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Research Article

# The Mycobiome of the Gut of Willow Wood Borer, *Xiphydria Prolongata* (Hymenoptera: Xiphydriidae): A Rich Source of Rare Yeasts

## Özgül DOĞAN[1], Ertan Mahir KORKMAZ[2], Mahir BUDAK[*2]

[1]Sivas Cumhuriyet University University, Vocational School of Health Services, 12345, Sivas, Türkiye
[2]Sivas Cumhuriyet University, Science Faculty, Molecular Biology and Genetics Department, 58140, Sivas, Türkiye

Özgül DOĞAN, ORCID No: 0000-0003-0182-8654, Ertan Mahir KORKMAZ, ORCID No: 0000-0003-0699-1354, Mahir BUDAK, ORCID No: 0000-0001-5610-486X
*Corresponding author e-mail: mbudak@cumhuriyet.edu.tr

**Abstract:** A high-throughput amplicon sequencing as a culture-independent approach was used to identify the gut mycobiome of the willow wood borer *Xiphydria prolongata*. The findings of this study are significant in terms of the insect-fungal interactions and indicate the unexpected richness of the mycobiome and the presence of many rare yeast species in the wood borer gut. A total of 40 fungal genera were found, and among them, only one endophytic fungus, *Daldinia* (Hypoxylaceae), has been previously reported in *Xiphydria*. *Zygosaccharomyces siamensis* is the most prevalent ascomycete species, while *Rhodosporidiobolus colostri* is the most abundant basidiomycetous yeast in *X. prolongata*. Some of the species identified here were known as very rare fungi such as *Skoua fertilis*, *Chaetomium nepalense, R. colostri,* and *Vustinia terrae*. This study is also the first report on *S. fertilis* and *V. terrae* in the insect gut flora. These funguses most likely aid in the digestion of lignocellulose in the gut of wood borer. Therefore, further research is required to know the source of acquisition and functional role of these yeast and their industrial potential.

## Odun Arısı *Xiphydria Prolongata*'nın (Hymenoptera: Xiphydriidae) Bağırsak Mikobiyomu: Nadir Mayalar İçin Zengin Bir Kaynak

**Öz:** Söğüt odun arısı *Xiphydria prolongata*'nın bağırsak mikobiyomunu tanımlamak için kültürden bağımsız bir yaklaşım olarak yüksek verimli amplikon dizileme kullanılmıştır. Bu çalışmanın bulguları böcek-fungal etkileşimleri açısından önem taşımakta ve mikobiyomun beklenmedik zenginliğini ve ağaç kurdu bağırsağında birçok nadir maya türünün varlığını göstermektedir. Toplam 40 mantar cinsi bulunmuştur ve bunlar arasında sadece biri endofitik mantar olan *Daldinia* (Hypoxylaceae) daha önce *Xiphydria*'da rapor edilmiştir. *Zygosaccharomyces siamensis* en yaygın askomiset türü iken, *Rhodosporidiobolus colostri X. prolongata*'da en bol bulunan bazidiomiset mayadır. Tanımlanan türlerden *Skoua fertilis*, *Chaetomium nepalense*, *R. colostri* ve *Vustinia terrae* çok nadir bulunan mantarlar olarak bilinmektedir. Bu çalışma aynı zamanda *S. fertilis* ve *V. terrae*'nın böcek bağırsak florasındaki ilk raporudur. Bu funguslar büyük olasılıkla odun kurdunun bağırsağındaki lignoselülozun sindirimine yardımcı olmaktadır. Bu nedenle, bu mayaların elde edilme kaynağı ve işlevsel rolü ile endüstriyel potansiyellerinin bilinmesi için daha fazla araştırma yapılması gerekmektedir.

## 1. Introduction

Xiphydriidae (Hymenoptera: Symphyta) is a relatively small but widely distributed family of wood borers, including 146 known species in two subfamilies, of which Xiphydrinae is the largest with 124 species in 24 genera (Taeger et al., 2018). Even though not much is known about the biology of the family, their members have long, cylindrical bodies, dome-shaped heads, and flexible, long necks. The most common genus is *Xiphydria*, which includes 36 described species. The female wood borers have a characteristically long ovipositor that projects posteriorly to drill into wood. The females lay their eggs in a variety of deciduous broad-leaved trees such as maple (*Acer* spp.), alder (*Alnus* spp.), hornbeam (*Carpinus* spp.), willow (*Salix* spp.), oak (*Quercus* spp.), birch (*Betula* spp.), aspen (*Populus* tremula), pulum (*Prunus* spp.) and lindens (*Tilia* spp.) (Smith, 1976 and 2008; Johansson & Larsson, 2020). The larvae bore into physiologically weakened or dead wood of branches, making them economically little important pests (Smith, 1976).

Although there is little information available on how the xiphydrid larvae interact with their host plants and symbiotic fungi, we know that similar to economically important siricid counterparts, the xiphydrid wood borers coexist as an obligatory nutritional symbiotic relationship with fungi in order to digest the complex tissues of their host plants, such as cellulose, making them economically valuable as wood destroying agents (Pažoutová et al., 2010). In this symbiosis, the female inoculates the spores of the xylobiont fungus she has carried from her native tree into the wood during oviposition (Pažoutová et al., 2013). Then, the spores increase in the larval galleries and spread into the adjacent wood, causing the wood to soften so that the larvae can consume the mycelium and tunnel through the tough components of the wood. But, to date, only the members of cramp balls *Daldinia* (Xylariaceae) consisting of wood-inhabiting pyrenomycetes with perithecia as symbionts of three xiphydrid species were reported using traditional culture techniques (Pažoutová et al., 2010 and 2013; Šrůtka et al., 2007). These are *D. decipiens* from *Xhiphydria camelus* and *X. longicollis*, *D. childiae* from *X. longicollis* and *X. prolongata*, and *D. hawksworthii* from *X. prolongata*.

To understand the role of these fungi in decomposing host plant polymers to their monomers and making them digestible, first of all, it is necessary to define the mycobiota here by focusing on the insect adult intestinal flora. Due to the limited available fungal symbiotic data to explore the evolution of insect and host plant interaction, in recent years, there has been an increasing interest in insect gut flora, and in understanding the roles of fungus in this interaction. Here, we want to explore the intestinal mycobiota of *Xiphydria prolongata*, also known as the "willow wood borer" because of its affinity for physiologically weakened willow trees as host plants (Smith, 1983; Taeger et al., 2018), collected from the host plant white willow (*Salix alba*) by isolating total DNA of intestinal flora of the female and utilizing ITS1 barcode region in amplicon sequencing as a culture-independent approach. Then, we used various metabarcoding analyses to simultaneously determine the presence and diversity of the previously described and/or novel fungal species.

## 2. Material and Methods

Female wood-boring bees were used in the study, as the core mycobiota is transferred from female bees by oviposition. The samples used in the study were collected from white willow in Sivas province between 2014 and 2019 and stored in absolute ethanol in a deep freezer below -20 °C until the study.

### 2.1. DNA extraction and sequencing

Each willow wood borer preserved in absolute ethanol was washed with sterile distilled water before genomic DNA isolation. Five insect intestines were separated for DNA isolation under a stereomicroscope. The tissues were then pooled in a new sterile 1.5 ml microcentrifuge tube and homogenized using a micropestle. Microbial DNA was extracted from the homogenized tissues with a standard salting-out procedure (Miller et al., 1988). Integrity and purity of the DNA extracts were assessed through visualized on 1% (w/v) agarose gel. ITS1 amplicon metabarcoding 2 x 250 paired-end sequencing was conducted on the Illumina MiSeq platform following the standard protocol at CUTAM (Sivas Cumhuriyet University).

## 2.2. Bioinformatics analysis

The Qiime2 version 2022.11.1 (Amir et al., 2017) workflow was used to conduct bioinformatics analyses. To evaluate the quality of the generated sequences, the built-in QIIME2 commands were used. The DADA2 v.2022.11.1 (Callahan et al., 2016) plugin was used to filter chimeras and perform sequence denoising and deduplication. Taxonomy assignment was carried out against the UNITE dataset (dynamic release) (Kõljalg et al., 2020) using the blast+ (Camacho et al., 2009) plugin. R software v.4.2.1 (R Core Team, 2022) was used for the presentation and organization of the results. The raw sequence data and codes are accessible at https://doi.org/10.5281/zenodo.7813125.

## 3. Results

A total of 33,110 reads were generated in each direction with read lengths of 250. The analyses were continued with forward reads because the reverse reads' quality scores were low. 23,500 reads made it through the filter after being cleaned up and checked for chimeras (dada2.tsv in supplementary). The deduplication analysis revealed a total of 439 distinct features (OTUs). 15270 out of 23500 sequences were identified as being from *X. prolongata* (features.csv in supplementary). Fungal species identified by molecular and bioinformatic techniques is reported in Table 1. In total, fungal species accounted for 51 of the 439 OTUs. One fungal species was unidentified representing 1.18% of the total fungal sequences. The majority of the found fungi, approximately sixty percent, were assigned to the Ascomycota, while the remaining forty percent were represented by Basidiomycota (Figure 1). However, the differences in both the fungal composition and abundance were observed in lower taxonomic levels (Table 1).
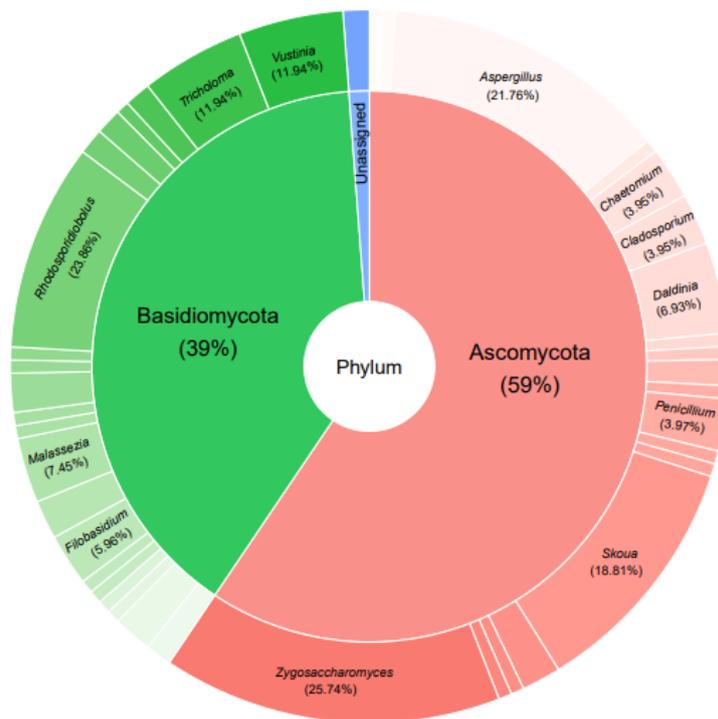


Figure 1. Relative abundance of fungi related to the willow wood borer *Xiphydria prolongata* (Hymenoptera: Xiphydriidae) at the generic level.

The Ascomycota is represented by six fungal classes, in which Saccharomycetes is the predominant with 15.9% of the total fungal sequences. This class is comprised of two genera including *Zygosaccharomyces* and *Candida*. The most represented species is *Zygosaccharomyces siamensis* with 14.71% of the total sequences (92.6% in this class). Eurotiomycetes is the second class representing 15.3% of the total reads with two genera (*Aspergillus* and *Penicillium*). In this class, the most frequently

encountered species is *Aspergillus ruber* with 11.18% of the total sequences (73.10% of the class). The third most common class is Leotiomycetes (14.13%) with five genera and seven species. The most abundant species is *Skoua fertilis* in this class, representing 75% of the reads obtained from this class. Sordariomycetes is also one of the abundant and important classes (7.65%) of the xiphydrid intestinal flora. This class is represented by five species (*Trichoderma citrinoviride*, *Chaetomium nepalense*, *Apiospora iberica*, *Daldinia childiae,* and *D. hawksworthii*), which are mostly able to degrade cellulose and hemicelluloses. Dothideomycetes and Taphrinomycetes are also found, consisting of 4.71% and 1.77% of all fungal reads, respectively (Table 1). On the other hand, the phylum Basidiomycota is represented by five classes and its reads are largely members of the Microbotryomycetes and Tremellomycetes classes, with equal representation (accounted for 25.89% of the total reads; Table 1). The first class includes six genera representing seven species, in which the predominated species is *Rhodosporidiobolus colostri*, with 9.41% of the total reads (72.7% in this class). The class Tremellomycetes is represented by nine species under seven genera and *Vustinia terrae* is the most abundant species. Agaricomycetes, Malasseziomycetes, and Cystobasidiomycetes are also observed in the reads obtained from the Basidiomycetes representatives, accounting for 10.59%, 2.94%, and 0.59% of all fungal reads, respectively. Agaricomycetes includes the reads from six species, in which the most abundant is *Tricholoma terreum,* followed by *Baeospora myosura*. In comparison, the other two classes are represented by only one species each: *Erythrobasidium* sp. and *Malassezia restricta* (Table 1).

Table 1. Relative abundance of fungal genera discovered from the gut of willow wood borer Xiphydria prolongata (Hymenoptera: Xiphydriidae) and their current taxonomic status according to the UNITE dataset (dynamic version)

| Species | Relative abundance | Phylum | Class | Order | Family |
|---|---|---|---|---|---|
| *Zygosaccharomyces siamensis* | 14.71 | Ascomycota | Saccharomycetes | Saccharomycetales | Saccharomycetaceae |
| *Aspergillus ruber* | 11.18 | Ascomycota | Eurotiomycetes | Eurotiales | Aspergillaceae |
| *Skoua fertilis* | 10.59 | Ascomycota | Leotiomycetes | Helotiales | Myxotrichaceae |
| *Rhodosporidiobolus colostri* | 9.41 | Basidiomycota | Microbotryomycetes | Sporidiobolales | Sporidiobolaceae |
| *Tricholoma terreum* | 4.71 | Basidiomycota | Agaricomycetes | Agaricales | Tricholomataceae |
| *Vustinia terrae* | 4.71 | Basidiomycota | Tremellomycetes | Cystofilobasidiales | Mrakiaceae |
| *Daldinia hawksworthii* | 3.53 | Ascomycota | Sordariomycetes | Xylariales | Hypoxylaceae |
| *Malassezia restricta* | 2.94 | Basidiomycota | Malasseziomycetes | Malasseziales | Malasseziaceae |
| *Cladosporium herbarum* | 2.35 | Ascomycota | Dothideomycetes | Capnodiales | Cladosporiaceae |
| *Chaetomium nepalense* | 2.35 | Ascomycota | Sordariomycetes | Sordariales | Chaetomiaceae |
| *Aspergillus flavus* | 1.76 | Ascomycota | Eurotiomycetes | Eurotiales | Aspergillaceae |
| *Baeospora myosura* | 1.76 | Basidiomycota | Agaricomycetes | Agaricales | Tricholomataceae |
| *Filobasidium wieringae* | 1.76 | Basidiomycota | Tremellomycetes | Filobasidiales | Filobasidiaceae |
| *Genolevuria tibetensis* | 1.76 | Basidiomycota | Tremellomycetes | Tremellales | Bulleraceae |
| *Penicillium aotearoae* | 1.18 | Ascomycota | Eurotiomycetes | Eurotiales | Aspergillaceae |
| *Penicillium thomii* | 1.18 | Ascomycota | Eurotiomycetes | Eurotiales | Aspergillaceae |
| *Taphrina sp* | 1.18 | Ascomycota | Taphrinomycetes | Taphrinales | Taphrinaceae |
| *Atheliaceae sp* | 1.18 | Basidiomycota | Agaricomycetes | Atheliales | Atheliaceae |
| *Sistotrema sp* | 1.18 | Basidiomycota | Agaricomycetes | Cantharellales | Hydnaceae |
| *Mrakia aquatica* | 1.18 | Basidiomycota | Tremellomycetes | Cystofilobasidiales | Mrakiaceae |
| *Tremellales sp* | 1.18 | Basidiomycota | Tremellomycetes | Tremellales | Tremellales |
| *Perusta sp* | 0.59 | Ascomycota | Dothideomycetes | Dothideales | Dothioraceae |
| *Dothideomycetes sp* | 0.59 | Ascomycota | Dothideomycetes | Dothideomycetes | Dothideomycetes |
| *Acrodontium neolitseae* | 0.59 | Ascomycota | Dothideomycetes | Mycosphaerellales | Mycosphaerellaceae |
| *Teratosphaeriaceae sp* | 0.59 | Ascomycota | Dothideomycetes | Mycosphaerellales | Teratosphaeriaceae |
| *Lachnum virgineum* | 0.59 | Ascomycota | Leotiomycetes | Helotiales | Hyaloscyphaceae |
| *Oidiodendron mellicola* | 0.59 | Ascomycota | Leotiomycetes | Helotiales | Myxotrichaceae |
| *Skoua asexualis* | 0.59 | Ascomycota | Leotiomycetes | Helotiales | Myxotrichaceae |
| *Monilinia baccarum* | 0.59 | Ascomycota | Leotiomycetes | Helotiales | Sclerotiniaceae |
| *Monilinia ssiori* | 0.59 | Ascomycota | Leotiomycetes | Helotiales | Sclerotiniaceae |
| *Pseudeurotiaceae sp* | 0.59 | Ascomycota | Leotiomycetes | Thelebolales | Pseudeurotiaceae |
| *Zygosaccharomyces favi* | 0.59 | Ascomycota | Saccharomycetes | Saccharomycetales | Saccharomycetaceae |
| *Candida zeylanoides* | 0.59 | Ascomycota | Saccharomycetes | Saccharomycetales | Saccharomycetales |
| *Trichoderma citrinoviride* | 0.59 | Ascomycota | Sordariomycetes | Hypocreales | Hypocreaceae |
| *Apiospora iberica* | 0.59 | Ascomycota | Sordariomycetes | Xylariales | Apiosporaceae |
| *Daldinia childiae* | 0.59 | Ascomycota | Sordariomycetes | Xylariales | Hypoxylaceae |
| *Taphrina tosquinetii* | 0.59 | Ascomycota | Taphrinomycetes | Taphrinales | Taphrinaceae |
| *Mycena metata* | 0.59 | Basidiomycota | Agaricomycetes | Agaricales | Mycenaceae |

Table 1. Relative abundance of fungal genera discovered from the gut of willow wood borer
Xiphydria prolongata (Hymenoptera: Xiphydriidae) and their current taxonomic status
according to the UNITE dataset (dynamic version) (continued)

| Species | Relative abundance | Phylum | Class | Order | Family |
|---|---|---|---|---|---|
| *Ceriporia purpurea* | 0.59 | Basidiomycota | Agaricomycetes | Polyporales | Irpicaceae |
| *Erythrobasidium sp* | 0.59 | Basidiomycota | Cystobasidiomycetes | Erythrobasidiales | Erythrobasidiaceae |
| *Microbotryum betonicae* | 0.59 | Basidiomycota | Microbotryomycetes | Microbotryales | Microbotryaceae |
| *Bannozyma yamatoana* | 0.59 | Basidiomycota | Microbotryomycetes | Microbotryomycetes | Chrysozymaceae |
| *Microbotryomycetes sp* | 0.59 | Basidiomycota | Microbotryomycetes | Microbotryomycetes | Microbotryomycetes |
| *Rhodotorula diobovata* | 0.59 | Basidiomycota | Microbotryomycetes | Sporidiobolales | Sporidiobolaceae |
| *Rhodotorula mucilaginosa* | 0.59 | Basidiomycota | Microbotryomycetes | Sporidiobolales | Sporidiobolaceae |
| *Sporidiobolales sp* | 0.59 | Basidiomycota | Microbotryomycetes | Sporidiobolales | Sporidiobolales |
| *Mrakia frigida* | 0.59 | Basidiomycota | Tremellomycetes | Cystofilobasidiales | Mrakiaceae |
| *Filobasidiaceae sp* | 0.59 | Basidiomycota | Tremellomycetes | Filobasidiales | Filobasidiaceae |
| *Filobasidium chernovii* | 0.59 | Basidiomycota | Tremellomycetes | Filobasidiales | Filobasidiaceae |
| *Naganishia albida* | 0.59 | Basidiomycota | Tremellomycetes | Filobasidiales | Filobasidiaceae |
| Unassigned fungi | 1.18 | | | | |

## 4. Discussion and Conclusion

Recent advances in the high-throughput sequencing technology and bioinformatics have enabled researchers to understand the role of the insect mycobiota in host nutrition, host development, and evolutionary and ecological processes with a growing number of metabarcoding studies (Lewis & Lizé, 2015; Brentassi et al., 2020; Vega & Biedermann, 2020). Some fungal taxa, predominantly yeasts, have been identified as important members of the insect gut mycobiota, by providing a significant survival and/or nutritional advantage to their hosts. These yeasts degrade high molecular weight molecules, produce nitrogen compounds, and play an important role in detoxification processes through facultative or obligate symbiotic associations (Malacrinò et al., 2015). Additionally, they have proven to be an important source of enzymes for various industries (Douglas, 2015).

The present study is a preliminary to investigate the intestinal mycobiota (fungal composition) of *X. prolongata* females using a metabarcoding approach based on the use of fungal ITS1 region, which is widely accepted as a barcode gene in discrimination of the fungal species (Malacrinò et al., 2015). The preference for a culture-independent technique in the present study has considerably expanded our knowledge of the mycobiome of *X. prolongata* and provided new insights into the symbiotic relationship between this mycobiome and host insect species. The findings of this study also indicated that the gut habitat of *X. prolongata* harbors an astonishing diversity of yeasts. Despite the fact that some of the species found in the present study comprise the wood borer core mycobiome, others may be diet-specific (Višňovská et al., 2020).

Here, 40 fungal genera were found associated with *X. prolongota* (Table 1), and among them, only one endophytic fungus, *Daldinia* belonging to the family Hypoxylaceae, has been previously reported in *Xiphydria* using culture-based techniques (Šrůtka et al., 2007; Pažoutová et al., 2010 and 2013). The asexual morphs of species of this saprophytic ascomycete genus that grow on decaying wood are known as symbionts of wood borers and also related to the species of some deciduous broad-leaved trees (Pažoutová et al., 2013). The identified species here are *D. childiae* and *D. hawksworthii*, which are reported as insect-related funguses (Pažoutová et al., 2013) and both have been extensively investigated in recent years in terms of ecology, symbiosis, genome, and bioactivity and biosynthesis of their precious metabolites (Šrůtka et al., 2007; Pažoutová et al., 2010; Kim et al., 2020 and 2022). On the other hand, the mycobiome of the female gut flora of *X. prolongata* is mostly represented by *Zygosaccharomyces siamensis* (14.71% of the total sequences, Table 1). This ascosporogenous yeast has also been observed in natural honey with high sugar concentrations collected from honey bees and stingless bees (Saksinchai et al., 2012; Chikano & Takahashi, 2020), indicating its ability to tolerate the environment with high osmotic pressures. The species is therefore used as a promising fungal lipid producer utilizing different carbon sources in biotechnology (Ilmi et al., 2023). *Aspergillus* and *Penicillium*, the ubiquitous saprophytic ascomycete genera present in natural habitats, may be detected rarely, but they have previously been reported from the gut flora of some moths and cockroaches (Bai et al., 2020; Zhang et al., 2020). Another most abundant species of the mycobiome is the very rare and pollen saprophyte fungus *Skoua fertilis* that belongs to Leotiomycetes. This species was only reported

twice from beebread of honey bee hives (Wynns, 2015), but this is the first investigation to find this less conspicuous species in the insect gut flora. In addition, we detected the ascomycete fungus *Chaetomium nepalense* commonly observed in deteriorating wood products and plays a significant role in the degradation of cellulose-based materials. Recent reports indicate the isolation of the members of *Chaetomium* from insect guts (Fujii et al., 2012; Samal et al., 2014), despite the genus's reputation as a soil fungus with high cellulolytic activity. This fungus most likely aids in the digestion of lignocellulose in the gut of wood borer.

The most common basidiomycetous yeast in the wood borer *X. prolongata* is the less noticeable fungus *Rhodosporidiobolus colostri*, with an average of 9.5% of total reads. This species has also been found in *Euploea* butterflies and the waste deposit of the attina ant *Acromyrmex lundii* nest (Masiulionis & Pagnocca, 2017; Lin et al., 2018), and it is used in the breakdown of lignin-derived aromatic compounds (Margesin et al., 2022). Another abundant basidiomycetous yeast within *X. prolongata* is *Vustinia terrae*, many rare yeast with orange-coloured pigmentation that belongs to Tremellomycetes (Kachalkin et al., 2019). This is also the first report of this species in the insect gut flora. Further studies are therefore necessary to know the source of acquisition and functional role of this yeast in *X. prolongata*. We have additionally found the lipophilic basidiomycetous yeast *Malassezia restricta*, which is a natural component of human and animal skin flora and has been extensively studied about skin diseases in recent years (Theelen et al., 2018). This species was also observed in the gut of beetles (Zhang et al., 2003), indicating that it is a natural member of the phytophagous insect gut mycobiome.

The close examination of the mycobiome of the *X. prolongata* enables us to enhance the knowledge regarding the diversity of fungi present in the gut community of this species and to analyze for the first time the mycobiome related to the female *X. prolongata*. The findings of this study are significant in terms of the insect-fungal interactions and indicate the unexpected richness of the mycobiome and the presence of many rare yeast species in the wood borer gut. Wood borers, like other reported phytophagous or pollinator insects, most likely use their gut microbiota to improve the usage of plant nutrients, which are more difficult for insects to degrade, thus allowing these insects to overcome the nutrient-poor features of food sources. This study may also serve as a guide for researchers to further explore the evolutionary and ecological relationships between *X. prolongata* and its mycobiome. As a result, more detailed studies are required to uncover the core mycobiota of wood borers through sampling from different life stages of the species.

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