Differential divergences of obligately insect-pathogenic *Entomophthora* species from fly and aphid hosts

Annette Bruun Jensen¹, Jørgen Eilenberg¹ & Claudia López Lastra²

1Department of Agriculture and Ecology, University of Copenhagen, Frederiksberg C, Denmark; and ²CEPAVE Centro de Estudios Parasitológicos y de Vectores, Universidad Nacional de La Plata, La Plata, Argentina

**Abstract**

Three DNA regions (ITS 1, LSU rRNA and GPD) of isolates from the insect-pathogenic fungus genus *Entomophthora* originating from different fly (*Diptera*) and aphid (*Hemiptera*) host taxa were sequenced. The results documented a large genetic diversity among the fly-pathogenic *Entomophthora* and only minor differences among aphid-pathogenic *Entomophthora*. The evolutionary time of divergence of the fly and the aphid host taxa included cannot account for this difference. The host-driven divergence of *Entomophthora*, therefore, has been much greater in flies than in aphids. Host-range differences or a recent host shift to aphid are possible explanations.

**Introduction**

All species within the fungal genus *Entomophthora* (Entomophthoromycotina: *Entomophthorales: Entomophthoraceae*) are obligate insect pathogens that, in nature, only grow and proliferate in their insect host. They belong to one of the oldest fungal lineages (Jensen et al., 1998; James et al., 2006), and to a family within this group of fungi where the obligate insect-pathogenic lifestyle is ancestral. *Entomophthora* species can be found in many different orders of insect hosts: Coleoptera (beetles), Diptera (flies, midge, gnats, etc.), Hemiptera (aphids, bugs), Hymenoptera (wasps), Neuroptera (lacewings, etc.), Plecoptera (stoneflies) and Thysanoptera (thrips) (Eilenberg et al., 1987; Balazy, 1993; Keller, 2002). However, each species is thought to have a fairly narrow host range, infecting host species belonging to the same insect order, genus or even a single host species. *Entomophthora* species, therefore, offer an excellent opportunity to study the coevolution between host and pathogen and how hosts have affected the divergence of the pathogen.

Several species of the genus *Entomophthora* (including *Entomophthora ferdinandii*, *Entomophthora grandis*, *Entomophthora muscae*, *Entomophthora scatophagae*, *Entomophthora schizophorae* and *Entomophthora syrphi*) infect dipterans within the derived dipteran fly clade Muscomorpha and they infect hosts in many different families within this clade (Keller, 2002). In comparison, only two *Entomophthora* species, *Entomophthora chromaphidis* and *Entomophthora planchoniana*, are known to infect aphids (Keller, 2002), and these species only infect aphids primarily within the family Aphididae (Barta & Cagán, 2006). Molecular analyses have recently confirmed the species status of most of the fly-pathogenic *Entomophthora* (Jensen et al., 2006), while it has been questioned whether the two aphid-pathogenic *Entomophthora* species are distinct, because they cannot be distinguished molecularly, phenotypically or by cultivation abilities (Freimoser et al., 2001). In addition, molecular analyses have revealed a high intraspecific variation within the fly-pathogenic *E. muscae*, with each host species harbouring its own fungal genotype (Jensen et al., 2001), while only minor differences in *E. planchoniana* from several different aphid species have been documented (Freimoser et al., 2001). Thus, the host specificity of fly-pathogenic *Entomophthora* seems to be higher than that for the aphid-pathogenic *Entomophthora*.  

---

**Correspondence:** Annette Bruun Jensen, Department of Agriculture and Ecology, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark. Tel.: +45 35 33 26 62; fax: +45 35 33 26 70; e-mail: abj@life.ku.dk

Received 9 March 2009; accepted 20 August 2009. Final version published online 30 September 2009.

DOI:10.1111/j.1574-6968.2009.01778.x

Editor: Michael Bidochka

**Keywords**

Entomophthorales; Entomophthoraceae; entomopathogenic fungi; evolution; Hemiptera, Diptera.
The fly infraorder Muscomorpha (also known as Cyclorrhapha) includes approximately 80 families with 65,000 recent species, and it is the largest and most diverse group of true flies. Muscomorpha are divided into two sections: Aschiza and Schizophora, with Schizophora being the largest section, comprised of approximately 50,000 species. Several of the basal Aschiza evolved in the Cretaceous, but radiation of Syrphidae (hoverflies) apparently first occurred in Early Tertiary, as suggested by numerous Baltic amber fossils (Eocene 57–65 Mya) (Gralgaldi & Engel, 2005). Schizophora originated in the Late Cretaceous, but their radiation was exclusively Tertiary and is probably contemporary with the major diversifications of angiosperm (flowering plants) lineages (Wiegmann et al., 2003).

The radiation of recent aphid tribes occurred in the Upper Cretaceous particularly for the family Aphididae, which is the largest aphid family including 2000–3000 recent species. It has been suggested that this significant radiation event took place as the aphid ancestors moved from gymnosperm hosts to woody angiosperms (von Dolen & Moran, 2000), although only a few aphid fossils have been found in Canadian amber from the Upper Cretaceous (75–80 Mya) (Heie & Wegierek, 1998). The first record is a termite infected by Entomophthorales (45–15 Mya). The first record is a termite infected by an entomophthoralean fungus based on the fungal external growth pattern showing several possible cystidia. No conidia can be seen, and so further fungal identification is difficult (Poinar & Poinar, 2005). The findings of Aphididae and Pemphigidae first appears in Baltic amber from the Eocene epoch in the early Tertiary (Heie & Wegierek, 1998).

Fossil records of fungal pathogens of insects are very rare (Poinar & Poinar, 2005), and only two fossil records of insects infected by entomophthoralean fungi have so far been described, both from Dominican Amber (approximately 45–15 Mya). The first record is a termite infected by an entomophthoralean fungus from the family Entomophthoraceae based on the shape of the primary and secondary conidia (Poinar & Thomas, 1982), and the second record is a fungus gnat from the Mycetophilidae, a family of very small primitive flies, infected by an entomophthoralean fungus based on the fungal external growth pattern showing several possible cystidia. No conidia can be seen, and so further fungal identification is difficult (Poinar & Poinar, 2005). The findings of Entomophthoraceae on different insect orders from prehistorical time support the hypothesis of the ancestral obligate insect-pathogenic lifestyle of the family Entomophthoraceae and shows that entomophthoralean fungi were able to exploit insects for nutrition and growth. Because of their obligate insect-pathogenic lifestyle, Entomophthora species are highly dependent on their host; thus, it is interesting to compare the divergence of these fungi with the evolutionary divergence of their fly and aphid hosts, as well as with other life-history traits.

In the current study, we sequenced three DNA regions of several Entomophthora specimens originating from different fly and aphid host species in order to investigate the impact of aphid or fly host species on the evolutionary divergence of the genus Entomophthora.

Materials and methods

Fungal material

Fungus-infected fly or aphid cadavers were sampled from various localities. The infected flies originated primarily in Denmark, but two infected flies were from Argentina and the United States, respectively. The infected aphids originated from Argentina, Denmark and Iceland. In addition, Entomophthora isolates from the ARSEF collection (ARS Collection of Entomopathogenic Fungi, Ithaca) were included. A list of isolates is given in Table 1.

The cadavers were placed in humid chambers on a glass slide to allow fungal spores (conidia) to be discharged. Thereafter, the cadavers were stored in 96% ethanol or in vitro cultures were isolated as described by Jensen et al. (2001). Fungal species were subsequently determined to the species level based on the morphology of primary and secondary conidia according to Keller (1987) and Humber (1997).

DNA extraction, PCR and sequencing

DNA was extracted from in vivo or in vitro materials either by chloroform/octanol as in Jensen et al. (2001) or by ammonium hydroxide extraction as in Jensen et al. (2008). PCR was performed on three loci: the internal transcribed spacer 1 (ITS 1) region, the first part of the 28S rRNA gene, also called large-subunit (LSU) rRNA, and a part of the glyceraldehyde-3-phosphate dehydrogenase gene (GPD). Fungal or entomophthoralean-specific primers that were either designed for either this or previous studies were used, in order to avoid amplification of any host-based DNA.

The PCR conditions were initial denaturation for 5 min at 96 °C, followed by 30–35 cycles with denaturation for 1 min at 96 °C, annealing for 1 min at 55–62 °C (ITS 55 °C, LSU 55 °C, GPD 62 °C), extension for 1 min at 72 °C and a final extension for 10 min at 72 °C. The PCR reactions were carried out in 50-µL volumes each, with 250 µM of each dNTP, 0.8 µM of each primer, 2.5 mM MgCl₂, 1 × buffer (10 mM Tris-HCl, pH 8.8 at 25 °C, 50 mM KCl, 0.1% Triton X-100), 1 U DyNaZyme II (Finnzymes, Espoo, Finland) and 1 µL of extracted DNA diluted 1:10 or 1:100. Two different forward primers were used in the amplification of the ITS I region for the aphid– and fly–Entomophthora system, respectively: ML2: 5′-GGCAACGATCATCAGTGA-3′ (aphid–Entomophthora) and ITS 5: 5′-GGAAGTAAAAGTCGTATCGATGAA-3′ (fly–Entomophthora) (White et al., 1990), and a single reverse primer Nu-5.8S-3′: 5′-AATCCGGTTCTTGC ATCGATGA-3′ (Jensen & Eilenberg, 2001). For the LSU
amplification, we used Nu-LSU-0018-5': 5'-GTAAGTTATTGAGTCAACAAGAG-3' (Jensen & Eilenberg, 2001) and LSU 0805: 5'-CAT ATG TCA CCA TCT TTC GG-3' (Kjøller & Rosendahl, 2000), and for the GPD amplification MFLgp d71F: 5'-GACAACCTTTGGTGTAGTCGAAGG-3' and MFL gp55R: 5'-ACWCGGAAGCCATACCGRT-3'. Before sequencing, the PCR products were purified using the GFXtm PCR DNA and Gel Purification Kit (Amersham Pharmacia). The purified PCR products were sent to MWG Biotech for sequencing in both directions using the above PCR primers.

**Sequence analysis**

The sequences were checked and aligned with BIOEDIT v7.0.8.0. Subsequently, sequence analyses were performed separately for each of the three loci using PHYLIP v3.6. Neighbour-joining analyses were performed with the Jukes–Cantor evolutionary model using DNADIST, and neighbour-branch procedures and parsimony analyses were performed using the DNAPARS procedure. Supports for internal branches were assessed by 1000 bootstrap replications using the SEQBOOT procedure.

### Host divergence

The evolutionary history of insects has been constructed by analysing the morphology of recent and fossil records, but the analyses of DNA sequences are now increasingly being applied. We estimated the time of divergence of the six fly families and the two aphid families from which we had obtained *Entomophthora*-infected individuals based on a search of the literature. These estimates were based on either fossil records or DNA sequence analyses (Table 2).

### Results

**Samples**

Twelve *Entomophthora*–fly host associations were obtained from 10 different dipteran host species representing six different families. Of these 12 fly–*Entomophthora* associations, six different *Entomophthora* species were recognized based on morphology. This included three different *Entomophthora* species pathogenic to the common house fly *Musca domestica*. Twelve *Entomophthora*–aphid associations were obtained from 12 different aphid host species.
Table 2. Period of divergence of the six fly families and the two aphid subfamilies included in this study, based on fossil or DNA sequence analyses

<table>
<thead>
<tr>
<th>Period</th>
<th>Epoch</th>
<th>Began Mya</th>
<th>Aschiza</th>
<th>Schizophora</th>
<th>Calyptratae</th>
<th>Acalyptratae</th>
<th>Aphyidoea</th>
<th>Pemphigidae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tertiary</td>
<td>Pliocene</td>
<td>5</td>
<td>Fossil* DNA†</td>
<td>Fossil† DNA‡</td>
<td>Fossil‡ DNA§</td>
<td>Fossil** DNA††</td>
<td>Fossil†† DNA‡‡</td>
<td>Fossil†† DNA‡‡</td>
</tr>
<tr>
<td></td>
<td>Miocene</td>
<td>24</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Oligocene</td>
<td>36</td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Eocene</td>
<td>57</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Paleocene</td>
<td>65</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Cretaceous</td>
<td>Late</td>
<td>100</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Early</td>
<td>146</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Divergence mostly took place in early Tertiary.

*Grimaldi & Engel (2005).
†Wiegmann et al. (2003).
‡Pont & de Carvalho (1997).
§Gaunt & Miles (2002).
¶Evenhuis (1994).
‖Michelsen (2000).
**Michelsen (1970).
††McAlpine (1970).
representing two families and three tribes. The aphid-pathogenic Entomophthora species were all identified as *E. planchoniana*, except for one isolate, which was designated *E. chromaphidis* and obtained from the ARSEF culture collection.

**Sequence analyses**

We successfully amplified and obtained good sequences of the three different loci, from many of the various Entomophthora–aphid and Entomophthora–fly host associations. ITS I was sequenced for all Entomophthora–aphid and Entomophthora–fly associations, LSU was sequenced for nine Entomophthora–aphid and eight Entomophthora–fly host associations and GPD was sequenced for three Entomophthora–aphid and five Entomophthora–fly host associations (Table 1).

The ITS I alignment included 292 positions with sequences of 258–283 bp, the LSU alignment included 787 positions with sequences of 774–781 bp and the GPD alignment included 191 positions with sequences of 191 bp. The phylogenetic analyses of the three loci resulted in trees with the same overall topology (Fig. 1). All aphid-pathogenic Entomophthora specimens clustered together, whereas the fly-pathogenic Entomophthora specimens represented three apparently different lineages, one with *E. schizophorae*, one including specimens with *E. muscae* phenotypes and a third more heterogeneous lineage including *E. grandis* and *E. syrphi*. The latter two lineages also formed a cluster in all three trees. All four major lineages were supported by high bootstrap values in all three sequence analyses (Fig. 1).

**Host divergence**

The divergence of the six fly and the two aphid families from which we had Entomophthora-infected individuals mostly took place in the early Tertiary (Table 2). The estimated divergence time of the aphid families *Aphididae* and *Pemphigidae* and the divergence of the six included fly families did not differ with respect to the prehistorical time in which they were estimated to have taken place.

**Discussion**

In the current study, we have shown a high genetic variation among the Entomophthora attacking flies, even at an intraspecific level, whereas only minor sequence differences were revealed within the aphid-pathogenic Entomophthora. Consequently, we conclude that true flies and aphids have had different impacts on the divergence of closely related pathogenic fungi from the genus Entomophthora. The evolutionary split within the two aphid families and within the fly families included all date back to between the late Cretaceous (approximately 80 Mya) and the early Tertiary (approximately 50 Mya). Thus, the evolutionary history of the host cannot in itself account for the different host-associated divergences, which have been much higher in the Entomophthora infecting flies than in Entomophthora infecting aphids.

Host-specific divergence of pathogens and parasites is commonly seen, and may evolve as a consequence of limited dispersal or adaptation (Timms & Read, 1999). If a pathogen does not come into contact with other host species, it can lead to a restricted host range as a result of a form of allopatric differentiation. Aphids often have a restricted host plant choice and being phloem feeders, they are rather immobile once they start feeding on plant sap, factors that would favour allopatric speciation of their pathogens. Interestingly, we did not find host-specific divergence among Entomophthora infecting aphids. Adult flies are rather mobile, and Entomophthora-infected individuals seek elevated positions, (e.g. tall plants) just before death, thus facilitating fungal spore dispersal (Roy et al., 2006). This host-altering manipulation increases the chance of Entomophthora spores reaching the cuticle of a new alternative host species. Allopatric differentiation is, therefore, not a likely explanation for the divergence we have detected in the Entomophthora–fly system.

Host-specific divergence might also arise due to adaptive specialization (van Tienderen, 1991). Trade-offs between adaptation to different hosts and the high cost of being a generalist can lead to a host-specific differentiation of pathogens in sympatry. Narrow host ranges, restricted to a single host species or genera, have been shown for fly-pathogenic Entomophthora (Jensen et al., 2001) and even though transmission of Entomophthora between different fly host species was possible under laboratory conditions, successful infection was often rather limited (Steinkraus & Kramer, 1987; Jensen et al., 2006). Successful transmission experiments with *E. planchoniana* between different aphid species have not been conducted yet, to our knowledge, but surveys have shown that *E. planchoniana* infects a large number of aphid species, in particular, from the family *Aphididae* (Barta & Caganˇ 2006). This, together with the sequence similarities of *E. planchoniana* from various aphid species documented in this study, suggests a broad host range. A narrow host range can potentially lead to bottleneck effects in periods where the host population is minimized, leading to a higher impact of genetic drift, which provides a possible explanation for the sequence difference between the aphid– and fly–Entomophthora systems.

An alternative explanation for the different degree of divergence could be that Entomophthora has more recently developed the ability to infect aphid hosts. In this scenario, Entomophthora attacking aphids have had less time to diverge than have the fly-pathogenic Entomophthora. Host jumps, involving a new host that is distantly related to the...
Fig. 1. Phylogenetic relationships of *Entomophthora* species infecting flies and aphids inferred from parsimony analysis of three different DNA regions: the ITS 1, part of the nuclear LSU rRNA gene (LSU) and part of the GPD. Bootstrap percentages over 50% from 1000 replicates are shown above each supported branch. The scale bar corresponds to 10 nucleotide changes. In all three phylograms, *Entomophthora* assorted into four major branches, one including all the aphid-pathogenic *Entomophthora* and three branches including the fly-pathogenic *Entomophthora*, largely corresponding to phenotypic groups.
original host, for example from another class or order, are known phenomena within fungi (Nikoh & Fukatsu, 2000). Even interkingdom host jumps have occurred as exemplified by multiple jumps within ascomycete clavicipitoid fungi. The common ancestor of Clavicipitaceae (in the broad sense) is suggested to have been an animal pathogen, but during the course of evolution, interkingdom host jumps between animal, plant and fungi have occurred (Spatafora et al., 2007).

Entomophthorales species are obligate insect pathogens and, as such, are tightly connected to their host. Therefore, one might hypothesize that the divergence of Entomophthora and their hosts has occurred in synchrony. Coevolution between parasites and pathogens is an established theory (Fahrenholz’s rule) (Eichler, 1948). However, in this study, we were not able to show strict coevolution with congruent phylogenies below the insect ordinal level. For example members of the fly family Muscidae were infected by several different Entomophthora species including representatives from each of the three major fly-pathogenic Entomophthora lineages. At the insect ordinal level, the Entomophthora species, however, seem to have coevolved with their hosts as evidenced by the clustering of all the fly-pathogenic Entomophthora in one separate clade. Because Entomophthora species from other host orders (e.g. Coleoptera, Hymenoptera or Thysanoptera) have not been included in this analysis, future work is, however, needed to pursue this pattern further.

Acknowledgements

Thanks are due to D.R. Nash and N.V. Meyling for helpful comments on this paper. A.B.J. acknowledges Velux and Vilum Kann Rasmussen Foundation and C.L.L. acknowledges CONICET (National Research Council of Argentina) and ANPCyT Grant (PICT 20337-04) for financial aid and partial support.

References


