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Daldinia decipiens and *Entonaema cinnabarina* as fungal symbionts of *Xiphydria* wood wasps

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ABSTRACT

The identity of symbiotic fungi associated with the *Xiphydria* spp. wood wasps was investigated using DNA analysis. The fungi were isolated from the mycangia of adult females of *X. camelus*, *X. prolongata* and *X. longicollis* reared from colonized logs of *Alnus glutinosa*, *Salix alba* and *Quercus robur*, respectively. Sequences of rDNA and β-tubulin were obtained. Phylogenetic analysis based on the NJ method showed that the isolates from *X. camelus* clustered with *Daldinia decipiens*, whereas those of *X. prolongata* belonged to *Entonaema cinnabarina*. In *X. longicollis*, both symbiotic fungi (*D. decipiens* and *E. cinnabarina*) have been found. Morphological characteristics of the anamorphs are presented. In cultures of *D. decipiens*, sympodial holoblastic, as well as annelidic, conidiation was observed. So far, fruit bodies of *D. decipiens* have only been recorded from *Betula* spp. whereas the host spectrum of its wasp vectors covers predominantly oaks and alders. Fruiting bodies of *E. cinnabarina* do not occur in Central Europe. This is the first report of *Entonaema* as a symbiotic fungus of siricid wood wasps.

Introduction

Forests in Europe are influenced by alteration of habitat as well as climatic changes, which is documented by numerous disease outbreaks or species 'declines', e.g. Dutch-elm disease and oak decline in central and southern Europe, *Fraxinus* decline in northern Europe, *Pinus* dieback in various areas in Europe, and *Ostrya* decline in southern Europe. Physiologically weakened broad-leaved trees are often the primary target of wood wasps (*Hymenoptera: Xiphydriidae*). In the Ukraine, the wood wasp *Xiphydria longicollis* caused mass dieback of oak stands (Dominik & Starczyk 1988). Another wood wasp, *X. camelus*, caused problems for newly planted street birch trees in Germany (Lehmann 2000).

There are four species of *Xiphydria* in Europe: *Xiphydria longicollis* (oak wood wasp) colonizes, according to Liston (1997), only *Quercus* and *Acer* spp., whereas Schimitschek (1935) observed massive occurrence on *Quercus* spp. in Austria as well as on *Acer campestre*, *Ulmus carpinifolia* and *Betula verrucosa*. Other hosts are *Alnus*, *Pyrus* and *Prunus*. According to Kraus (1997), *X. longicollis* does not occur on *Fraxinus* species. This species has been spreading in continental Europe and in Britain since the 1980s, corresponding to the worsening health of broadleaved trees (Shaw & Liston 1985; Halstead 1992; Liston 1997). In Germany, *X. longicollis* was rare before the 1960s, and even in its recent spreading, it prefers warmer regions (Kraus 1997). In the Czech Republic, *X. longicollis* was recorded first in South Moravia only (Schimitschek 1935); Kraus (1997) mentions Czech specimens from *B. verrucosa* from 1983. Since 1990, the oak wood wasp has become widespread in the whole country (Pádr 1990).

X. camelus (alder wood wasp) was found predominantly in floodplain forests on *Alnus* and *Betula*, but also on *Quercus*, *Ulmus*, *Populus*, and *Prunus*. Its distribution reaches from

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Europe to Siberia. It has been observed in local outbreaks on *Alnus glutinosa* that had been weakened by *Phytophthora*, by low soil water levels (Taeger & Blank 1998), or by long-term flooding of stands (in Czechia during floods in 2002).

X. prolongata (willow wood wasp) colonizes various broad-leaved trees, predominantly *Salix*, *Populus*, and *Ulmus*, but also *Alnus* and *Carpinus*. It has been implicated as a passive carrier of watermark disease of willows. The rare *X. picta* occurs mainly on *Alnus* (Eichhorn 1982).

Females of most species of the taxonomically related siricid wasps (*Hymenoptera*: *Siricidae*; with the exception of the genus *Xeris*) carry symbiotic fungi in their mycangia. The fungi are inoculated in the wood during oviposition and serve as cellulose-digesters and/or nutrition for siricid larvae. These fungi belong to basidiomycetous genera *Stereum*, *Amylostereum* or *Cerrena*.

Most of the work done on siricids and their symbionts concerns *Sirex noctilio* damaging pines. *Siricidae* and their fungal symbionts are native to the northern hemisphere. In these regions, these insect–fungal complexes are considered to be secondary pests that attack sick or already dying trees. However, *S. noctilio* and its fungal symbiont, *A. areolatum*, which have been introduced into Australasia (1900–1950s), South America (1980s) and South Africa (1994), now pose a threat to the softwood industries of these regions (summarized in Slippers *et al.* 2002). Symbiotic basidiomycetes of the genus *Amylostereum* were identified and their co-evolution with *Sirex* wasps, population genetics, phylogeny and distribution have been researched (Vasiliauskas *et al.* 1999; Tabata *et al.* 2000; Slippers *et al.* 2001, 2002, 2003).

In comparison with the state of research on *Sirex*, the information concerning *Xiphydria* wasps and their symbionts is rather scarce. The taxonomy and phylogeny of these fungi have received little attention in the past. *Xiphydria* females carry xylariaceous ascomycetes. Cartwright (1938) cultivated the fungus from mycangium of *X. prolongata* and noted the macroscopic similarity of the culture with *Daldinia concentrica*. This view was shared also by Francke-Grosmann (quoted as personal communication by Schimitschek) and Schimitschek (1974) who found *Daldinia*-like fungi in *X. camelus* and *X. longicollis*. Kajimura (2000) reported a *Daldinia*-like fungus in a Japanese wood wasp *X. ogasawarai*. However, during the time between the first and last report, no attempt was made to identify the symbiotic fungi in any *Xiphydria* species.

Daldinia spp. occur also as endophytes (or more precisely latent pathogens) inside trees, remaining dormant (Petrini & Petrini 1985) until wood decay and/or perithecial stroma formation is triggered by drying out, whether this may be caused by climatic stress or by a forest fire (Boddy 1994; Johannesson *et al.* 2000). It is supposed that *Daldinia* spp. ascospores and conidia from perithecial stromata spread on neighbouring trees through fungivorous insects and via the wind (Johannesson *et al.* 2001).

Daldinia taxonomy is based on ascospore morphology and ornamentation (detected by SEM), morphology of fruiting bodies, and the appearance of mycelium and conidiation type on oatmeal agar (Ju *et al.* 1997). Analysis of pigments and secondary metabolites provides additional information (Stadler *et al.* 2001a, 2004a).

Entonaema differs from *Daldinia* by stromata with an interior that is hollow, partially gelatinous, and filled with liquid in which the perithecia develop in a single layer beneath a thin brightly coloured crust. (Rogers 1981). The anamorph, observed in culture, is *Nodulisporium* (Stadler *et al.* 2004a). *Entonaema* was considered a genus with a tropical distribution (Rogers 1981), but more recently, records were reported from southern Bulgaria (Benkert 1993) and southwestern France (Stadler *et al.* 2004a). Rogers (1981) mentioned a collection from Estonia, which he considered dubious. There is no record of *Entonaema* from the Czech Republic.

The taxonomy of *Daldinia* and related genera has been revised recently using molecular techniques that have been applied to elucidate the phylogeny and population structures (Johannesson *et al.* 2000, 2001) and the phylogenetic relationships between genera (Hsieh *et al.* 2005; Triebel *et al.* 2005). Cryptic speciation appears to occur inside *D. eschholzii* and *D. concentrica* that has been overlooked using traditional methods of identification (Stadler *et al.* 2004b).

In this study, fungal symbionts collected from three *Xiphydria* species were isolated and their taxonomic placement and phylogenetic position inside *Xylariales* were identified using DNA analysis.

Material and methods

Fungal and plant material

Samples of *Quercus robur* colonized by *Xiphydria longicollis* larvae were collected at Libický luh forest near Velký Osek (close to river Labe). Samples of *Alnus glutinosa* containing *X. camelus* were collected in the floodplain forest, Kančí obora (Moravia). *Salix alba* with *X. prolongata* was sampled in floodplain forest in the Litovelské Pomoraví (Moravia) reserve. Several specimens of *Daldinia* fruiting bodies and their respective living cultures were used for the comparison (Table 1). All isolates were maintained on slants of 2 % malt extract agar (MEA; Difco, Detroit, MI) at 4 °C. Representative isolates are deposited in the Culture Collection of Fungi (CCF) and in the herbarium of the National Museum (PRM), both in Prague (Table 1).

Isolation of symbionts

The wasps were reared from tree trunks. The logs (0.8 m long, 0.1–0.3 m diam) were cut from colonized trees in January to February and incubated in cage nets and polyethylene bags at −2 °C to −4 °C. In April, they were transferred to 20–26 °C and sprinkled bi-weekly with sterile water (important for completing wood wasp development). Emerging female wood wasps were killed by ethylacetate vapour and dissected on paraffin plates. Their mycangia were exposed by a short tug of tweezers onto the ovipositor and the contents transferred by a fine needle on MEA plates. The number of colony forming units in a single mycangium was assessed using the dilution method. Ten mycangia of *Xiphydria longicollis* were placed each in 1 ml sterile water, vigorously vortexed for 5 min, and the resulting suspension diluted and plated on MEA. Colony counts were made after 3–5 d.

Table 1 – Isolates used in the study

Species	Isolate/herbarium specimen	Accession no.		Source	Host tree	Location
		rDNA	β-tubulin			
Daldinia concentrica	CCF3625/PRM 857194	AM292046	-	Fruiting body	Trunk of *Quercus robur*	UK, Stansted, 2005
D. concentrica	CCF3626/PRM 857195	AM292045	-	Fruiting body	Burnt log of *Tilia* sp.	Czech Republic, Holasovice, Štemplovec 1996
D. fissa	CCF3627/PRM 857196	AM292038	-	Fruiting body	Broad-leaved tree*	Czech Republic, Jazevčí reserve, White Carpathians, 2004
D. decipiens	CCF3628 (A)	AM292039	AM292036	*Xiphydria longicollis*	*Q. robur*	Czech Republic, Velký Osek, Libický luh, 2005
D. decipiens	CCF3629 (L29_1)	AM292040	-	*X. longicollis*	*Q. robur*	Czech Republic, Velký Osek, Libický luh, 2005
D. decipiens	CCF3630 (10_11)	AM292041	-	*X. longicollis*	*Q. robur*	Czech Republic, Velký Osek, Libický luh, 2005
D. decipiens	CCF3631 (C1)	AM292042	AM292037	*X. camelus*	*Alnus glutinosa*	Czech Republic, Břeclav, Kančí obora, 2005
D. decipiens	CCF3632 (R)	-	-	Fruiting body	*A. glutinosa*	Czech Republic, Třeboň, 2005
Entonaema cinnabarina	CCF3633 (B)	AM292043	-	*X. longicollis*	*Q. robur*	Czech Republic, Velký Osek, Libický luh, 2005
E. cinnabarina	CCF3634 (P1)	AM292044	-	*X. prolongata*	*Salix alba*	Czech Republic, Litovelské Pomoraví reserve, near Litovel, 2005

* Fallen tree in a forest composed of oaks and hornbeams.

Isolations from fruiting bodies

Ascospores were released from the perithecial layer into sterile distilled water using a sterile needle and plated on MEA. Alternately, pieces of the stroma from the inside the fruiting body were plated and the resulting mycelium subcultured.

Microscopy

Samples were mounted in 1 % Cotton blue in lactophenol and observed using a JENAVAL microscope (Zeiss, Jena) and a Nikon SMZ-1B stereo zoom microscope equipped with a Camedia C-5060 WZ camera (Olympus, Hamburg). Spore measurements were done digitally on 50–100 conidia and 40–60 ascospores using the software QuickPHOTO Camera 2.2.

DNA analysis

DNA was purified from young (preferably uncoloured) cultures grown on cellophane laid onto MEA agar plates using UltraClean Microbial DNA Isolation Kit (Mo-Bio Laboratories, Solana Beach, CA) according to the manufacturer's manual. Alternatively, a piece of the inner stroma of a fruiting body was used. Nuclear rDNA containing the ITS regions (ITS1 and ITS2) was amplified with the primers ITS5 and ITS4s (White *et al.* 1990; Kretzer *et al.* 1996) and part of the β-tubulin gene with the primers T1 and T22 (O'Donnell & Cigelnik 1997) in a Mastercycler Gradient (Eppendorf, Hamburg) as follows: one cycle of 3 min at 95 °C, 30 s at 55 °C and 1 min at 72 °C, 30 cycles of 30 s at 95 °C, 30 s at 55 °C and 1 min at 72 °C and one cycle 30 s at 95 °C, 30 s at 55 °C and 10 min at 72 °C. The reaction mix consisted of PCR buffer (Finnzymes, Oy), 2 mM deoxynucleotides, 2 pmol of each primer and 1 U DynaZyme (Finnzymes, Oy) and 5–50 ng DNA in 25 μl total volume. Amplified fragments were purified using Wizard DNA Clean-up System (Promega, Madison, WI) and custom-sequenced at the Faculty of Sciences, Charles University, Prague.

Phylogenetic relationships

RAPD analysis using primer 8F (GCTCTGAGATTGTTCCGGCT) was performed according to Pažoutová & Frederickson (2005). Sequences of rDNA and β-tubulin were aligned using the MUSCLE (Edgar 2004) web interface (http://phylogenomics.berkeley.edu/cgi-bin/muscle/input_muscle.py) with manual corrections in BioEdit 7.0.5 (Hall 1999). The data set of ITS rDNA sequences consisted of 571 sites, 113 of them variable. The data set of β-tubulin sequences consisted of 1447 sites, 322 of them variable. All phylogenetic analyses were performed using MEGA3 (Kumar *et al.* 2004). NJ trees were inferred

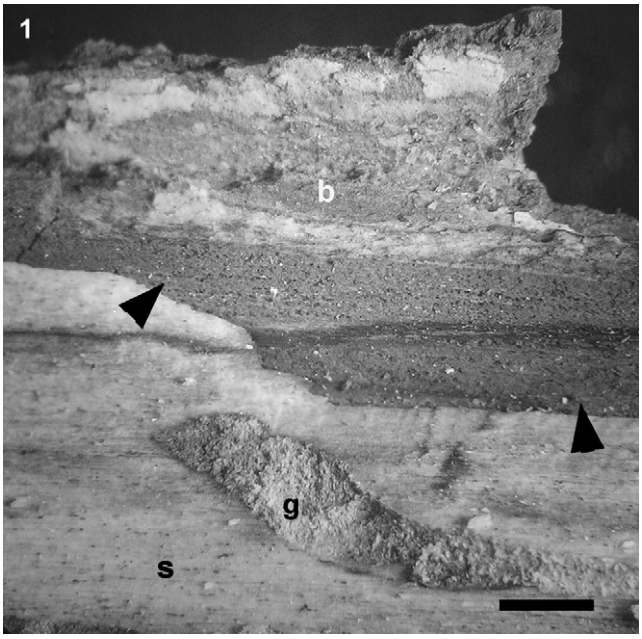

Fig 1 – Alder log colonized by *Xiphydria camelus* and its symbiotic fungus. Arrows indicate layers of black stromatous cells at the interface between bark and wood; b, bark; g, larval gallery; s, clusters of stromatous cells inside tracheae. Bar = 1 cm.

from a Kimura-2 parameter distance matrix. The distance for each pair of sequences was computed, ignoring only those gaps that were involved in the comparison ('Pairwise-Deletion' option in MEGA3). Statistical support for clades was obtained with 500× BS.

Results and discussion

Growth of the fungi in the wood

After the wasps emerged, the growth of the symbiotic fungi inside the incubated logs was observed. The fungi had spread through the wood from the galleries. A layer of stromatous cells was found also at the boundary between the bark and wood (Fig 1). Dark branched stromatous cells formed aggregates inside the tracheae (Fig 2), effectively clogging

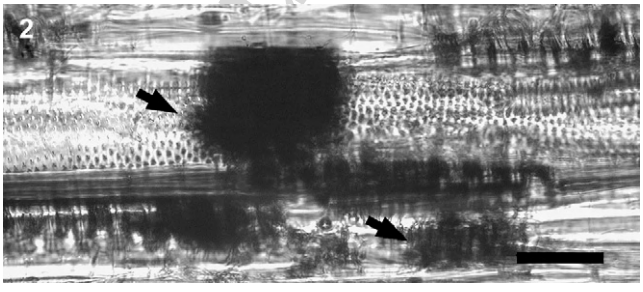

Fig 2 – Clusters of stromatous cells of *Daldinia decipiens* inside alder tracheae (arrows). Bar = 50 μm.

Fig 3 – *Xiphydria prolongata* female. Arrow shows the localization of mycangia at the proximal end of the ovipositor. Bar = 1 mm.

transpiration. The appearance of all symbiotic fungi in this stage of development was indistinguishable.

On the alder log from Třeboň, several small fruiting bodies with a stipe developed on an upper cut end of a caged specimen during incubation. Their development stopped before perithecial formation so that no ascospores were available for measurement.

Mycangia of Xiphydria *spp.*

In *Xiphydria* females, paired mycangia were found directly below the subgenital plate (Fig 1), immediately at both sides of vagina and the ovipositor entry. A reservoir of clear mucus was located in the last segment of the abdomen (Figs 3–4). The mucus was colourless in *X. longicollis* and *X. prolongata*, and orange to reddish in *X. camelus* (also noted in Eichhorn 1982). Mycangia were oblong to obovoid, tapering distally (Fig 5). The size of mycangia was not proportional to the size of the female; this was especially so in *X. camelus*. The smallest mycangia were found in *X. camelus*, the biggest in *X. longicollis*, those of *X. prolongata* were of intermediate size. Mycangia of *X. longicollis* contained yeast-like and pseudomycelial propagules (Fig 6) amounting to 10^6–10^7 colony forming units per female. Dilution experiments have shown that the mycangium content was pure culture of a single fungus.

Morphological and molecular identification of the symbionts

All isolates of *Xiphydria* spp. were sorted to two morphotypes (A and B) with minimal infragroup variability in RAPD pattern, but differing from each other (data not shown). All of the 45 *X.*

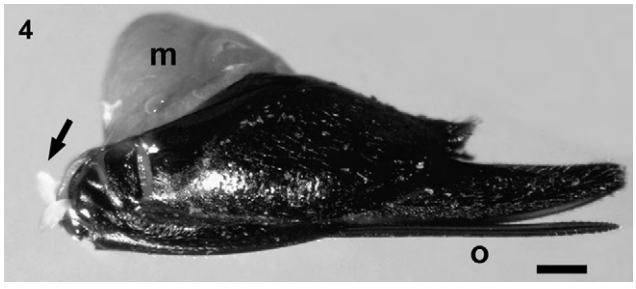

Fig 4 – Detached abdomen of *Xiphydria camelus*. Arrow indicates a pair of mycangia; m, mucus reservoir; o, ovipositor. Bar = 500 μm.

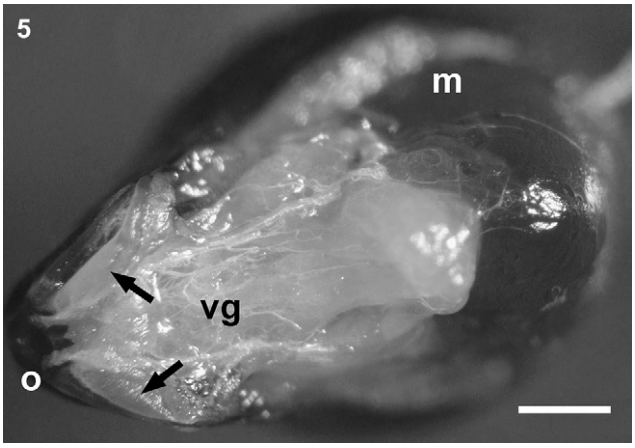

Fig 5 – Mycangia of *Xiphydria camelus* of the detached abdomen in transverse view. Arrows indicate mycangia; m, mucus reservoir; o, base of the ovipositor; vg, vagina. Bar = 500 μm.

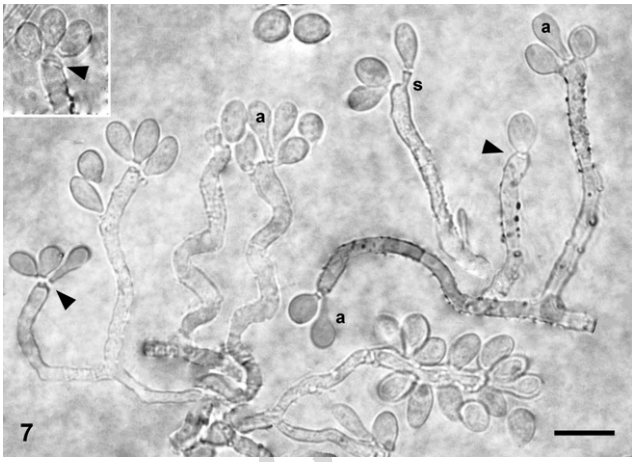

Fig 7 – *Daldinia decipiens* from *Xiphydria camelus*. Culture on MEA 3-wk old. Arrows indicate annelidic conidiation; s, stipe remnant after broken conidium; a, conidia with attenuated base. Bar = 10 μm.

camelus females shared the same morphotype, which was identical in RAPD pattern with the isolate from a fruiting body from the alder log (morphotype A). Morphotype B was exclusively associated with two adults of *X. prolongata*. Fifteen *X. longicollis* females were examined and two types of fungi were found. Eleven females carried fungi from morphotype A. Four females contained fungus resembling the symbiont of *X. prolongata* (Fig 9, Table 2).

From the isolates representative of all groups, sequences of the ITS-rDNA regions were obtained. Comparison with *Daldinia* sequences from databases and fruiting body specimens collected in our laboratory revealed that representatives of group A were close, but not identical, to *D. petriniae*. Sequences of rDNA belonging to species related to *D. petriniae* (*D. singularis, D. decipiens*) were not available in the database, only parts of their β-tubulin gene. Therefore, this region of the representatives of group A was also sequenced. The closest relative was *D. decipiens* (Fig 10) (Hsieh *et al.* 2005), which also corresponded to the morphology of immature fruiting bodies (presence of a stipe) (Stadler *et al.* 2001b).

Sequences of rDNA from symbiotic *D. decipiens* isolates were not identical, which suggests the existence of different lineages, even across the host and vector species; the sequence of isolate CCF3630 of *X. longicollis* was identical to that of isolate CCF3631 from *X. camelus*. The only difference between the 829 bp β-tubulin sequences of isolate CCF3628 from *X. longicollis* and isolate CCF3631 from *X. camelus*, was a single G–C transversion.

Surprisingly, rDNA sequences of a B morphotype clustered with that of a species of another genus, *Entonaema cinnabarina* (Fig 11). Sequences of morphotype B from *X. longicollis* and *X. prolongata* differed in three positions out of 580.

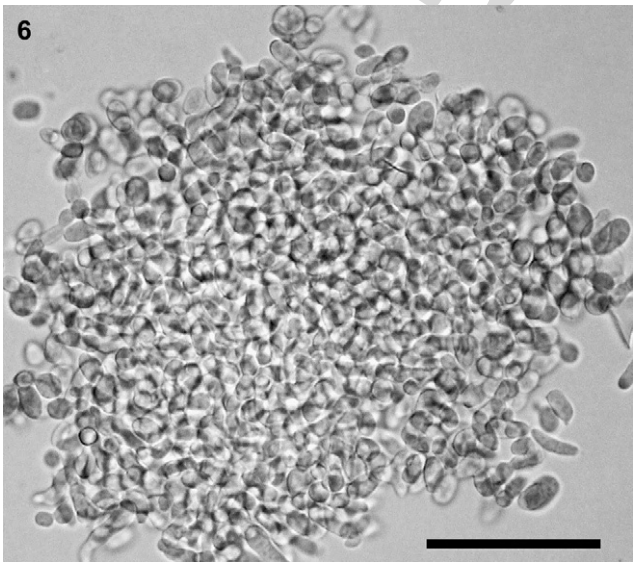

Fig 6 – Propagules of symbiotic fungus from a mycangium. Bar = 50 μm.

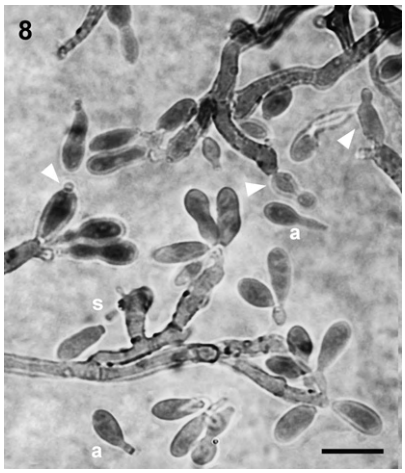

Fig 8 – *Entonaema cinnabarina* from *Xiphydria prolongata*. Culture on MEA 3-wk old. Arrows indicate secondary conidiation; s, stipe remnant after broken conidium; a, conidia with attenuated or pin-shaped base. Bar = 10 μm.

Table 2 – Spore measurements of *Daldinia* and *Entonaema* spp.

	Daldinia fissa CCF3627	*D. concentrica* CCF3626	*D. concentrica* CCF3625	*D. decipiens*[a]	*Entonaema cinnabarina* CCF3633	*E. cinnabarina* CCF3634 (dacryoid)	*E. cinnabarina* CCF3634 (pin-shaped)
CONIDIA							
Length (μm)							
Mean	7.8	7.4	7.6	6.8	7.2	8.1	10.6
S.D.	0.9	0.9	0.8	0.8	0.7	0.9	1.4
Minimum	5.9	5.6	5.4	5.2	6.4	6.3	8.7
Maximum	9.5	11.7	10.1	9.6	9.4	9.9	13.5
Width (μm)							
Mean	5.1	3.5	3.8	4.3	4.5	3.7	3.8
S.D.	0.5	0.3	0.4	0.5	0.6	0.4	0.4
Minimum	4.0	2.7	2.9	2.5	3.6	2.8	2.9
Maximum	6.0	4.1	4.7	5.7	6.9	4.9	4.8
ASCOSPORES	PRM 857196	PRM 857195	PRM 857194				
Length (μm)							
Mean	12.7	16.6	12.7				
S.D.	0.7	1.1	1.1				
Minimum	10.5	13.8	9.8				
Maximum	14.2	18.6	15.8				
Width (μm)							
Mean	6.5	8.1	6.2				
S.D.	0.4	0.6	0.5				
Minimum	5.8	6.8	5.5				
Maximum	7.5	9.4	7.6				

a Averaged from all isolates

***Daldinia decipiens* (morphotype A)**

Substrate: *Xiphydria camelus*, and *X. longicollis*; on alder and oak.

Felty colonies darkening with age, often producing yellowish (citrine) to green–brown pigments in agar. Stromatous cells and rough conidiophores of *Sporothrix* and *Nodulisporium* type have been observed. Conidiation type was mixed; both sympodial holoblastic and annelidic conidiation were observed. Conidia were hyaline, ovoid to dacryoid, often with an attenuated base (Fig 7, Table 2). The latter separated from the conidiogenous cell either as a whole, or a septum was formed in the attenuated part, and the conidium broke off leaving a short stipe.

***Entonaema cinnabarina* (morphotype B)**

Substrate: *Xiphydria longicollis* and *X. prolongata*; on oak and willow.

Black colonies with a prevalence of dark stromatous cells and a release of brown–black pigment into agar. After one to three weeks, initially white then darkening aerial mycelium with mostly *Nodulisporium/Sporothrix* type of rough conidiophores

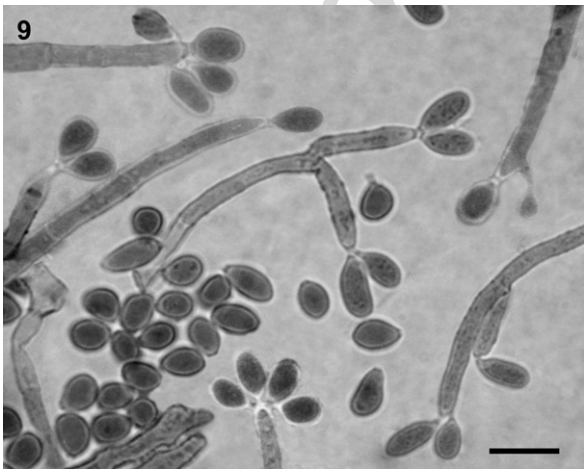

Fig 9 – *Entonaema cinnabarina* from *Xiphydria longicollis*. Culture on MEA 3-wk old. Bar = 10 μm.

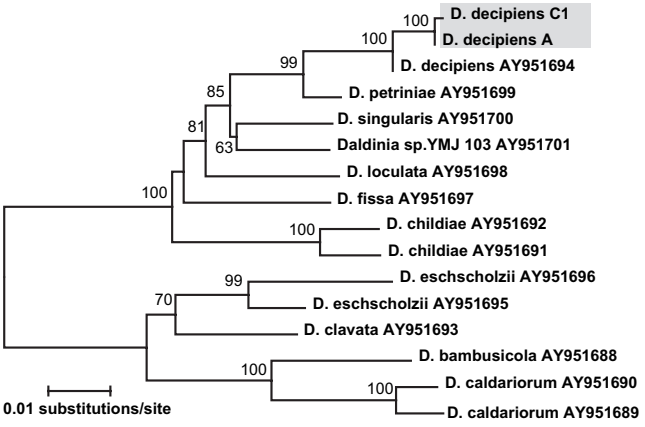

Fig 10 – Phylogenetic position of *Xiphydria* symbiotic fungi inferred from β-tubulin sequences. NJ tree was based on Kimura-2 parameter distance matrix. BS supports over 50 % are given on the respective clades. Sequences obtained in this study are highlighted in grey.

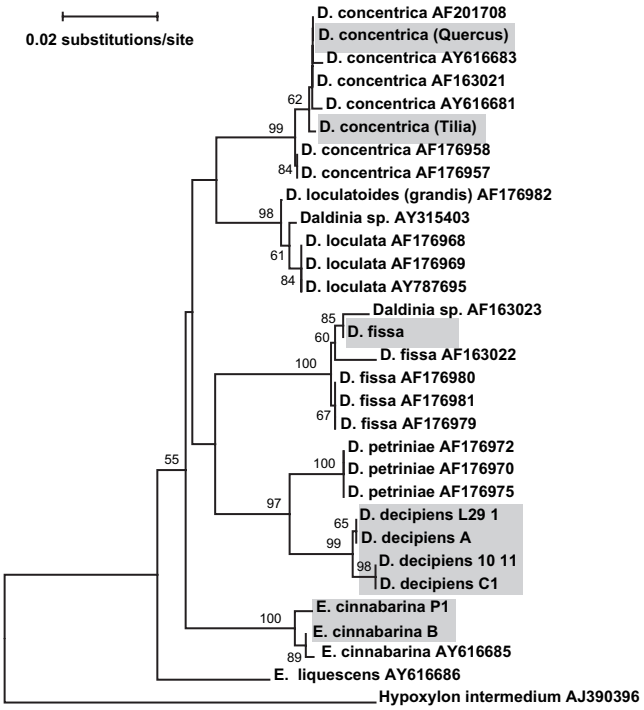

Fig 11 – Phylogenetic position of *Xiphydria* symbiotic fungi inferred from ITS rDNA sequences. NJ tree was based on Kimura-2 parameter distance matrix. BS supports over 50 % are given on the respective clades. *Hypoxylon intermedium* sequence was used as an outgroup. Sequences obtained in this study are highlighted in grey.

was formed on the clusters of stromatous cells. Conidiation was sympodial and holoblastic. We observed dacryoid conidia with a truncated base as well as longer conidia with an attenuated base or shaped like a bowling pin. Occasionally, septa were formed in the attenuated part. Sometimes these elongated spores produced secondary conidia (Fig 8, Table 2). The isolates from *Xiphydria longicollis* formed smaller conidia of the dacryoid shape and an attenuated base was seldom found (Fig 9, Table 2).

Two species of xylariaceous symbiotic fungi, *Daldinia decipiens* and *Entonaema cinnabarina*, have been isolated from three *Xiphydria* species. Except for one record, *E. cinnabarina* has been considered a warm region species. We have supposed that an association of *X. longicollis* with *E. cinnabarina* may reflect that the oak wood wasp is spreading to Central and Northern Europe from the south, where *E. cinnabarina* fruiting bodies occur. However, the association of *E. cinnabarina* with *X. prolongata* contradicts this assumption, as there is no documented south–north migration of this wasp species. The 'latent' presence of symbiotic *E. cinnabarina* in Central and Northern Europe may explain the single Estonian record of a fruiting body from 1967 (Rogers 1981).

The association of *Daldinia*-related fungi with xiphydriid wasps was found in Europe and Asia. The identity of the Asian symbiont is unknown, but the colony appearance on potato-dextrose agar documented by Kajimura (2000) does not resemble *D. decipiens* as we have seen it on the same medium.

So far, fruit bodies of *D. decipiens* have only been recorded from *Betula* spp. (Stadler *et al.* 2001a, b) in Denmark, Sweden, and various provinces of Germany, whereas the host spectrum of its wasp vectors covers predominantly oaks and alders, and fewer birches. It seems that the ability of intense growth inside tracheae after an inoculation by wasps does not lead to equally successful fructification on the colonized substrate.

Wood wasps ensure that the fungal symbiont is reliably transferred to suitably weakened host trees. Such trees attract many wood wasp females, therefore the presence of isolates with different mating factors is to be expected. Larval galleries penetrate the whole tree and the symbiotic fungus spreads from the galleries through tracheae, so that a contact of different mycelia followed by heterokaryosis and fruiting body formation might occur. Indeed, we have observed an incomplete fructification of *D. decipiens* on an incubated alder log, arrested by the loss of natural humidity of the wood that cannot be fully replaced by sprinkling. However, it is not known whether *D. decipiens* is a heterothallic species; heterothallism has so far only be proven in *D. loculata* (Guidot *et al.* 2003).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mycres.2006.10.006

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