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# Low intraspecific genetic diversity indicates asexuality and vertical transmission in the fungal cultivars of ambrosia beetles

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ABSTRACT

Ambrosia beetles farm ascomycetous fungi in tunnels within wood. These ambrosia fungi are regarded asexual, although population genetic proof is missing. Here we explored the intraspecific genetic diversity of *Ambrosiella grosmanniae* and *Ambrosiella hartigii* (Ascomycota: Microascales), the mutualists of the beetles *Xylosandrus germanus* and *Anisandrus dispar*. By sequencing five markers (ITS, LSU, TEF1α, RPB2, β-tubulin) from several fungal strains, we show that *X. germanus* cultivates the same two clones of *A. grosmanniae* in the USA and in Europe, whereas *A. dispar* is associated with a single *A. hartigii* clone across Europe. This low genetic diversity is consistent with predominantly asexual vertical transmission of *Ambrosiella* cultivars between beetle generations. This clonal agriculture is a remarkable case of convergence with fungus-farming ants, given that both groups have a completely different ecology and evolutionary history.

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## 1. Introduction

Insect agriculture evolved once in ants, once in termites and at least twelve times in wood-boring weevils (Curculionidae: Scolytinae and Platypodinae), the so-called ambrosia beetles (Mueller et al., 2005; Jordal and Cognato, 2012). While fungus-farming ants and termites collect substrate to grow their fungal mutualists within the nests, ambrosia beetles live within wood, which serves as substrate for their cultivars. Despite these ecological differences, there are some striking similarities: new nests of ants and beetles are founded by single individuals, which transmit the fungal cultivars from their parental nest by vertical transmission (Francke-Grosmann, 1967; Korb and Aanen, 2003; Himler et al., 2009). Furthermore, in termites, where two individuals found the colony, in some species one of the two founding individuals transmits the fungus vertically (Korb and Aanen, 2003). This corresponds to clonal farming across many farmer generations, as all vertically transmitted fungi appear asexual. By contrast, the horizontally acquired symbionts of most other termite species undergo regular meiosis and sexual recombination (Mueller et al., 2005; de Fine Licht et al., 2006; Nobre et al., 2011). In theory vertical symbiont transmission is expected to strengthen the mutualism by linking the fitness between host and symbiont, whereas horizontal transmission may lead to the deterioration of the partnership (Frank, 1997).

For ambrosia beetles both vertical transmission and asexuality of fungal cultivars are based on circumstantial evidence, however. First, specialized fungal-spore-carrying organs (i.e., mycetangia; Francke-Grosmann, 1956, 1967) were regarded as evidence for sole

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vertical cultivar transmission, although beetle nests typically co-occur at high densities, which may also facilitate horizontal propagation of cultivars between neighbouring nests. Second, beetle cultivars have long been assumed asexual, because sexual states remained unknown (Harrington, 2005). The latter has been proven wrong by recent discoveries of a sexual state (Musvuugwa et al., 2015) and two mating types in the *Raffaelea* lineage (Ascomycota: Ophiostomatales) of ambrosia beetle-associated fungi (Wuest et al., 2016). However, even though sex is possible it remains unknown to what extent this affects the genetic population structure of those fungal symbionts.

Like other farming insects, ambrosia beetles are obligately dependent on fungi as their sole food source. The best studied ambrosia beetles are found in the inbreeding tribe Xyleborini, which contains about 1300 species (Farrell et al., 2001). These beetles bore tunnel systems in the wood of recently dead or dying trees and inoculate the walls of the tunnels with fungal spores. Glands in their spore-carrying mycetangia ensure specificity of the fungal inoculum during foundation of a new nest (Francke-Grosmann, 1967; Schneider and Rudinsky, 1969). Typical fungal cultivars of ambrosia beetles are species in the genera *Raffaelea* and *Ambrosiella* (Ascomycota: Ophiostomatales and Microascales). Related to plant pathogens, both genera are polyphyletic and it appears that domestication by beetles has occurred several times independently (Cassar and Blackwell, 1996; Jones and Blackwell, 1998; O'Donnell et al., 2015). While the association of ambrosia beetles with *Raffaelea* symbionts appears usually quite loose (i.e., often several different *Raffaelea* species are found within one beetle species and also in a single nest; e.g. Harrington et al., 2010), associations with *Ambrosiella* symbionts are tighter (i.e., only a single ambrosia fungus per nest and species; Mayers et al., 2015).

Research has mainly focused on the identity of ambrosia fungi and only a single study has investigated genetic variation of a *Raffaelea* symbiont (Wuest et al., 2016). *Raffaelea lauricola*, associated with the Redbay ambrosia beetle *Xyleborus glabratus* showed very low genetic variation in its invasive range in the US, but higher diversity in its native Asian range, where also two mating types were discovered. The mating system in the independently evolved and probably more beetle-specific lineage of *Ambrosiella* symbionts remains unstudied so far, however. If the latter indeed are truly asexual and predominantly vertically transmitted between beetle generations, this would lead to low intraspecific variation. Alternatively, sexuality associated with horizontal exchange of cultivars is expected to lead to a higher degree of genetic variation (Charlesworth and Willis, 2009).

Here we tested the hypothesis that genetic variation within and between *Ambrosiella* symbiont populations is low. This was done by comparing the amount of genetic variation by sequencing five polymorphic genetic markers (ITS, LSU (Schoch et al., 2012), TEF1α (Stielow et al., 2015), RPB2, and β-tubulin), which have been used successfully to confirm high genetic variation and frequent horizontal exchange of fungal cultivars in *Macrotermes* fungus-farming termites (de Fine Licht et al., 2006; Nobre et al., 2011) and other non-mutualistic fungal species (Johannesson et al., 2001). Two species of *Ambrosiella* were collected from two beetle species from seven different populations across Europe. *Ambrosiella grosmanniae* was isolated from the ambrosia beetle *Xylosandrus germanus,* which originates from Asia, and *Ambrosiella hartigii* was isolated from the pear blight beetle *Anisandrus dispar*, a species endemic to Europe.

## 2. Materials and methods

### 2.1. Beetle collection and fungal extractions

We collected beetles and their symbionts between May and July 2014 at six different locations in six different countries (for details see Fig. 1). The fungi from an additional population in Switzerland were collected in 2012. Additional samples for *A. dispar* beetles were collected in Wageningen, the Netherlands in May 2015. Beetles were trapped using ethanol (96%) baited traps. After collection we immediately stored living beetles in 1.5 ml Eppendorf tubes with a small piece of wet tissue. *X. germanus* and *A. dispar* beetles were stored at 4 °C until they were used for fungal extraction. Prior to fungal extraction the beetles were surface sterilized by dipping them briefly in 70% ethanol and rinsing them afterwards with sterile demineralized water. We isolated fungi by first grinding individual beetles in 1 ml of sterile PBS buffer solution (1:10 dilution), vortexing of the mixture and spreading 200 μl of the pure or diluted (10 ×, 20 ×, 50 ×, 100 ×) mixtures on SMEA plates (3% malt extract, 1.5% agar and 100 ppm streptomycin added after autoclaving) with a metal hockey. Plates were incubated in the dark at 25 °C until fungal colonies appeared. When present, two to three CFUs of all suspected *Ambrosiella* morphotypes were picked and purified on MEA (3% malt extract, 1.5% agar) for molecular identification and sequencing of the different markers. In total we isolated 35 *Ambrosiella* strains from 31 collected beetles. Three additional fungal isolates from *X. germanus* from the USA were provided by T.C. Harrington. An extra ITS sequence of a fungal isolate from a Swiss *X. germanus* beetle was added to the alignment.

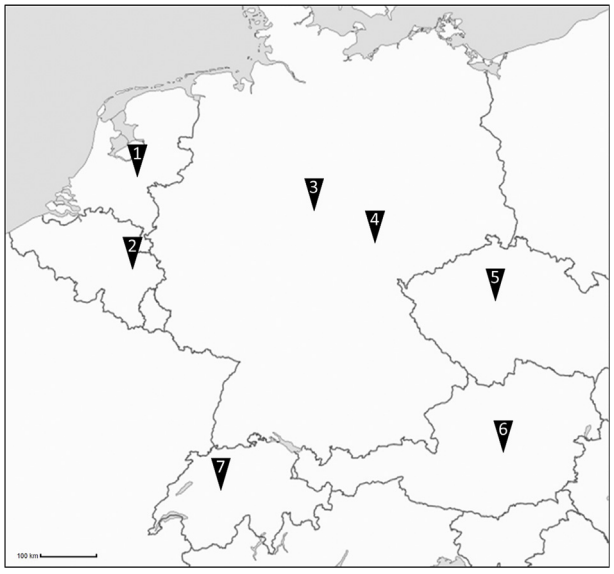


**Fig. 1. Origin of the fungal isolates from Europe**. Pin number (1) Wageningen, NL, (51°58′44.1″N 5°42′31.0″E) (2) Comblain-au-Pont, BE, (50°28′31.91″N 5°35′26.47″E) (3) Reinhausen (51°27′36.4″N 9°59′54.1″E) and Göttingen (51°34′13.3″N 9°58′24.5″E), DE, (4) Jena (50°59′18.0″N 11°44′44.3″E), DE, (5) Prague (50°01′30.7″N 14°28′07.5″E), CZ, (6) Gesäuse (47°36′28.4″N 14°37′13.4″E), AT, (7) Bern (46°54′37.5″N 7°20′34.1″E), CH. Modified map of NordNordWest, Wikimedia Commons, licensed by CreativeCommons (CC BY-SA 3.0), URL: https://creativecommons.org/licenses/by-sa/3.0/deed.de.

### 2.2. DNA extraction, sequencing and phylogenetic analyses

Pure cultures of all *Ambrosiella* morphotypes were kept for 7–10 d on MEA plates with cellophane so that the mycelium could be easily harvested. We extracted DNA by placing around 1 g of mycelium in a 1.5 ml Eppendorf tube with glass beads. After freezing in liquid nitrogen the tubes were shaken for 1 min in a Beadbeater machine; this step was repeated once. After grinding, 100 μl of 5% Chelex100 and 10 μl of proteinase K (20 mg/ml) were added. Dilutions were vortexed and incubated for 30 min at 56 °C and for

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