

**The microbial community associated with the
ambrosia beetle *Xyleborinus saxesenii*
(Coleoptera: Scolytinae)
and its influence on the growth of the
mutualistic fungus**

Masterarbeit

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1. Summary

Ambrosia beetles of the weevil subfamily Scolytinae cultivate fungi in their excavated wood galleries. The most elaborate of beetle - fungus interactions is true fungus farming or fungiculture. Members of the species - rich subtribe Xyleborini are the most advanced fungiculturists among the ambrosia beetles. Agriculture usually is associated with sociality, which also applies to the Xyleborini, which show a haplodiploid sex determination and regular sib mating. Especially *Xyleborinus saxesenii* lives in societies comparable to the ones of truly social insects like bees or wasps. An obligate beetle - fungus relationship (mutualismus) evolved several times independently in weevils. Ambrosia beetles cannot feed from wood they live in, but solely receive their nutrients feeding on ambrosia fungi, which are members of the heterogeneous group of ophiostomatoids that include the anamorphic genus *Ambrosiella*. The ambrosial layers of these fungi on the gallery walls are maintained by tending and browsing by the beetles which appear to inhibit the invasion of harmful foreign fungi.

Additionally, the beetles are able to induce and enhance the growth of their mutualistic ambrosia fungi within their tunnel systems. Previously, this phenomenon could be observed between the alnus ambrosia beetle *Xylosandrus germanus* and its associated fungus in pure culture. However, the mechanisms of this growth - promoting effect are not known yet. An involvement of secretions by the beetles is assumed. Furthermore, microbial endosymbionts in *X. saxesenii* could also play a crucial role, but are still underexplored.

This is the first study to investigate the endosymbiotic microbial community of this beetle. Bacterial tag - encoded FLX amplicon pyrosequencing (bTEFAP) revealed *Sphingobacterium* and *Ochrobactrum* as the two most abundant bacterial endosymbionts which could also be isolated from the digestive tract and cultured on agar medium. Furthermore, endosymbiotic *Candida* yeasts and a variety of cultivatable gut symbionts including *Pseudomonas* and *Bacillus* could be detected. Phylogenetic analyses revealed a monophyletic clustering of associated *Candida* indicating a specific relationship as well as a distinctive clade within the Saccharomycetales or even a new *Candida* species. Most of the microbes might play a nutritional role for the beetles as do the ambrosia fungus *Ambrosiella xylebori*, isolated from a lab gallery, as well as the ectosymbiotic 'weedy' fungi *Aspergillus* and *Penicillium*.

No effects of the beetles on the growth of *A. xylebori* could be detected, which may be because this fungus usually is not a mutualist of *X. saxesenii*. It can be considered as an auxiliary ambrosia fungus and remarkably, was positively stimulated in growth by an

Aspergillus. However, this mold fungus might be a life threatening passenger as it invades galleries and infests beetles as well.

In summary, this study gave the first extensive insights in the microbial community associated with the ambrosia beetle *Xyleborinus saxesenii*. It showed, that the complex of associated microorganisms is remarkably divers, which is probably also true for their interactions. Many more studies will be necessary until we understand how ambrosia beetles sustainably culture fungi in their wooden galleries.

2. Zusammenfassung

Ambrosiakäfer sind Rüsselkäfer aus der Unterfamilie der Borkenkäfer (Scolytinae) und leben in Tunnelsystemen oder Galerien im Kernholz von Bäumen. Diese Käfer sind bemerkenswert, da sie dort „aktive Pilzzucht“ betreiben - sonst nur von pilzzüchtenden Termiten und Ameisen bekannt. Soweit man weiß, ist diese Art von Pilzanbau bei den Xyleborini am höchsten entwickelt. Diese sehr artenreiche Untertribus ist geprägt durch eine haplodiploide Geschlechtsbestimmung sowie regulären Geschwisterbruten. Der daraus resultierende hohe Verwandtschaftsgrad und die stark weibchenlastigen Geschlechtsverhältnisse innerhalb einer Galerie fördern Verwandtenselektion, die soziale Verhaltensweisen begünstigt, die speziell beim untersuchten Kleinen Holzbohrer (*Xyleborinus saxesenii*) sehr zahlreich sind. Pilzzucht ist innerhalb der Rüsselkäfer mehrmals unabhängig entstanden. Pilz und Käfer sind obligatorisch voneinander abhängig: für das Insekt dient der Pilz als einzige Nahrung, der Pilz wiederum wird vom Käfer verbreitet und ist ohne dessen Pflege nicht überlebensfähig. Die meisten dieser „Ambrosiapilze“ gehören zur heterogenen Gruppe der ophiostomatoiden Pilze, zu denen auch die anamorphe Gattung *Ambrosiella* zählt. Als Ambrosiarasen an den Galeriewänden bilden sie die Hauptnahrungsquelle für die Käfer, welche das Holzgewebe nicht verdauen können. Diese Pilzwände oder – farmen werden durch regelmäßiges Abgrasen gehegt und gepflegt, um dem Eindringen fremder Schadpilze entgegenzuwirken.

Die Käfer selbst können das Wachstum ihrer mutualistischen Pilze innerhalb ihrer Galerien induzieren und anregen. Dieses Phänomen konnte kürzlich zwischen dem Schwarzen Nutzholzborkenkäfer (*Xylosandrus germanus*) und Reinkulturen seines assoziierten Pilzes beobachtet werden. Die zu Grunde liegenden Mechanismen sind jedoch bisher nicht bekannt. Sehr wahrscheinlich spielen Sekrete der Käfer eine entscheidende Rolle. Zudem könnten auch endosymbiontische Mikroorganismen einen Beitrag leisten, diese sind jedoch in Ambrosiakäfern bisher wenig erforscht.

Summary / Zusammenfassung

Dies ist die erste Studie, die sich mit der endosymbiontischen mikrobiellen Zusammensetzung in *X. saxesenii* beschäftigte. Mit Hilfe von High - Throughput - Sequenzierung (bTEFAP: bacterial tag – encoded FLX amplicon pyrosequencing) konnten die beiden häufigsten bakteriellen Endosymbionten, *Sphingobacterium* und *Ochrobactrum*, identifiziert werden. Diese konnten auch aus dem Darm des Käfers isoliert und auf Agar – Nährmedium in Kultur gebracht werden. Des Weiteren konnten endosymbiontische Hefen der Gattung *Candida*, sowie weitere fungale und bakterielle Darmsymbionten, einschließlich *Pseudomonas* und *Bacillus*, identifiziert und kultiviert werden. Phylogenetische Analysen der assoziierten *Candida* Gattung ergaben eine monophyletische Gruppierung, was nicht nur auf eine spezifische Interaktion, sondern auch auf einen eigenständigen Cluster innerhalb der Saccharomycetales oder gar einer neuen *Candida* Spezies hinweisen könnte. Die meisten der detektierten Mikroorganismen haben wahrscheinlich die Funktion einer Nahrungsquelle, so auch der Ambrosiapilz *Ambrosiella xyleborini*, der aus einer Laborgalerie von *X. saxesenii* isoliert werden konnte. Selbige Rolle spielen womöglich auch die ektosymbiontisch assoziierten „Schimmelpilze“ *Aspergillus* und *Penicillium*.

Es konnte kein Effekt des Käfers auf den Pilz *A. xylebori* festgestellt werden. Das könnte daran liegen, dass dieser normalerweise nicht mit *X. saxesenii* mutualistisch assoziiert ist. Es wird angenommen, dass es sich dabei um einen „Neben – Ambrosiapilz“ handelt. Dessen Wachstum konnte allerdings in bemerkenswerter Weise von *Aspergillus* induziert und angeregt werden. Dieser Schadpilz wird jedoch auch als lebensbedrohlicher „Passagier“ betrachtet, da er zusammen mit anderen invasiven Pilzen Käfergalerien und die Insekten selbst befallen und eliminieren kann.

Der Einblick in die endosymbiontische Gemeinschaft und die weiteren assoziierten (Mikro-) Organismen konnten einen weiteren Teil des komplexen Käfer – Pilz Mutualismus enthüllen. Dieser muss wohl als Multi - Partner - Symbiose verstanden werden, die sich im Ganzen als stabiles, in der Zusammensetzung jedoch als variables und relativ kurzlebige Gefüge charakterisieren lässt.

3. Introduction

3.1 Insect - bacteria symbiosis

Insects are the most abundant and diverse animal class on earth (MAY, 1988). They are associated with diverse symbiotic microorganisms, both inside their bodies (endosymbionts), here even intracellular as well as outside (ectosymbionts) their bodies (BUCHNER, 1965). Their success and the facility to occupy a diverse range of ecological niches are closely connected to mutualistic associations. Endosymbiotic microbes reveal an expanded genetic repertoire that may account for mechanisms leading to (life - long) beneficial effects on the insect host (HARRIS et al., 2010). Especially bacteria play important roles, as an estimated 10 - 20% of insect species depend on intracellular bacterial mutualists for their viability and reproduction (DOUGLAS, 1989; WERNEGREN et al., 2009). These so called primary endosymbionts often synthesize and provide key nutrients that are lacking in the hosts' unbalanced diet (e.g. plant sap or vertebrate blood) (BAUMANN et al., 1999; MORAN & BAUMANN, 2000; FELDHAAR & GROSS, 2009) or defend against pathogens, parasitoids or predators. Such defensive mutualisms have been in the focus of an increasing number of studies (CURRIE et al., 1999; KELLNER, 2002; KALTENPOTH et al., 2005; SCOTT et al., 2008; TEIXEIRA et al., 2008; BROWNLIE & JOHNSON, 2009; XIE et al., 2010; KALTENPOTH et al., 2010). Proteobacteria and predominantly Actinobacteria, a group of gram - positive bacteria with high GC - content, are often involved in defensive symbioses. Proteobacteria, for example are associated with beetles (KELLNER, 2002; PIEL, 2002; CARDOZA et al., 2006), dipterans (TEIXEIRA et al., 2008) and bugs (MORAN et al., 2005; OLIVER et al., 2003; FERRARI et al., 2004; SCARBOROUGH et al., 2005; MAHADAV et al., 2008); Actinobacteria, also known as actinomycete bacteria, defend leaf - cutter ants (CURRIE et al., 1999; HAEDER et al., 2009; OH et al., 2009a), bark beetles (CARDOZA et al., 2006; SCOTT et al., 2008; OH et al., 2009b) and beewolves (KALTENPOTH et al., 2005) against detrimental microorganisms by producing antibiotics (KALTENPOTH, 2009). Some species of the ant and bark beetle hosts are known for fungus cultivation.

3.2 Fungus farming insects with emphasis on the fungiculture of ambrosia beetles

Fungus - farming and general agriculture in insects evolved independently in three insect lineages and can be found in leaf-cutter ants, termites and ambrosia beetles, whereas this

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behavior has arisen only once in ants and termites, respectively, and at least seven times independently in the scolytine beetles (FARRELL et al., 2001; MUELLER et al., 2005).

The relationship between wood-colonizing insects and wood-inhabiting fungi is one of the most successful symbioses among eukaryotes (VEGA & BLACKWELL, 2005). It arose independently in wood wasps, lymexilid beetles, passalid beetles, and many times within the weevils (Curculionidae), in the subfamilies Scolitinae and Platypodinae, known collectively as bark and ambrosia beetles. The most elaborate of beetle - fungus interactions is true (active) fungus farming, or fungiculture (HULCR & DUNN, 2011). This is a feature, which – within the bark beetles – only occurs in ambrosia beetles. These live in nutritional symbiosis with their mutualistic fungi, known as ambrosia fungi (term was given by SCHMIDBERGER, 1836) and cultivate them in their tunnel systems in woody tissues of weakened or dead trees (BEAVER, 1989; MUELLER et al., 2005). All stages of ambrosia beetles feed either solely on the ambrosia fungus mycelium (mycetophagy) or on a mixture of the mycelium and the sapwood tissue of the penetrated tree host (xylomycetophagy) (ROEPER, 1995).

3.3 Sociality in ambrosia beetles: the subtribe Xyleborini

Agriculture is usually associated with sociality, which also applies to ambrosia beetles (KENT & SIMPSON, 1992; KIRKENDALL et al., 1997; MUELLER et al., 2005; PEER & TABORSKY, 2007). The origin of fungiculture in fungus-growing ants and termites is based on the division of labor (MUELLER et al., 2005), whereas ambrosia beetles originate from solitary or colonial ancestors, and their fungus agriculture may have co-evolved with sociality (KIRKENDALL et al., 1997; MUELLER et al., 2005; PEER & TABORSKY, 2007; BIEDERMANN & TABORSKY, 2011).

The most advanced fungiculturists among the ambrosia beetles are members of the highly social subtribe Xyleborini (FARRELL et al. 2001; JORDAL, 2002; BIEDERMANN et al., 2009). It is species-rich and the members show haplodiploidy and obligatory sib mating (PEER & TABORSKY, 2005). Probably all of the 1200 species show high levels of inbreeding (KIRKENDALL, 1993). The high relatedness and haplodiploidy combined with an extremely female-biased sex ratio are predisposing factors to advanced sociality (PEER & TABORSKY, 2007; BOURKE, 2011). In one member, *Xyleborinus saxesenii* Ratzeburg, all colony members contribute to divergent tasks like gallery maintenance and brood care and there is an active behavioral task specialization between larvae and adults, which is unique for holometabolous insects. Additionally, task sharing in *X. saxesenii* is unequal between the sexes and age classes

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(BIEDERMANN & TABORSKY, 2011). Males are short-lived and flightless (NORRIS, 1979) and they do not seem to contribute intensely to specific tasks in the beetle galleries (BIEDERMANN & TABORSKY, 2011). These recent observations on beetle behavior inside galleries were enabled by using an artificial laboratory rearing technique (Figure 1) (SAUNDERS & KNOKE, 1967; BIEDERMANN et al., 2009).

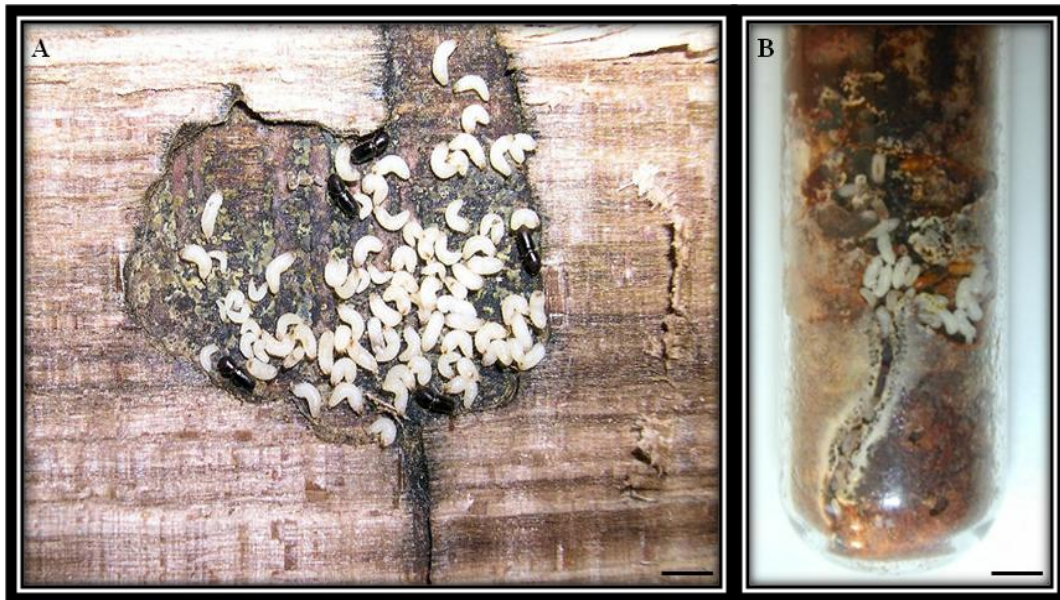


Figure 1. The ambrosia beetle *Xyleborinus saxesenii*. **A:** Opened brood chamber of *Xyleborinus saxesenii* with the entrance tunnel at the bottom. All larval stages and adult females are visible. The yellowish layers on the wall are fungi, whose penetrating hyphae cause the wood to stain black. **B:** An artificial medium containing lab gallery with beetles and larvae. Scale bars = 2 mm. (After BIEDERMANN, 2007).

Females disperse and excavate new breeding galleries in the wood; with their mycetangia (or mycangia; singular: mycetangium / mycangium) (BATRA, 1963; FRANCKE-GROSMANN, 1966), specialized ectodermal glandular cavities / pockets or modifications of mandibles and guts for fungus storage and fungus transmission (NUNBERG, 1951; FRANCKE-GROSMANN, 1956), they transfer the fungal spores from the natal gallery to a new gallery to establish an ambrosia fungus cultivation (FRANCKE-GROSMANN, 1956; FRANCKE-GROSMANN, 1967; BATRA, 1963). These evolved structures indicate an adaptation of the beetles to their mutualists and can also be found in other xylophagous (wood feeding) and obligatory fungus associated insects, like wood wasps. Usually they are present only in one sex, mainly in females (BATRA, 1963; FRANCKE-GROSMANN, 1966, 1967) and are not always essential for the successful transmission of the fungi, however (BEAVER, 1989). Unlike other Xyleborini, *X. saxesenii* females do not carry their mutualistic fungi in the mycetangium but in the foregut in the form of micromycelia (FRANCKE-GROSMANN, 1975).

3.4 Functional roles in the beetle - fungus symbiosis

To understand the beetle-fungus relationship in more detail, the roles of involved organisms have to be considered. The fungal symbionts extract nutrients from xylem and other surrounding tissues, providing a diet for the beetles that is much richer in proteins and nitrogen than pure wood (FRENCH & ROEPER, 1973; BEAVER, 1989; HULCR & DUNN, 2011). The fungal diet is crucial for the physiology and development of the beetles. By decomposing wood the fungi produce and provide organic molecules such as amino acids, vitamins and steroids (KOK, 1979; NORRIS, 1979; BEAVER, 1989).

The fungi profit from the association by a reliable transmission provided by the beetles, the dispersal of the spores and inoculation into new habits suitable for their growth. Additionally, they appear to recycle urea and uric acid, the nitrogen rich excretes of the Xyleborini (BATRA, 1963; ABRAHAMSON & NORRIS, 1969; KOK, 1979; NORRIS, 1979). Ambrosia beetles and their fungi evolved obligate symbioses and both partners cannot be found / survive without each other (BEAVER, 1989).

3.5 The ambrosia community with emphasis on the characteristics of ambrosia fungi

Except for two identifications of basidiomycetes from ambrosia beetle galleries (BATRA, 1972; HSIAU & HARRINGTON, 2003), almost all beetle associated fungi belong to the Ascomycota, more precisely to the clade composed of the polyphyletic asexual (anamorph) ophiostomatoid genera *Ambrosiella* and *Raffaelea* and close relatives (BATRA, 1967; CASSAR & BLACKWELL, 1996; JONES & BLACKWELL, 1998; GEBHARDT et al., 2005). The specificity of the ambrosia associations is not well known. However, most beetle species have a single fungus as the dominant symbiont, but the 'ambrosia' community usually includes species of filamentous fungi, yeasts and bacteria (BATRA, 1963; FRANCKE-GROSMANN, 1967; HAANSTAD & NORRIS, 1985).

An important characteristic of the ophiostomatoid ambrosia fungi is their pleomorphism. The growth form in the galleries usually differs from that in the mycangia, where the fungi appear to be micromycelial or yeast-like and slow-growing. In the wood and in the beetle galleries, the fungal growth is mycelial, when not tended by the beetles and 'ambrosial', when tended on the walls (FRANCKE-GROSMANN, 1956, 1975; BATRA, 1963; NORRIS, 1979; BEAVER, 1989; SIX, 2003). Fungal hyphae form an 'ambrosial' layer, a regular, erect palisade of tightly packed conidiophores. This layer lines the walls of the tunnels and galleries and is formed only in the presence of the beetles, which can easily browse it (FRENCH & ROEPER, 1972b;

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BEAVER, 1989). This modified, ambrosial growth form of the fungi can be considered as an adaptation to their insect mutualists for efficient grazing by the beetles (BIEDERMANN, 2007).

3.6 Beyond mutualism: fungal growth enhancement and inhibition of invaders

The ambrosia fungus is obligatory dependent on the care of the beetles. This lifetime care and maintenance of the fungal layers prevent the growth of invading harmful microbes (FRANCKE-GROSMANN, 1967; BEAVER, 1989; BIEDERMANN, 2007), as browsing and feeding on the ambrosia fungi promote their growth and additionally inhibits the growth of foreign fungi (BROWNE, 1961; BATRA, 1966). This antagonism is only effective as long as the beetles are active and the ambrosia fungus is in full vigor. Over time, the ambrosia growth declines and old or abandoned galleries are soon invaded and overgrown by foreign fungi, turning the gallery walls blackish (FRANCKE-GROSMANN, 1967).

Fungal growth enhancement as well as the prevention of invading antagonists may be attained through the involvement of secretions (FRANCKE-GROSMANN, 1956, 1967; FRENCH & ROEPER, 1972a). Recently, *X. saxesenii* and other Xyleborini have been observed to release secretions over their gut and mouth. Those have not been further analyzed up till now, but they could contain nutrients as well as fungal growth promoting and antimicrobial substances (BIEDERMANN, 2007). In another scolytine beetle, the spruce beetle *Dendroctonus rufipennis*, oral secretions were detected to contain bacteria (Actinobacteria, Proteobacteria and Firmicutes) that inhibited antagonistic fungi (CARDOZA et al., 2006). FRANCKE-GROSMANN (1956) already supposed that the formation of pure ambrosial mats in new brood galleries is induced by secretions of beetles and larvae. Furthermore, the ambrosial growth also seems to be induced and enhanced by physical contact with the beetles (WHITNEY, 1971; FRENCH & ROEPER, 1972b). Recently, a growth enhancement of pure culture ambrosia fungus, isolated from galleries of *Xylosandrus germanus*, could be observed when beetles being placed on the fungal layer (BIEDERMANN, unpublished data).

3.7 Assumption of an endosymbiotic microbial community

In the present study, I investigated the endosymbiotic microbial community associated with *X. saxesenii*, not only due to the fact, that there is only little knowledge about bacteria involved in this beetle-fungus mutualism, but rather because the presence of bacterial and / or fungal endosymbionts can be assumed, as a wide range of insects is associated with endosymbiotic

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microorganisms, often playing a crucial role. The scolytine bark beetle *Dendroctonus frontalis* harbors antibiotic-producing actinomycetes of the genus *Streptomyces* for maintaining beneficial microbes (SCOTT et al., 2008). Furthermore, in the xyleborine member *Xyleborus ferrugineus* a *Staphylococcus* species was detected on female ovaries, showing to mediate parthenogenetic reproduction of the females (PELEG & NORRIS, 1972; NORRIS & CHU, 1980). Recently, bacteria of the genus *Gordonia* could be detected in *X. saxesenii* (BIEDERMANN, 2007). They are known to show an interesting and diverse metabolism, as they are able to degrade e.g. environmental pollutants (ARENKOTTER et al., 2004). They belong to the suborder Corynebacterineae, a group within the actinobacterial order Actinomycetales (STACKEBRANDT et al., 1997). Moreover, a wide range of unidentified bacteria is known to be part of the symbiotic microbial complex of ambrosia beetles (BATRA, 1963; HAANSTAD & NORRIS, 1985). In the *X. saxesenii* – ambrosia fungus mutualism, endosymbiotic bacteria may also play a crucial role as potential producers of bioactive compounds or they could be involved in the nitrogen cycling of the beetles and / or the fungi.

3.8 Objectives

All these findings suggest that the already fascinating ambrosia beetle - fungus relationship could be extended to a third symbiont level. This project addresses the very questions of

- identifying the endosymbionts of *X. saxesenii*
- their respective roles
- their cultivation
- their potential nitrogen - fixation ability
- the effect of the beetle on the ambrosia fungus growth
- the mechanisms of a potential effect
- investigation of chemical beetle and fungal compounds possibly being involved in ambrosia fungus growth promotion and / or antimicrobial defense

4. Material and methods

4.1 Beetle breeding and ambrosia fungus cultivation

Adult females of *Xyleborinus saxesenii* were reared and kept in lab - galleries. These are glass tubes filled with artificial medium that mainly contain sawdust (BIEDERMANN et al., 2009); tubes were kept at 26°C in a temperature cabinet.

Ambrosia fungus was isolated from a lab-gallery; a tunnel section with fungal layers on the wall was excavated from the artificial medium and added to a malt agar plate (1 % malt extract, 2 % agar), which was incubated at 26°C for some days. Risen ambrosia fungus was transferred to a fresh malt agar plate obtaining a pure culture. A continuous re-plating maintained the fungus cultivation. For cultivation in liquid medium, mycelial layer was scraped off the agar plate and added to 1 % malt extract, allowing the mycelium to grow at 26°C.

4.2 DNA extraction

Beetles were used as a whole or were dissected for gut isolation. Beetle material and small fungus-overgrown malt agar pieces were crushed in liquid nitrogen and subjected to DNA extraction using the Epicentre MasterPure™ DNA extraction kit (Epicentre, Madison, USA). For beetle and bacterial DNA extraction, lysozyme (100 mg/ml) (Sigma-Aldrich, St. Louis, USA) was added and the sample was incubated at 37°C for 30 min. The RNase step was skipped, centrifugation steps were at room temperature and DNA was resuspended in Low TE - buffer (10 mM Tris/HCl, 0.1 mM EDTA).

Some alternative treatments were performed to yield utilizable fungal biomass. Agar pieces were heated up to approximately 90°C until agar was melted or treated with agar (agarose) degrading GP-buffer of the peqGold MicroSpin Gel Extraction Kit (Pepqab, Erlangen, Germany) under heat condition (~ 65°C). A short centrifugation step was included to pellet fungal biomass, followed by removing melted agar and / or buffer. Subsequently, DNA extraction was proceeded with kit protocol or fungal biomass was washed in 1x PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.47 mM KH₂PO₄, pH 7.4), before buffer Y1 (1 M sorbitol, 0.1 M EDTA), containing lyticase (1 mg/ml) (Sigma-Aldrich), was added. After incubation at 37°C for 30-60 min and removing Y1 buffer, DNA extraction proceeded according the kit protocol.

4.3 Bacterial tag - encoded FLX amplicon pyrosequencing (bTEFAP)

The community of endosymbiotic bacteria of *X. saxesenii* was sequenced by using the bTEFAP method (DOWD et al., 2008), based on 454 sequencing. DNA of 10 adult female *X. saxesenii* was pooled, purified using the SoilMasterTM DNA Extraction Kit (Epicentre) and quantified in a Nanodrop spectrophotometer (ND-1000) (Pqlab). The DNA pool was sent to the Research and Testing Laboratory (Lubbock, TX, USA) for 454-sequencing service. Obtained sequences were denoised (= reducing the amount of erroneous OTUs) using the denoiser algorithm (denoise_wrapper.py) of Qiime (quantitative insights into microbial ecology) software (Version 1.3.0) (CAPORASO et al., 2010). OTUs (operational taxonomical units) were picked by multistep OTU picking (cdhit and uclust).

4.4 PCR screening

Presence and identification of bacterial and fungal symbionts of *X. saxesenii* were assessed by polymerase chain reaction (PCR). For evaluation of beetle endosymbionts, DNA from ten single beetles was pooled and used as PCR template. For single screenings and species identification, DNA from single organisms was used. Amplification was performed in a gradient thermal cycler (UnoCycler) (VWR, Darmstadt, Germany) in a total reaction volume of 12.5 µl containing 1 µl template, 1x PCR buffer (10 mM Tris/HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.1 % Triton X-100), 2.5 mM MgCl₂, 2 mM dNTPs, 10 pmol/µl of each primer, and 0.5 U of *Taq* DNA polymerase (VWR). Universal primer pairs used for amplification are listed in following table (Table 1).

Table 1. Universal primers used for PCR amplification.

<i>Primer (direction)</i>	<i>Sequence (5'-3')</i>	<i>Target gene</i>	<i>Product size [bps]</i>	<i>Annealing Temperature [°C]</i>	<i>Reference</i>
fD1 (fwd) rP2 (rev)	AGAGTTTGATCCTGGCTCAG ACGGCTACCTTGTTACGACTT	16S rRNA of most Eubacteria	~ 1500	60	WEISBURG et al., 1991
LS1 (fwd) LR5 (rev)	AGTACCCGCTGAACTTAAG TCCTGAGGGAACTTCG	LSU (28S) rRNA of fungi	~ 900	68 - 58	HAUSNER et al., 1993; VILGALYS & HESTER, 1990

The eubacterial 16S rRNA gene was PCR amplified using the fD1 / rP2 primer set. Cycle parameters were as follows: an initial denaturation step at 94°C for 3 min, followed by 32 cycles of 94°C for 40 s, 60°C for 1 min and 72°C for 1 min. A final extension step of 72°C for

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4 min was included. Fungal 28S nuclear large subunit (LSU) rRNA was PCR amplified with primers LS1 and LR5 with following touchdown cycling reaction conditions: initial denaturation at 94°C for 2 min, 94°C for 1 min, 68°C for 1 min (- 0.7°C per cycle down to 58°C) for 13 cycles, followed by 26 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min and a final extension at 72°C for 6 min (modified from GIBSON & HUNTER, 2009).

All PCR products were assessed by gel electrophoresis using GelRed™ (Biotium, Hayward, USA) stained 1.5 % agarose gel. Documentation and processing of gel pictures was performed with GeneSnap image acquisition software (Version 7.09.06) (Syngene, Cambridge, UK).

4.5 Molecular cloning

Amplicons of *X. saxesenii* DNA pool were cloned into cloning vector pSC-A-amp/kan following the StrataClone PCR Cloning Kit (Stratagene / Agilent Technologies, Santa Clara, USA) protocol. PCR products were ligated to Topoisomerase I - charged vector arms. The vector contained a β -galactosidase α -fragment coding sequence (*lacZ'*) for blue-white screening. For transformation, competent *Escherichia coli* K12 cells were used. Transformants were cultivated on LB agar (1 % tryptone, 1 % NaCl, 0.5 % yeast extract, 2 % agar) plates containing ampicillin (100 μ g/ml) (Roth, Karlsruhe, Germany) and 2 % X-gal (Zymo Research, Irvine, USA). After overnight incubation at 37°C, white colonies were picked and screened for successful ligation (DNA insert) by colony PCR with M13 forward (5'-TGTAACGACGGCCAGT-3') and M13 reverse primer (5'-GGAAACAGCTATGACCATG-3') according to the following PCR program: 94°C for 3 min, 32 cycles of 94°C for 40 s, 55°C for 1 min and 72°C for 1 min. A final extension step of 72°C for 4 min was included.

4.6 Sequencing

PCR products were sequenced after cloning or without cloning (directly). Positive clones (with the right DNA insert) were purified according the protocol of peqGold MicroSpin Cycle Pure Kit (Peqlab) and sequenced monodirectionally with M13 forward or M13 reverse primer. Amplicons of individual DNA samples were directly and bidirectionally sequenced (after purifying) by using Sanger's method (SANGER et al., 1977) at the Entomology Department of

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the Max Planck Institute for Chemical Ecology with same primers used for PCR amplification (Table 1 & 2).

Sequences were analyzed and edited with Geneious (Version 5.3.4) (Biomatters, Auckland, New Zealand) and submitted to BLAST (Basic Local Alignment Search Tool) (ALTSCHUL et al., 1990) at the GenBank database (<http://blast.ncbi.nlm.nih.gov>) to be identified.

4.7 PCR - investigation of specific bacterial and fungal endosymbionts

Preliminary work by the author unveiled bacteria of the genus *Burkholderia*. For confirmation, the 16S rRNA gene was amplified with BKH primers (Table 2) with the following PCR program: initial denaturation at 94°C for 4 min, 40 cycles of 94°C for 40 s, 52°C for 40 s and 72°C for 1.5 min; final extension at 72°C for 10 min.

Candida yeasts were identified by sequencing of bacterial clones. The fungal 18S rRNA gene was amplified with NS1 / NS4 primer pair (Table 2) with following parameters: 3 min at 94°C, followed by 35 cycles of 94°C for 30 s, 62°C for 45 s and 72°C for 1 min, and a final extension time of 7 min at 72°C.

Specific bacterial primers were designed to detect the two most abundant OTUs obtained from bTEFAP sequencing, *Sphingobacterium* and *Ochrobactrum* (Table 2) via diagnostic PCR. Sequences served as templates for primer design with Primer3 (Version 0.4.0) (<http://frodo.wi.mit.edu/primer3/>). The oligonucleotides were chosen to match a variable region of the 16S rRNA of the putative endosymbiotic bacterial genera. The specificity of the primers was checked against the 16S rRNA sequences from the Ribosomal Database Project (<http://rdp.cme.msu.edu/using>) (MAIDAK et al., 2001) with the Probe Match tool. *Sphingobac* and *Ochrobac* primers were used for PCR amplification of the bacterial 16S rRNA genes. Amplification parameters were as follows: 3 min at 94°C, followed by 35 cycles of 94°C for 30 s, optimal annealing temperature (Table 2) for 45 s and 72°C for 1 min, and a final extension time of 7 min at 72°C.

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Table 2. Specific primers used for PCR amplification.

<i>Primer (direction)</i>	<i>Sequence (5'-3')</i>	<i>Target gene</i>	<i>Product size [bps]</i>	<i>Annealing Temperature [°C]</i>	<i>Reference</i>
BKH 143 (fwd) BKH 1434 (rev)	TGGGGGATAGCYCGGCG TGCGGTTAGRCTASCYACT	16S rRNA of <i>Burkholderia</i>	~ 1300	52	OPELT et al., 2007
NS1 (fwd) NS4 (rev)	GTAGTCATATGCTTGTCTC CTTCCGTC AATTYCTTTAAG	18S rRNA of fungi (yeasts)	~1100	62	modified from WHITE et al., 1990
Sphingobac (fwd) Sphingobac (rev)	TAACGCGTGAGCAACCTACC CTCAGAGCCCCTAGACATCG	16S rRNA of <i>Sphingobacterium</i>	185	65	This work
Ochrobac1 (fwd) Ochrobac1 (rev)	AATACCGTATGTGCCCTTCG CGTGTCTCAGTCCCAGTGTG	16S rRNA of <i>Ochrobactrum</i>	147	58	This work

4.8 Phylogenetic analysis

Partial sequences of the 26S rRNA (large subunit) of the *Candida* endosymbionts and other representative yeast sequences from the GenBank database were aligned in Geneious using the default settings and subjected to GARLI (Genetic Algorithm for Rapid Likelihood Inference) (ZWICKL, 2006) web service (<http://molecularevolution.org/software/phylogenetics/GARLI>) for a maximum likelihood (ML) analysis. A GTR+G+I (general time reversible with gamma distribution of rates and proportion invariant) nucleotide substitution model was used with 1000 replicates and with a $-\ln L = 3928.8520$. Bootstrap analysis was also conducted in GARLI, using 1000 replicates to calculate the consensus tree (50 % majority-rule), which was generated in PAUP*4.0 (SWOFFORD, 1998).

A bayesian estimation of the phylogeny was performed using MrBayes 3.1.2 (HUELSENBECK & RONQUIST, 2001; RONQUIST & HUELSENBECK, 2003) with the default value of four simultaneous Markov chains. Temperature parameters were set to a value of 0.2 and the Markov chain Monte Carlo (MCMC) length was 3,000,000 generations with resampling taking place every 50 generations. A consensus tree was calculated out of 60000 trees (samplefreq = 100). Markov chain Monte Carlo chains ran until a convergence stop value of 0.01 was reached. The estimation of the phylogenetic uncertainty was indicated with posterior probabilities (only values > 0.5). Bayesian bootstrap values were included in the ML tree (only values > 0.7).

4.9 Fluorescence *in situ* hybridization (FISH)

FISH was performed to investigate the occurrence of endosymbionts in *X. saxesenii* using probes labelled with fluorescent dyes. Universal yeast probe PF2 was used for *Candida* and genus-specific probes Sphingobac and Ochrobac were used for endosymbiotic *Sphingobacterium* and *Ochrobactrum*, respectively (Table 3). The Sphingobac probe corresponds to the designed Sphingobac reverse primer and the Ochrobac probe is reverse complement to Ochrobac1 forward primer (Table 1). Both were labelled with Cy3 fluorescent dye at the 5'-end. The unspecific eubacterial probe EUB338 and the *Coriobacterium*-specific probe Cor653 were used as positive and negative controls, respectively (Table 3).

Table 3. Fluorescent probes used for FISH.

<i>Probe</i>	<i>Sequence (5'-3')</i>	<i>Direction</i>	<i>5'-modification</i>	<i>Target</i>	<i>Reference</i>
EUB338	GCTGCCTCCCGTAGGAGT	rev.	Cy3 / Cy5	16S rRNA of Eubacteria	AMANN et al. (1990)
Cor653	CCCTCCCMTACCGGACCC	rev.	Cy3 / Cy5	16S rRNA of <i>Coriobacterium</i> & <i>Collinsella</i>	HARMSSEN et al. (2000)
PF2	CTCTGGCTTCACCCTATTC	rev.	Cy3	18S rRNA of yeasts	KEMPF et al. (2000)
Sphingobac	CTCAGAGCCCCTAGACATCG	rev.	Cy3	16S rRNA of <i>Sphingobacterium</i>	This work
Ochrobac	CGAAGGGCACATACGGTATT	rev.	Cy3	16S rRNA of <i>Ochrobactrum</i>	This work

For FISH on gut suspensions, isolated guts of *X. saxesenii* were crushed in 1x PBS. The gut suspension was spread onto eight-field microscope slides, dried at ~50°C and dehydrated in 50 % ethanol. Hybridization was performed in a moist chamber with different fluorescent probes (50 ng/μl) in hybridization buffer (900 mM NaCl, 20 mM Tris/HCl pH 8.0, 0.01 % SDS) at 50°C for 90 min. With PF2 probe, hybridization was at 46°C for 60 min (KEMPF et al., 2005). Subsequently, the slides were washed once with washing buffer (100 mM NaCl, 20 mM Tris/HCl pH 8.0, 5 mM EDTA pH 8.0, 0.01 % SDS) and then covered with the same buffer and incubated for 20 min at 50°C (KALTENPOTH et al., 2010). Finally, the slides were rinsed with distilled water and air dried in the dark before sealing with DAPI-containing SlowFade® (Invitrogen, Carlsbad, USA).

For hybridization within the beetle gut, an isolated organ was fixed in 70 % ethanol, dehydrated in 100 % acetone and embedded in cold-polymerizing resin (Technovit 8100) (Heraeus Kulzer, Wehrheim, Germany) in accordance to the manufacturer's instructions.

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Sections (5-7 µm thick) were obtained with a steel knife on a rotary microtome (Microm HM355S) (Thermo Scientific, Waltham, USA) and mounted on Polysine[®] microscope slides (Thermo Scientific). Hybridization and following steps were performed as described above.

For positive *Candida* controls, a small amount of the *Candida* layer on the malt agar culture plate and 5µl from liquid yeast culture medium (malt extract) was spread onto eight-field microscope slides and dried for 20 min at 45°C. The slides were dehydrated in 50 %, 70 % and 100 % ethanol for 3 min each (modified from BÖREKÇI et al., 2010). Hybridization was performed at 46°C for 60 min (KEMPF et al., 2005). Following steps were performed as described above.

Slides were examined under an upright fluorescence microscope (AxioImager Z1) (Zeiss, Oberkochen, Germany) and photographed with an AxioCam MRm colour camera (Zeiss). Pictures were processed with the AxioVision software (Lite Edition 4.8.1) (Zeiss).

4.10 Bioassays

To investigate a direct influence of *X. saxesenii* on the growth of the ambrosia fungus, a standardized bioassay was performed with untreated and treated beetles. The latter were dipped in 1 % SDS and 70 % ethanol subsequently. Individual beetles were placed separately under small hoods made of sterile filter tips on malt agar plates containing the pure culture of ambrosia fungus for one night. Hoods without beetles were used as control fields (Figure 2). After one day, hoods and beetles have been removed from agar plates and pictures have been taken from the fields using a binocular – microscope. In case of changes in fungal growth, monitoring and photographing was prolonged. As a direct observer the author could evaluate the fungal growth immediately. However, for an objective and analyzable evaluation, pictures showing beetle fields (where a beetle was put before) and control fields (without beetles) were compared and fungal growth was evaluated by non - informed outside observers. Data was analyzed using a Chi - squared test (χ^2) with the statistical software BiAS (Version 8.2 01/2007).

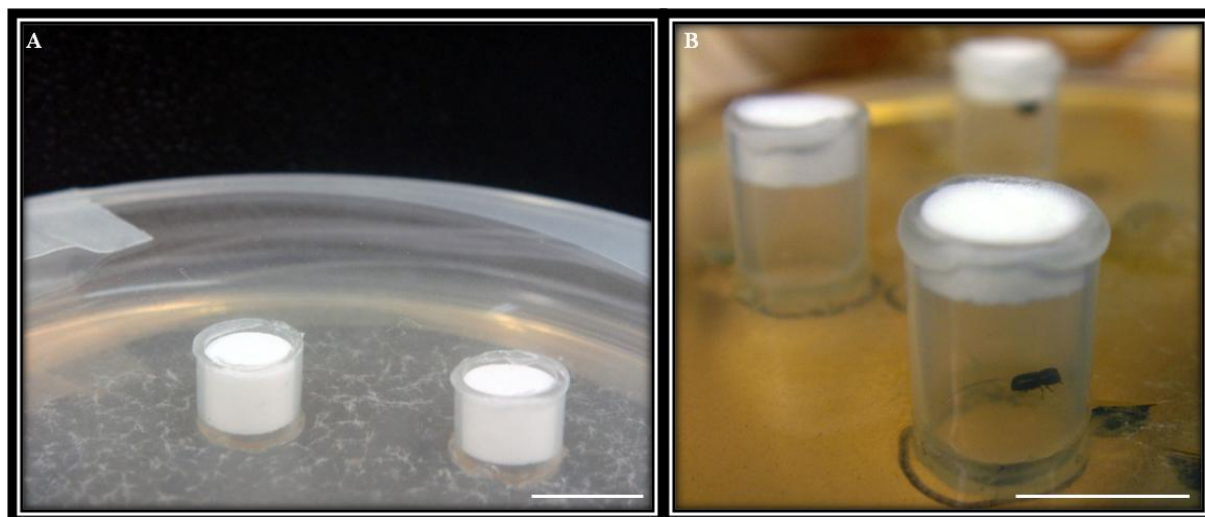


Figure 2. Bioassay setup. **A:** Filter tip hoods on the ambrosia fungus, covering a malt agar plate (white layer). **B:** *Xyleborinus saxesenii* under the hood and an empty hood (control). Scale bars = 8 mm.

4.11 Secretion extraction

For extraction of potential surface (cuticular) and internal beetle secretions, 10 whole beetles and 10 crushed beetles were incubated in methanol (just enough to cover material completely) in a 1.5 ml vial for approximately 1 hour and with agitation. Methanol was transferred in a new vial, evaporated with argon and readopted in ~50 μ l methanol. Extracts were stored at -20°C until analysis. No extraction method was used for ambrosia fungi as the liquid malt extract medium containing fungal hyphae was used as extract.

4.11.1 Agar - diffusion tests

Extracts were tested for antimicrobial activity against potential pathogens for the ambrosia beetle. Filter paper discs (\varnothing 6mm) were added on culture agar plates of fungal (*Aspergillus tamari*, *Penicillium* sp.) and bacterial (*Acinetobacter baylyi*, *E. coli*) antagonists. 10 - 15 μ l of beetle and fungal extracts were added to the discs. An equal amount of methanol and different antibiotics (including antifungal reagents) were used as a negative and positive control, respectively (Table 4).

Table 4. Antibiotics used for antimicrobial tests.

<i>Antibiotic (producer)</i>	<i>Working concentration</i>
ampicillin (Roth)	100 μ g/ml
kanamycin (Serva, Heidelberg, Germany)	50 μ g/ml
nalidixic acid (Sigma-Aldrich)	15 μ g/ml
nystatin (Invitrogen-Gibco)	100 U/ml

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Based on the same concept, a growth-enhancement test was performed with ambrosia fungus on malt agar plates. Beetle extracts as well as nystatin (positive control) and methanol (negative control) were added to discs.

4.12 Cultivation of symbiotic bacteria and fungi

As the isolation and cultivation of the mutualistic ambrosia fungus of a *X. saxesenii* gallery was already mentioned (2.1), here, the focus is on microbes originating from the ambrosia beetle itself. Growth of symbionts from the beetle surface was achieved by putting a single beetle on a malt agar plate which was incubated for some days at 26°C. Risen microbes were transferred onto new plates to get pure cultures. For cultivation of gut symbionts, beetles were dissected for gut isolation and the intestinal tract was crushed in 1x PBS. Gut suspension was spread on different agar media (Table 5), containing antibiotic and antifungal reagents and incubated at 26°C for 1-3 days (until medium-sized single colonies were visible) under aerobic and anaerobic conditions. The latter were generated by using Anaerocult®A reagent (Merck, Darmstadt, Germany). For Sf-900 agar, a serial plate dilution (until 10⁻⁴) of the gut suspension was done.

Table 5. Media used for cultivation of microbial symbionts.

<i>Medium</i>	<i>Preparation</i>	<i>Antibiotic</i>
malt agar	1% malt extract, 2% agar	-
powdered chitin agar	0.07% K ₂ HPO ₄ , 0.03% KH ₂ PO ₄ , 0.05% MgSO ₄ ·5 H ₂ O, 0.001% FeSO ₄ ·7 H ₂ O, 0.0001% ZnSO ₄ , 0.0001% MnCl ₂ , 0.5% chitin suspension (8-10%), 2% agar (modified from HSU & LOCKWOOD, 1975)	ampicillin (100 µg/ml)
nutrient agar	0.5% peptone, 0.3% meat extract, 1.5% agar	cycloheximide (100 µg/ml) (Santa Cruz Biotechnology, Santa Cruz, USA)
R2A agar	0.05% yeast extract, 0.05% peptone, 0.05% casein hydrolyzate, 0.05% glucose, 0.05% starch, 0.03% K ₂ HPO ₄ , 0.0024% MgSO ₄ , 0.03% sodium pyruvate, 1.5% agar (after REASONER & GELDREICH, 1985)	cycloheximide (100 µg/ml)
SF-900II agar	SF-900II serum free medium (Invitrogen-Gibco), 3% agar	cycloheximide (100 µg/ml)
trypticase soy yeast extract agar (TSYE)	3% trypticase soy broth (1.7% trypton, 0.3% pepton, 0.25% glucose, 0.5% NaCl, 0.25% K ₂ HPO ₄), 0.3% yeast extract, 1.5% agar	cycloheximide (100 µg/ml)
yeast glucose (YG)	0.5% yeast extract, 0.4% glucose, 0.1% NaCl, 1.5% agar	nystatin (100 U/ml)

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For identification of ectosymbionts, DNA was extracted, PCR-amplified with corresponding primer pairs and sequenced (see 4.2; 4.3; 4.6).

Gut symbionts were re-plated two times. That means colonies were transferred first on corresponding fresh plates and second on R2A agar plates by the single streak method. Single bacterial colonies were picked in PCR lysis solution [67 mM Tris-Cl pH 8.8, 16.6 mM $(\text{NH}_4)_2\text{SO}_4$, 5mM β -mercaptoethanol, 6.7 mM MgCl_2 , 6.7 μM EDTA pH 8.0, 1.7 μM SDS] (SAMBROOK & RUSSEL, 2001), frozen at -80°C for 15 min, defrosted at room temperature, heated at 95°C for 10 min and cooled down to room temperature before used as templates for colony PCR with fd1 / rP2 and LS1 / LR5 primer pairs (see 4.3). Amplicons were directly sequenced (see 4.6).

Symbiotic gut fungi, cultivated on chitin-agar, were cut with agar pieces and subsequently treated with GP-buffer of the peqGold MicroSpin Gel Extraction Kit (Peqlab), followed by DNA extraction, PCR-amplification and direct sequencing (see 4.2; 4.3; 4.6).

4.13 Acetylene reduction assay (ARA)

Galleries were tested for potential nitrogen (N_2) fixation using the acetylene reduction assay (DILWORTH, 1966; KOCH & EVANS, 1966; SLOGER & SILVER, 1967; STEWART et al., 1967; HARDY et al., 1968). This test is based on the reduction of acetylene (C_2H_2) to ethylene (C_2H_4), catalyzed by the nitrogenase enzyme responsible for N_2 - fixation. The activity of the enzyme is determined by the extent of reduction of C_2H_2 to C_2H_4 .

Six empty reference tubes and five galleries were sealed with a rubber septum and paraffin film. Acetylene was generated by adding 10 rocks of calcium carbide (CaC_2) in a 50 ml flask filled with H_2O and immediately covered with a balloon [$\text{CaC}_2 + 2 \text{H}_2\text{O} = \text{C}_2\text{H}_2 + \text{Ca}(\text{OH})_2$]. Argon was used as an internal standard to quantify the reduction of acetylene and especially compensate for variation in test tube volume as well as injection volume. An initial atmosphere of 50 % acetylene and 5 % argon was created in another balloon (20 ml acetylene, 2 ml argon, 18 ml air). After determination of air volume in the beetle galleries (4.5 - 7 ml) and reference tubes (14 ml), ~ 20 % of the volume was removed (so the tubes are not over pressurized) with a gas - tight syringe (Hamilton, Switzerland) and replaced by the equal volume of acetylene-argon mixture so that the test tubes contained an initial atmosphere of 10 % acetylene and 1 % argon. Samples were analyzed by coupled capillary gas chromatography - mass spectrometry (GC - MS) with a Varian 450 - GC gas chromatograph (Varian, Middelburg, Netherlands; now: Agilent Technologies, Böblingen, Germany), equipped with a DB-5MS capillary column (Agilent Technologies) and coupled to a Varian

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240 - MS mass spectrometer. 25 μ l of gas mixture of each tube were successively injected in the gas chromatograph with injector at 50°C and oven at 35°C. The first injection was at time zero (t_0). Further injections followed after 1, 2, 3, 5, 6 and 24 hours ($t_1 - t_{24}$). The software Varian MS Workstation for Windows (Version 6) was used for data acquisition. There was no detection of ethylene possible, as it has the same mass as nitrogen and the mass spectrometer cannot distinguish between them. Thus, only the decline of acetylene was measured as acetylene / argon quotients. To assess an effect between time and treatments, the acetylene / argon ratios were subjected to a repeated - measures ANOVA with the SPSS 17.0 software.

5. Results

5.1 Bacterial and fungal endosymbionts of *Xyleborinus saxesenii*

It has to be clarified in advance, that *Burkholderia* - bacteria were not further investigated as neither the diagnostic PCRs nor the 454 sequences showed any conclusive results.

bTEFAP enabled an insight in the bacterial community within the beetle showing the top eight unique and ubiquitous genera (or closest taxonomic designation) with *Sphingobacterium* and *Ochrobactrum* emerging as the two most abundant endosymbionts (Figure 3). With a percentage of 35.6 % and 28.1 %, respectively, of all sequences (12357), they appeared as the two predominant bacterial endosymbionts. In total, the sequencing results unveiled 40 genera (OTUs) of 27 families and 7 classes (full list see appendix 1).

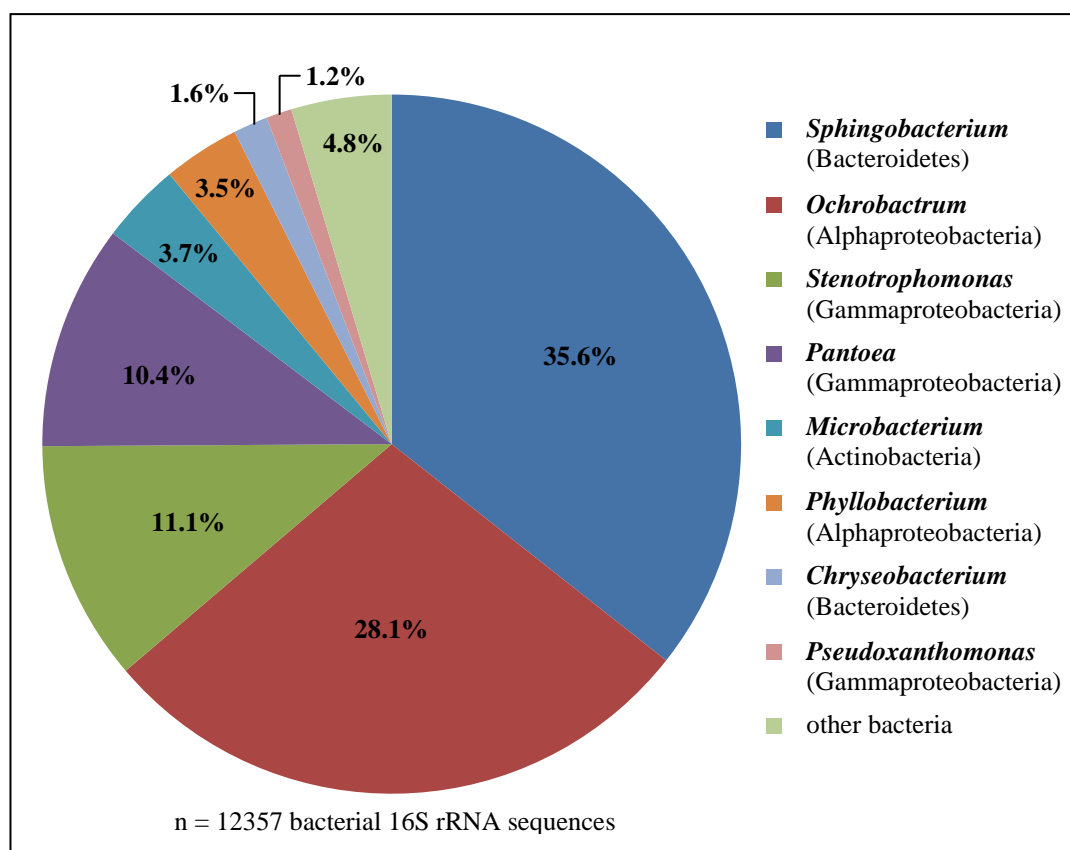


Figure 3. Composition of the endosymbiotic bacterial community of *X. saxesenii* as revealed by bTEFAP - 454 sequencing. Only genera are shown with sequence percentages above 1 %.

Diagnostic PCRs with specific primers designed to match a variable region of the 16S rRNA of *Sphingobacterium* and *Ochrobactrum* revealed 100 % ‘infection’ rates, meaning 10 / 10 PCR products of whole beetle DNA samples showing positive signals.

Sequencing of *E. coli* clones containing fungal 28S rRNA (LSU) inserts revealed sequences of the yeast genus *Candida* (Ascomycota: Saccharomycetes) which was found in 100 % of *X.*

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saxesenii (10 / 10). Overall, 22 clones were sequenced and 17 sequences were identified as *Candida* sp. Showing at least 96 % similarity. Those sequences were compared with GenBank sequences of yeasts known to be associated with other ambrosia beetles and with low identity sequences from the same BLAST hit. Phylogenetic analysis based on the partial 26S (28S) rRNA gene sequence revealed that the ‘*Candida* clones’ cluster as a monophyletic, polytomous group (90 % posterior probability) within the order Saccharomycetales. Among *Candida* clones, 26S rRNA gene sequence similarity was relatively high (96 – 99 %) with their closest relatives, *C. trypodendroni* and *C. insectorum* (Figure 4).

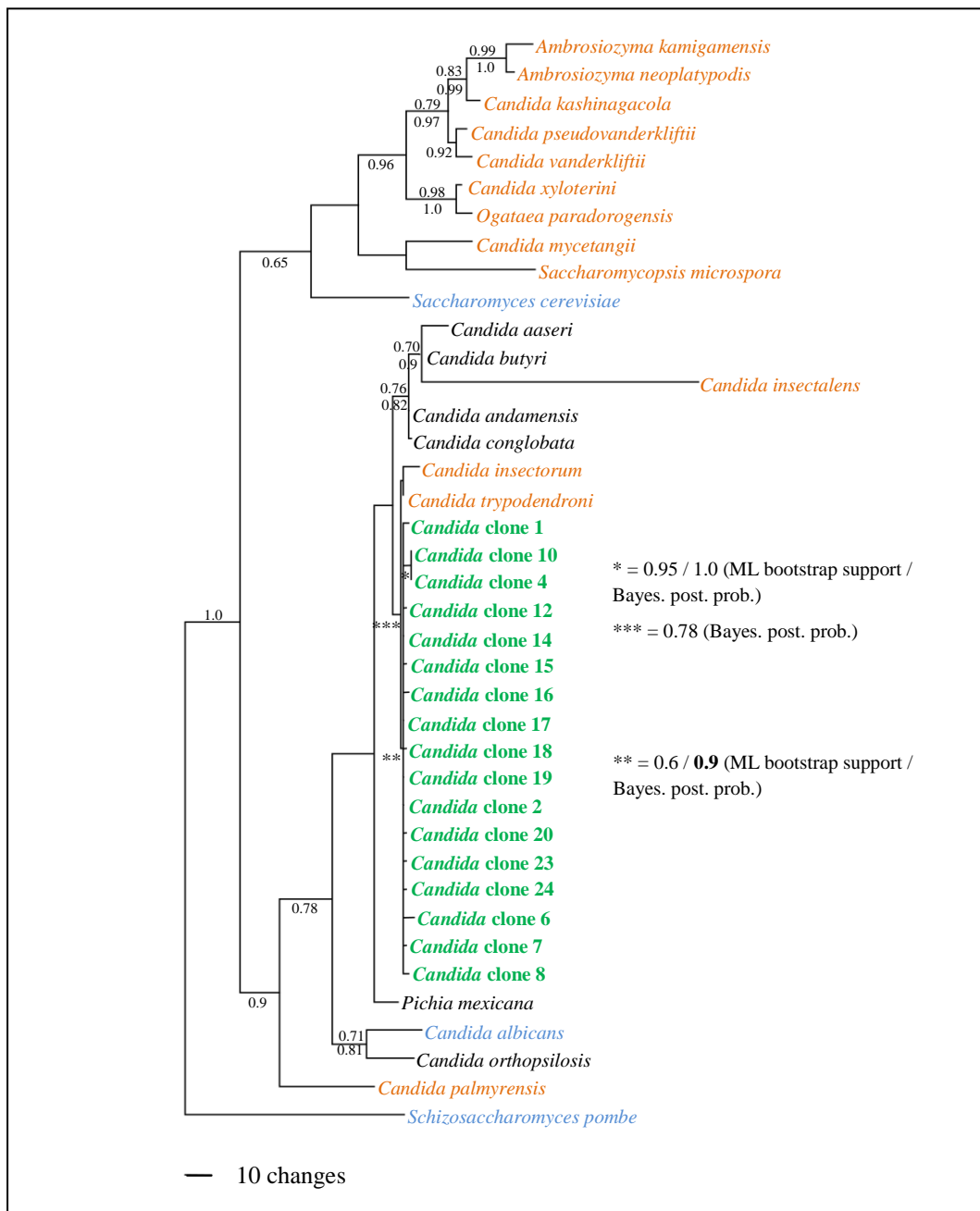
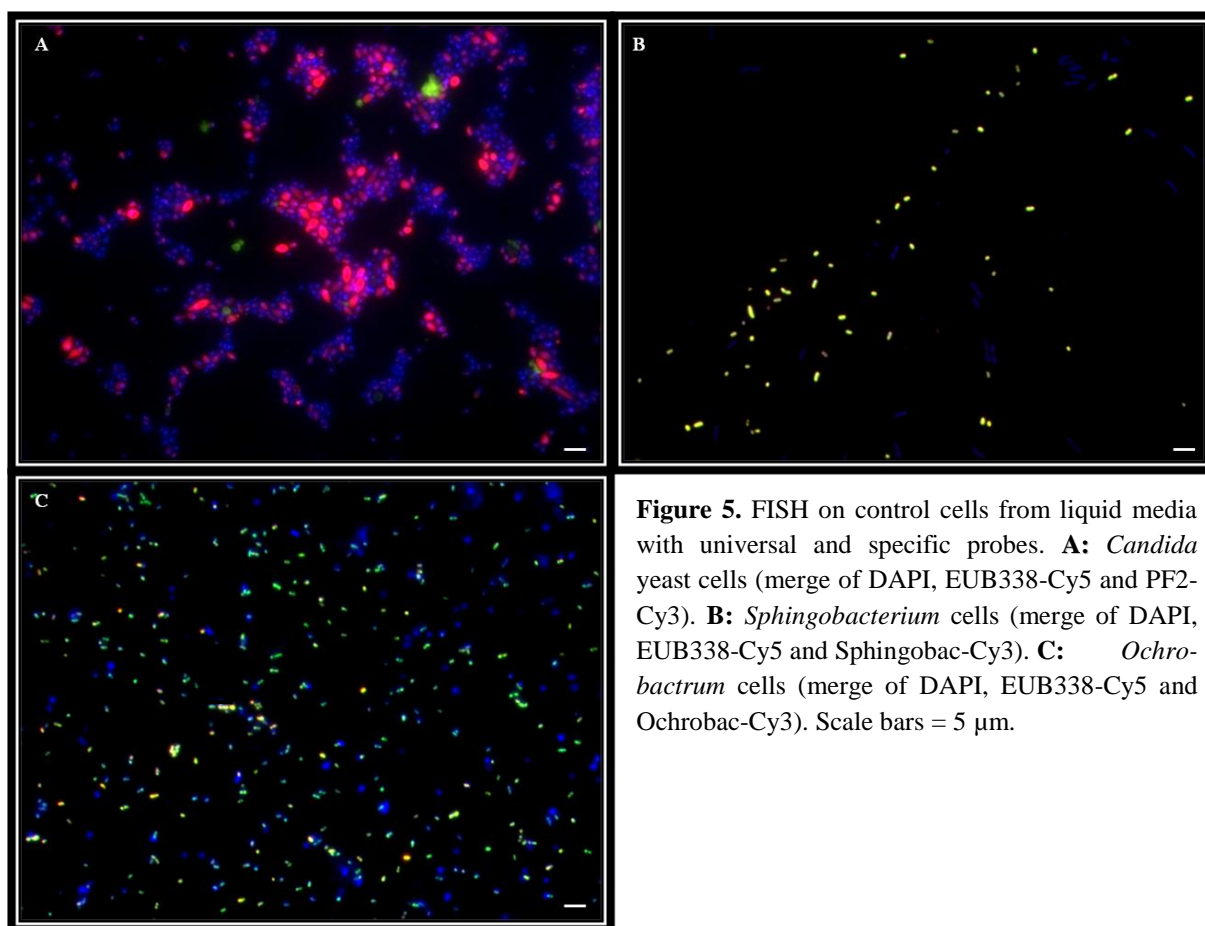


Figure 4. Phylogenetic position of endosymbiotic *Candida* of *X. saxesenii* (green) based on 22 26S (28S) rRNA gene sequences (605 bps, maximum likelihood heuristic search, 50 % majority – rule consensus tree of 1000 replicates). The tree is rooted with *Schizosaccharomyces pombe* as outgroup. Bootstrap supports of at least 70 % are indicated at nodes as maximum likelihood (ML) bootstrap values (1000 replicates) above and / or Bayesian posterior probabilities (59402 replicates) below branches. Yeasts known to be associated with other ambrosia beetles are indicated in orange, common yeasts in blue and other close taxonomic designations in black.

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5.2 FISH

For localization of the endosymbionts FISH was conducted on semi-thin sections of the beetle gut with a combination of fluorescence labeled (Cy3, Cy5) universal (EUB338) and specific (PF2, *Sphingobac*, *Ochrobac*) probes. To test the specificity of the fluorescent probes and to get an idea of the morphology of endosymbiotic cells, positive controls from pure liquid media were examined. While *Candida* appeared in spherical to elongated cell shapes (~ 3 μm long), cells of both bacteria genera appeared as short rods (1 – 2 μm long). Not all cells hybridized with the probes, thus, only the DAPI stain is visible (Figure 5.) Furthermore, FISH on *Candida* cells with nonsense probes Cor653-Cy5 and Cor653-Cy3 revealed no results (not shown).



FISH with the combination of Cy5 – stained eubacterial probe EUB338 and Cy3 – stained all yeast probe PF2 revealed a remarkable affection of *Candida* in the gut lumen (Figure 6).

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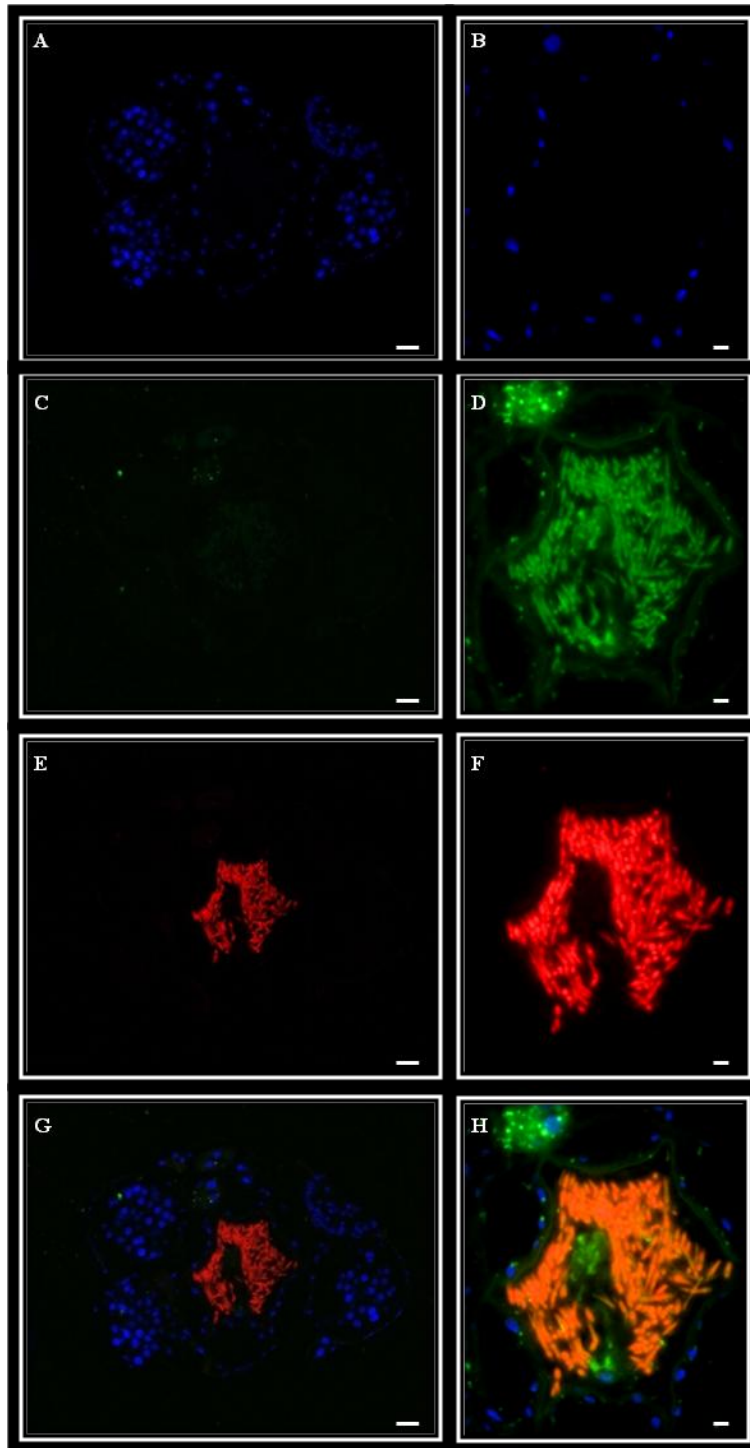


Figure 6. FISH on gut semi-thin sections with eubacterial probe EUB338-Cy5 and yeast probe PF2-Cy3. Elongated *Candida* cells appear only the gut lumen (center part). Adjacent epithelial cells indicate bacteria. **A, B:** DAPI. **C, D:** EUB338-Cy5. **E, F:** PF2-Cy3. **G, H:** merge. Scale bars: left column = 50 µm; right column = 10

Other gut sections showed *Candida* in spherical cells as well as in long shaped hyphae within the gut lumen, where also bacteria appeared (Figure 7). Furthermore, unknown and remarkable big egg – shaped structures could also be detected.

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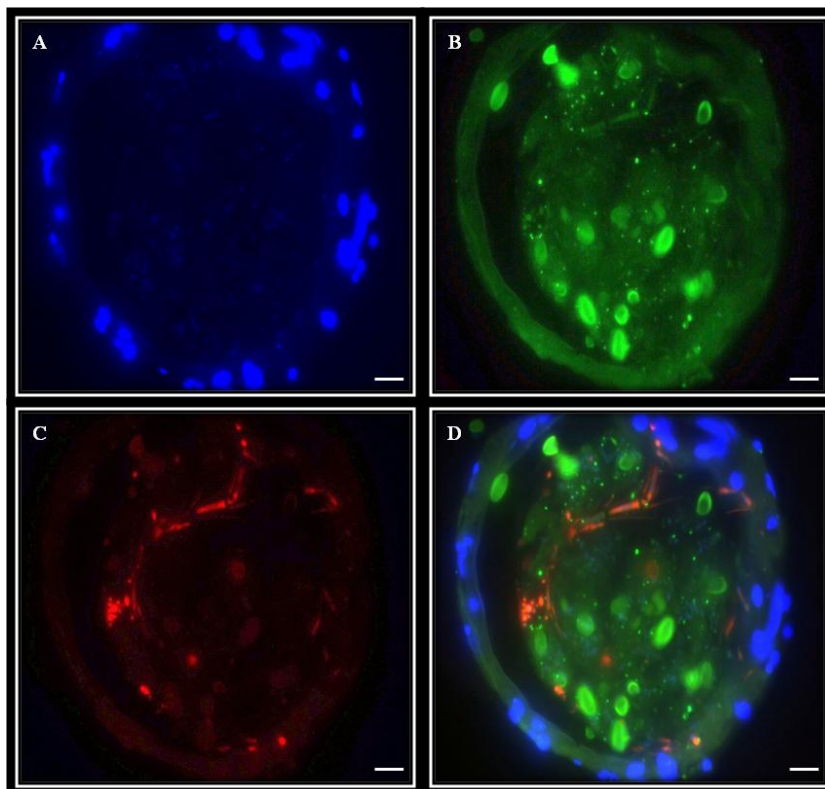


Figure 7. FISH on gut semi-thin sections with eubacterial probe EUB338-Cy5 and yeast probe PF2-Cy3. *Candida* cells appear in two morphologies (spherical and elongated) and with bacteria as well as egg-shaped structures within the gut lumen. **A:** DAPI. **B:** EUB338-Cy5. **C:** PF2-Cy3. **D:** merge. Scale bars = 10 μm .

There were no conclusive results of *in situ* hybridizations with both specific *Sphingobac* and *Ochrobac* probes. Some positive signals could be detected on the margin of the gut lumen, but also beyond the sections, however (see appendix 3 and 4).

5.3 The ambrosia fungus, the microbial gut community and other associated micro - organisms of *X. saxesenii*

The ambrosia fungus which was isolated from the *X. saxesenii* gallery and cultivated on malt agar plates was identified by direct sequencing. However, there were strong hits for *Ambrosiella xylebori* as well as *Ceratocystis adiposa*, both with a 100 % query coverage / 99 % maximum identity, as indicated in the BLAST hit. PCR on gut DNA with bacterial primers unveiled a higher bacterial affection within the gut compared to residual beetle DNA. For isolation of the microbial gut community, gut suspensions (crushed beetle gut in PBS) were spread on different cultivation media and finally it was possible to cultivate both bacterial and fungal gut symbionts on 7 different media in total, some of them under

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aerobic as well as anaerobic conditions. The beetle gut microbiota was characterized using direct sequencing of PCR products of pure culture colonies. Only bacterial as well as yeast or yeast like colonies were considered. Both universal bacterial and fungal primers were used for sequencing. Overall, 6 different bacterial genera including *Sphingobacterium* and *Ochrobactrum* as well as diverse fungi including yeasts could be identified, some of them on species level (confirmed by query coverage and maximum identity values of GenBank) (Table 6). See appendix (2) for a complete list showing sequence lengths and first BLAST hits.

Table 6. Gut microbiota of *X. saxesenii* cultivatable on different media. Results base on GenBank entries after BLASTing consensus sequences of each sample.* = sequences were not obtained from pure culture colonies; TSYE = trypticase soy yeast extract agar; NutrA = nutrient agar; YG = yeast glucose agar; Sf900 = serum free medium agar; ChitinA = powdered chitin agar; MaltA = malt agar; -O₂ = anaerobic; query cov. = query coverage; max. ident. = maximum identity.

sample	original medium	organism(s)	phylum/class	query cover. [%]	max. ident. [%]
B1_1	R2A	<i>Bacillus simplex</i>	Firmicutes	100	99
B1_3	R2A	<i>Bacillus simplex</i>	Firmicutes	100	99
B2_7	R2A (-O ₂)	<i>Enterococcus</i> sp.	Firmicutes	45	85
B1_6	TSYE	<i>Sphingobacterium siyangense</i>	Bacteroidetes	100	99
B1_8	TSYE	<i>Bacillus simplex</i>	Firmicutes	100	99
B1_10	NutrA	<i>Bacillus simplex</i>	Firmicutes	99	99
B1_11	NutrA	<i>Pseudomonas putida</i>	Gammaproteobacteria	100	99
B1_12	NutrA	<i>Pseudomonas putida</i>	Gammaproteobacteria	100	99
B1_13	NutrA	<i>Enterococcus mundtii</i>	Firmicutes	100	90
B1_14	NutrA	<i>Enterococcus durans</i>	Firmicutes	99	95
B2_3	NutrA	<i>Enterococcus mundtii</i>	Firmicutes	100	99
B1_15	YG	<i>Stenotrophomonas maltophilia</i>	Gammaproteobacteria	100	92
B1_18	YG	<i>Ochrobactrum</i> sp.	Alphaproteobacteria	100	98
B1_19	YG	<i>Enterococcus</i> sp.	Firmicutes	63	98
B2_1	YG	<i>Enterococcus</i> sp.	Firmicutes	71	96
B2_5	YG (-O ₂)	<i>Enterococcus mundtii</i>	Firmicutes	100	99
B1_21	SF900	<i>Bacillus</i> sp.	Firmicutes	61	96
B1_22	SF900	<i>Bacillus simplex</i>	Firmicutes	100	99
B1_23	SF900	<i>Bacillus simplex</i>	Firmicutes	100	99
B1_26	SF900	<i>Stenotrophomonas</i> sp.	Gammaproteobacteria	61	95
B1_27	SF900	<i>Stenotrophomonas</i> sp.	Gammaproteobacteria	89	93
F1_11	NutrA	<i>Cladosporium</i> sp. / <i>Davidiella</i> sp.	Dothideomycetes	90	98
F1_12	NutrA	<i>Alternaria</i> sp.	Dothideomycetes	84	93
F1_22	SF900	div. Chaetothyriales	Eurotiomycetes	100	98
F1_23	SF900	<i>Cladosporium</i> sp.	Dothideomycetes	97	86
F1 ChA	ChitinA	div. yeasts	Ascomycota	97	92
F2 ChA	ChitinA	<i>Malassezia</i> sp.	Exobasidiomycetes	100	99
Gut fungus 1*	MaltA	<i>Ophiostoma stenoceras</i>	Sordariomycetes	100	98
Gut fungus 2*	MaltA	<i>Petriella</i> sp.	Sordariomycetes	99	99

Results

Furthermore, *Penicillium* (identification based on morphology, not confirmed by sequencing) as well as an unidentified ambrosia fungus and white ‘star’ shaped fungal colonies could be observed on malt agar plates after adding gut suspension (Figure 8). The white colonies could be identified by direct sequencing as *Ophiostoma stenoceras* and / or *Petriella* sp.

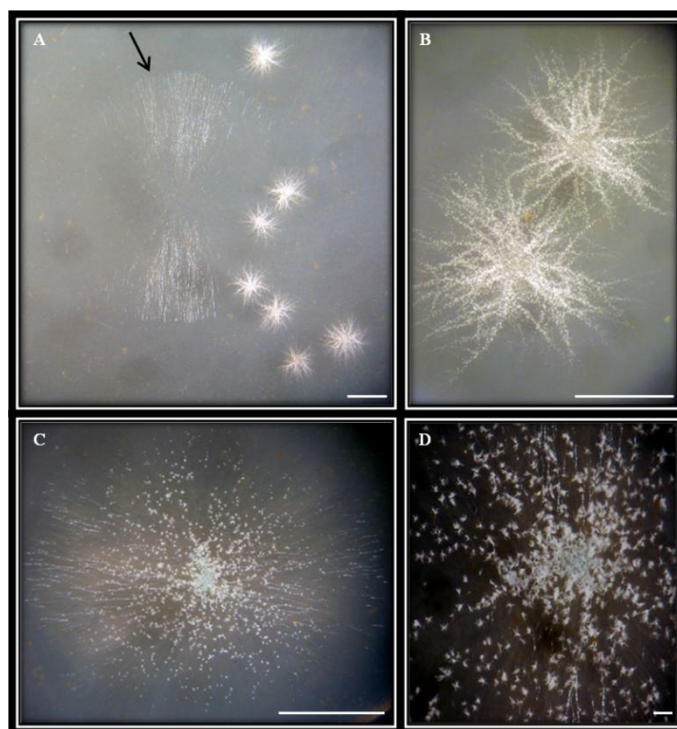


Figure 8. *Penicillium* sp. and *Ophiostoma stenoceras* / *Petriella* sp. from the gut of *X. saxesenii* on malt agar. **A, B:** Mycelium of an unidentified ambrosia fungus (A, arrow) and the white ‘star’ shaped colonies of *O. stenoceras* / *Petriella* sp. **C, D:** *Penicillium* sp. Scale bars: A, C = 1 mm; B = 0.5 mm; D = 0.1 mm.

Furthermore, other microbes than endosymbionts associated with *X. saxesenii* could be perceived. Fungal growth could be observed after a single beetle was placed on a malt agar plate to move freely. After approximately 24 hours two different ‘mold fungi’ could be recognized as well as a yeast (-like) colony. Direct sequencing identified the first mold as *Aspergillus flavus* or *Aspergillus oryzae* (both with 100 % query cov. / 100 % max. ident.), the second as *Penicillium* sp. (100 % query cov. / 99 % max. ident.) and the yeast as *Candida* sp. (100 % query cov. / 96 % max. ident.). This *Candida* sample was highly similar - if not identical - to the endosymbiotic *Candida* clones (not shown).

Furthermore, an enhanced growth of (probable) ambrosia fungus surrounding *Aspergillus* could be observed (Figure 9).

Results

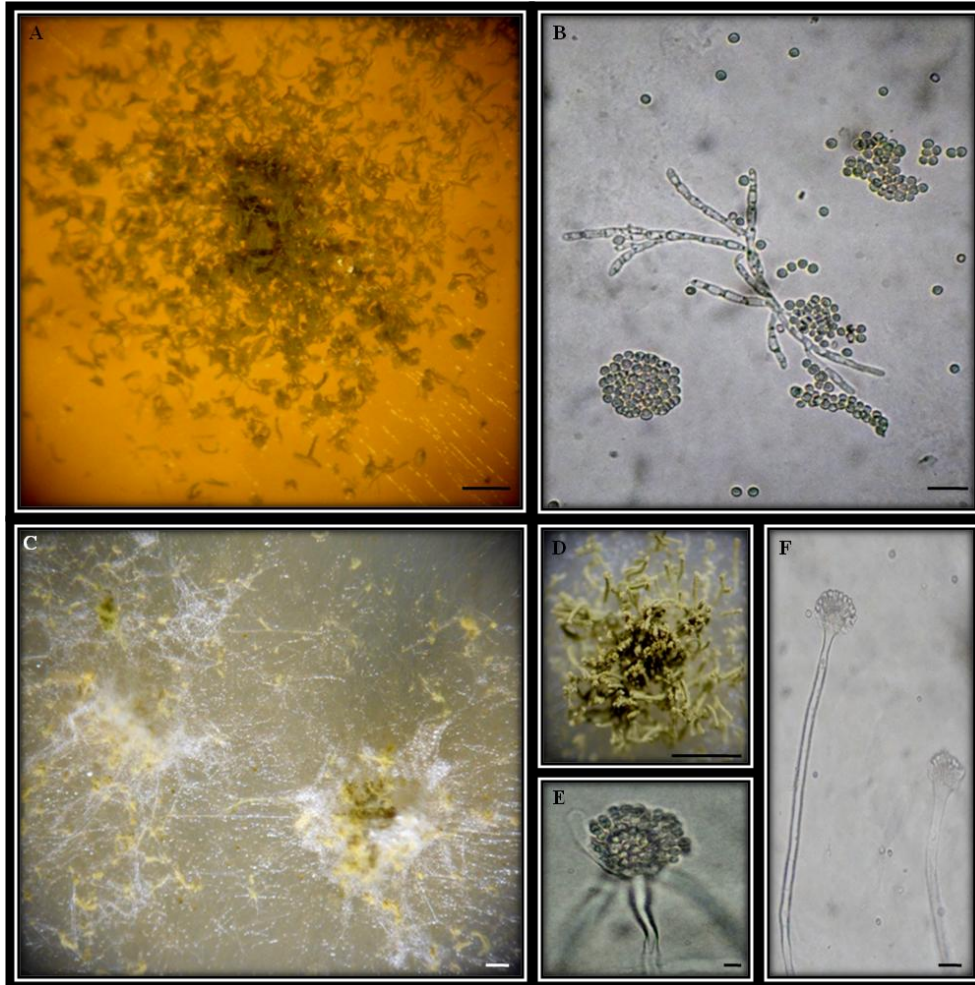


Figure 9. *Penicillium* sp. and *Aspergillus flavus* (or *oryzae*) associated with *X. saxesenii*. **A, B:** *Penicillium* sp. on malt agar and under the microscope. **C–F:** *Aspergillus flavus* / *oryzae* on malt agar and under the microscope. Enhanced growth of ambrosia fungus (white) could be observed in close proximity to *Aspergillus* (C). Scale bars: A, C, D = 0.5 mm; B, E = 10 μ m; F = 20 μ m.

Sometimes, beetles were invaded and overgrown by *Aspergillus* and other mold fungi, especially when they were on (ambrosia fungus) plates for a longer period (Figure 10). Those mold fungi could also be observed in old and / or abandoned galleries (not shown).

Results

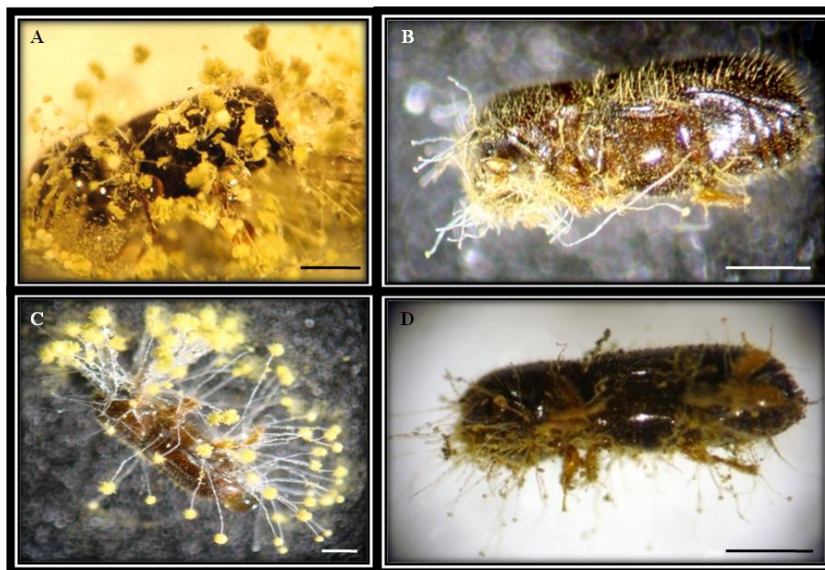


Figure 10. *X. saxesenii* overgrown by *Aspergillus* and other invasive fungal pathogens. Scale bars = 0.5 mm.

5.4 Bioassays

To test if there is a direct influence of *X. saxesenii* on the growth of *Ambrosiella xylebori* / *Ceratocystis adiposa*, bioassays were conducted. A first series of bioassays (#1 – #10) was not standardized. There were more than one control fields as well as beetle fields per plate and bioassays proceeded for different, undefined time periods (between 1 and 6 days). Picture dates varied as well. After beetle contact, new white hyphae of the ambrosia fungus emerged forming dense layers covering greater parts of the malt agar plate before turning dark over time. Furthermore, as beetles were not ‘washed’ (treated with SDS and ethanol) foreign ‘weedy’ fungi could be observed on 8 out of 10 plates. The growth of those fungi (mostly with *Aspergillus* phenotype) was accompanied by light to mainly strong growth enhancement of the ambrosia fungus within the beetle fields (Figures 11, 12).

Results

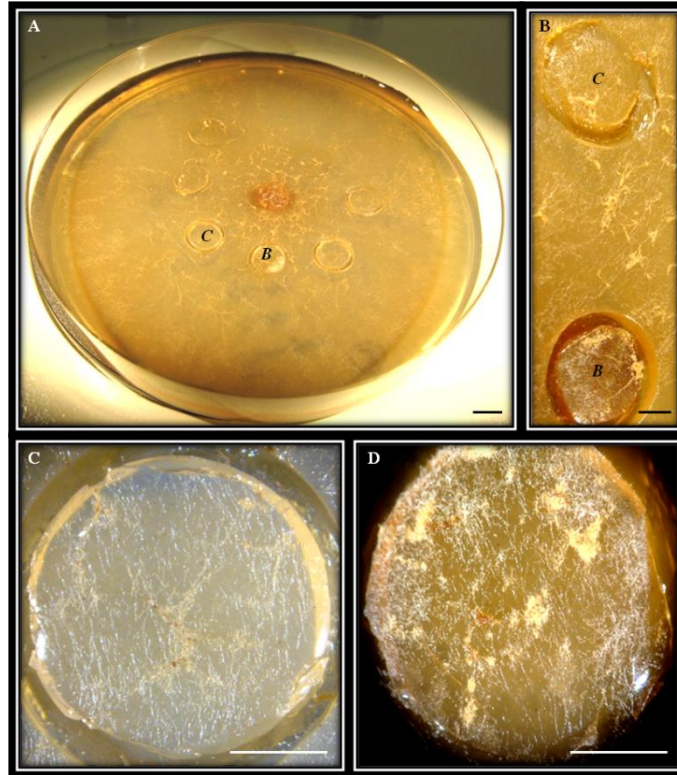


Figure 11. Growth of ambrosia fungus *Ambrosiella xylebori* / *Ceratocystis adeposa* after bioassays. **A:** Malt agar plate with different fields. **B:** Control field (C) and beetle field (B). **C:** Control field with ambrosia fungus growth. **D:** Beetle field with enhanced ambrosia fungus growth. Bioassays proceeded between 1 and 7 days. Pictures were taken up to 7 days after bioassays. Scale bars: A = 6 mm; B-D = 2 mm.

Results

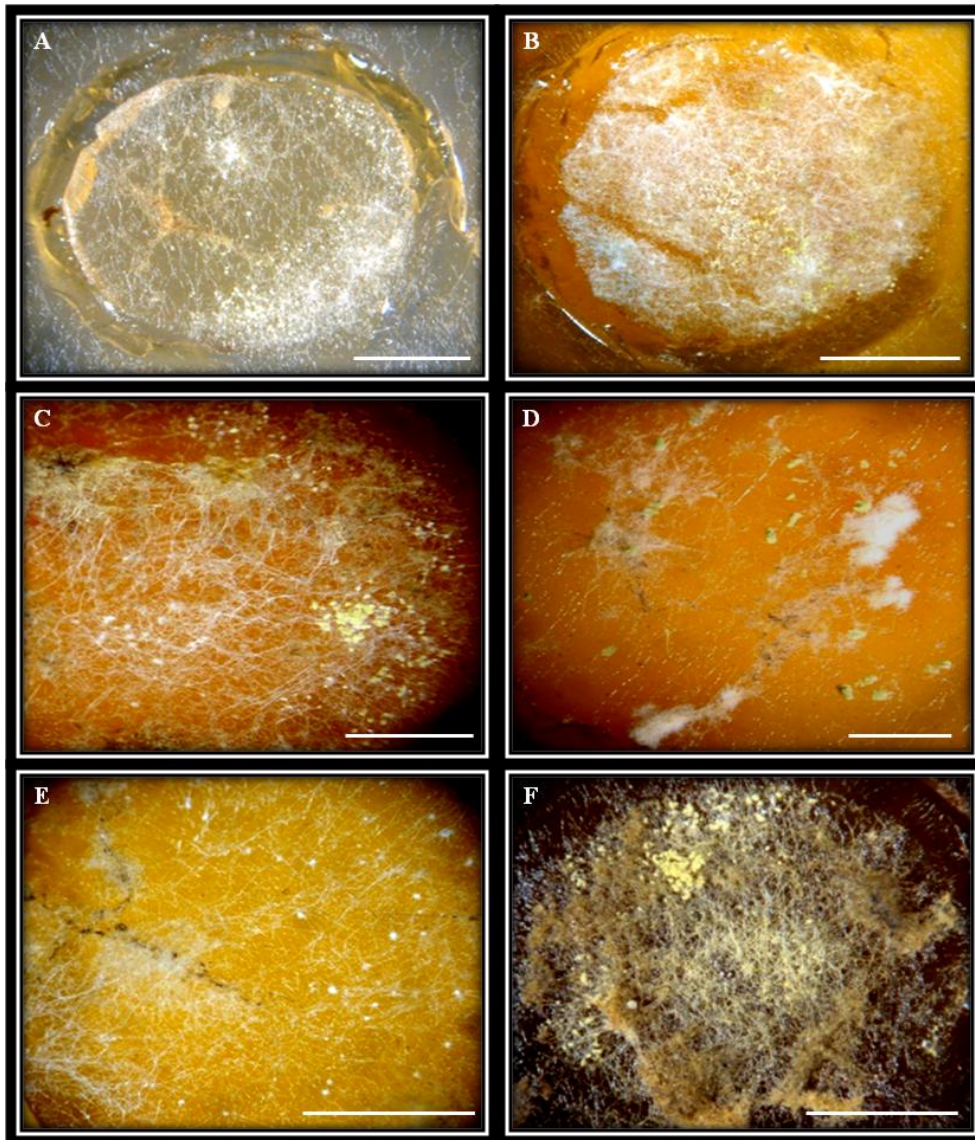


Figure 12. Enhanced and new induced growth of ambrosia fungus *Ambrosiella xylebori* / *Ceratocystis adeposa* appearing in white layers accompanied by some mold fungi. **A, B, C, D, F:** The predominant ‘invasive’ fungus is *Aspergillus* (light green). Over time, the ambrosia fungus and the overgrown agar fields turn dark (**F**). **C, E:** Emerging white colonies similar to those of *O. stenoceras* / *Petriella* sp. Bioassays proceeded and were monitored between 1 and 7 days. Scale bars = 2 mm.

Differences between control fields and beetle fields were assessed by comparing representative pictures and were confirmed statistically (Chi - squared test). Overall, every independent observer voted for stronger fungal growth on the beetle fields and in 7 out of 8 (87.5 %) evaluations these differences were significant (see χ^2 - and p - values in Table 7).

Results

Table 7. Evaluation of ambrosia fungus growth in not standardized bioassays. Eight independent observers compared the fungal growth (strong or light in sense of density of hyphae / mycelium) on control fields and beetle fields with the aid of representative pictures showing the two different fields. Vote options: 1, pro beetle field, 2, pro control field, 3, nondescript. Particular votes are shown as well as χ^2 – and p – values generated in the chi – squared test. Only vote options 1, and 2, were considered in the test. Df = 1. * = significant, ** = very significant, *** = highly significant.

observer χ^2 -test	1	2	3	4	5	6	7	8
beetle	10	16	17	7	16	15	19	14
control	2	1	5	3	3	4	4	2
χ^2	5.33	13.24	6.55	1.6	8.89	6.37	9.78	9.00
p	0.021 *	0.0003 ***	0.011*	0.206	0.003 *	0.012 *	0.002 **	0.003 **

The second series of bioassays (#11 - #47) was standardized. There were two fields (beetle / control) per plate, beetles were removed after one day and pictures were taken more frequently and regularly. Additionally, some beetles were treated with SDS and ethanol directly prior to the bioassays leading to a non-attendance of mold fungi. If beetles were not treated, other fungi like *Aspergillus* appeared (not shown). At first sight, there was no difference regarding the intensity of ambrosia fungus growth on both fields (Figure 13). However, some plates already showed a strong growth of ambrosia fungus even without beetle contact.

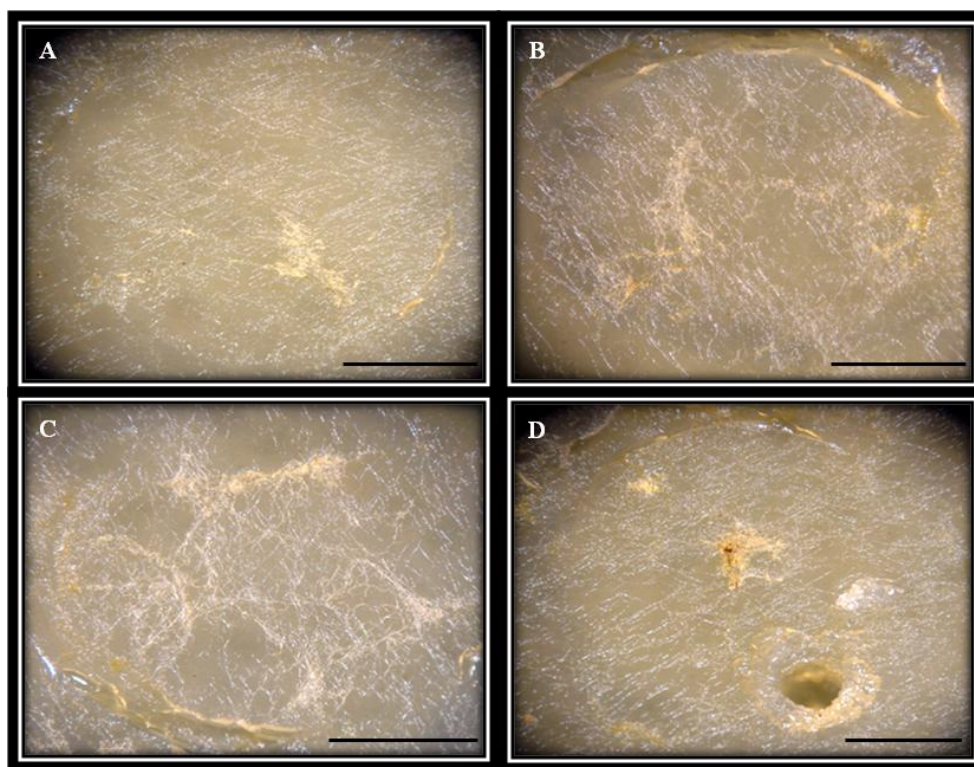


Figure 13. Growth of ambrosia fungus *Ambrosiella xylebori* / *Ceratocystis adiposa* after standardized bioassays. **B, D:** Neither a growth enhancement of the ambrosia fungus nor emerging invasive fungi could be detected on beetle fields, where some individuals dug a tunnel in the agar (D). **A, C:** control fields. Bioassays proceeded one day and were photographed one day later. Scale bars = 2 mm.

Results

It was very hard to evaluate the fungal growth at all and to detect differences. The analysis of the evaluation revealed more votes for the control fields indicating a slight stronger growth of the ambrosia fungus. In three cases (37.5 %) the votes for the control fields were significant (see χ^2 - and p - values in Table 8).

Table 8. Evaluation of ambrosia fungus growth in standardized bioassays. Eight independent observers compared the fungal growth on control fields and beetle fields with the aid of representative pictures showing the two different fields. Vote options: 1, pro beetle field, 2, pro control field. Particular votes are shown as well as χ^2 - and p - values generated in the chi - squared test. Df = 1. * = significant, ** = very significant, *** = highly significant.

χ^2 -test \ observer	1	2	3	4	5	6	7	8
beetle	11	14	9	11	14	17	14	15
control	26	22	27	26	23	20	23	22
χ^2	6.08	1.78	9.00	6.08	2.19	0.24	2.19	1.32
p	0.014*	0.182	0.003**	0.014*	0.139	0.622	0.139	0.250

Furthermore, on the majority of malt agar plates unidentified mites (at least one) could be observed, regardless whether beetles were treated with SDS and ethanol before. Those arachnids were observed to feed on the ambrosia fungus as well, which made it difficult to evaluate the fungal growth.

5.5 Secretions and agar - diffusion tests

Beetle secretions may be responsible for the prevention of invading antagonists as well as for the growth enhancement of ambrosia fungi. To investigate potential compounds produced by the beetles and fungi, beetle methanol extracts and fungal extracts were tested for antimicrobial activity on pure culture plates of potential pathogens (*Aspergillus tamari*, *Penicillium* sp.; *Acinetobacter baylyi*, *E. coli*). None of the tests showed any effect of the extracts, whereas both positive (various antibiotics) and negative controls (methanol) showed an inhibiting effect and no effect, respectively (not shown).

A growth enhancement test using beetle extracts as potential growth stimulants of the ambrosia fungus *Ambrosiella xylebori* / *Ceratocystis adiposa* also failed to show any effect (not shown).

5.6 Acetylene reduction assay

To test a potential N_2 - fixation ability of the galleries, an acetylene reduction assay was conducted. The reduction of acetylene (C_2H_2) to ethylene (C_2H_4) is catalyzed by the enzyme nitrogenase, which is also responsible for N_2 - fixation and whose activity is determined by the extent of the reduction reaction. As C_2H_4 could not be detected, the decline of C_2H_2 was measured as quotients of acetylene and argon gas (Figure 15), which was used as an internal standard for the quantification of the reduction process. This detection is equitable with a quantitative estimation of N_2 - fixation.

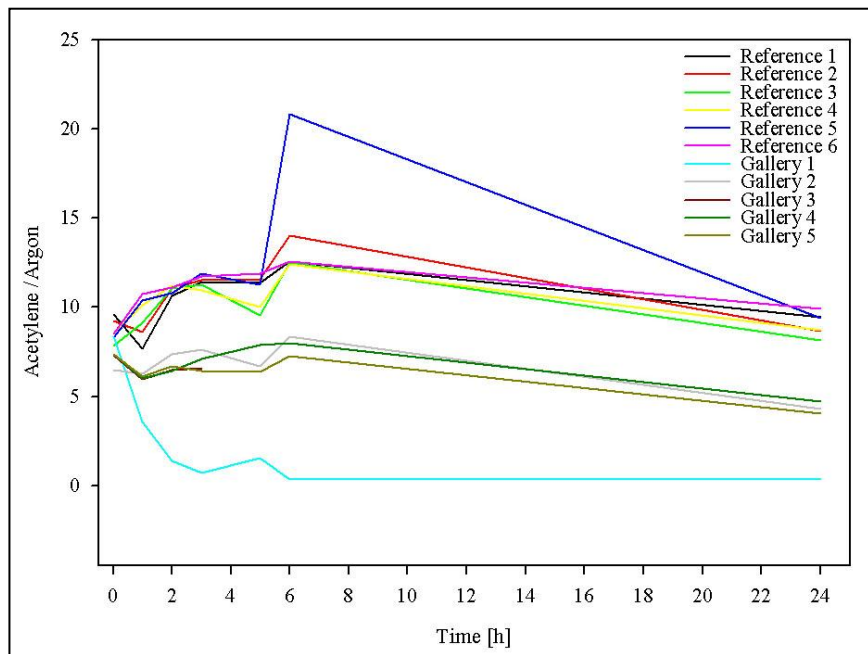


Figure 14. Progress of the acetylene reduction assay showing acetylene / argon ratios of different beetle galleries and empty reference tubes over time (t_0 , t_1 , t_2 , t_3 , t_5 , t_6 , t_{24}). For gallery 3, only the ratios of $t_0 - t_2$ could be measured due to subsequent collapse of the sealing septum.

Although the curve shape of beetle gallery 1 showed a different progress (light blue line in Figure 15), this sample was not taken into account anymore. The means of the acetylene / argon ratios of all references and galleries (2 - 5) over time are plotted in Figure 16.

Results

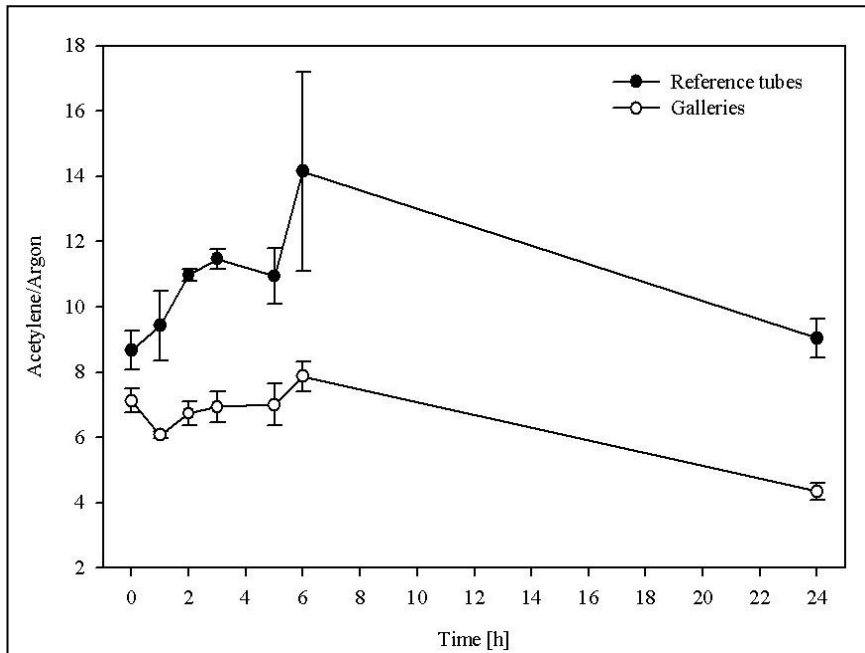


Figure 15. Changes of acetylene / argon ratios (means) between beetle galleries and reference tubes over time (t_0 , t_1 , t_2 , t_3 , t_5 , t_6 , t_{24}). For gallery 3, only the ratios of $t_0 - t_2$ could be measured due to subsequent collapse of the sealing septum. Error bars represent standard deviations.

For an assessment of time and / or treatment effects on the acetylene - argon ratios, statistical analyses (repeated - measures ANOVAs with multivariate tests) were conducted. Two different options were considered:

1. all 7 measure times (t_0 , t_1 , t_2 , t_3 , t_5 , t_6 , t_{24}) plus all references and 3 galleries (without gallery 1 due to probable leak and gallery 3 due to missing measure times)
2. 3 measure times (t_0 , t_1 , t_2) plus all references and 4 galleries (without gallery 1)

The first ANOVA (within-subject factor 'time', between-subject factor 'treatment') revealed a significant effect of time (Wilks' Lambda: $F_{6,2} = 19.48$, $p = 0.05$) and a more significant effect of factor interaction (time * treatment; Wilks' Lambda: $F_{6,2} = 35.46$, $p = 0.028$) on the acetylene - argon ratios.

If regarding only 3 measure times, a second ANOVA revealed a strong significant effect of time (Wilks' Lambda: $F_{2,7} = 19.641$, $p = 0.001$) as well as a strong significant effect of factor interaction (Wilks' Lambda: $F_{2,7} = 17.81$, $p = 0.002$) on the ratios.

6. Discussion

6.1 Predominant microbial endosymbionts of *Xyleborinus saxesenii*

6.1.1 The bacterial genera *Sphingobacterium* and *Ochrobactrum*

454 sequencing revealed the endosymbiotic bacterial community of *Xyleborinus saxesenii* with *Sphingobacterium* (Bacteroidetes) and *Ochrobactrum* (Alphaproteobacteria) as the two predominant genera. These could also be isolated on culture medium from the beetle digestive tract.

Sphingobacteria usually are environmental bacteria (YABUUCHI et al., 1983; SHIVAJI et al., 1992; LIU et al., 2008); but they could also be isolated from human clinical specimens (HOLMES et al., 1982). However, *Sphingobacterium* is also known as a gut symbiont of insects, e.g. Hymenoptera (JAFFE et al., 2007; MATALON et al., 2007), Diptera (LINDH, 2007; GEIGER et al., 2011) and beetles (Coleoptera). A xylanase gene was described from *Sphingobacterium* sp., isolated from the gut of wood – inhabiting larvae of the long - horned beetle *Batocera horsfieldi* (Cerambycidae; ZHOU et al., 2009). Xylanases are digestive-enzymes capable of breaking down hemicelluloses and could previously be detected in larvae of *X. saxesenii* as well (DE FINE LICHT & BIEDERMANN, in press). However, the functional role of Sphingobacteria symbionts has not been further investigated yet. As gut symbionts they could be involved in nutrient provision. This genus is also known to feature high concentrations of sphingophospholipids (YANO et al., 1982, 1983; YABUUCHI et al., 1983). In one species, a mannose - possessing sphingophospholipid was described (NAKA et al., 2003). The sugar monomer mannose is toxic for some insects, especially for bees (Apidae) and the common wasp *Vespa vulgaris* (FRISCH, 1934; STAUDENMAYER, 1939; SOLS et al., 1960; DE LA FUENTE, 1986). Future analyses would need to identify *Sphingobacterium* on the species level and to investigate a potential production of this kind of sphingolipids.

The second bacterial endosymbiont candidate, *Ochrobactrum*, usually occurs in diverse habitats including soil, plants and their rhizosphere, (waste) water, animals and humans (BATHE et al., 2006). Some species are of medical relevance (JELVEH & CUNHA, 1999; MOLLER et al., 1999). However, this genus could also be detected in insects. In Diptera (VOLF et al., 2002), it may contribute to larval development (AHMAD et al., 2006) and in herbivore larvae of some Lepidoptera, *Ochrobactrum* sp. is known, amongst other bacteria, to be involved in the biosynthesis of *N*-Acylamino acids (SPITELLER et al., 2000), which could be used by the insects as biosurfactants for digestion (COLLATZ & MOMMSEN, 1974). SCHÄFER et

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al. (1996) showed *Ochrobactrum* sp. being a symbiotic bacterium of the termite gut and being involved in the degradation of hemicellulose.

As both bacterial genera, *Sphingobacterium* and *Ochrobactrum*, showed a clear infection within the whole beetle organism (100 % infection rate), they are supposed as endosymbionts of adult females of *Xyleborinus saxesenii*. However, for confirmation, more individuals would have to be tested and the localization within the beetle host should be further unveiled by a more elaborate FISH method. Weak signals of PCR products of beetle gut DNA, amplified with specific primers, indicated the presence of both genera in the digestive tract of *X. saxesenii*. Here, they could be accordingly identified as *Sphingobacterium siyangense* and *Ochrobactrum* sp. As *S. siyangense* has been originally isolated and described from farm soil (LIU et al., 2008) and *Ochrobactrum* also comprises species detected in the environment, both genera may just represent environmental bacteria, taken up orally by the beetle in the gallery.

6.1.2 Further bacterial endosymbionts, including gut symbionts

The bacterial members of the beetle gut community which could be cultivated on different agar media and revealed by direct sequencing of bacterial 16S rRNA were identified as *Pseudomonas (putida)*, *Bacillus (simplex)*, *Enterococcus (mundtii)* and *Stenotrophomonas (maltophilia)*. The latter genus could also be detected by 454 sequencing as well as the common insect - symbiont *Pantoea*.

In general, all those genera and species are known to be associated with insects and especially bark beetles. However, some are more common symbionts than others. Pseudomonads are commonly associated with insects and often appear to be useful and beneficial. In *Tetraponera* ants, they may be involved in nitrogen cycling (VAN BORM et al., 2002) as in general, this genus possesses the ability of nitrogen fixation (GHIGLIONE et al., 2000). In some staphylinid beetles, the bacterium is known to produce pederin (KELLNER, 2002; PIEL, 2002; PIEL et al., 2004), a compound with potent antitumor and antiviral activities (NARQUIZIAN & KOCIENSKI, 2000). In this study, *Pseudomonas putida* could be identified in the gut. Usually, it can be found in soils, waters and plants (YILMAZ et al., 2006; MURATOGLU et al., 2011; SILBY et al., 2011). It is further known to be applicable to bioremediation (NELSON et al., 2002; WEINEL et al., 2002). However, this species also appeared to infect insects, including beetles (STEINHAUS, 1963; BUCHER, 1981; SEZEN & DEMIRBAG, 1999; YAMAN et al., 1999; SCHNEIDER & DORN, 2001; MURATOGLU et al., 2011), like the spruce bark beetle *Dendroctonus micans* (YILMAZ et al., 2006). The genus *Dendroctonus* is a common host for

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harboring a number of endosymbiotic bacteria, including *Pseudomonas*. This bacterium is mostly accompanied by *Bacillus* spp. (CARDOZA et al., 2009; MORALES-JIMENÉZ et al., 2009; ADAMS et al., 2010), including *B. simplex* (ADAMS et al. 2011b), which could be identified in this study. Those two genera are considered to belong to the most frequently reported bacteria associated with *Dendroctonus* (ADAMS et al., 2011b) with *Bacillus* generally being the most commonly found bacterial genus in insect guts (ERTÜRK & DEMIRBAG, 2002; WENZEL et al., 2002). One species each (*P. viridiflava*; *B. pumilus*) is capable to produce antifungal metabolites to inhibit antagonistic fungi (MARTIN, 1987; MOEIN & RUST, 1992; BOTTONE & PELUSO, 2003). For example, *B. pumilus* can be found in oral secretions of *Dendroctonus rufipennis* to hinder harmful fungi from invading their galleries (CARDOZA et al. 2006). However, the host range of both genera is not limited. *Bacillus* bacteria, especially sporeforming species, are known to live in a symbiotic relationship with a number of insects (NICHOLSON, 2008) potentially playing a nutritional role, as it could be shown in cockroaches (FEINBERG et al, 1999). The bacterial taxa *Stenotrophomonas* and *Pantoea* generally are widespread in (the guts of) insects, especially in wood - boring beetles (DELALIBERA et al., 2005, 2007). The type species of the genus *Stenotrophomonas*, *S. maltophilia*, is known as a human pathogen causing serious infections (DENTON & KERR, 1998; GILLIGAN et al., 2003). Previously, it was called *Pseudomonas maltophilia* and *Xanthomonas maltophilia* (PALLERONI & BRADBURY, 1993). It is ubiquitous in aqueous environments, soil and rhizosphere of crop plants (DEBETTE & BLONDEAU, 1980; LAMBERT & JOOS, 1989; BERG et al., 1994, 1999; HEUER & SMALLA, 1999). It also appears to play a biotechnological role as it was applicable as a biological control agent of fungal plant pathogens in agriculture (ELAD et al., 1987; LAMBERT et al., 1987; BERG et al., 1996) and bioremediation (NAWAZ et al., 1993; BINKS et al., 1995; WANG et al., 1997). In insects, *S. maltophilia* could show an inhibitory effect against the primary bacterial pathogen of honey bees, *Paenibacillus larvae* (EVANS & ARMSTRONG, 2006). Furthermore, it could be detected in a *Culex* mosquito (PIDIYAR et al., 2004), the pea aphid *Acyrtosiphon pisum* (ATEYYAT 2008) and in the linden borer *Saperda vestita* (DELALIBERA et al., 2005). Members of the genus *Pantoea* are associated with plants and some are known as phytopathogens (CHA et al., 1997; HAUBEN et al., 1998). Some *Pantoea* species could also be found in the gut of plant-feeding insects as common bacterial components of the microbiota. They are present in the diverse insect orders Lepidoptera (BRODERICK et al., 2004) and Orthoptera (DILLON & CHARNLEY, 1995). *Pantoea* bacteria are also known to be associated with insects feeding on nitrogen - poor food sources like termites, wood wasps and bark beetles, where they provide an important source of diet as they are

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capable of cellulose degradation and nitrogen fixation (BENEMANN, 1973; BRIDGES, 1981; BRIDGES et al., 1984; OHKUMA et al., 1999; VASANTHAKUMAR et al., 2006; MORALES-JIMENÉZ et al., 2009; ADAMS et al., 2011a). Also females of the phytophagous ambrosia beetle *Anisandrus dispar* showed bacterial association, including *Pantoea* spp. (BUCINI et al., 2005). This genus was not part of the gut community of *X. saxesenii*, but so was *Enterococcus mundtii*, a further representative of the Firmicutes class, besides *Bacillus*. This and other species usually are primary associated with plants (DEVRIESE et al., 1993). However, further members of the *Enterococcus* genus have also been reported in the gut of wood termites (THOLEN et al., 1997) and wood - boring beetles (SCHLOSS et al., 2006; VASANTHAKUMAR et al., 2006).

The bacterial genus *Gordonia* (Actinobacteria), which could be identified in *X. saxesenii* by BIEDERMANN (2007), was not frequently detected in this study, as there was only one congruent OTU hit in the 454 sequencing results.

6.1.3 Endosymbiotic *Candida*

Beside a wide range of bacteria being part of the microbial community in *X. saxesenii*, fungal endosymbionts could be identified as well by sequencing clones containing fungal 28S rRNA. The yeast genus *Candida* was unveiled as the predominant endosymbiotic fungus; it could also be detected in other ambrosia beetles (VAN DER WALT, 1972; HAANSTAD & NORRIS, 1985; KURTZMANN & ROBNETT, 1998; KURTZMANN, 2000; ENDOH et al., 2008, SUH & ZHOU, 2010). *Candida* and other genera are associated with beetles, like wood - ingesting passalids, showing the ability to ferment and assimilate xylose, a major component of hemicelluloses (MARTIN, 1987; NARDON & GRENIER, 1989; SUH et al, 2003). Yeasts, including *Candida*, are also commonly isolated from bark beetles (GANTER, 2006; ADAMS et al., 2008), where they show the ability to degrade saccharides like trehalose and pectin (LEFAY et al., 1969) and to modify pheromones (LEUFVÉN et al., 1984). Furthermore, anobiid beetles are able to live in cigarette packs because even the tobacco is detoxified by yeast - like fungi (DOWD 1989, 1991). In ambrosia beetles, associated yeasts can be found mostly in addition to the (primary) ambrosia fungi and could be isolated from adult (female) beetles, their mycangia and galleries (BAKER, 1963; BATRA, 1967; VAN DER WALT, 1972; HAANSTAD & NORRIS, 1985; BEAVER, 1989; KINUURA, 1995; GANTER, 2006). *Candida* spp. and other yeasts have been classified as auxiliary fungi which are likely ingested secondarily by the ambrosia beetles, thus appearing

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also in the gut (BAKER, 1963; BATRA, 1966). The functional roles of yeasts as part of the microbial complex associated with ambrosia beetles have not been clarified until now.

Genetic analysis of the partial 26S (28S) rRNA gene sequences of endosymbiotic fungi revealed that *X. saxesenii* harbors *Candida* yeasts grouping together in a monophyletic clade (support of 90 % Bayesian posterior probability) within the ascomycetous order Saccharomycetales. Among *Candida* clones, 26S rRNA gene sequence similarity was relatively high (96 – 99 %) with their closest relatives, *C. trypodendroni* and *C. insectorum*, which are both known to be associated with (ambrosia) beetles (VAN DER WALT, 1972; KURTZMANN & ROBNETT, 1998). Although they are not clearly separated from other species, their high relatedness could indicate a distinctive clade within the Saccharomycetales. Thus, the *Candida* clones could represent a new species, showing a specific association to *X. saxesenii*, as the genus *Xyleborinus* is monophyletic as well, easily defined by their conical scutellum (COGNATO et al., 2011).

However, to unveil the specificity of the *Candida* – *X. saxesenii* association and to test a potential co-evolution of both organisms, elaborate comparative phylogenetic analyses should be investigated in the future.

6.1.4 Functional roles of endosymbionts

Although there are many studies on (gut) microbiota of insects, few of them are on bark and ambrosia beetles (JOUVENAZ & WILKINSON, 1970; MOORE, 1972; TANADA & KAYA, 1993, VASANTHAKUMAR et al., 2006). Insects with restricted diets of limited nutrition, like bark beetles and other wood boring insects, rely on their gut symbionts. Bacteria and fungi help in nitrogen processing, sulfate assimilation, fatty acid metabolism and help to contribute deficient sterols, vitamins, digestive enzymes and essential amino acids to their insect host (DOWD, 1992; LUNDGREN & LEHMAN, 2010). Like most xylomycetophagous ambrosia beetles *X. saxesenii* feeds on a mixture of wood inhabiting fungi introduced into the gallery system by the beetle and the sapwood tissue of the penetrated host tree (BEAVER, 1989; ROEPER, 1995). The saprophagous fungal symbiont degrades cellulose and lignin and extracts nutrients from surrounding tissues, providing a (nitrogen) rich diet for the beetles, which is crucial for beetle development and physiology (FRENCH & ROEPER, 1973; KOK, 1979; NORRIS, 1979; BEAVER, 1989). Furthermore, fungi may also be involved in the conversion of host tree chemicals into beetle pheromones (BEAVER, 1989).

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I claim the (gut) microbiota detected in *X. saxesenii* plays a supportive role in nutrient processing / providing and may produce bioactive compounds, as there are many members of Proteobacteria and Firmicutes. Those bacterial phyla are known, besides Actinobacteria, to be crucial for the defense of their insect host organisms by antagonizing enemies, like microbes, viruses and parasitoid insects (KELLNER, 2002; OLIVER et al., 2003; FERRARI et al., 2004; MORAN et al., 2005; SCARBOROUGH et al., 2005; CARDOZA et al., 2006; MAHADAV et al., 2008; TEXEIRA et al., 2008). As the defensive mechanisms used by supportive Actinobacteria often are characterized by production of antifungal or antibacterial compounds (CURRIE et al., 1999; KALTENPOTH et al., 2005; HAEDER et al., 2009; OH et al., 2009a,b), they remain poorly understood for Proteobacteria and Firmicutes. Besides Gammaproteobacteria producing specific toxins (KELLNER, 2002; PIEL, 2002; OLIVER et al., 2003; MORAN et al., 2005), there are Proteobacteria known to stimulate the growth and reproduction of beetle symbiotic fungi in the presence of a chemical tree host compound (ADAMS et al., 2009). In future studies, the focus should be more on localizing the endosymbiotic microbes, e.g. with molecular techniques like FISH or by scanning electron microscopy (SEM), and further on exploring the potential role of cultivated symbionts in mediating the *X. saxesenii* – ambrosia fungus mutualism by using symbiont pairing bioassays and chemical analyses. These methods would be more achievable than creating symbiont - free beetles and galleries, e.g. by treatment with antibiotics, as the ambrosia beetle – fungus mutualistic system is very complex.

As only cultivatable gut symbionts could be identified in this study, the two predominant genera *Sphingobacterium* and *Ochrobactrum* included, those microbes are probably not obligate. However, this should not diminish their relevance. Furthermore, some species which could be detected in the gut of *X. saxesenii* were not part of the entire bacterial community and vice versa; but here it is because most endosymbiotic microorganisms cannot be cultivated. For a more precise assessment of the gut microbiota, next - generation sequencing of the gut DNA is recommended.

The endosymbiotic microbiota might be transient and the composition may change continuously dependent on the developmental stage of the insect, the associated ambrosial complex within the gallery, and maybe the invaded tree. However, there might always be predominantly occurring organisms indicating a stable infection as that could be the case for the endosymbiotic *Candida* species. To investigate potential differences of the composition of the endosymbiotic microbiota and possibly concomitant effects on their host individuals, it would be necessary to examine beetle males and larvae of one species additionally. Studies on

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larvae could also unveil questions of acquirement and transmission of the endosymbionts as well as the stability of the symbiont affection during insect development (metamorphosis).

Nevertheless, some genera may represent environmental contaminants and live as commensals, relying on nutrients digested by host or fungal enzymes.

Furthermore, it would be interesting to screen the ambrosia fungus for bacterial endosymbionts. As already mentioned fungi are capable of many degrading and detoxifying mechanisms because of specific enzymes. However, endofungal bacteria could also be involved as they could contribute to those chemical pathways by providing their enzymes. Thus, they could be the solely responsible metabolic force initiating the processing of the beetles` nutritional basis.

Determining the endosymbiotic microbial community of ambrosia or bark beetles or general of potential invasive insects can allow the development of new approaches for biological control (LI et al., 2005). A wide range of invasive symbioses between wood – boring insects and fungi is shifting to prolific tree – killing and emerges as a new and currently uncontrollable threat for forest ecosystems, as well as fruit and timber industries worldwide (HULCR & DUNN, 2011).

6.1.5 Localization of endosymbionts

The bacterial (*Sphingobacterium* and *Ochrobactrum*) and fungal endosymbionts (*Candida*) are present in the whole beetle organism and could be assessed by PCRs with specific primers. However, they could also be detected by FISH with specific oligonucleotide probes in the beetle gut. Positive controls showed the bacterial organisms of both genera as short rods. Not all cells hybridized with the probes and could only be seen stained with DAPI. Some clustered in groups and appeared bigger and not rod - shaped. Alternatively, a contamination cannot be excluded and cells may represent another microbial organism. Unfortunately, it was not possible to detect *Sphingobacterium* and / or *Ochrobactrum* cells in the gut. Positive signals could be detected on the gut sections, but those were rather due to contamination / pollution and represented only artifacts. The difficulty of interpreting the fluorescent signals does not equal an absence of the bacterial endosymbionts, but as there were bacteria - specific bands visible (although weak) after PCR on gut DNA, they should be present in the digestive tract. It might be possible, that they appear in a particular gut part, which was not tested; generally, it was not possible to specify different gut sectors. However, the bacterial probes should be further checked for their specificity and maybe newly designed

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as well as FISH should be conducted on whole beetle sections (longitudinal) and on hemolymph. A general problem was the autofluorescence emitted from the sections, which should be tried to reduce.

Candida cells appeared as ovoid ‘yeast’ cells when grown in liquid malt extract. In the gut sections, they showed their di – or polymorphic character as mostly hyphal growth forms were visible in the gut lumen. Additionally, further big ovoid structures could be detected, showing autofluorescence. They may represent spores, like blastospores, chlamydospores, ascospores or conidia of fungi, which are part of the ambrosia complex *X. saxesenii* feeds on. In some cases, spore – like structures seemed to possess two nuclei which could be due to germination. For example, germinated conidia of *Ophiostoma*, which could also be detected within the beetle gut, have more than one nucleus if they are septated (HOFSTEN, 1959). However, nothing is known about germinating fungal spores within beetle guts. Furthermore, unknown bacteria are indicated in the adjacent gut epithelial cells.

To confirm the *Candida* specificity of FISH, it would be necessary to design oligonucleotide probes, which are exclusively specific to the *Candida* genus, as in the present study only an ‘all yeast probe’ was used. In combination, a general fungal probe should be used.

6.2 The isolated ambrosia fungus and other associated microorganisms of *X. saxesenii*

6.2.1 *Ambrosiella xylebori* as an auxiliary ambrosia fungus

The ambrosia fungus which was isolated from the *X. saxesenii* gallery and cultivated on malt agar plates could not be clearly identified by direct sequencing. Partial 28S rRNA sequences equaled those of *Ambrosiella xylebori*, as well as *Ceratocystis adiposa*, both showing 99 % similarity. *X. saxesenii* usually is associated with its primary ambrosia fungus *Ambrosiella sulfurea* (or *sulphurea*), which is usually transmitted in the form of a micromycelium in the beetle hind gut (FRANCKE-GROSMANN, 1975). *A. sulfurea* was first described by BATRA (1967) as he could isolate this fungus from mycangia, tunnels and pupal cells of *X. saxesenii*. This fungus was detected as the predominant ambrosia fungus of this beetle in every corresponding study until now. However, BATRA (1963) pointed out, that the fungus associated with *X. saxesenii* in North America is different from the one associated with this beetle in Europe. *A. xylebori* usually is associated with *Xylosandrus compactus*, *X. crassiusculus* and *Corthylus columbianus* (BRADER, 1964; VON ARX & HENNEBERT, 1965; BATRA, 1967) and was never found associated with *X. saxesenii* until now. Those three host species predominantly appear in North America (WOOD, 1982; WOOD & BRIGHT, 1992).

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The genetic analysis of the present study did not allow a clear and distinct identification of the ambrosia fungus because the DNA sequences of *A. xylebori* are close to those of *Ceratocystis adiposa* (PAULIN-MAHARDY et al., 2002; HARRINGTON, 2009; MASSOUMI ALAMOUTI et al., 2009; SIX et al. 2009). Species of *Ambrosiella* associated with ambrosia beetles belong to the Ophiostomatales order and are anamorphic (BATRA, 1967; GEBHARDT et al., 2005; HARRINGTON et al., 2010), meaning there are no sexual reproductive stages, *Ceratocystis* is a teleomorphic genus (there are mostly no asexual stages) and appears to be placed with members of the Microascales (ALEXOPOULOS et al., 1996; MASSOUMI ALAMOUTI, 2009; HARRINGTON et al., 2010). However, the type species *A. xylebori* is phylogenetically placed within the Microascales as well (PAULIN-MAHARDY et al., 2002; GEBHARDT et al., 2005). But as *Ceratocystis* species have less specific relationships with their beetle hosts than do members of Ophiostomatales, which are always associated with Scolytid bark beetles (KIRISITS, 2004; MASSOUMI ALAMOUTI et al., 2009), the ambrosia fungus of *X. saxesenii* in this study and in the sense of having been isolated from a lab gallery, is designated as *A. xylebori*.

Generally, it was considered only one species specific fungus, the primary ambrosia fungus, being tightly associated with a particular ambrosia beetle species (FRANCKE-GROSMANN, 1956, 1967; BATRA, 1963, 1966; BEAVER, 1989). However, the occurrence of *A. xylebori* in association with *X. saxesenii* is the first one detected and could challenge this hypothesis. GEBHARDT and colleagues (2004) had similar findings and BATRA (1966) also queried this hypothesis after detecting (young adults of) ambrosia beetles feeding on non-specific ambrosia fungi or non – ambrosia fungi, calling them secondary or auxiliary (ambrosia) fungi or secondary microflora. The most common examples of auxiliary ambrosia fungi are *Ceratocystis* spp. (BATRA, 1985). In this study, *A. xylebori* is considered to be a (transitory) secondary ambrosia fungus of *X. saxesenii* or to be part of the ambrosia complex or community associated with the beetle, which, according to NORRIS (1965, 1979) and HAANSTAD & NORRIS (1985), can be seen as a multi – species complex or a supraspecies, additionally including bacteria. However, this observation has to be confirmed in future studies. Furthermore, the detection of a second, non identified ambrosia fungus in older *X. saxesenii* galleries after hibernation by FRANCKE-GROSMANN (1975) possibly suggests *A. xylebori* as well. The standard yellowish color of the fungus and its darkening into a ‘dark olive’ (BATRA, 1967) on the agar plate after a while could also be indicative for this species. However, the color could also refer to the primary ambrosia fungus of *X. saxesenii*, which is *A. sulfurea*. It was quite difficult to isolate DNA in an evaluable concentration from the

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fungus, but the sequence was applicable for analysis and did not lead to any hit for *A. sulfurea*. For future studies, it would be important to solitary isolate and cultivate this ambrosia fungus with regard to investigations testing the reciprocal effects of both involved organisms.

A true species specific and mutualistic ambrosia fungus cannot naturally survive outside the mycangia or tunnel systems and cannot be transmitted without the symbiotic beetle (BATRA, 1966; GEBHARDT et al., 2004). The problem of isolation and identification of the primary ambrosia fungus is also based on the fact that generally, auxiliary fungi are easy to culture and thus, some have been mistaken for primary ambrosia fungi (BATRA, 1979, 1985). Furthermore, most primary fungi usually show, if successfully cultured on laboratory agar, mycelial growth without sporulation and formation of an ambrosial morphology (BATRA, 1963). Typically, ambrosia fungi are dimorphic: they grow as the ambrosial (yeast - like) form or as mycelium (BATRA, 1963; NORRIS, 1979; BEAVER, 1989; SIX, 2003). Ambrosial cells, under appropriate conditions, give rise to vegetative mycelium and vice versa. This phenomenon is referred to as pleomorphism (BATRA, 1985). However, the ambrosial growth form has to be controlled and can be induced by the beetles (FRANCKE-GROSMANN 1956, 1967; WHITNEY, 1971; FRENCH & ROEPER, 1972).

6.2.2 Associated yeasts

As long ago as 1950, PEKLO & ŠATAVA could induce ambrosial growth of the cultivated fungus of *Xyleborus dispar* with pure cultures of a nitrogen fixing yeast of the genus *Torula* (*Torulopsis*). Although several genera of yeasts (e.g. *Candida* and *Pichia*) show mutualistic associations with bark and ambrosia beetles (CALLAHAM & SHIFRINE, 1960; BAKER, 1963; FRANCKE-GROSMANN, 1967; WHITNEY, 1971, 1982; HAANSTAD & NORRIS, 1985), relatively little is known of their effects on the beetle host. In all developmental stages, associations with yeasts, which are commonly carried in the mycangia and in pits of the exoskeleton of adult Scolytid beetles (WHITNEY & FARRIS, 1970; WHITNEY & COBB, 1972; FURNISS et al., 1990, 1995; LEWINSOHN et al., 1994), could be demonstrated. These fungi densely colonize the walls of beetle galleries and pupal chambers as well. Yeasts might affect the insect directly by supplying nutrients (such as vitamins) or by degrading potentially toxic host tree compounds. They may also produce behavioral chemicals (CALLAHAM & SHIFRINE, 1960; BRAND et al., 1976, 1977; BRIDGES et al., 1984). In this study, growth of a yeast fungus could be detected, after the beetle was placed on a malt agar plate. It could be identified as *Candida*

with a sequence very similar to the sequences of the endosymbiotic *Candida* species. Thus, this yeast genus is considered to be both ecto – and endosymbiotically associated with *X. saxesenii*. The potential ability of ambrosia beetle associated yeasts to induce and stimulate the growth of principal ambrosia fungi (WEBB, 1945; PEKLO & ŠATAVA, 1950; BAKER & KREGER-VAN RIJ, 1964) should be approached in future studies.

6.2.3 Other associated fungi and mites

In this study, it has been shown, that *X. saxesenii* does not feed only on one fungus / nutritional source. The occurrence of *A. xylebori* and the ectosymbiotic association with *Candida*, which might originate from the nutritional ambrosia complex in the gallery, may confirm this hypothesis. Furthermore, *Candida* could also be detected in the gut as well as some various unspecific fungal gut symbionts and two fungi known to be wood – inhabiting (*Ophiostoma stenoceras*; *Petriella sordida*) (ROBAK, 1932; DAVIDSON, 1966; ABBOTT, 2002; AGHAYEVA et al., 2004). Thus, some of those fungi could occur as digested food residues and some may just represent environmental contaminants of the wood galleries. Especially the appearance of the genera *Ophiostoma* (Ophiostomatales), which is closely related to *Ambrosiella* (MASSOUMI ALAMOUTI et al., 2009), and *Petriella* (Microascales), is not accidental, as their spores are dispersed by insects in general (WINGFIELD et al., 1993; ABBOTT, 2000) with bark beetles being well known vectors of fungi (SIX, 2003; KIRISITS, 2004, ZHOU et al., 2006). In the same context, the presence of mites is not surprising either. Unidentified mite individuals could be detected in the galleries as well as on ambrosia fungus agar plates after bioassays and on plates containing gut suspension. Cleaning treatment of the beetles did not hinder their appearance, thus, they were inconspicuous, but omnipresent concomitants. They could be observed feeding on the ambrosia fungus (*A. xylebori*) as well on other fungi (*Aspergillus*). Generally, mites are associated with some bark beetles and have been observed transporting, disseminating, and feeding on (ophastomatoid) fungal species (MOSER et al., 2005). Those fungivorous mites often live in are phoretic relationship with the beetles and can be found all over the insect's body without being injurious to the beetle (MOSER & ROTON, 1971; CARDOZA et al., 2008). In this study, mites could not be detected on the beetle's body, however. The appearance on gut solution might be indicative of the beetle (accidentally) feeding on mite eggs and / or larvae while browsing the fungal layers or grooming other members of the gallery. *X. saxesenii* has not been observed to be associated with mites until now.

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Besides the discussed ambrosia fungus and yeasts, some other fungi could be found associated with *X. saxesenii* and they could be identified as *Aspergillus flavus* or *A. oryzae* and a *Penicillium* species. The growth was observed after individual beetles were placed on malt agar plates, containing neither antibiotics nor antimycotics. Both genera have been detected and isolated in other ambrosia beetles (BATRA, 1963; MACLEAN & GIESE, 1968, WHITNEY, 1982; KIRSCHNER, 2001; HENRIQUES et al., 2006; JANKOWIAK & BILAŃSKI, 2007; JANKOWIAK & ROSSA, 2008; QI et al., 2011). The regular browsing of the ambrosial layer in the gallery performed by the beetles and their larvae usually inhibits the invasion of foreign fungi (BROWNE, 1961; BATRA, 1966). However, if the gardening insects are removed or if the tunnels and galleries are abandoned, the ‘ambrosial garden’ as well as the beetles themselves are quickly overrun by invading fungi (FRANCKE-GROSMANN, 1967; BEAVER, 1989), including *Aspergillus* and *Penicillium*. Those fungi, often regarded as mold or ‘weed’ fungi, normally coexist at low levels along with the crop and maybe used as a supplementary food source; thus, some of them share no more than a commensal relationship with the beetles (LEACH et al., 1940; BATRA & BATRA, 1967; MACLEAN & GIESE, 1968; BATRA, 1979; NORRIS, 1979; BEAVER, 1989; QUI et al., 2011). Nevertheless, if the dominance of the primary ambrosia fungus in the galleries is lost to the mold fungi, they occur as a real threat as some species especially of *Aspergillus* and *Penicillium* show entomopathogenic abilities. Their invading and infesting character could be observed and confirmed as the topside of some lab galleries were covered with a mold fungus layer and single *X. saxesenii* individuals were overgrown and killed mostly by *Aspergillus*, when they were on a malt agar plate for a longer period.

In this study, both genera are supposed as ectosymbionts, as they (and their spores, respectively) probably are attached to the beetle surface and carried around. Further findings in bioassays suggest a positive effect of *Aspergillus* (see 4.3.1). Furthermore, *Penicillium* sp. also appeared as a gut symbiont, emerging on malt agar plates containing gut suspension. This indicates a feeding of the beetle on this fungus. However, an active ingestion could not be observed.

Future investigations could focus on the composition of whole gallery system associated microbiota in comparison to the (endosymbiotic) beetle microbiota.

6.3 In - vitro experiments and analysis of potential secretions

6.3.1 Bioassays

Browsing and feeding on the ambrosial layer as well as the physical contact seems to enhance and induce the growth of the ambrosia fungus. In *in - vitro* experiments, BIEDERMANN (unpublished) could observe a significant induction of ambrosial growth of the mutualistic fungus of *Xylosandrus germanus* by adult females, pupae and larvae of *X. germanus* and *Anisandrus sayi*. The supply of nutrients, probably mainly excretes of the beetles and larvae, as well as secretions and physical contact is beneficial for the growing fungal layer (WHITNEY, 1971; FRENCH & ROEPER, 1972b; BIEDERMANN, 2007).

To investigate an effect of *Xyleborinus saxesenii* on its mutualistic fungus, similar bioassays were conducted. As already discussed, *Ambrosiella xylebori* was isolated from galleries and reared in pure agar culture, representing not the actual and true primary ambrosia fungus of this beetle. Being aware of that, a significant effect in the bioassays is not necessarily expected; and de facto, there was no effect of *X. saxesenii* on the fungal growth, as the experiments have shown with the control fields showing a stronger growth. This was more due to the selected placement of the hoods (pipette tip pieces), as the agar was not overgrown uniformly with ambrosia layer. However, there was an effect, when mold fungi like *Aspergillus* emerged. This was only the case, if the beetles' surface, where those fungi (spores) are supposed to be attached. Most often, the ambrosia fungal growth was enhanced and new white fungal mycelia appeared and covered great parts of the malt agar plate. Growth enhancement or stimulation means a denser mycelium compared to a control fungus field. The growth induction could be observed near *Aspergillus*, indicating a positive effect of the mold fungus. Actually, it was not checked microscopically, if the ambrosia fungus actually showed an 'ambrosial' growth form (erect palisades of tightly packed conidiophores). The white color of new induced hyphae of *Ambrosiella xylebori* seems to be in accordance with the colony description by BATRA (1967). As the *Aspergillus* fungus could probably be the carcinogenic aflatoxin producing species *A. flavus*, which is classified as 'risk - group - 2 (S2) - organism', further investigations including creating pure cultures were not possible / allowed.

Why might *Aspergillus* affect the growth of an ambrosia fungus as it naturally occurs as an invading enemy of both the beetle and the mutualistic fungus? In the galleries, ambrosia fungi usually show an enhanced growth only due to regular browsing by their mutualistic beetle. Nothing is known in particular, about their behavior, when infected by invading fungi. It is

possible, that the presence of a microbial antagonist boosts their growth as well. Furthermore, fungal exoenzymes could also be involved in producing favorable, non toxic compounds.

6.3.2 Secretions and extracts

The mycangia of the ambrosia beetles, highly evolved and specialized structures (invaginations of the exoskeleton) for housing and transmission of ambrosia fungus spores, are lined with secretory gland cells (BATRA, 1963; SCHNEIDER & RUDINSKY, 1969a; SIX, 2003). Those sequester secretions, which are thought to serve to protect mycangial spores from desiccation, provide nourishment for proliferating fungal propagules and determine the fungal growth in the mycangia (BATRA, 1963; ABRAHAMSON et al., 1967, FRANCKE-GROSMANN, 1967; SCHNEIDER & RUDINSKY, 1969a,b; HAPP et al., 1971; BATRA, 1985; SIX, 2003). However, the origin of secretions of both adult and larval beetles, responsible for the formation of pure ambrosial mats in a (new) gallery (FRANCKE-GROSMANN, 1956, 1958, 1975; FRENCH & ROEPER, 1972a) may be different. Furthermore, the inhibition of contaminating and invading fungi may also be attained through the evolvment of beetle secretions (HAPP et al., 1971., SCHNEIDER, 1976; CARDOZA et al., 2006; SCOTT et al., 2008). As secretions by themselves could not be observed and were not available, beetle methanol extracts were investigated and applied to antimicrobial and growth stimulation tests (agar diffusion tests). The extracts are supposed to contain exocrine and endocrine secretions of the beetle with potential bioactive features. However, none of the antimicrobial tests against probable harmful microorganisms showed any effect of the extracts, nor did the growth enhancement test with the ambrosia fungus *A. xylebori*. However, adult beetles of *X. saxesenii* as well as their larvae could be observed to excrete liquid substances over the gut and the mouth (BIEDERMANN, 2007). Thus, further investigations should focus on beetle and fungal secretions and / or excretions, as they might contain antibiotic compounds, which may be produced and provided by bacterial endosymbionts, as well as growth stimulating substances.

6.4 Test for nitrogen fixation

Some bark beetles, but apparently not ambrosia beetles, are associated with bacterial symbionts, which are able to fix atmospheric nitrogen (N₂) (BRIDGES, 1981). Results from preliminary work of the author (not shown) indicated the genus *Burkholderia* (Betaproteobacteria) associated with *X. saxesenii*. Those bacteria mainly appear in the soil

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and rhizosphere but some species are plant and animal pathogens or symbionts (ESTRADA-DE LOS SANTOS et al., 2001). Interestingly, there are also some diazotrophic species, which have been found in natural endophytic association, e.g. with grasses (MATTOS et al., 2005), and even insect-*Burkholderia* associations (KIKUCHI et al., 2005, 2007, 2010; MARTINSON et al., 2011). However, in this present study, screenings with specific PCR assays as well as the 454 sequencing results did not confirm the presence of this bacteria genus. Nitrogen fixation in insects, especially in their guts, has been demonstrated extensively in termites (NARDI et al., 2002) but nitrogen – fixing bacteria could also be identified in (the guts of) bark beetles (BRIDGES, 1981; BRIDGES et al., 1984; VASANTHAKUMAR et al., 2006; MORALES-JIMENÉZ et al., 2009). Nothing is known about fungi capable of N₂ - fixation, so far.

To test potential nitrogen fixing ability of *X. saxesenii* and their cultivated fungi in their galleries, an acetylene reduction assay (ARA) was conducted.

As ethylene could not be detected by the gas – chromatograph, the decline of acetylene (C₂H₂) was indicated by the acetylene / argon rations. No significance can be concluded from the assay. The progress of the assay probably indicates a decline of acetylene, with a significant effect of time. A slight increase within the first hours, followed by a continuous decrease can be observed in both the reference tubes and the beetle galleries. The empty reference tubes were expected to show no changes in the ratios. Instead, they showed higher rations in the beginning. A fast diffusion of argon gas might be responsible for the initial increase. Alternative inert gas carriers, like helium or carbon dioxide could be more suitable. Some gallery tubes could not be taken into account because they were leaky. Therefore, another sealing method and other tubes / vials, respectively, should be used for future analyses. This assay would have to be more concrete and comparable; according to MORALES-JIMENÉZ and colleagues (2009), ARA could be performed in single live adults (and larvae) of *X. saxesenii*, incubated in closed vessels containing acetylene – enriched atmosphere, and with autoclaved insects used as a negative control. It would be advantageous to directly detect ethylene to assess nitrogenase activity of single insects. The approach could be adapted to the gallery systems as well.

Alternatively, potential nitrogen – fixing bacteria (of the beetle gut) should be isolated in nitrogen – free liquid and solid medium.

7. References

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8. List of abbreviations and formulas

Ø	diameter
#	number
~	approximately
°C	degree Celsius
5 H ₂ O	pentahydrate
7 H ₂ O	heptahydrate
16S rRNA	16S ribosomal ribonucleic acid
ANOVA	analysis of variance
ARA	acetylene reduction assay
bp(s)	base pair(s)
C ₂ H ₂	acetylene
C ₂ H ₄	ethylene
ca.	circa
CaC ₂	calcium carbide
Ca(OH) ₂	calcium hydroxide
cm	centimeter
Cy3/Cy5	cyanine (fluorescent dye)
dH ₂ O	distilled water
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
EDTA	ethylenediamine tetraacetic acid
e.g.	abbreviation for the Latin phrase “ <i>exempli gratia</i> ”, meaning “for example”
et al.	abbreviation for the Latin phrase “ <i>et alii</i> ”, meaning “and others”
EtOH	ethanol
fem.	Female

List of abbreviations and formulas

FeSO ₄	iron(II) sulfate
FISH	fluorescence <i>in situ</i> hybridization
fwd.	forward
GC	gas chromatograph(y)
HCl	hydrogen chloride
KCl	potassium chloride
K ₂ HPO ₄	dipotassium phosphate
KH ₂ PO ₄	monopotassium phosphate
l	liter
LB	Luria Bertani
LSU	large subunit
MgCl ₂	magnesium chloride
MgSO ₄	magnesium sulfate
µg	microgram
µl	microliter
µM	micromolar
min	minute
ml	milliliter
mM	millimolar
MnCl ₂	manganese(II) chloride
MS	mass spectrometer/spectrometry
(NH ₄) ₂ SO ₄	ammonium sulfate
N ₂	nitrogen
NaCl	sodium chloride
PAUP	phylogenetic analysis using parsimony
PBS	phosphate buffered saline
PCR	polymerase chain reaction

List of abbreviations and formulas

pmol	picomole
®	registered trademark symbol
rev.	reverse
rpm	revolutions per minute
S	Svedberg (unit used for sedimentation coefficients)
s	second
SDS	sodium dodecyl sulfate
sp.	species
TE	tris-EDTA
™	unregistered trademark symbol
Tris	tris(hydroxymethyl)-amino-methane
U	unit(s)
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside
ZnSO ₄	zinc sulfate

Appendix

Appendices

<i>Sequences</i>	<i>%</i>	<i>log</i>	<i>Genus</i>	<i>Family</i>	<i>Phylum/Class</i>
4402	35,62353	1,551736992	<i>Sphingobacterium</i>	Sphingobacteriaceae	Bacteroidetes
3478	28,14599	1,449416531	<i>Ochrobactrum</i>	Brucellaceae	Alphaproteobacteria
1376	11,13539	1,046705387	<i>Stenotrophomonas</i>	Xanthomonadaceae	Gammaproteobacteria
1285	10,39896	1,016990081	<i>Pantoea</i>	Enterobacteriaceae	Gammaproteobacteria
461	3,730679	0,571787879	<i>Microbacterium</i>	Microbacteriaceae	Actinobacteria
438	3,54455	0,549561064	<i>Phyllobacterium</i>	Phyllobacteriaceae	Alphaproteobacteria
197	1,594238	0,202553179	<i>Chryseobacterium</i>	Flavobacteriaceae	Bacteroidetes
147	1,189609	0,075404288	<i>Pseudoxanthomonas</i>	Xanthomonadaceae	Gammaproteobacteria
83	0,671684	-0,172834954	<i>Rhizobium</i>	Rhizobiaceae	Alphaproteobacteria
81	0,655499	-0,183428028	<i>Bacillus</i>	Bacillaceae	Firmicutes
79	0,639314	-0,194285955	<i>Mycobacterium</i>	Mycobacteriaceae	Actinobacteria
75	0,606943	-0,216851783	<i>Devosia</i>	Hyphomicrobiaceae	Alphaproteobacteria
53	0,428907	-0,367637177	<i>Roseomonas</i>	Acetobacteraceae	Alphaproteobacteria
37	0,299425	-0,523711323	<i>Pseudomonas</i>	Pseudomonadaceae	Gammaproteobacteria
24	0,194222	-0,711701805	<i>Pedobacter</i>	Sphingobacteriaceae	Bacteroidetes
21	0,169944	-0,769693752	<i>Bradyrhizobium</i>	Bradyrhizobiaceae	Alphaproteobacteria
13	0,105204	-0,977969694	<i>Bosea</i>	Bradyrhizobiaceae	Alphaproteobacteria
11	0,089018	-1,050520362	<i>Curtobacterium</i>	Microbacteriaceae	Actinobacteria
11	0,089018	-1,050520362	<i>Novosphingobium</i>	Sphingomonadaceae	Alphaproteobacteria
10	0,080926	-1,091913047	<i>Defluviobacter</i>	Phyllobacteriaceae	Alphaproteobacteria
10	0,080926	-1,091913047	<i>Sphingomonas</i>	Sphingomonadaceae	Alphaproteobacteria
8	0,064741	-1,18882306	<i>Propionibacterium</i>	Propionibacteriaceae	Actinobacteria
7	0,056648	-1,246815007	<i>Actinomyces</i>	Actinomycetaceae	Actinobacteria
7	0,056648	-1,246815007	<i>Hansschlegelia</i>	Methylocystaceae	Alphaproteobacteria
6	0,048555	-1,313761796	<i>Legionella</i>	Legionellaceae	Gammaproteobacteria
5	0,040463	-1,392943042	<i>Pelomonas</i>	Comamonadaceae	Betaproteobacteria
5	0,040463	-1,392943042	<i>Hymenobacter</i>	Cytophagaceae	Bacteroidetes
5	0,040463	-1,392943042	<i>Methylobacterium</i>	Methylobacteriaceae	Alphaproteobacteria
3	0,024278	-1,614791792	<i>Dietzia</i>	Dietziaceae	Actinobacteria
3	0,024278	-1,614791792	<i>Massilia</i>	Oxalobacteraceae	Betaproteobacteria
2	0,016185	-1,790883051	<i>Escherichia</i>	Enterobacteriaceae	Gammaproteobacteria
2	0,016185	-1,790883051	<i>Peptoniphilus</i>	Incertae Sedis XI	Firmicutes
2	0,016185	-1,790883051	<i>Arthrobacter</i>	Micrococcaceae	Actinobacteria
2	0,016185	-1,790883051	<i>Acinetobacter</i>	Moraxellaceae	Gammaproteobacteria
2	0,016185	-1,790883051	<i>Streptococcus</i>	Streptococcaceae	Firmicutes
2	0,016185	-1,790883051	<i>Microcoleus</i>		Cyanobacteria
1	0,008093	-2,091913047	<i>Brevibacterium</i>	Brevibacteriaceae	Actinobacteria
1	0,008093	-2,091913047	<i>Gordonia</i>	Gordoniaceae	Actinobacteria
1	0,008093	-2,091913047	<i>Pseudonocardia</i>	Pseudonocardiaceae	Actinobacteria
1	0,008093	-2,091913047	<i>Wolbachia</i>	Rickettsiaceae	Alphaproteobacteria

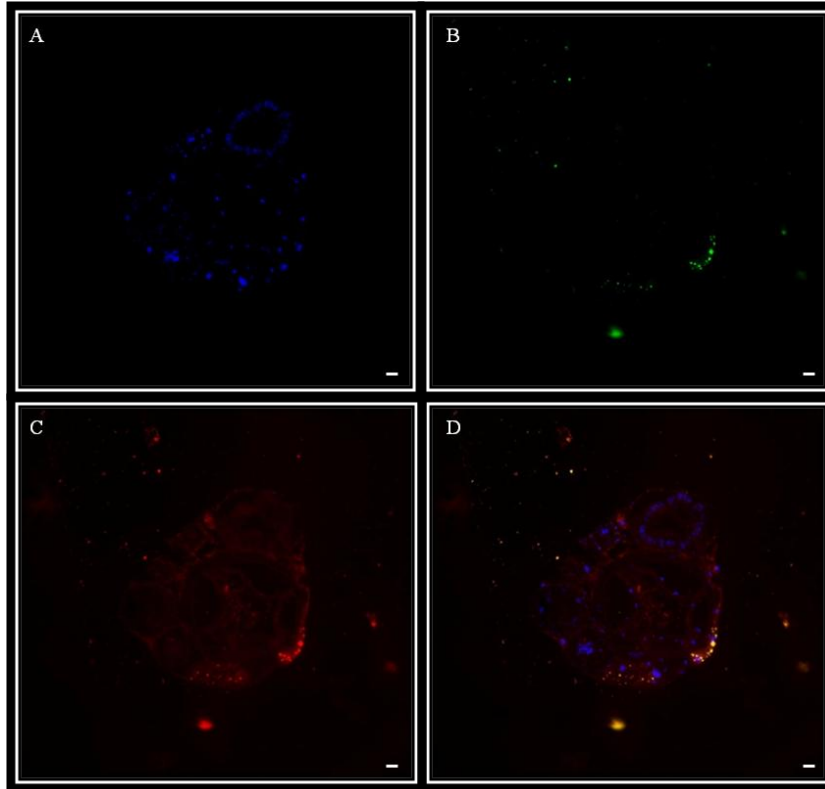
Appendix 1: Endosymbiotic OTUs revealed by bTEFAP sequencing.

Appendix

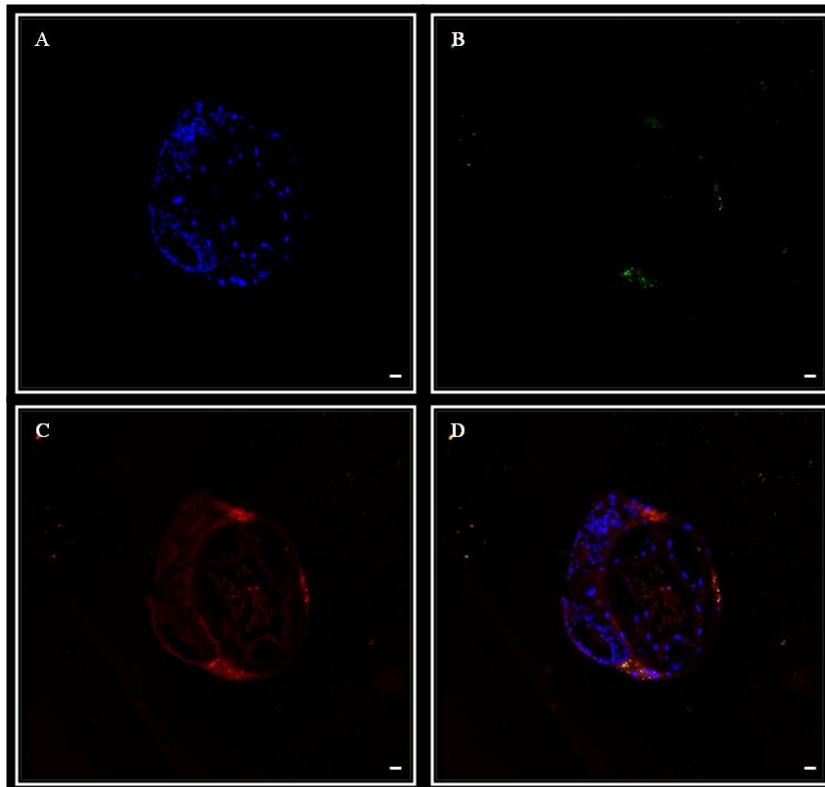
Sample	Seq Primer	Length [bps]	original Medium	Organism(s)	1st BLAST Hit	Query Coverage [%]	Max Ident [%]
BI_1 R2A1	fD1/rP2	1394	R2A	<i>Bacillus simplex</i>	<i>Bacillus simplex</i> strain FJAT-10590 16S ribosomal RNA gene, partial sequence	100	99
BI_3 R2A2	fD1/rP2	1398	R2A	<i>Bacillus simplex</i>	<i>Bacillus simplex</i> strain Bi19 16S ribosomal RNA gene, partial sequence	100	99
BI_6 TSYE1	fD1/rP2	1008	TSYE	<i>Sphingobacterium siyangensis</i>	<i>Sphingobacterium siyangensis</i> strain YRL14 16S ribosomal RNA gene, partial sequence	100	99
BI_8 TSYE2	fD1/rP2	1382	TSYE	<i>Bacillus simplex</i>	<i>Bacillus sp.</i> MHS035 16S ribosomal RNA gene, partial sequence	100	99
BI_10 NutrA1	fD1/rP2	1397	Nutrient Agar	<i>Bacillus simplex</i>	<i>Bacillus simplex</i> strain MX6 16S ribosomal RNA gene, partial sequence	99	99
BI_11 NutrA2	fD1/rP2	1094	Nutrient Agar	<i>Pseudomonas putida</i>	<i>Pseudomonas sp.</i> cbp1-3 16S ribosomal RNA gene, partial sequence	100	99
BI_12 NutrA2	fD1/rP2	1089	Nutrient Agar	<i>Pseudomonas putida</i>	<i>Pseudomonas sp.</i> cbp1-3 16S ribosomal RNA gene, partial sequence	100	99
BI_13 NutrA3	fD1/rP2	223	Nutrient Agar	<i>Enterococcus mundtii</i>	<i>Enterococcus mundtii</i> strain Ag5 16S ribosomal RNA gene, partial sequence	100	90
BI_14 NutrA3	fD1/rP2	1071	Nutrient Agar	<i>Enterococcus durans</i>	<i>Bacterium</i> 1/10/17 16S ribosomal RNA gene, partial sequence	99	95
B2_3 NutrA3	fD1/rP2	1401	Nutrient Agar	<i>Enterococcus mundtii</i>	<i>Enterococcus mundtii</i> strain AT3 16S ribosomal RNA gene, partial sequence	100	99
BI_15 YG1	fD1/rP2	703	YG	<i>Stenotrophomonas maltophilia</i>	<i>Enterococcus mundtii</i> sp. DJM4B9 16S ribosomal RNA gene, partial sequence	100	92
BI_18 YG2	fD1/rP2	1070	YG	<i>Ochrobactrum sp.</i>	<i>Ochrobactrum sp.</i> Ti_2_7 16S ribosomal RNA gene, partial sequence	100	98
BI_19 YG3	fD1/rP2	625	YG	<i>Enterococcus sp.</i>	<i>Enterococcus mundtii</i> strain Ag5 16S ribosomal RNA gene, partial sequence	63	98
B2_1 YG3	fD1/rP2	1050	YG	<i>Enterococcus sp.</i>	<i>Enterococcus durans</i> strain KLDS 6.0643 16S ribosomal RNA gene, partial sequence	71	96
BI_21 Sf1	fD1/rP2	607	SF900-Agar	<i>Bacillus sp.</i>	<i>Bacillus simplex</i> strain CBG_LBIQ 16S ribosomal RNA gene, partial sequence	61	96
BI_22 Sf1	fD1/rP2	1378	SF900-Agar	<i>Bacillus simplex</i>	<i>Bacillus simplex</i> strain B31 16S ribosomal RNA gene, partial sequence	100	99
BI_23 Sf2	fD1/rP2	1381	SF900-Agar	<i>Bacillus simplex</i>	<i>Bacillus simplex</i> strain B31 16S ribosomal RNA gene, partial sequence	100	99
BI_26 Sf4y	fD1/rP2	709	SF900-Agar	<i>Stenotrophomonas sp.</i>	<i>Stenotrophomonas sp.</i> AVO 65 clone 2 16S ribosomal RNA gene, partial sequence	61	95
BI_27 Sf4	fD1/rP2	755	SF900-Agar	<i>Stenotrophomonas sp.</i>	<i>Stenotrophomonas sp.</i> 45 16S ribosomal RNA gene, partial sequence	89	93
B2_5 YG_anaerob	fD1/rP2	1391	YG	<i>Enterococcus mundtii</i>	<i>Enterococcus mundtii</i> strain AT3 16S ribosomal RNA gene, partial sequence	100	99
B2_7 R2A_anaerob	fD1/rP2	812	R2A	<i>Enterococcus sp.</i>	<i>Enterococcus mundtii</i> strain Ag5 16S ribosomal RNA gene, partial sequence	45	85
FL_11 NutrA2	LS1/LR5	448	Nutrient Agar	<i>Cladosporium sp. / Davidiella sp.</i>	<i>Cladosporium ramotenellum</i> strain CPC 18224 28S ribosomal RNA gene, partial sequence	90	98
FL_12 NutrA2	LS1/LR5	164	Nutrient Agar	<i>Alternaria sp.</i>	<i>Ascomycota sp.</i> ARIZ PAMAn1-1 internal transcribed spacer 1, partial sequence; 5	84	93
FL_22 Sf1	LS1/LR5	818	SF900-Agar	div. Chaetothyriales	<i>Knufia cryptophthalidica</i> strain DAOM 216555 28S ribosomal RNA gene, partial sequence	100	98
FL_23 Sf2	LS1/LR5	587	SF900-Agar	<i>Cladosporium sp.</i>	<i>Cladosporium sp.</i> PMA1 28S ribosomal RNA gene, partial sequence	97	86
ChitA F1	LS1/LR5	302	Chitin Agar	div. yeasts	<i>Candida sp.</i> BCMU BX03 genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 26S rRNA,	97	92
ChitA F2	LS1/LR5	837	Chitin Agar	<i>Malassezia sp.</i>	<i>Malassezia cf. restricta</i> HN3127 28S large subunit ribosomal RNA gene, partial sequence	100	99
Cut_fungus B1	LS1/LR5	841	malt agar	<i>Ophiostoma stenoceras</i>	<i>Ophiostoma stenoceras</i> isolate AFTOL-ID 1038 28S large subunit ribosomal RNA	100	98
Cut_fungus B2	LS1/LR5	923	malt agar	<i>Petriella sp.</i>	<i>Petriella sordida</i> large subunit ribosomal RNA gene, partial sequence	99	99
			malt agar	<i>Penicillium sp.</i>			

Appendix 2: Microbes isolated from the gut of *X. saxesenii* on different media.

Appendix



Appendix 3: FISH on gut semi-thin sections with eubacterial probe EUB338-Cy5 and *Sphingobacterium* – specific probe (Cy3). **A:** DAPI. **B:** EUB338-Cy5. **C:** Sphingobac-Cy3. **D:** merge. Scale bars = 10 μ m.



Appendix 4: FISH on gut semi-thin sections with eubacterial probe EUB338-Cy5 and *Ochrobactrum* – specific probe (Cy3). **A:** DAPI. **B:** EUB338-Cy5. **C:** Ochrobac-Cy3. **D:** merge. Scale bars = 10 μ m.

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Erklärung

Hiermit erkläre ich, WOLFGANG FABIG, die vorliegende Masterarbeit selbstständig und ohne Verwendung anderer als der angegebenen Hilfsmittel und Quellen angefertigt zu haben. Diese Arbeit wurde bisher weder in dieser noch in ähnlicher Form in einem anderen Prüfungsverfahren vorgelegt. Ich erkläre weiterhin, dass ich außer meinem Bachelor of Science in Biologie an der Universität Bayreuth keine akademischen Grade erworben oder zu erwerben versucht habe.

Jena, den xx. November 2011