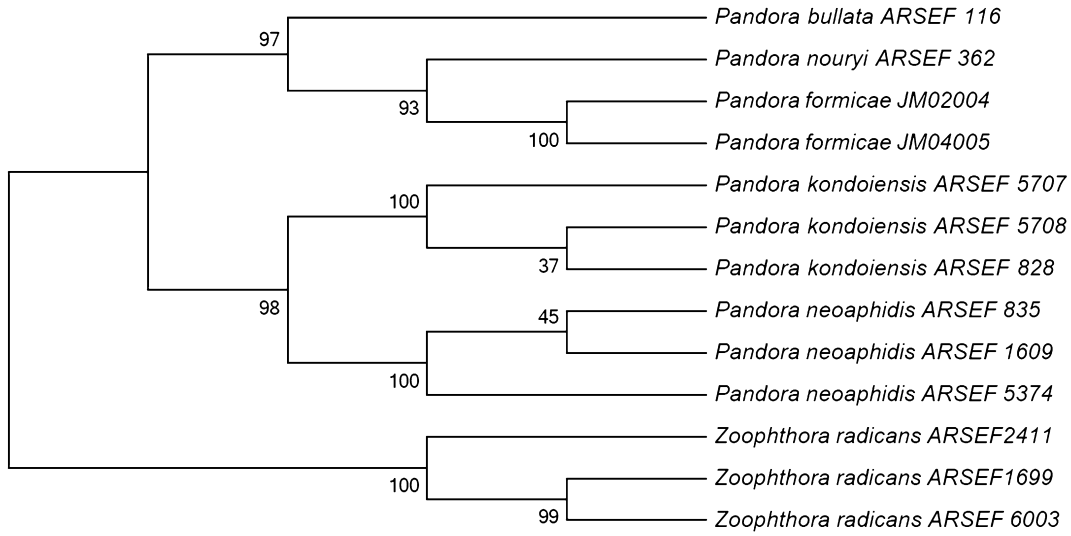

Fig. 4. One of three most parsimonious trees based on sequences of ribosomal RNA gene containing Internal Transcribed Spacer 1 (ITS1), 5.8S and ITS 2 regions, using Maximum Parsimony. Values on branches indicate bootstrap support (1000 replicates). There were 558 nucleotide positions in the analyzed dataset. Within the genus *Pandora*, *P. formicae* isolates form a branch with *P. nouryi* and *P. bullata*.

length is of primary importance, like in the case of *Entomophthora muscae* in cabbage flies (Thomsen and Eilenberg, 2000). It could also be that resting spores in nature are produced very rarely by *P. formicae*, and the specific combination of high humidity and stable temperature in the cups, together with decreasing day length, led to formation of these resistant structures.

As compared with life cycles of other entomophthoralean fungi infecting non-social hosts, *P. formicae*/*F. polyctena* system shares some of the main overall features: conidial production from fungus-killed hosts attached to vegetation and a seasonal trend of increasing fungal prevalence towards autumn, with also increasingly frequent resting spore production during autumn (Eilenberg and Michelsen, 1999). However, the social insect system with foraging labor casts gives a unique insight into spatio-temporal relationship between host, pathogen and environment, and could point to specific behavioral of physiological traits of the host that affect susceptibility. It is yet to be studied more in detail, how the significant differences in prevalence between ants sampled around nest, on trails and more scattered are related to behavior, working task, or age before infection of ant hosts.

4.2. Phylogenetic analysis

Analysis of ITS sequences confirmed the position of Danish ant-infecting fungus within the genus *Pandora*. In addition, morphological features of the fungus observed in Denmark are similar to those observed on *Pandora* infections in *Formica* ants in Germany, Poland, Russia and the Netherlands (Bałazy and Sokołowski, 1977;

Boer, 2008; Loos-Frank and Zimmermann, 1976; Marikovsky, 1962). We have therefore decided to use the name *P. formicae*, even though the possible synonymy of *P. formicae* and *P. myrmecophaga*, and historical antecedence of the latter epithet has motivated some other researchers to use the name *P. myrmecophaga.* Below we elaborate on reasons for our choice and summarize briefly the taxonomic history of ant-infecting entomophthoralean fungi.

4.3. Note on nomenclature

Since the fungus infecting ants and causing summit disease was first described by P. I. Marikovsky, in 1962 on *F. rufa*, there was little doubt that the fungal causing agent belonged to the order Entomophthorales (even though it was identified as *Alternaria tenuis*). Distinctive host manipulation behavior, timing of disease development and presence of active conidia discharge and conspicuous rhizoids all fit with this group of entomopathogenic fungi (Marikovsky, 1962).

However, it was not until 1969 that similar infections in *Lasius* ants in Switzerland were observed by Turian and Wuest, and the fungus informally named *Entomophthora myrmecophaga*. In the Swiss fungus, there were no rhizoids observed (Turian and Wuest, 1969).

In 1976, Loos-Frank and Zimmermann observed entomophthoralean mycosis in *F. pratensis* in Germany, and elegantly described disease development, but they used a non-specific name *Entomophthora* sp. (Loos-Frank and Zimmermann, 1976). Bałazy and Sokołowski tried to fulfil this taxonomical gap; they sampled fungus-killed ants of the *F. rufa* group (*F. rufa* and *F. polyctena*) in Poland (Bałazy and Sokołowski, 1977). They supplemented available knowledge on the fungus with morphological observations and measurements; they also observed conspicuous rhizoids forming a fascicle growing out of the ventral thorax. The authors suggested that the ant-infecting fungus showed features of the genus *Zoophthora sensu lato* which included the sub-genus *Pandora* (Batko, 1964).

Turian and Wuest subsequently published a taxonomical description of a fungus infecting *Formica* (*Serviformica*) *fusca* in Switzerland, and formally named it *Zoophthora myrmecophaga* (Turian and Wuest, 1977). In the description, the authors included rough-walled resting spores. However, our interpretation of the published photographs of the globose structures, similarly to Humber and Ben-Ze'ev's (1981) interpretation, is that they are most likely plant pollen grains. The authors also noted that they observed some fine, thin hyphal strings growing out from all body parts that fixed ants to the surface. They specifically stated that the absence of rhizoids was one of the differences between the fungus they observed and the one observed in Germany by Loos-Frank and Zimmermann (1976). The authors also pointed to differences in conidial morphology between their fungus and the, yet undescribed, German fungus. Since there was no type specimen of *P. myrmecophaga* designated, illustrations in the publication have to be treated as type for this name; the type locality is a forest near Geneva, Switzerland. This first formal description of the fungus is considered incomplete and therefore invalid.

All this created a confusing situation: fungi observed in *Formica* from Germany, Poland and Russia all had conspicuous rhizoids forming a fascicle growing from ants' ventral thorax only, and no resting spores were observed. Those described from Switzerland, from *Lasius* and *Formica*, had no rhizoids or few rhizoids, and rough-walled resting spores.

In a monograph of the genus *Erynia* (subgenus of *Zoophthora sensu lato* raised to generic level), Humber and Ben-Ze'ev (1981) addressed the issue by giving the new epithet *formicae* to the fungus described in Poland and Germany, now *Erynia formicae* Humber & Bałazy, and re-describing the Swiss fungus as *Erynia myrmecophaga* (Turian & Wuest) Humber, with a new, formally improved Latin description (Humber and Ben-Ze'ev, 1981), thus validating the epithet.

Keller made an attempt to synonymize the two species in his monograph (1991), stating that he had been sampling a few kilometers from *E. myrmecophaga* type locality, and saw no substantial differences between fungi infecting ants there and those described by Loos-Frank and Zimmermann (1976), or Bałazy and Sokołowski (1977). He therefore proposed to use *E. myrmecophaga* for all ant-infecting fungi, claiming it was unnecessary to ever describe *E. formicae* (Keller, 1991). Similarly, Bałazy in his monograph of Entomophthorales (1993) used the name *Zoophthora myrmecophaga*, claiming that there is only one species of ant-infecting entomophthoralean fungus, but also admitting that synonymization attempts remain invalid, including those by Keller (Bałazy, 1993).

Entomophthoralean fungi have since then undergone many genus-level reorganizations, and now both species are included in *Pandora*: as *P. formicae*, a new combination made by Humber (1989), and *P. myrmecophaga*, a new combination made by Keller (Keller and Petrini, 2005). It should be noted that the name *P. myrmecophaga* seems to be the one favored be the few scientists that have made more observations of ant mycoses since then (Boer, 2008; Csata et al., 2013).

We decided to use the name *P. formicae* for the fungus infecting *F. polyctena* in Denmark, because available descriptions all suggest this choice. One of the few differentiating characteristics is the presence or absence, and morphology of fungal attachment structures: *P. myrmecophaga* does not form a fascicle of rhizoids, and *P. formicae* does. Even though the epithet "myrmecophaga" has the advantage of priority, none of the attempts to formally include rhizoid-producing fungi (described from Germany and Poland, and later from Switzerland) were sufficient to finalize the synonymization. Therefore, the only available name for rhizoid-forming fungi is *P. formicae*. Even though there are many similarities between the fungus we observe and the one described by Turian and Wuest (1977), these differences in description are too pronounced to justify the use of the name *P. myrmecophaga*. Turian and Wuest (1977) specifically stated that the lack of thick rhizoids distinguished *P. myrmecophaga* from the German fungus; also, the morphology of resting spores they present differs greatly from the smooth walled resting spores that we report here for *P. formicae*, which further supports our conclusion. Also, page number priority from the first monograph that contains valid description of both names (Humber and Ben-Ze'ev, 1981) also belongs to the epithet *formicae*. Nonetheless, more work on ant-infecting species across different geographical regions and hosts, and on relation between morphological characters and genetic structure in these fungi will be needed to conclude if there is in fact one or more ant killers among the Entomophthorales.

Acknowledgements

We would like to thank Danish National Research Foundation (grant DNRF57) and University of Copenhagen (PhD stipend for JM) for funding. Antoine Lecoq is thanked for his help in translating articles from French, and proof-reading the manuscript. We are also grateful to professor Richard Humber and two anonymous reviewers for critically reviewing the manuscript and for helpful comments. JM, ABJ and JE planned the experiments, JM collected and analyzed the data, JM, ABJ and JE wrote the paper.

References

Andersen, S.B., Gerritsma, S., Yusah, K.M., Mayntz, D., Hywel-Jones, N.L., Billen, J., Boomsma, J.J., Hughes, D.P., 2009. The life of a dead ant: the expression of an

adaptive extended phenotype. Am. Nat. 174, 424–433. http://dx.doi.org/10.1086/603640.
Bałazy, S., 1993. Flora of Poland, Fungi (Mycota). Entomophthorales, Wydawnictwa Inst. Bot. Pan., Kraków, vol. 24. Polish Academy of Sciences, W. Szafer Institute of Botany, Kraków.
Bałazy, S., Sokołowski, A., 1977. Morphology and biology of *Entomophthora myrmecophaga*. Trans. Br. Mycol. Soc. 68, 134–137. http://dx.doi.org/10.1016/S0007-1536(77)80172-1.
Batko, A., 1964. On the new genera *Zoophthora* gen. nov., *Triplosporium* (Thaxter) gen. nov. and *Entomophaga* gen. nov. (Phycomycetes: Entomophthoraceae). Bull. Acad. Pol. Sci. Ser. Sci. Biol. 12, 323–326.
Boer, P., 2008. Observations of summit disease in *Formica rufa* Linnaeus, 1761 (Hymenoptera: Formicidae). Myrmecol. News 11, 63–66.
Buhl, J., Hicks, K., Miller, E.R., Persey, S., Alinvi, O., Sumpter, D.J.T., 2009. Shape and efficiency of wood ant foraging networks. Behav. Ecol. Sociobiol. 63, 451–460. http://dx.doi.org/10.1007/s00265-008-0680-7.
Carruthers, R.I., Ramos, M.E., Larkin, T.S., Hostetter, D.L., Soper, R.S., 1997. The *Entomophaga grylli* (Fresenius) Batko species complex: its biology, ecology, and use for biological control of pest grasshoppers. Mem. Entomol. Soc. Canada 129, 329–353.
Christe, P., Oppliger, A., Bancala, F., Castella, G., Chapuisat, M., 2002. Evidence for collective medication in ants. Ecol. Lett. 6, 19–22. http://dx.doi.org/10.1046/j.1461-0248.2003.00395.x.
Coenen-Stass, D., Schaarschmidt, B., Lamprecht, I., 1980. Temperature distribution and calorimetric determination of heat production in the nest of the wood ant, *Formica polyctena* (Hymenoptera, Formicidae). Ecology 61, 238–244.
Collingwood, C.A., 1979. The Formicidae (Hymenoptera) of Fennoscandia and Denmark. Scandinavian Science Press.
Cremer, S., Armitage, S.A.O., Schmid-Hempel, P., 2007. Social immunity. Curr. Biol. 17, R693–R702. http://dx.doi.org/10.1016/j.cub.2007.06.008.
Csata, E., Czekes, Z., Eros, K., Német, E., Hughes, M., Csosz, S., Markó, B., 2013. Comprehensive survey of Romanian myrmecoparasitic fungi: new species, biology and distribution. North West. J. Zool. 9, 23–29.
Domisch, T., Finér, L., Neuvonen, S., Niemelä, P., Risch, A.C., Kilpeläinen, J., Ohashi, M., Jurgensen, M.F., 2009. Foraging activity and dietary spectrum of wood ants (*Formica rufa* group) and their role in nutrient fluxes in boreal forests. Ecol. Entomol. 34, 369–377. http://dx.doi.org/10.1111/j.1365-2311.2009.01086.x.
Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32, 1792–1797. http://dx.doi.org/10.1093/nar/gkh340.
Eilenberg, J., Michelsen, V., 1999. Natural host range and prevalence of the genus *Strongwellsea* (Zygomycota: Entomophthorales) in Denmark. J. Invertebr. Pathol. 73, 189–198. http://dx.doi.org/10.1006/jipa.1998.4795.
Eilenberg, J., Thomsen, L., Jensen, A.B., 2013. A third way for entomophthoralean fungi to survive the winter: slow disease transmission between individuals of the hibernating host. Insects 4, 392–403. http://dx.doi.org/10.3390/insects4030392.
Ellis, S., Robinson, E.J.H., 2014. Polydomy in red wood ants. Insectes Soc. 61, 111–122. http://dx.doi.org/10.1007/s00040-013-0337-z.
Frouz, J., 2000. The effect of nest moisture on daily temperature regime in the nests of *Formica polyctena* wood ants. Insectes Soc. 47, 229–235. http://dx.doi.org/10.1007/PL00001708.
Frouz, J., Finer, L., 2007. Diurnal and seasonal fluctuations in wood ant (*Formica polyctena*) nest temperature in two geographically distant populations along a South - North gradient. Insectes Soc. 54, 251–259. http://dx.doi.org/10.1007/s00040-007-0939-4.
Glare, T.R., Milner, R.J., Chilvers, G.A., 1989. Factors affecting the production of resting spores by *Zoophthora radicans* in the spotted alfalfa aphid, *Therioaphis trifolii f. maculata*. Can. J. Bot. 67, 848–855. http://dx.doi.org/10.1139/b89-114.
Hajek, A.E., Shimazu, M., 1996. Types of spores produced by *Entomophaga maimaiga* infecting the gypsy moth *Lymantria dispar*. Can. J. Bot. 74, 708–715. http://dx.doi.org/10.1139/b96-089.
Hajek, A.E., Humber, R.A., 1997. Formation and germination of *Entomophaga maimaiga* azygospores. Can. J. Bot. 75, 1739–1747.
Hajek, A.E., Bauer, L., McManus, M.L., Wheeler, M.M., 1998. Distribution of resting spores of the *Lymantria dispar* pathogen *Entomophaga maimaiga* in soil and on bark. Biocontrol 43, 189–200.
Humber, R.A., Ben-Ze'ev, I., 1981. Erynia (Zygomycetes: Entomophthorales): validations and new species. Mycotaxon 13, 506–516.
Humber, R.A., 1989. Synopsis of a revised classification for the Entomophthorales (Zygomycotina). Mycotaxon 34, 441–469.
Kalsbeek, V., Mullens, B.A., Jespersen, J.B., 2001. Field Studies of *Entomophthora* (Zygomycetes: Entomophthorales)—induced behavioral fever in *Musca domestica* (Diptera: Muscidae) in Denmark. Biol. Control 21, 264–273. http://dx.doi.org/10.1006/bcon.2001.0943.
Keller, S., 1987. Observations on the overwintering of *Entomophthora planchoniana*. J. Invertebr. Pathol. 50, 333–335. http://dx.doi.org/10.4241/kyubyochu.2.51.
Keller, S., 1991. Arthropod-pathogenic Entomophthorales of Switzerland. II. *Erynia*, *Eryniopsis*, *Neozygites*, *Zoophthora* and *Tarichium*. Sydowia 43, 39–122.
Keller, S., Petrini, O., 2005. Keys to the identification of the arthropod pathogenic genera of the families Entomophthoraceae and Neozygitaceae (Zygomycetes), with descriptions of three new subfamilies and a new genus. Sydowia 57, 23–53.
Loos-Frank, B., Zimmermann, G., 1976. Über eine dem *Dicrocoelium*-Befall analoge Verhaltensänderung bei Ameisen der Gattung *Formica* durch einen Pilz der Gattung Entomophthora. Z. Parasitenkd. 49, 281–289.
Maitland, D.P., 1994. A parasitic fungus infecting yellow dungflies manipulates host perching behaviour. Proc. Roy. Soc. B: Biol. Sci. 258, 187–193. http://dx.doi.org/10.1098/rspb.1994.0161.
Małagocka, J., Grell, M.N., Lange, L., Eilenberg, J., Jensen, A.B., 2015. Transcriptome of an entomophthoralean fungus (*Pandora formicae*) shows molecular machinery adjusted for successful host exploitation and transmission. J. Invertebr. Pathol. 128, 47–56. http://dx.doi.org/10.1016/j.jip.2015.05.001.
Marikovsky, P.I., 1962. On some features of behavior of the ants Formica rufa L. infected with fungous disease. Insectes Soc. IX, 173–179.
Nielsen, C., Hajek, A.E., Humber, R.A., Bresciani, J., Eilenberg, J., 2003. Soil as an environment for survival of aphid-pathogenic Entomophthorales. Biol. Control 28, 92–100. http://dx.doi.org/10.1016/S1049-9644(03)00033-1.
Pontoppidan, M.-B., Himaman, W., Hywel-Jones, N.L., Boomsma, J.J., Hughes, D.P., 2009. Graveyards on the move: the spatio-temporal distribution of dead *Ophiocordyceps*-infected ants. PLoS ONE 4, e4835. http://dx.doi.org/10.1371/journal.pone.0004835.
R Core Team, 2013. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. <http://www.R-project.org/>.
Reznikova, Z., 2011. Division of labour and communication at the individual level in highly social *Formica* ants (Hymenoptera, Formicidae). Russ. Entomol. J. 20, 315–319.
Rosengren, R., 1977. Foraging Strategy of Wood Ants (*Formica Rufa* Group). I. Age Polyethism and Topographic Traditions. Societas pro fauna et flora Fennica.
Roy, H.E., Steinkraus, D.C., Eilenberg, J., Hajek, a.E., Pell, J.K., 2006. Bizarre interactions and endgames: entomopathogenic fungi and their arthropod hosts. Annu. Rev. Entomol. 51, 331–357. http://dx.doi.org/10.1146/annurev.ento.51.110104.150941.
Savolainen, R., Vepsalainen, K., 1989. Niche differentiation of ant species within territories of the wood ant *Formica polyctena*. Oikos 56, 3–16.
Scorsetti, A.C., Jensen, A.B., López Lastra, C., Humber, R.A., 2012. First report of *Pandora neoaphidis* resting spore formation in vivo in aphid hosts. Fungal Biol. 116, 196–203. http://dx.doi.org/10.1016/j.funbio.2011.11.002.
Sosnowska, D., Bałazy, S., Prishchepa, L., Mikulskaya, N., 2004. Biodiversity of arthropod pathogens in the Białowieża forest. J. Plant Prot. Res. 44, 313–321.
Steinkraus, D.C., 2006. Factors affecting transmission of fungal pathogens of aphids. J. Invertebr. Pathol. 92, 125–131. http://dx.doi.org/10.1016/j.jip.2006.03.009.
Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739. http://dx.doi.org/10.1093/molbev/msr121.
Thomsen, L., Eilenberg, J., 2000. *Entomophthora muscae* resting spore formation in vivo in the host *Delia radicum*. J. Invertebr. Pathol. 76, 127–130. http://dx.doi.org/10.1006/jipa.2000.4961.
Turian, G., Wuest, J., 1969. Mycoses à Entomophthoracées frappant des populations de Fourmis et de Drosophiles. Mitt. Schweiz. Entomol. Gesell. 42, 197–201.
Turian, G., Wuest, J., 1977. Description complementaire de *Zoophthora* (*Entomophthora*) myrmecophaga Turian & Wuest, agent d'une mycose chez *Serviformica fusca* L. Mitt. Schweiz. Entomol. Gesell. 50, 285–289.
Watson, D.W., Mullens, B.A., Petersen, J.J., 1993. Behavioral fever response of *Musca domestica* (Diptera: Muscidae) to infection by *Entomophthora muscae* (Zygomycetes: Entomophthorales). J. Invertebr. Pathol. http://dx.doi.org/10.1006/jipa.1993.1003.
White, T., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR Protocols: A Guide to Methods and Applications 18 (1), 315–322.
http://www.dmi.dk/vejr/arkiver/normaler-og-ekstremer/klimanormaler-dk/ (accessed: 2016-10-21)