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RESEARCH LETTER

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

Annette Bruun Jensen[1], Jørgen Eilenberg[1] & Claudia López Lastra[2]

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

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

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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; Bałazy, 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 & Cagáň, 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*.

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The fly infraorder Muscomorpha (also known as Cyclorrapha) 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) (Grimaldi & 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). Ancestors of the Aphidoidea families *Aphididae* and *Pemphigidae* were part of this radiation based on molecular and morphological phylogenies (Moran *et al.*, 1993; von Dolen & Moran, 2000), but fossil evidence 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 erynoid *Entomophthorales* 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 *Entomophthorales* 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 $MgCl_2$, 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′-GGCAACGGATCATCATGTAA-3′ (aphid–*Entomophthora*) and ITS 5: 5′-GGAAGTAAAAGTCGT AACAAG G-3′ (fly–*Entomophthora*) (White *et al.*, 1990), and a single reverse primer Nu-5.8S-3′: 5′-ACTACGTTCTTC ATCGATGA-3′ (Jensen & Eilenberg, 2001). For the LSU

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Table 1. List of the *Entomophthora* species used in this study with host taxon, origin, GenBank accession numbers and code (See Fig. 1)

				GenBank accession numbers			
Entomophthora species	Isolate	Host species (family)	Origin	ITS I	LSU	GPD	Code
Aphids							
E. chromaphidis	ARSEF 1860	*Acyrthosiphon kondoi* (*Aphididae*)	Australia	GQ285848		GQ285884	Echro Akon
E. planchoniana	*In vivo*	*Aphis fabae* (*Aphididae*)	Denmark	GQ285849	GQ285873		Eplan Afab
E. planchoniana	*In vivo*	*Aphis sambuci* (*Aphididae*)	Denmark	GQ285850	GQ285874		Eplan Asam
E. planchoniana	*In vivo*	*Capitophorus elaeagni* (*Aphididae*)	Argentina	GQ285851	GQ285875	GQ285885	Eplan Cela
E. planchoniana	*In vivo*	*Chaetosiphon fragaefolii* (*Aphididae*)	Argentina	GQ285852	GQ285876		Eplan Cfra
E. planchoniana	*In vivo*	*Elatobium abietinum* (*Aphididae*)	Iceland	GQ285853	GQ285877		Eplan Eabi
E. planchoniana	*In vivo*	*Macrosiphum rosae* (*Aphididae*)	Denmark	GQ285854			Eplan Mros
E. planchoniana	*In vivo*	*Myzus persicae* (*Aphididae*)	Argentina	GQ285855			Eplan Mper
E. planchoniana	ARSEF 6252	*Ovatus crataegarius* (*Aphididae*)	Denmark	GQ285856	GQ285878	GQ285886	Eplan Ocra
E. planchoniana	*In vivo*	*Rhopalosiphum padi* (*Aphididae*)	Denmark	GQ285857	GQ285879		Eplan Rpad
E. planchoniana	*In vivo*	*Sitobion avenae* (*Aphididae*)	Denmark	GQ285858	GQ285880		Eplan Save
E. planchoniana	*In vivo*	*Prociphilus xylostei* (*Pemphigidae*)	Denmark	GQ285859	GQ285881		Eplan Pxyl
Flies							
E. ferdinandii	ARSEF 6918	*Coenosia tigrina* (*Muscidae*)	Denmark	GQ285860			Efer Ctir
E. ferdinandii	*In vivo*	*Musca domestica* (*Muscidae*)	Argentina	GQ285861	GQ285882		Efer Mdom
E. ferdinandii	KVL 99-87	*Pegoplata infirma* (*Anthomyiidae*)	Denmark	GQ285862			Efer Pinf
E. grandis	ARSEF 6701	*Myospila meditabunda* (*Muscidae*)	Denmark	GQ285863	DQ481229		Egra Mmed
E. muscae	ARSEF 6815	*Delia radicum* (*Anthomyiidae*)	Denmark	GQ285864	DQ481225	GQ285887	Emus Drad
E. muscae	ARSEF 5954	Anthomyiidae sp. (*Anthomyiidae*)	Denmark	GQ285865		GQ285888	Emus Antsp
E. muscae s str	ARSEF 6132	*Musca domestica* (*Muscidae*)	Denmark	GQ285866	DQ481224	GQ285889	Emus Mdom
E. scatophagae	ARSEF 6704	*Scatophaga stercoraria* (*Scatophagidae*)	Denmark	GQ285867	DQ481226	GQ285890	Esca Sster
E. schizophorae	ARSEF 2541	*Chamaepsila rosae* (*Psilidae*)	Denmark	GQ285868			Eschi Cros
E. schizophorae	ARSEF 5348	*Musca domestica* (*Muscidae*)	USA	GQ285869	GQ285883	EF434863	Eschi Mdom
E. schizophorae	ARSEF 6817	*Pollenia rudis* (*Calliphoridae*)	Denmark	GQ285870	DQ481228		Eschi Prud
E. sp.	*In vivo*	*Oscinella frit* (*Chloropidae*)	Denmark	GQ285871			Esp Ofrit
E. syrphi	ARSEF 5955	*Melanostoma mellinum* (*Syrphidae*)	Denmark	GQ285872	DQ481230	GQ285891	Esyr Mmel

ARSEF, ARS Collection of Entomopathogenic fungi; KVL, Entomopathogenic Fungal Culture Collection at Department of Agriculture and Ecology, Faculty of Life Sciences, University of Coppenhagen.

amplification, we used Nu-LSU-0018-5′: 5′-GTAGTTATTC AAATCAAG CAA G-3′ (Jensen & Eilenberg, 2001) and LSU 0805: 5′-CAT AGT TCA CCA TCT TTC GG-3′ (Kjøller & Rosendahl, 2000), and for the GPD amplification MFLgp d71F: 5′-GACAACTTTGGTGTAGTCGAAGG-3′ and MFL gpd55R: 5′-ACWCKGAAAGCCATACCRGT-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 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

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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

			True flies												Aphids			
			Aschiza		Schizophora													
					Calyptratae								Acalyptratae		Aphidoidea			
			Syrphidae		*Muscidae*		*Scatophagidae*		*Anthomyiidae*		*Calliphoridae*		*Chloropidae*		*Aphididae*		*Pemphigidae*	
Period	Epoch	Began Mya	Fossil[*]	DNA[†]	Fossil[‡]	DNA[§]	Fossil[¶]	DNA	Fossil[‖]	DNA	Fossil[**]	DNA[§]	Fossil[¶]	DNA	Fossil[††]	DNA[‡‡]	Fossil[††]	DNA[‡‡]
Tertiary	Pliocene	5																
	Miocene	24			+													
	Oligocene	36				+												
	Eocene	57	+			+	+		+				+		+		+	
	Paleocene	65		+		+						+						
Cretaceous	Late	100		+		+					+					+		+
	Early	146																

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).
[**] McAlpine (1970).
[††] Heie & Wegierek (1998).
[‡‡] Von Dolen & Moran (2000).

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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 & Cagáň 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

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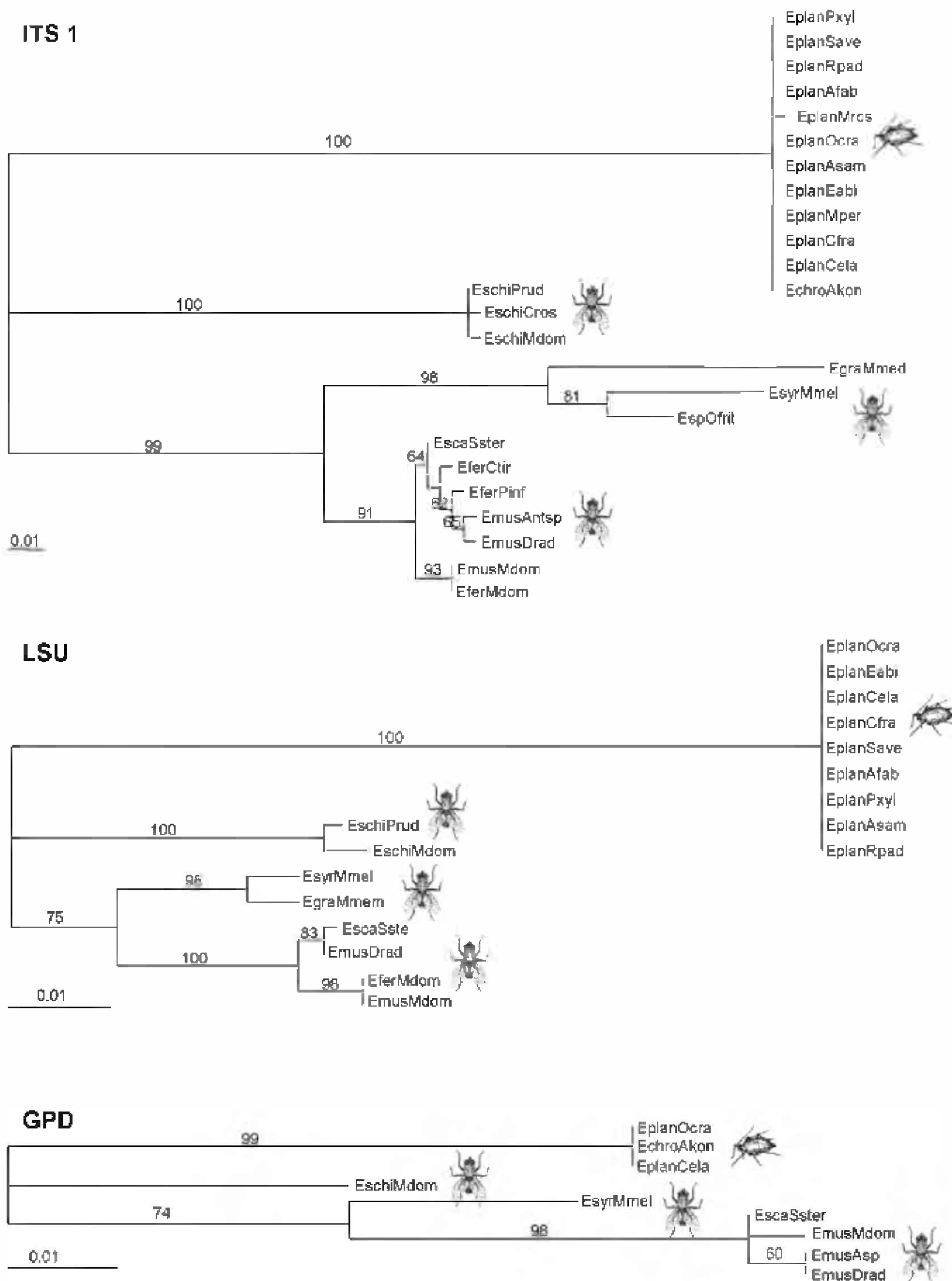

Fig. 1. Phylogenetic relationships of *Entomophthora* species infecting flies and aphids inferred from parsimony analysis of three different DNA regions: the ITS 1, part of the nuclear LSU rRNA gene (LSU) and part of the GPD. Bootstrap percentages over 50% from 1000 replicates are shown above each supported 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.

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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).

Entomophthora 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* into three groups, and of all the aphid-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.

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