Identification of the ambrosia fungus of *Xyleborus monographus* and *X. dryographus* (Coleoptera: Curculionidae, Scolytinae)

Heiko Gebhardt1,*, Dominik Begerow1, and Franz Oberwinkler1

The scolytid ambrosia beetles *Xyleborus monographus* and *X. dryographus* were investigated to identify their nutritional ambrosia fungi. The examination of the oral mycetangia of the beetles, the specialized organs for fungal transport, revealed the dominant occurrence of *Raffaelea montetyi*, a fungus that was also predominant in the beetle tunnels in the immediate vicinity of the feeding larvae. *R. montetyi* was previously known only as the ambrosia fungus of the platypodid ambrosia beetle, *Platytopus cylindrus*. These beetle species inhabit the same habitat, mainly trunks of oaks in the Western Palearctic. The possibility of an exchange of the symbiotic fungus between the ambrosia beetles within their common breeding place is discussed. Consequently, the previous hypothesis of a species-specific association of a single ambrosia fungus with a single beetle species is questioned. A phylogenetic analysis based on DNA sequences classified *R. montetyi* within the Ophiostomatales of the ascomycetes. The investigation of conidiogenesis of *R. montetyi* by SEM supported this taxonomic placement and showed the development of the conidia by anellidic percurrent proliferation, identical to the conidiogenesis reported for many anamorph states of the Ophiostomatales.

Attacks of host trees by forest beetles, such as the Scolytinae and Platypodinae (Coleoptera: Curculionidae), are followed by colonization of the trees by a multitude of different fungi that are loose or close associates of these insects. The wood boring scolytid and platypodid beetles and their fungi, referred to as ambrosia fungi, form a symbiotic interaction with mutual adaptation of the symbionts. The adults bore extensive galleries into the sapwood of their host trees, where their progeny complete their life cycle protected from the external environment. On the tunnel walls the wood-inhabiting ambrosia fungi form a thin continuous layer. The beetles cannot utilize wood as a primary nutritional substrate and all developmental stages depend on the ambrosia fungi as their chief source of food. (e.g. Batra 1963, Beaver 1989, Francke-Grosmann 1956).

Different fungal species can form the ambrosia fungus layer simultaneously in the vicinity of one coleopterous species. Several non-specific fungi, the auxiliary ambrosia fungi, can appear in association with different beetle species. In contrast, a highly species-specific fungus, the primary ambrosia fungus, is constantly associated with a particular beetle species (Batra 1966, 1985, Beaver 1989). Generally, this fungus cannot survive outside the tunnel systems without the symbiotic beetle. One reason is a strong sensitivity against even short periods of desiccation (Zimmermann & Butin 1973). During the short flight phases, when the adult beetles search for a new breeding substrate, the primary ambrosia fungus has to be protected and is stored during these transmission events within an invaginated cuticular structure of the beetle, the mycetangium (Batra 1963, Francke-Grosmann 1956, 1967).

Species of ambrosia fungi are difficult to distinguish as they lack clearly defining morphological features. The similarity among species may be due to convergent evolution of this group of imperfect fungi. However, the use of ribosomal DNA sequence data has become a useful tool for inferring fungal phylogeny. Ambrosia fungi of the genera *Ambrosiella* Arx & Hennebert and *Raffaelea* Arx & Hennebert are indicated to be non-monophyletic and closely related to the different clades of the polyphyletic ophiostomatoid fungi (Blackwell & Jones 1997, Cassar & Blackwell 1996, Farrell et al. 2001, Jones & Blackwell 1998, Rollins et al. 2001).

These ophiostomatoid fungi, including the monophyletic genus *Ophiostoma* H. Sydow & P. Sydow (Ophiostomatales), comprise several convergently developed groups, united by their morphology and by the production of spores well adapted for insect dispersal. Ascomata with elongated perithecial necks produce sticky ascospores that are transmitted on the surfaces of the insects or by passage through their digestive tracts (Malloch & Blackwell 1993).

The close symbiotic relationship between ambrosia beetles and fungi has been known for more than 150 years (Hartig 1844). However, for most species of ambrosia beetles the associated fungi have been neither characterized nor described.

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Tab. 1: Numbers of adult female beetles of the genera *Platypus* and *Xyleborus* and gallery samples collected from *Quercus robur* in different localities in southwest Germany 2001–2002

<table>
<thead>
<tr>
<th>beetle species</th>
<th>Fungus isolated from</th>
<th>Number of samples</th>
<th>Geographic origin</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. cylindrus</em> (Fabr.)</td>
<td>galleries</td>
<td>10</td>
<td>Karlsruhe</td>
<td>June 2001</td>
</tr>
<tr>
<td></td>
<td>beetles</td>
<td>5</td>
<td>Pfalz</td>
<td>Oct. 2001</td>
</tr>
<tr>
<td><em>X. dryographus</em> (Ratzeb.)</td>
<td>galleries</td>
<td>15</td>
<td>Pfalz</td>
<td>June 2001</td>
</tr>
<tr>
<td></td>
<td>beetles</td>
<td>20</td>
<td>Karlsruhe</td>
<td>Jan. 2002</td>
</tr>
<tr>
<td><em>X. monographus</em> (Fabr.)</td>
<td>galleries</td>
<td>10</td>
<td>Karlsruhe</td>
<td>June 2001</td>
</tr>
<tr>
<td></td>
<td>beetles</td>
<td>40</td>
<td>Karlsruhe</td>
<td>Jan. 2002</td>
</tr>
<tr>
<td></td>
<td>beetles</td>
<td>25</td>
<td>Pfalz</td>
<td>May 2002</td>
</tr>
<tr>
<td></td>
<td>galleries</td>
<td>12</td>
<td>Freiburg</td>
<td>July 2002</td>
</tr>
</tbody>
</table>

For *X. dryographus*, neither the associated ambrosia fungi nor the position of the mycetangium have been reported. For *X. monographus*, a species with a paired oral mycetangium (SCHIEDL 1964), imprecise and contradictory results have been obtained regarding the identity of the involved ambrosia fungi: FRANCKE-GROSSEMMANN (1967) suggested *Ambrosiella sulphurea* Batra as a possible symbiont while KOWALSKI (1991) reported an undescribed species of the genus *Raffaelea* from tunnel systems of *X. monographus* from Poland and SCHIEDL (1964) mentioned a species of the genus *Mortierella* after investigations in Austria.

We decided to investigate the fungus-beetle interactions of a number of European species of ambrosia beetle and describe here our results for *X. monographus*, *X. dryographus* and *Platypus cylindrus*. On the basis of our results we discuss some general hypotheses regarding the symbiotic interrelationships of ambrosia beetle and fungi.

**Material and methods**

**Collection of beetles and wood samples with galleries.** Material was collected from Biénwald near Lauterburg and Schaidt (Pfalz), Hardwald (northern Karlsruhe) and forests near Weisweil (northern Freiburg) all located in southwest Germany. Logs of *Quercus robur* L. (20–35 cm in diameter) infested by *X. monographus* and/or *X. dryographus* as well as *P. cylindrus* were cut. The recently collected logs were disinfected as described by BATRA (1985). Cross-sections were sawn (about 4 cm long) and were immediately treated with a disinfected wood chisel to open the tunnel systems of the beetles in the sapwood. Wood chips cut from the samples and containing beetle tunnel sections were placed into sterile Petri dishes for observation. Adult females from the tunnel systems were also taken for fungal isolation. Additional females were collected from trunks of oaks during their short flight-periods.

**Histology of the beetles.** 25 adult females of *X. dryographus*, collected during the flight-period (June 2000, Karlsruhe), were fixed in Bouin-solution, dehydrated with isopropanol, and embedded in paraffin (58 °C melting-point). They were sectioned at 20 μm and stained in a diluted aceton solution of Giemsa stain, and mounted in Euparal.

**Isolation of the ambrosia fungus.** Isolations of the ambrosia fungi were made from the galleries found in infested logs or from the mycetangia of adult females (Tab. 1). Within the genus *Xyleborus* ambrosia fungi are stored exclusively in the mycetangia of female beetles.

For isolating the fungus from tunnel walls, sections of the gallery systems, where the larvae graze on the fungus, were taken. A piece of the ambrosia fungus layer was transferred by a sterile needle onto a glass slide covered with a fine layer of PDA (potato-dextrose agar) or YEEM (yeast-extract-malt extract agar) media (BATRA 1967). The slides were incubated in moist Petri dishes. They were observed daily with a light microscope using sterile cover-glasses. To obtain pure cultures, the mycelium of growing colonies was then transferred to agar plates.

Each of the beetles used to isolate the mycetangial fungi was first processed through the fractional-sterilization method of FRANCKE-GROSSEMMANN (1956). The method was modified reducing the incubation period (BATRA 1985) and using Petri dishes with autoclaved oak wood shavings embedded in water agar instead of Petri dishes with moist filter paper. The small part of a living beetle containing the mycetangium was crushed and the contents dispersed on glass slides covered with a fine layer of agar. The slides were observed using a light microscope, and the fungal contents of the mycetangia were transferred onto new glass slides covered with a fine layer of agar. They were observed daily until mycelium of growing colonies was transferred to agar plates to obtain pure cultures. All cultures were incubated in the dark at room temperature.

**Fungal morphology.** The appearance of the fungus was studied in numerous samples taken from gallery systems and from cultured material from the different media. Samples were mounted in water on slides and observed using a light microscope. For comparison, cultures of *Raffaelea montetyi, R. ambrosiae* and *R. arxii* were obtained from CBS (Utrecht). Pure cultures on 2 % malt extract agar (MEA) plates were used for
scanning electron microscopy (SEM), taken from the ex-type strain of *R. montetii* (CBS 463.94) and from isolations of a mycetangium from *X. monographus* (Karlsruhe). After fixation in 2% glutaraldehyde in 0.1 M cacodylate buffer, the material was postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h, then washed with distilled water, and dehydrated in a graded ethanol series. The fixed material was critical point dried, coated with gold palladium, and examined using a Cambridge Stereoscan 250 MK 2 scanning electron microscope.

**DNA extraction, amplification and sequencing.** The strains of fungi used for DNA analyses are listed in Table 2. Genomic DNA was extracted from pure cultures using Qiagen DNeasy Plant Kit™ according to the manufacturer’s instructions. The SSU rDNA region was amplified according to the protocol of White et al. (1990) using the primer pairs NS1/NS4 and NS3/NS6. The templates were purified using the Qiagen PCR Purification Kit. BigDye® Terminator v. 3.1 Cycle Sequencing Kit was used in combination with a 3100 Genetic Analyser to determine the sequences of both strands using the PCR-primers and following the manufacturer’s protocol.

**Phylogenetic analyses.** The DNA sequences of the fungi listed in Table 2 were aligned with the following published sequences from the GenBank nucleotide sequence databases: *Hypomyces chrysospermus* Tul. & C. Tul. M89993, *Leucostoma persoonii* (Nitschke) Höhn M83259, *Microascus cirrosus* Curzi M89994, *Opophyllum ulmi* (Buissman) Nannf. M83261, *Pleospora rudis* Berl. U00975 and *Taphrina wiesneri* (Rathay) Mix D12531. An alignment of about 1400 bp was created using MEGALIGN of the Lasergene package. A Bayesian approach using Markov chains Monte Carlo (MCMC) as implemented in MrBayes 2.01 (Huelsenbeck & Ronquist 2001) was used for phylogenetic analyses. Four incrementally heated simultaneous Monte Carlo Markov chains were run over 1,000,000 generations using the general time reversible model of DNA substitution with gamma distributed substitution rates, random starting trees and random starting parameters for the DNA substitution model. Trees were sampled every 100 generations resulting in an overall sampling of 10000 trees. From those trees that were sampled after the process had reached stationarity, a 50% majority rule consensus tree was computed to obtain estimates for the a posteriori probabilities. This Bayesian approach to phylogenetic analysis was repeated several times, always using random starting trees and random starting values for the model parameters to test the reproducibility of the results.

**Results**

**Mycetangia.** Examination of the paraffin sections from *X. dryographus* females demonstrated an oral position at the base of the mandibles (Fig. 1) for the paired mycetangium (Fig. 2). The chitinous mycetangium wall is covered with small denticles.

**Fungal associates.** The gallery systems of the investigated beetle species showed a discoloration of the tunnel walls. In older parts of the galleries and in the entrance shaft, different fungi, particularly ophiostomatoid fungi and moulds were invariably present. In contrast, tunnel sections where the larvae occurred showed a single predominant fungus. The fungus grew with tightly packed hyphae bearing conidia at the apices.
of the conidiophores. Cultures of the predominant fungus were identified as *Raffaelea montetyi* Morelet after a comparison with the ex-type strain (CBS 463.94).

The oral mycetangia of both species contained tightly packed, highly vacuolated yeast-like cells. From these cells germination occurred within the next day after inoculation on glass slides covered with a fine layer of culture medium (Fig. 3). Conidiophores were produced within two days, and in each case the fungus was identified as *Raffaelea montetyi*.

Spores and cells of additional fungi were found sporadically among cells of *R. montetyi* in the mycetangia of the beetles. It was uncertain whether they derived from mycetangia or represented fungal contaminations from the beetle’s pharyngial cavity that is adjacent to the mycetangia. However, in more than 60% of the samples of individuals, mycetangia of the two *Xyloborus* species appeared as a pure isolate of *R. montetyi*.

Microscopical observations of the galleries (Karlsruhe) and mycetangia (Pfalz) of *P. cylindrus* confirmed *R. montetyi* as the predominant associated ambrosia fungus.

**Fungal morphology.** The conidiophores of *R. montetyi* are mononematous on the culture medium used, while in the galleries of their associated beetles they formed fascicles. Scanning electron micrographs of *R. montetyi* showed prominent annellations at the apices of the conidiogenous cells (Figs. 5, 6). The annellations varied in each sample from dense to extensive as well as nodulose.

**Phylogenetic analyses.** The phylogenetic relationships of the fungus species isolated in this study were investigated using DNA sequencing of nuclear rDNA sequences from the SSU region to confirm the phylogenetic relationship of *R. montetyi*. About 1400 nucleotid positions were sequenced for each taxon. Among the three strains of *R. montetyi* nucleotid differences were not detected. For *R. ambrosiae* the NS3-NS6 product contained an insertion of an additional 411 bp. By comparison with the consensus sequence of Gargas et al. (1995) the site of this insertion was identified as position 989. Insertions or introns at this site have not been found previously.

The data set constructed comprises 18 taxa and 1321 bp of DNA sequence for each isolate. Representative species were selected of known ambrosia fungi of the genera *Raffaelea* and *Ambrosiella*. A repeated Bayesian phylogenetic analysis yielded consistent tree topologies and *a posteriori* probabilities (Fig. 4). Within the Ophiostomatales the *Ophiostoma* species grouped into two clusters, both supported by a high *a posteriori* probability (> 99%). All the selected ambrosia fungi grouped closely together (100% *a posteriori* probability) into only one of these clusters, which also contains *O. cucullatum*, *O. piceaeperdum*, and *O. serpens*. Between the three *Raffaelea* species the topology of the DNA sequence analysis reflects a distinction. *R. montetyi* resolved as a close relative to *A. sulphurea* (Fig. 4).

**Discussion**

**Primary ambrosia fungus association.** Norris (1965, 1979) and Haanstad & Norris (1985) showed that a complex of fungus species and additional bacteria is probably involved in a mutualistic relationship with each ambrosia beetle with respect to the utilization of wood as a primary nutritional substrate. They called these groups a multi-species complex or a superspecies. However, in a series of examples the dominance of one particular fungus, the primary ambrosia fungus, has been demonstrated in the symbiotic associations of the beetles (e.g. Baker 1963, Batra 1966, Francke-Gromman 1963, 1967, Kinulra 1995). Also in our present investigation
Fig 4: Bayesian inference of phylogenetic relationships of Ophiostoma, Ambrosiella and Raffaelea. A Monte Carlo Markov chain analysis of a 1321 bp alignment of nuclear rDNA sequences from the SSU region using the general time reversible model of DNA substitution with gamma distributed substitution rates, random starting trees, and random starting parameters for the substitution model was used. Shown is the majority rule consensus tree resulting from 9,000 trees sampled after the process had reached stationarity. The topology was rooted with Taphrina wiesneri. Numbers on the branches are estimates for a posteriori probabilities. Branch lengths are scaled in terms of expected numbers of nucleotide substitutions per site.
a single fungus colonized with tightly packed hyphae the parts of the galleries populated by the feeding larvae. The occurrence of the fungus in the immediate vicinity of the larvae is significant, because ambrosia beetles ingest most of their nutrients during the larval stage. Additionally the dominant occurrence of this fungus in the mycetangia of the investigated beetles confirms the close biological association between the partners. The primary ambrosia fungus of Xyleborus monographus and X. dryographus was identified as Raffaelea montetyi.

R. montetyi has previously been described as a mycetangial ambrosia fungus of the beetle Platypus cylindrus (Curculionidae: Platypodinae) (Morelet 1998). The Scolytinae and Platypodinae have generally been considered to be two independent families within the Curculionoidea and their current grouping as two subfamilies within the family Curculionidae is widely accepted (Kuschel 1995, Marvaldi 1997). Recent assignment, however, has rejected the independent status of the Platypodinae and has suggested their placement as an apomorphic derivative of the Scolytinae (Kuschel et al. 2000, Farrell et al. 2001, Marvaldi 2002). Although the relationship between the Scolytinae and Platypodinae is still questioned, the platypodid beetle P. cylindrus and the two scolytid beetles investigated here are not closely related.

This occurrence of R. montetyi in association with these three ambrosia beetles challenges the hypothesis (Francke-Grosman 1956, 1963, 1967, Batra 1963) of a species-specific association of a single ambrosia fungus with a single beetle species. Batra (1966) himself questioned this hypothesis after finding under artificial conditions that several species of ambrosia and nonambrosia fungi could provide adequate nutrition for one species of ambrosia beetle. However, in nature, these beetles are usually associated with one specific fungus. The transport of a nutritional fungus in the mycetangium of the beetle could function so as to guarantee a stable symbiotic association. Secretions of glandular cells associated with the mycetangia may prevent the establishment of nonsymbiotic fungi. In addition, the associated fungi are known to multiply in the mycetangia of their respective symbiotic beetle (Beaver 1989, Francke-Grosmann 1967).

Cross contamination of fungal symbionts may occur when more than one beetle species inhabits an individual tree. An exchange of associated symbiotic fungi could then occur between the galleries of different beetle species, where the insect tunnels cross or lie adjacent to one another. The growth of ambrosia fungi through the woody tissue of their host plants has been observed: Ambrosiella ferruginea (Math.-Käärik) Batra grew a distance of up to 15 cm (Mattheisen-Käärik 1953, Francke-Grosmann 1956). In contrast to the slow growth of most ambrosia fungi, R. montetyi grows rapidly (Morelet 1998) possibly promoting its establishment in adjacent tunnels. Because the three investigated beetle species share a common habitat, trunks of oaks in the Western Palaeartic (Postner 1974), frequent exchanges between the fungi become occasional and this could prevent specificity of the fungus with its symbiotic beetle.

Generally, a single primary ambrosia fungus is found consistently with a single ambrosia beetle species. An impressive example of specificity was shown for Xylosandrus germanus, a species which naturally occurs in Asia, and which has been introduced into many different areas of the world. Ambrosiella hartii remains the primary ambrosia fungus of the beetle in its present distribution across Asia (Batra 1967), Europe (Francke-Grosmann 1958) and North America (French & Roep 1972). In contrast, P. cylindrus carries R. ambrosiae in Great Britain (Baker 1963, von Arx & Hennebert 1965). While isolates from mycetangia and galleries in France and Portugal contained in R. montetyi (Morelet 1998). In the present study R. montetyi was found as the single primary am-

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brosia fungus of *P. cylindrus* in south-west Germany. Consequently, various primary ambrosia fungi could be found in association with a specific ambrosia beetle in different geographical areas. This could perhaps explain the contradictory results regarding the ambrosia fungus of *Xyleborus monographus*. However, the report of *Mortierella* as a primary ambrosia fungus of this beetle (Schedl 1964) may be inaccurate and based on isolations of a contaminating fungus.

A list of nutritional fungi and their associated beetles (Batra 1967) suggested that certain species of ambrosia fungi are associated with unrelated beetle species. However, these data relate to auxiliary ambrosia fungi not primary ambrosia fungi, or are based on speculation. Furthermore, differentiation based only on light microscopic observation is often inconclusive, because of the reduced morphology of ambrosia fungi.

**Morphology.** Previous observations of *R. montetyi* based on light microscopy indicated sympodial production of the conidia (Morelet 1998), as was indicated for genus *Raffaelea* (Batra 1967, Von ARX & Hennebert 1965). The scanning electron micrographs show the conidial development occurring through annelidic pericentral proliferation and consequently identical to that found in anamorph genera of the Ophiostomatales: *Hyalorhinochliadiella* Upadhyay & Kendrick, *Leptographium* Lagerb. & Melin and *Pestotum* Crane & Schenk (Benade 1995, Kendrick 1962, Okada et al. 1997, Seifert & Okada 1993). Because of the mononematous and hyaline conidiophores the species is morphologically close to *Hyalorhinochliadiella*.

Conidium development constitutes an important taxonomic character for anamorphic fungi (Hughes 1953) and the named findings necessitate further study of the type species of this genus, *R. ambrosiae*, to re-assess the taxonomy of the genus in relation to the Ophiostomatales.

**Phylogeny studies.** Cladistic analysis of characters derived from nuclear encoded small subunit (18S) rDNA sequences revealed that species of the ambrosia fungus genera *Raffaelea* as well as *Ambrosiella* are polyphyletic (Blackwell & Jones 1997, Cassar & Blackwell 1996, Farrell et al. 2001, Jones & Blackwell 1998, Rollins et al. 2001). Data from the present analysis based on ribosomal DNA sequence data (18S) position *R. montetyi* within the Ophiostomatales, which are separated into two clusters. Together with the additional ambrosia fungi included, *R. montetyi* was placed in one cluster together with *O. serpens, O. piceaerumpand* and *O. cucullatum*. These Ophiostoma species, with anamorphs assignable to *Pestotum* and *Leptographium*, have conidia developing mainly by annelidic pericentral proliferation, consequently identical to the conidiogenesis of *R. montetyi*.

The congruence between the presented DNA-based phylogeny and morphological characters of *R. montetyi* is additionally supported by knowledge of the vector relationships within the genus *Ophiostoma*. Even these fungi depend on dispersal by arboreal insects. As well as non species-specific vectoring, transmission within the evolved mycetangia of scolytid beetle species is regularly found (Whitney 1982). These developmental stages are similar to the symbiotic associations of species of the genus *Raffaelea*, which are found to exhibit specialized morphological adaptations, since they lack a telemorph state.

As an evaluation of the previous phylogenies shows, the different lineages of ambrosia fungi appear to have arisen independently on several evolutionary occasions (Blackwell & Jones 1997) and do not correspond in all parts with the phylogeny of the associated ambrosia beetles (Farrell et al. 2001). Host switching of ambrosia fungi may account for points of incongruence between the beetle and fungal phylogenies.

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