DISSERTATION | DOCTORAL THESIS

Titel | Title

In the tree farm of *Azteca* ants: A step forward in understanding the biodiversity and dynamics of ant-made patches in the stem of *Cecropia* trees

verfasst von | submitted by

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A mi familia,

que siempre ha sido y será

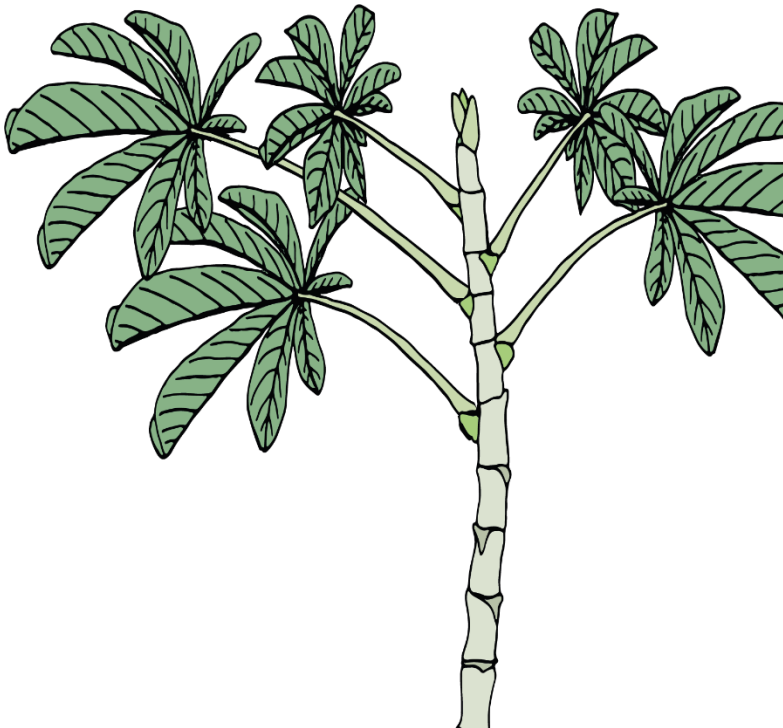
la fuerza motriz de todos mis logros

Table of Contents

General Introduction

The symbionts of a symbiont: complex communities associated with tropical ant-plant mutualisms

Veronica Barrajon-Santos



The continuum of symbiosis: the case of social insects

All living organisms continuously interact with other species in their environment. Thus, it is not surprising that biotic interactions play fundamental roles in biodiversity and ecosystem functioning [1, 2]. When organisms from different species interact closely over extended periods, their relationship can be defined as symbiotic [3]. Symbiosis encompasses a continuum of interactions based on the degree and type of association (Figure 1) [4]. These range from facultative relationships to highly specialized, coevolved, and interdependent partnerships [5]. They also span from parasitic or pathogenic (beneficial-harmful) via commensal (beneficial-neutral) to mutualistic (mutually beneficial) [6]. Where a symbiont falls along the continuum depends on ever-changing biotic and environmental variables [6]. Given that, a symbiotic relationship could undergo one or more shifts between antagonism and cooperation during their co-evolutionary history [7, 8]. While the mechanisms behind bipartite symbiotic relationships have been thoroughly studied since the 19th century (e.g. lichens, coral-algae associations, mycorrhizae) [3, 9, 10], little is known about the remarkably complex multipartite interactions revealed by molecular-based techniques in many associations [11–14].

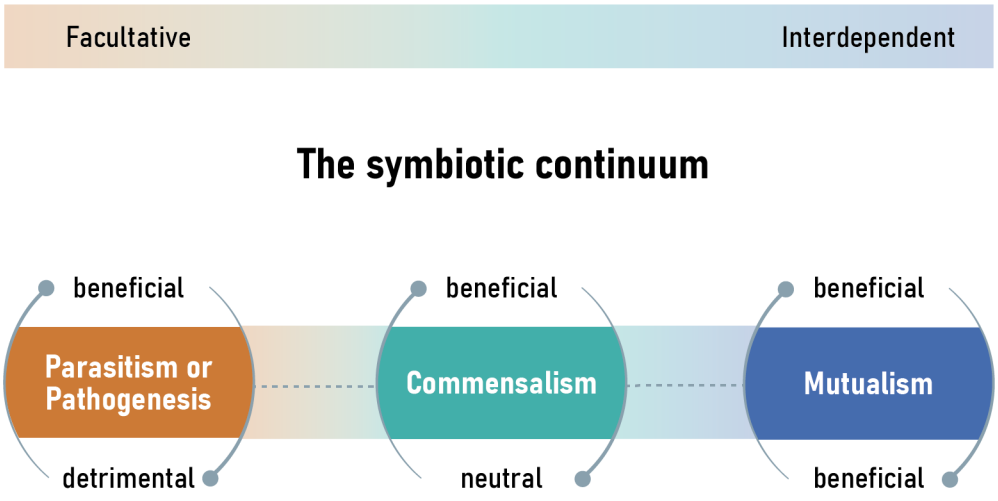


Figure 1. The symbiotic associations based on the degree and type of association.

Recent studies have uncovered an unprecedented complexity in the diversity and interactions within the nests of eusocial insects like ants (Formicidae:Hymenoptera), termites (Blattodea), and bees (Hymenoptera) [15]. These colonies, numbering from dozens to millions of individuals [16, 17], are known to fiercely protect their colony, and with it, the premises of their nests [18]. From large soldier casts in termites and some ant species [19] to the coordinated mass stinging response of honey bees [20], social insects have evolved specialized defence mechanisms, often involving self-sacrificial behaviours [21]. Given the formidable nature of these defences, it would seem unlikely that any intruders could infiltrate such fortified environments without the colony's consent. Unexpectedly, recent research has shown that a notably diverse array of organisms —including bacteria, fungi, nematodes, mites, dipterans, coleopterans, and hemipterans— commonly inhabit social insect nests [15, 22–27]. How these organisms managed to coexist within such heavily defended environments is surprising: are they invited guests, or have they found ways to evade detection and bypass the colony's defences? While many are likely invited, some have surprisingly managed to imitate the colony's odour, and thus, chemically hide within the nest [15, 28].

In some cases, the co-evolution between social insects and their symbionts has evolved beyond mutualism, leading to domestication [29–31]. For instance, ants often tend hemipterans, primarily scale insects (Coccomorpha) and aphids (Aphidoidea), like "cattle" on plants [32]. The honeydew they secrete, derived from plant sap, is harvested by ant workers as a carbohydrate-rich food source [33, 34]. Moreover, the fungal cultivars grown by termites, leaf-cutting ants and ambrosia beetles are the best-studied examples of domestication by insects, particularly as a form of agriculture [35–37]. These insects cultivate fungi within their nest, nourishing them with plant material [36, 38]. In return, the fungi serve as the colony's primary food source [39–41]. These examples make it evident that social insects do much more than simply invite these symbionts into their nests. They can actively bring them in and exert control over their nutrition, fitness, and even, in some cases, their reproduction [30].

Despite the repeated detection of complex communities in a multitude of social insect nests [22, 42–45], true agriculture has been confirmed only in a few [36]. Thus, the question remains if domestication is more widespread in social insects than we think from our anthropocentric perspective.

Ants as mutualistic symbionts of plants

Tropical forests are known to harbour the vast majority of Earth's terrestrial biodiversity [1, 46, 47]. Their unprecedented high species richness leads to even more ubiquitous and complex biotic interactions than in any other biome [1, 47]. A perfect example of such complexity is the widespread ant-plant mutualisms found across tropical regions worldwide [48, 49]. In these relationships, the host plant offers specialized nesting spaces known as domatia —hollow stems, thorns, petioles, leaf pouches or swollen tuber-like organs— along with food resources like food bodies, pearl bodies or extrafloral nectaries [50–52]. In return, ants protect their hosts from herbivorous insects and competitors, and in some cases, supply them with nutrients [51, 53–55]. While the relationship is facultative for the myrmecophytic plant [54, 56, 57], the ants are entirely dependent on the plant for their survival, making them obligate symbionts [51, 58].

Originating in the Mesozoic era, likely during the Cretaceous, the ant-plant associations emerged from arboreal foraging lineages with partially or fully plant-based diets [59]. Then, these lineages gradually co-evolved with plants through increasing interdependence until the actual ant-plant mutualisms [58, 59]. Despite being limited to the Tropics, this partnership evolved in over 100 genera of angiosperms (e.g., *Cecropia, Macaranga, Hirtella* and *Vachellia*) and 40 genera of ants (e.g., *Azteca, Crematogaster, Allomerus and Pseudomyrmex,* respectively*)* [49, 60]. As their macroevolutionary assemblage was highly dynamic, these associations exhibit varying degrees of specialization and different ecological interactions [58].

Since Janzen (1966) first described the mutualistic nature of an ant-plant association (*Vachellia-Pseudomyrmex* interaction) [61], an increasing number of organisms have been gradually found cohabiting with the ant colony in the domatia. Among those, a

conspicuous group of filamentous fungi from a monophyletic clade within the Chaetothyriales order are known to grow in the majority of ant-plant mutualisms [62]. While numerous investigations have consistently proved that these fungi establish a highly specialized symbiotic relationship with the ant-plants [62–68], the nature of their ecological interactions remains uncertain. In addition to Chaetothyriales, other organisms, including bacteria, nematodes, hemipterans, mites, and dipterans, have also been identified in the domatia [69–74]. Although many studies have attempted to elucidate the dynamics and ecological interactions among players in this complex ecosystem, it remains unclear possibly due to their focus on one-to-one interactions.

The *Azteca – Cecropia* association in the Tropics of America

Cecropia plants, commonly known as “yagrumo”, “guarumo” or “trumpet tree”, are pioneer and fast-growing trees from the Urticaceae family (Figure 2).

Figure 2. *Cecropia peltata* trees located next to a road in La Gamba, Puntarenas (Costa Rica). On the right side, the adaxial (above) and abaxial (below) sides of one of the leaves. (Photos by: Veronica Barrajon-Santos)

This genus ranges from Mexico to southern Brazil, including the Caribbean islands, where it occupies nearly every terrestrial habitat. *Cecropia* is especially prominent alongside the riverbanks, roads and in disturbed areas, such as secondary forests and agricultural lands [75, 76]. This genus comprises 61 species, of which 45 are likely myrmecophytes [76]. While diverse ant species forage on or even nest in young *Cecropia* plants (e.g., *Camponatus* sp., *Crematogaster* sp. and *Pseudomyrmex* sp.) [60], only species from the *Azteca* and *Neoponera* genera have co-evolved with this plant in a mutualistic relationship [77]. The latest is notably rare and it has only been recorded inhabiting *Cecropia insignis* plants in Costa Rica.

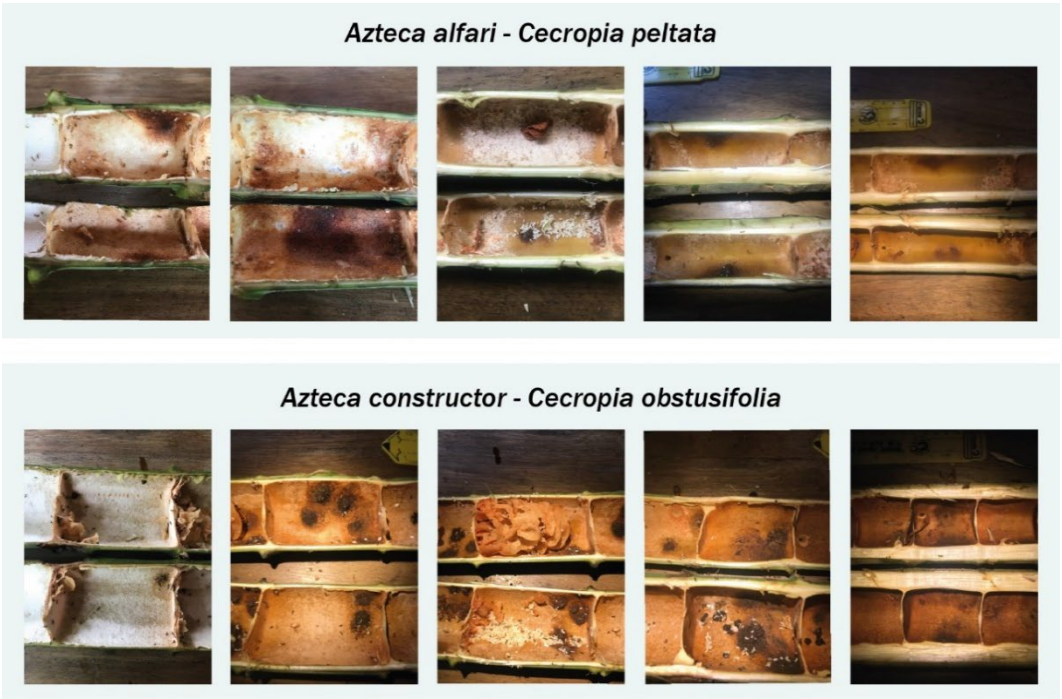


Figure 3. *Azteca* colonies inhabiting the hollow stem of *Cecropia* trees.
(Photos by: Veronica Barrajon-Santos)

The genus *Azteca* (Hymenoptera: Formicidae), belonging to the subfamily Dolichoderinae, is distributed in lowland habitats of the Tropics and Subtropics of America [78]. These ants are strictly arboreal and usually make large colonies of active and aggressive ants [79]. Among the 113 identified species, at least 13 species have been documented to establish an obligate mutualism with *Cecropia* [77]. Remarkably, the genus *Azteca* has never been found in mutualistic association with any other plant genera [78].

The *Azteca-Cecropia* mutualism is one of the most ubiquitous and prominent associations of the Neotropics realm [80], which makes it an ideal model system for investigating the evolution and ecology of ant-plant mutualisms. In fact, this association has been the subject of extensive investigation by many scientists from different backgrounds including naturalists, botanists, entomologists, evolutionary biologists and ecologists for a long time [51, 55, 82–89, 56, 57, 60, 67, 68, 77, 79, 81].

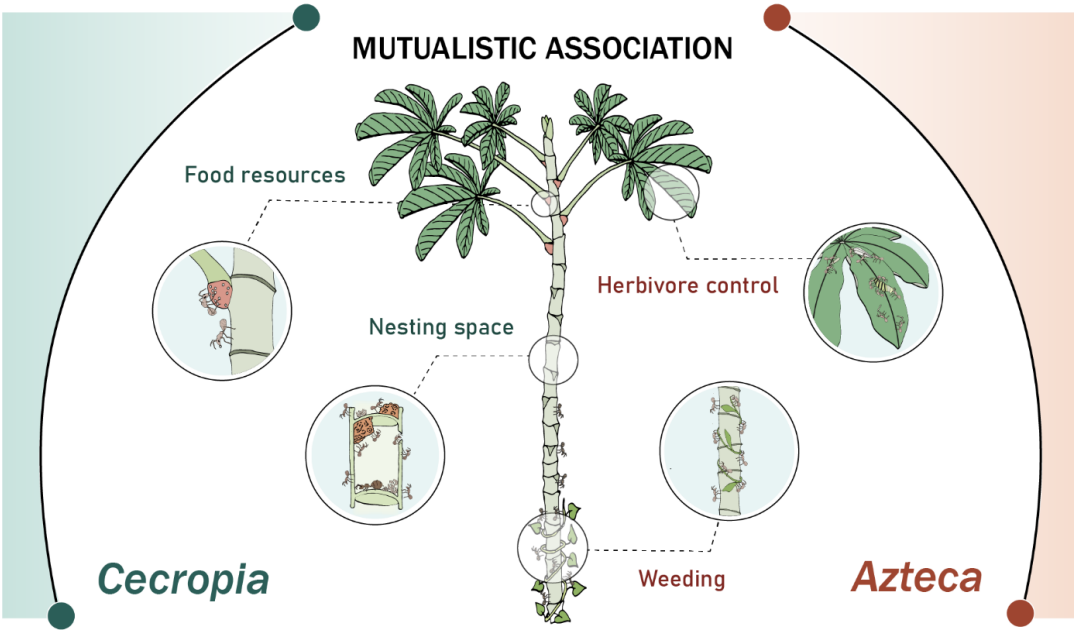


Figure 4. Principles of the *Azteca-Cecropia* mutualism.

In this relationship, *Azteca* ants live inside the hollow stems of *Cecropia* (Figure 3) and are supplied with glycogen-rich Müllerian bodies produced by the plant in specialized structures, known as trichillia, at the base of its leaves (Figure 4) [84, 90]. In exchange, *Azteca* ants fiercely defend their host plant by weeding climbing vines and killing herbivorous insects they find feeding on leaves (Figure 4) [91, 92]. In some cases, *Azteca* ants even repair damage to the trunk using parenchyma mixed with glandular secretions [83].

During their 8 million years of co-evolution [77], most *Azteca* workers have ceased foraging outside their host plant, which means that the colony is entirely sustained by the resources they find on the tree [82, 93]. While food bodies are the primary food source of their larvae, *Azteca* workers access a broader range of resources. Like

many ant species [94], *Azteca* ants are often found tending mealybugs (Hemiptera: Pseudococcidae) and scale insects (Hemiptera: Coccidae) to obtain carbohydrate-rich honeydew [78]. Additionally, the domatia host a complex community of organisms, including bacteria, chaetothyrialean fungi and bacterivorous nematodes [42, 67, 70, 89]. These organisms are especially abundant in ant-built organic matter piles defined as "patches". Although patches are found in ant-plant mutualisms worldwide (e.g., *Petalomyrmex/Leonardoxa* in tropical Africa*, Pseudomyrmex/Triplaris* in tropical Central & South America, *Philidris/Dischidia* in tropical SE Asia, see Mayer et al., 2023 [64]), the role of these structures and their associated organisms remains unclear.

The *Azteca* ant colony development and the formation of patches

The colonization process of a *Cecropia* sapling by an *Azteca* ant queen starts right after mating by chewing into the hollow stem through the prostoma —a well-defined oval depression in the plant wall designed to facilitate the opening process and prevent damage to the vascular bundles— [57, 95]. Once inside, the foundress queen seals the hole again with plant tissue possibly to ensure a safe environment at the start of the new colony [80, 96]. Remarkably, other queens are often found trying to form their colonies in the same plant and even in the same internode [80]. Although some colonies are pleometrotic (i.e., cooperative) at their founding stage, only one queen will manage to successfully establish [97]. Thus, one could expect that foundress queens prioritizes the eggs laying over any other activity. Surprisingly, a study showed that *Azteca* foundress queens lay the eggs only after building the “initial patch” with scratched parenchyma tissue (Figure 5A) [96]. In addition, this study showed that while forming the patch, the queen inoculates it with patch organisms brought from the mother colony in its infrabuccal pocket, a filter organ that allows the separation of liquid food from bigger particles (Figure 5B). When the first workers emerge, they reopen the prostoma and start harvesting food bodies.

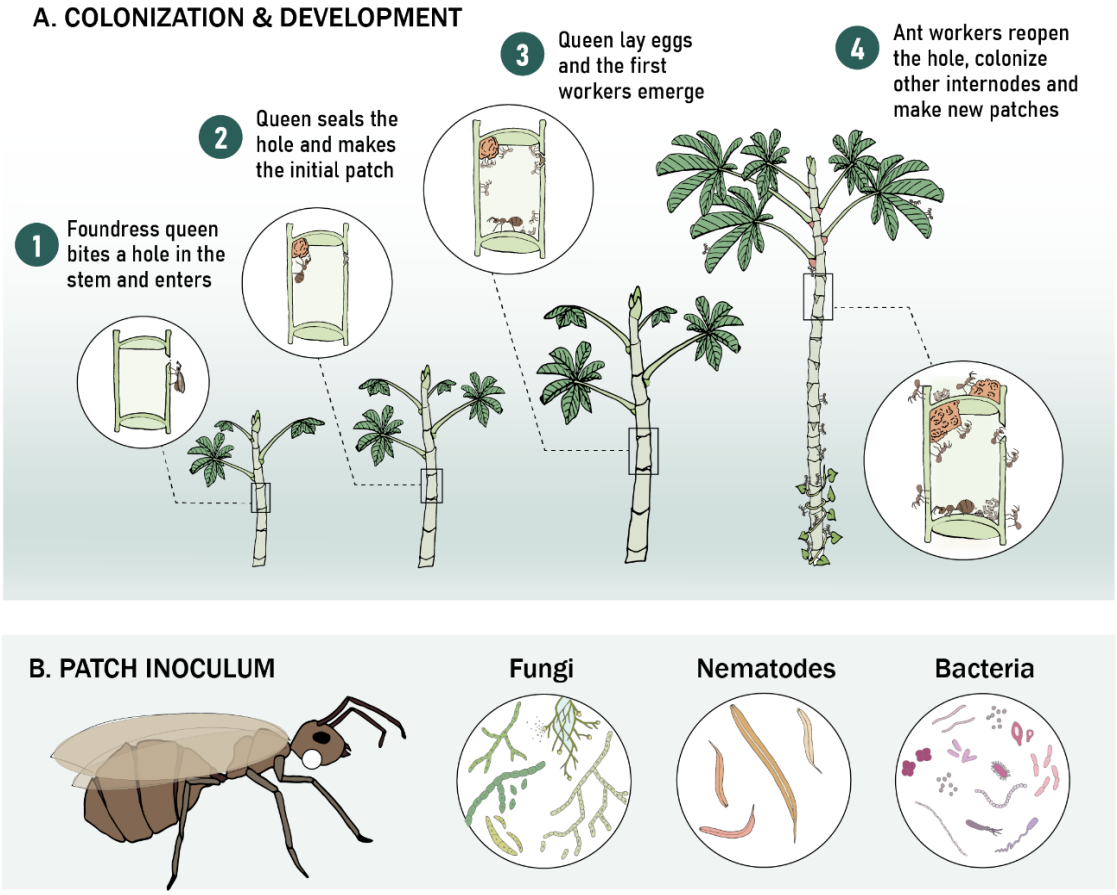


Figure 5. Colonization and development of an *Azteca* colony in a *Cecropia* tree

As the worker force grows, they expand into other internodes of the tree (5-10 months after colony foundation) [98], and make new patches in nearly every internode they colonize, even those containing brood (Figure 5A). At this point, the workers diversify the substrates deposited on the patches, adding not only parenchyma tissue but also plant trichomes, leaves of mosses, ant faeces and pellets regurgitated from the infrabuccal pocket and the bodies of dead nestmates and other insects [70, 99]. As the plant grows, the colony gradually occupies the new grown internodes of the host plant. During this migration, the ant workers collect the "mature" patch material from older domatia, transport it to the recently colonized internodes and mix it with the parenchyma tissue possibly for the formation of new patches (personal field observations). Based on extensive scientific observations [67, 68, 96], it is evident that patches are present in the domatia of every established *Azteca* colony. However, the questions that remains are: why are these ants making the patches? and what does the patch community lives from?

PhD thesis outline

Multiple lines of evidence related with the ant colony development strongly suggest that patches play essential roles in the *Azteca-Cecropia* mutualism. First, the foundress queen makes the initial patch even before laying eggs by scratching off parenchyma tissue and inoculating patch organisms (fungi, nematodes and bacteria) brought from the mother colony. Second, the ant workers make patches in almost every internode they colonize, including those with brood. Third, the ants continuously manure the patches by adding diverse plant- and animal-based waste material including their own faeces and dead nest mates. Last, the ant workers actively transfer the mature patch material from old internodes to younger ones. If these structures are so relevant throughout the life cycle of the ant colony, what is their function?

The general research hypothesis of the present PhD thesis was that the ant-made patches found in the *Azteca-Cecropia* mutualism function as nutrient recycling spots, equivalent to the compost piles made by humans. However, to elucidate the potential purposes of these striking structures, it was crucial to first understand the biodiversity and dynamics of the microbial and nematodes communities inhabiting the patches. The bacterial communities were previously analysed by 16S rRNA amplicon sequencing in a publication not included in this thesis [70]. Then, in Chapter I, the fungal diversity and community composition of the same *Azteca-Cecropia* patches as for bacteria was investigated using amplicon sequencing of the internal transcribed spacer 2 (ITS2) region. These samples included patches from different stages of ant colony development and from closely related ant species. In addition to the previously detected Chaetothyriales, Chapter I revealed a highly diverse fungal community, with genera such as *Fusarium*, *Moesziomyces*, *Mucor*, *Blakeslea* and *Pleiocarpon* frequently appearing in the amplicon data. Similar to the bacteria in the patches, the fungal communities were different and more diverse in the multiple established patches in well-developed colonies than in the initial patch at colony foundation. These results suggest a successional progression of microbial communities during ant colony growth, likely driven by substrate diversification over time and the introduction of new organisms from the environment as ant workers begin foraging on the tree. As shown for bacteria, the composition of fungal communities was influenced by the ant

species rather than the plant species, which suggests the ant colony as the main driver shaping such communities. Based on these findings, the potential role of fungi as an alternative or supplementary food source for ant larvae was discussed.

To add to the understanding of patch communities, in Chapter II, the diversity and composition of nematodes was examined by combining amplicon sequencing of18S rRNA gene and morphologically-based identification methods. Unlike fungi and bacteria, nematode diversity in the initial patches created by ant queens during colony foundation remained consistent when compared to established patches. These findings strongly support the transmission of nematodes from mother to daughter colonies and across patches within the same colony. In addition to the previously identified bacterivorous Rhabditida nematodes (e.g., *Diploscapter* and *Sclerorhabditis*), two other nematode groups with different feeding strategies from the orders Tylenchida (e.g., *Aphelenchoides*) and Dorylaimida (e.g., *Mesodorylaimus*) were detected. Despite sharing the same geographical region, closely related ant species harboured distinct nematode communities in their patches, suggesting that each ant species creates a unique nest environment that fosters the development of different communities. Overall, these findings suggest that nematodes may contribute positively to the patches by providing a range of ecosystem services, which could enhance the stability and functioning of the patch-associated microbiota.

Following the thorough dissection of the patch communities in the *Azteca-Cecropia* association, in Chapter III, the activity and metabolic potential of patch bacterial communities for degrading polysaccharide-rich substrates was investigated. By conducting isotope-based activity assays with patch samples, the hypothesized ability of patch communities to metabolize the recalcitrant cellulose and chitin found in the patch substrates was demonstrated. Then, to evaluate the genetic mechanisms related to these degradation processes, metagenome sequencing of patches was performed from which 214 MAGs with a completeness higher than 80% were obtained. This analysis showed that a rich and diverse genetic repertoire involved in polysaccharide breakdown is widely distributed among the reconstructed 214 MAGs representing the bacterial microbiome of ant-made patches. Based on these results, potential bacterial players (e.g., Chitinophagaceae, Comamonadaceae, Opitutaceae,

Lachnospiraceae) in the decomposition of such organic matter in the patches were suggested.

To conclude, in the general discussion of the thesis, I showed that the *Azteca* ants have indeed engineered a system that facilitates the microbial degradation of plant- and insect-based waste material, analogous to composting by humans. Then, I described how is the composting practice followed by *Azteca* ants. Finally, I summarized the nutrient fluxes and trophic interactions that are so far known in the *Azteca-Cecropia* complex and discussed the potential purposes of patch making in these exceptional ants.

References

1. Dáttilo W, Rico-gray V. Ecological networks in the Tropics. Cham, Switzerland: Springer International Publishing; 2018.

2. Thompson RM, Brose U, Dunne JA, Hall RO, Hladyz S, Kitching RL, et al. Food webs: Reconciling the structure and function of biodiversity. Trends Ecol Evol. 2012;27:689–97.

3. De Bary A. Die Erscheinung Der Symbiose. Die Erscheinung der Symbiose. 1879;:115–42.

4. Paracer S, Ahmadjian V. Symbiosis: an introduction to biological associations. 2nd edition. Oxford University Press, USA; 2000.

5. Artavia-León A, Pacheco-Leiva M, Moya-Román C, Rodríguez-Hernández N, Pinto-Tomás AA. Ant microbial symbionts are a new model for drug discovery. Drug Discov Today Dis Model. 2018;28:27–33.

6. Dimijian GG. Evolving together: the biology of symbiosis, part 1. Baylor Univ Med Cent Proc. 2000;13:217–26.

7. Sachs JL, Skophammer RG, Regus JU. Evolutionary transitions in bacterial symbiosis. Proc Natl Acad Sci U S A. 2011;108 SUPPL. 2:10800–7.

8. Herrera P, Schuster L, Wentrup C, König L, Kempinger T, Na H, et al. Molecular causes of an evolutionary shift along the parasitism–mutualism continuum in a bacterial symbiont. Proc Natl Acad Sci U S A. 2020;117:21658–66.

9. Rayner MC. Obligate symbiosis in *Calluna vulgaris*. Ann Bot. 1915;29:97–133.

10. Yonge CM. Experimental analysis of the association between invertebrates and unicellular algae. Biol Rev. 1944;19:68–80.

11. Tedersoo L, Bahram M, Zobel M. How mycorrhizal associations drive plant population and community biology. Science. 2020;367.

12. van Oppen MJH, Blackall LL. Coral microbiome dynamics, functions and design in a changing world. Nat Rev Microbiol. 2019;17:557–67.

13. Baker CCM, Bittleston LS, Sanders JG, Pierce NE. Dissecting host-associated

communities with DNA barcodes. Philos Trans R Soc B Biol Sci. 2016;371.

14. Rodrigues A, Mueller UG, Ishak HD, Bacci M, Pagnocca FC. Ecology of microfungal communities in gardens of fungus-growing ants (Hymenoptera: Formicidae): a year-long survey of three species of attine ants in Central Texas. FEMS Microbiol Ecol. 2011;78:244–55.

15. Parmentier T. Guests of social insects. In: Encyclopedia of Social Insects. 2019.

16. Bourke AFG. Colony size, social complexity and reproductive conflict in social insects. J Evol Biol. 1999;12:245–57.

17. Delabie JHC, Fresneau D, Pezon A. Notes on the ecology of *Thaumatomyrmex* spp. (Hymenoptera: Formicidae: Ponerinae) in Southeast Bahia, Brazil. Sociobiology. 2000;36:571–84.

18. Abbot P. Defense in social insects: diversity, division of labor, and evolution. Annu Rev Entomol. 2022;67:407–36.

19. Tian L, Zhou X. The soldiers in societies: Defense, regulation, and evolution. Int J Biol Sci. 2014;10:296–308.

20. López-Incera A, Nouvian M, Ried K, Müller T, Briegel HJ. Honeybee communication during collective defence is shaped by predation. BMC Biol. 2021;19:1–16.

21. Shorter JR, Rueppell O. A review on self-destructive defense behaviors in social insects. Insectes Soc. 2012;59:1–10.

22. Rutkowski D, Weston M, Vannette RL. Bees just wanna have fungi: A review of bee associations with nonpathogenic fungi. FEMS Microbiol Ecol. 2023;99:1–16.

23. Quevillon LE, Hughes DP. Pathogens, parasites, and parasitoids of ants: a synthesis of parasite biodiversity and epidemiological traits. bioRxiv. 2018;:384495.

24. Sudhaus W. The guild of saprobiontic nematodes associated with ants (Formicoidea). Ecol Montenegrina. 2016;7:600–13.

25. Hofstetter RW, Moser JC. The role of mites in insect-fungus associations. Annu Rev Entomol. 2014;59:537–57.

26. Rodrigues A, Passarini MRZ, Ferro M, Nagamoto NS, Forti LC, Bacci M, et al. Fungal communities in the garden chamber soils of leaf-cutting ants. J Basic Microbiol. 2014;54:1186–96.

27. Kanzaki N, Liang WR, Chiu CI, Yang CT, Hsueh YP, Li HF. Nematode-free agricultural system of a fungus-growing termite. Sci Rep. 2019;9:8917.

28. Howard RW, McDaniel CA, Blomquist GJ. Chemical mimicry as an integrating mechanism: Cuticular hydrocarbons of a termitophile and its host. Science. 1980;210:431–3.

29. Leal-dutra CA, Vizueta J, Baril T, Kooij PW, Rødsgaard-jørgensen A. Genomic signatures of domestication in a fungus obligately farmed by leafcutter ants. Mol Biol Evol. 2024;41.

30. Schultz TR, Gawne R, Peregrine PN. The convergent evolution of agriculture in humans and insects. MIT Press; 2022.

31. Purugganan MD. What is domestication? Trends Ecol Evol. 2022;37:663–71.

32. Sodano J, Oufiero CE, Schneider SA, Lapolla JS. Scale insect (Hemiptera: Coccomorpha)

morphology is transformed under trophobiosis. Ann Entomol Soc Am. 2024;117:49–63.

33. Nelson AS, Mooney KA. The evolution and ecology of interactions between ants and honeydew-producing hemipteran insects. Annu Rev Ecol Evol Syst. 2022;53:379–402.

34. Fischer MK, Shingleton AW. Host plant and ants influence the honeydew sugar composition of aphids. Funct Ecol. 2001;15:544–50.

35. Vanderpool D, Bracewell RR, McCutcheon JP. Know your farmer: Ancient origins and multiple independent domestications of ambrosia beetle fungal cultivars. Mol Ecol. 2018;27:2077–94.

36. Biedermann PHW, Vega FE. Ecology and evolution of insect – fungus mutualisms. Annu Rev Entomol. 2020;65:431–55.

37. Mueller UG, Gerardo NM, Aanen DK, Six DL, Schultz TR. The evolution of agriculture in insects. Annu Rev Ecol Evol Syst. 2005;36:563–95.

38. Conlon BH, O'Tuama D, Michelsen A, Crumière AJJ, Shik JZ. A fungal symbiont converts provisioned cellulose into edible yield for its leafcutter ant farmers. Biol Lett. 2022;18.

39. Sidde Gowda DK, Rajagopal D. Association of *Termitomyces* spp. with fungus growing termites. Proc Anim Sci. 1990;99:311–5.

40. Weber NA. Gardening ants, the attines. American Philosophical Society; 1972.

41. Diehl JMC, Kowallik V, Keller A, Biedermann PHW. First experimental evidence for active farming in ambrosia beetles and strong heredity of garden microbiomes. Proc R Soc B. 2022;289:20221458.

42. Lucas J, Madden AA, Penick CA, Epps MJ, Marting PR, Stevens JL, et al. *Azteca* ants maintain unique microbiomes across functionally distinct nest chambers. Proc R Soc B. 2019;286:20191026.

43. Turillazzi S, Meriggi N, Cavalieri D. Mutualistic relationships between microorganisms and eusocial wasps (Hymenoptera, Vespidae). Microorganisms. 2023;11:1340.

44. de Paula GT, Menezes C, Pupo MT, Rosa CA. Stingless bees and microbial interactions. Curr Opin Insect Sci. 2021;44:41–7.

45. Kaltenpoth M, Engl T. Defensive microbial symbionts in Hymenoptera. Funct Ecol. 2014;28:315–27.

46. Pillay R, Venter M, Aragon-Osejo J, González-del-Pliego P, Hansen AJ, Watson JEM, et al. Tropical forests are home to over half of the world's vertebrate species. Front Ecol Environ. 2022;20:10–5.

47. Brown JH. Why are there so many species in the tropics? J Biogeogr. 2014;41:8–22.

48. Mckey D, Davidson DW. Ant-plant symbioses in Africa and the Neotropics: history, biogeography and diversity. In: Biological relationships between Africa and South America. New Haven: Yale University Press; 1993. p. 568–606.

49. Davidson DW, Mckey D. The evolutionary ecology of symbiotic ant-plant relationships. J Hymenopt Res. 1993;2:13–83.

50. Fischer RC, Richter A, Wanek W, Mayer V. Plants feed ants: Food bodies of myrmecophytic *Piper* and their significance for the interaction with *Pheidole bicornis* ants. Oecologia. 2002;133:186–92.

51. Mayer VE, Frederickson ME, Mckey D, Blatrix R. Current issues in the evolutionary ecology of ant – plant symbioses. 2014;:749–64.

52. O'Dowd DJ. Pearl Bodies as ant food: An ecological role for some leaf emergences of tropical plants. Biotropica. 1982;14:40.

53. Michelangeli FA. Ant protection against herbivory in three species of *Tococa* (Melastomataceae) occupying different environments. Biotropica. 2003;35:181–8.

54. Heil M, McKey D. Protective ant-plant interactions as model systems in ecological and evolutionary research. Annu Rev Ecol Evol Syst. 2003;34:425–53.

55. Janzen DH. Allelopathy by myrmecophytes: The ant *Azteca* as an allelopathic agent of *Cecropia*. Ecology. 1969;50:147–53.

56. Vasconcelos SB. F and HL. The *Azteca-Cecropia* association: Are ants always necessary for their host plants ? Assoc Trop Biol Conserv. 2016;36:641–6.

57. Janzen DH. Dissolution of mutualism between *Cecropia* and its *Azteca* ants. Biotropica. 1973;5:15–28.

58. Chomicki G, Ward PS, Renner SS. Macroevolutionary assembly of ant/plant symbioses: *Pseudomyrmex* ants and their ant-housing plants in the neotropics. Proc R Soc B. 2015;282:20152200.

59. Nelsen MP, Ree RH, Moreau CS. Ant–plant interactions evolved through increasing interdependence. Proc Natl Acad Sci U S A. 2018;115:12253–8.

60. Longino JT. *Azteca* ants in *Cecropia* trees: taxonomy, colony structure, and behaviour. In: Cutler C, Huxley D, editors. Ant-plant interactions. Oxford University Press; 1991. p. 271–88.

61. Janzen DH. Interaction of the bull's-horn acacia (Acacia cornigera L.) with an ant inhabitant (Pseudomyrmex ferruginea F.Smith) in eastern Mexico. In: The University of Kansas Science Bulletin. 1967.

62. Vasse M, Voglmayr H, Mayer V, Gueidan C, Nepel M, Moreno L, et al. A phylogenetic perspective on the association between ants (Hymenoptera: Formicidae) and black yeasts (Ascomycota: Chaetothyriales). Proc R Soc B. 2017;284:20162519.

63. Voglmayr H, Mayer V, Maschwitz U, Moog J, Djieto-Lordon C, Blatrix R. The diversity of ant-associated black yeasts: insights into a newly discovered world of symbiotic interactions. Fungal Biol. 2011;115:1077–91.

64. Mayer VE, Voglmayr H, Blatrix R, Orivel J, Leroy C. Fungi as mutualistic partners in ant-plant interactions. Front Fungal Biol. 2023;4 October:1213997.

65. Teixeira MM, Moreno LF, Stielow BJ, Muszewska A, Hainaut M, Gonzaga L, et al. Exploring the genomic diversity of black yeasts and relatives (Chaetothyriales, Ascomycota). Stud Mycol. 2017;86:1–28.

66. Moreno LF, Mayer V, Voglmayr H, Blatrix R, Benjamin Stielow J, Teixeira MM, et al. Genomic analysis of ant domatia-associated melanized fungi (Chaetothyriales, Ascomycota). Mycol Prog. 2019;18:541–52.

67. Nepel M, Voglmayr H, Blatrix R, Longino JT, Fiedler K, Schönenberger J, et al. Ant-cultivated Chaetothyriales in hollow stems of myrmecophytic *Cecropia* sp. trees – diversity and patterns. Fungal Ecol. 2016;23:131–40.

68. Nepel M, Pfeifer J, Oberhauser FB, Richter A, Woebken D, Mayer VE. Nitrogen fixation by diverse diazotrophic communities can support population growth of arboreal ants. BMC Biol. 2022;20:135.

69. Esquivel A, Abolafia J, Hanson P, Pinto A. A new species of nematode, *Sclerorhabditis neotropicalis* sp.n. (Rhabditida), associated with *Azteca* ants in *Cecropia obtusifolia*. Nematropica. 2012;42:163–9.

70. Nepel M, Mayer VE, Barrajon-Santos V, Woebken D. Bacterial diversity in arboreal ant nesting spaces is linked to colony developmental stage. Commun Biol. 2023;6:1217.

71. Quate LW, Brown B V. Revision of neotropical Setomimini (Diptera: Psychodidae: Psychodinae). Contributions in Science; 2004.

72. Lindquist EE, Moraza ML. A new genus of mites of the subfamily Platyseiinae associated with *Azteca* ant galleries in *Cecropia* trees in Costa Rica (Acari: Mesostigmata: Blattisociidae). Acarologia. 2016;56:293–319.

73. Blatrix R, Bouamer S, Morand S, Selosse MA. Ant-plant mutualisms should be viewed as symbiotic communities. Plant Signal Behav. 2009;4:554–6.

74. Lucas J, Bill B, Stevenson B, Kaspari M. The microbiome of the ant-built home: the microbial communities of a tropical arboreal ant and its nest. Ecosphere. 2017;8:e01639.

75. Skutch AF. The most mospitable tree. Sci Mon. 1945;60:5–17.

76. Berg CC, Roselli PF, Davidson DW. *Cecropia*. Flora Neotrop. 2005;:1–230.

77. Gutiérrez-Valencia J, Chomicki G, Renner SS. Recurrent breakdowns of mutualisms with ants in the neotropical ant-plant genus *Cecropia* (Urticaceae). Mol Phylogenet Evol. 2017;111:196–205.

78. Longino JT. A taxonomic review of the genus *Azteca* (Hymenoptera: Formicidae) in Costa Rica and a global revision of the aurita group. Zootaxa. 2007;1491:1–63.

79. Marting PR. Collective Personality in the *Azteca-Cecropia* Mutualism. 2018.

80. Longino JT. Geographic Variation and Community Structure in an Ant-Plant Mutualism: *Azteca* and *Cecropia* in Costa Rica. Biotropica. 1989;21:126.

81. Folgarait PF, Johnson HL, Davidson DW. Responses of *Cecropia* to Experimental Removal of Mullerian Bodies. Funct Ecol. 1994;8:22–8.

82. Vasconcelos HL, Casimiro AB. Influence of *Azteca alfari* ants on the exploitation of *Cecropia* trees by a leaf-cutting ant. Biotropica. 1997;29:84–92.

83. Wcislo A, Graham X, Stevens S, Toppe JE, Wcislo L, Wcislo WT. *Azteca* ants repair damage to their *Cecropia* host plants. J Hymenopt Res. 2021;88:61–70.

84. Bischof S, Umhang M, Eicke S, Streb S, Qi W, Zeeman SC. *Cecropia peltata* accumulates starch or soluble glycogen by differentially regulating starch biosynthetic genes. Plant Cell. 2013;25:1400–15.

85. Oliveira KN, Coley PD, Kursar TA, Kaminski LA, Moreira MZ, Campos RI. The effect of symbiotic ant colonies on plant growth: A test using an *Azteca-Cecropia* system. PLoS One. 2015;10:e0120351.

86. Müller F. Ueber das Haarkissen am Blattstiel der Imbauba (*Cecropia*), das Gemüsebeet der Imbauba-Ameise. Jenaische Zeitschrift fur Medizin und Naturwiss. 1876;10:281–6.

87. Schupp EW. *Azteca* protection of *Cecropia*: ant occupation benefits juvenile trees. Oecologia. 1986;70:379–85.

88. Andrade JC, Carauta JPP. The *Cecropia-Azteca* association : A case of mutualism? Biotropica. 1982;14:15.

89. Morera J, Mora-Pineda G, Esquivel A, Hanson P. Detection, ultrastructure and phylogeny of *Sclerorhabditis neotropicalis* (Nematoda: Rhabditidae) nematodes associated with the *Azteca* ant-*Cecropia* tree symbiosis. Rev Biol Trop. 2018;66:368–80.

90. Rickson FR. Anatomical development of the leaf trichilium and Müllerian bodies of *Cecropia peltata* L. Am J Bot. 1976;63:1266–71.

91. Marting PR, Wcislo WT, Pratt SC. Colony personality and plant health in the *Azteca-Cecropia* mutualism. Behav Ecol. 2018;29:264–71.

92. Folgarait PJ, Davidson DW. Antiherbivore defenses of myrmecophytic *Cecropia* under different light regimes. Oikos. 1994;71:305–20.

93. Longino JT. Ants of Costa Rica. Key to *Azteca* of Costa Rica. 2007. https://ants.biology.utah.edu/genera/azteca/key.html.

94. Starr CK. Encyclopedia of Social Insects. Springer; 2021.

95. Busch JS, Mayer VE. Surface structures of *Cecropia* plants as landmarks for orientation of ant foundress queens ? Acta ZooBot Austria. 2019;156:135–44.

96. Mayer VE, Nepel M, Blatrix R, Oberhauser FB, Fiedler K, Schönenberger J, et al. Transmission of fungal partners to incipient *Cecropia*-tree ant colonies. PLoS One. 2018;13:e0192207.

97. Verhaag M, Verhaagh M. *Pachycondyla luteola* (Hymenoptera, Formicidae), an inhabitant of *Cecropia* trees in Peru. Andrias. 1994;13:215–24.

98. Choe JC, Perlman DL. Social conflict and cooperation among founding queens in ants.pdf. In: The Evolution of Social Behaviour in Insects and Arachnids. Cambridge University Press; 1997. p. 392–406.

99. Barrajon-Santos V, Nepel M, Hausmann B, Voglmayr H, Woebken D, Mayer VE. Dynamics and drivers of fungal communities in a multipartite ant-plant association. BMC Biol. 2024;22:112.

Overview of Manuscripts

Frist author manuscripts included in this PhD thesis

Manuscript I

Title: Dynamics and drivers of fungal communities in a multipartite ant-plant association

Authors: Veronica Barrajon-Santos, Maximilian Nepel, Bela Hausmann, Hermann Voglmayr, Dagmar Woebken and Veronika E. Mayer

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Manuscript II

Title: Hidden guests of ant-plant mutualisms: unravelling the nematode dynamics in the nest of the *Azteca-Cecropia* complex

Authors: Veronica Barrajon-Santos, Maximilian Nepel, Bela Hausmann, Dagmar Woebken and Veronika E. Mayer

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Author contributions: V.E.M., M.N. and D.W. designed the research. V.B.S., V.E.M. and M.N. performed the research. V.B.S. and B.H. contributed with analytical tools. V.B.S. and B.H. processed and analysed the data; V.B.S analysed and interpreted the results. V.E.M. and D.W. provided significant intellectual contribution. V.B.S wrote the original draft of the manuscript. All authors contributed to the final version of the manuscript and approved it for publication.

Manuscript III

Title: Composting in an ant-plant nest? Activity and metabolic potential of ant-associated microbial communities for degrading polysaccharide-rich substrates

Authors: Veronica Barrajon-Santos, Veronika E. Mayer, Maximilian Nepel, Joana Séneca, Dominik Zöllner, Michael Poulsen, Thomas Rattei and Dagmar Woebken

Status: Manuscript in preparation for publication

Author contributions: V.E.M., M.N. and D.W. designed this study. V.B.S., V.E.M. and M.N. conducted fieldwork and collected the samples. V.B.S., J.S., D.Z. and T.R. contributed with analytical tools. J.S. performed sample preparation and sequencing. V.B.S. and J.S. processed the metagenomics data. D.W., V.E.M., M.P. and T.R. provided significant intellectual contribution. V.B.S analysed the data and wrote the original draft of the manuscript. All authors contributed to the final version of the manuscript and approved it for publication.

Additional manuscripts not included in this PhD thesis

Manuscript IV

Title: Bacterial diversity in arboreal ant nesting spaces is linked to colony developmental stage

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Chapter I

Dynamics and drivers of fungal communities in a multipartite ant-plant association

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Abstract

Background: Fungi and ants belong to the most important organisms in terrestrial ecosystems on Earth. In nutrient-poor niches of tropical rainforests, they have developed steady ecological relationships as a successful survival strategy. In tropical ant-plant mutualisms worldwide, where resident ants provide the host plants with defence and nutrients in exchange for shelter and food, fungi are regularly found in the ant nesting space, inhabiting ant-made dark-coloured piles ("patches"). Unlike the extensively investigated fungus-growing insects, where the fungi serve as the primary food source, the purpose of this ant-fungi association is less clear. To decipher the roles of fungi in these structures within ant nests, it is crucial to first understand the dynamics and drivers that influence fungal patch communities during ant colony development.

Results: In this study, we investigated how the ant colony age and the ant-plant species affect the fungal community in the patches. As model we selected one of the most common mutualisms in the Tropics of America, the *Azteca-Cecropia* complex. By amplicon sequencing of the internal transcribed spacer 2 (ITS2) region, we analysed the patch fungal communities of 93 *Azteca* spp. colonies inhabiting *Cecropia* spp. trees. Our study demonstrates that the fungal diversity in patches increases as the ant colony grows, and that a change in the prevalent fungal taxa occurs between initial and established patches. In addition, the ant species significantly influences the composition of the fungal community in established ant colonies, rather than the host plant species.

Conclusions: The fungal patch communities become more complex as the ant colony grows, due to an acquisition of fungi from the environment and a substrate diversification. Our results suggest a successional progression of the fungal communities in the patches during ant colony growth and place the ant colony as the main driver shaping such communities. These findings demonstrate the unexpectedly complex nature of ant-plant mutualisms in tropical regions at a micro scale.

Keywords: Ant-plant mutualism, fungal communities, *Azteca*, *Cecropia*, insect-fungus interactions, tropical ecosystems, community dynamics, ant-made patches

Background

Plants, ants and fungi are key players in terrestrial ecosystems all over the world. While the role of plants is obvious, ants and fungi are often less understood. However, both groups have an enormous biomass [1, 2], and provide numerous important ecosystem functions. Ants turn and aerate the soil by digging nests and tunnels and contribute considerably to nutrient redistribution through scavenging large amounts of carrion and plant debris [3, 4]. Recent studies indicate that they are likely to be functionally non-replaceable in their foraging roles in tropical rainforests [4]. Fungi, with an estimated > 3 million species [5], are key players in soils being the dominant decomposers of the complex components of plant debris such as cellulose and lignin. While fungi are regularly found affecting the health of plants and animals as pathogens [6], they have also established mutualistic relationships with a wide range of organisms (e.g. lichens, mycorrhizae, insect-cultivated fungal gardens) [7–9].

In habitats where nutrient availability is notoriously low, like in tropical rainforests [10, 11], steady relationships between arthropods and fungi seem to be a recurrent survival strategy [12]. These interactions often have nutritional implications where arthropods either feed on fungi or indirectly benefit from their fungal enzymatic activity [9, 13–16]. In mutualistic associations, fungi are often rewarded with the dispersal of spores and constantly supplied with plant material as substrate [9, 17, 18]. Termites (Blattodea, Termitidae) and leaf-cutter ants (Hymenoptera, Formicidae) are examples for such mutualisms; the insects cultivate basidiomycetes for decomposing plant material they cannot digest themselves and feed on nutrient-rich fungal nodules [15, 19–22]. Similarly, ambrosia beetles (Coleoptera, Curculionidae) maintain complex fungal communities in their nests and use them as sole food source [16, 23].

In arboreal ants, and particularly in those that maintain mutualistic interactions with their hosting tree, a tripartite ant-plant-fungi association has been regularly documented [24–26]. Since the early 20th century, slow-growing fungi, most of them from the order Chaetothyriales (Eurotiomycetes), have been repeatedly detected in the plant cavities used by the ants as nesting spaces (domatia) [27–30]. Unlike the mutualistic relationships between fungi and termites, leaf-cutter ants or bark beetles,

the purpose of the association between ants and domatia-inhabiting fungi is less obvious as the host plant already provides nutrient resources (e.g. food bodies or extrafloral nectar) to the ant colony [31–34]. By next generation sequencing, several investigations recently showed that, in addition to Chaetothyriales, there is a highly diverse fungal community inhabiting the domatia of different ant-plant associations [35–37]. These studies have shown that the fungal community composition varies spatially between differently used nest chambers of the same host plant and is also different from the surrounding soil.

However, we are still lacking crucial information about the dynamics of fungal communities associated with ant-plant mutualisms. To study this, we chose the *Azteca-Cecropia* association as a model system. The interplay between the pioneer trees *Cecropia* spp. (Urticaceae) and their partner ants *Azteca* spp. (Formicidae, Dolichoderinae) is one of the most widespread and successful mutualisms in the Tropics of America [38]. *Azteca* ants defend their host plant against herbivores, phytopathogens and plant competitors [39–43]. In return, *Cecropia* trees provide ants with a nesting space inside the hollow stem (domatium) and plant-derived food bodies known as Müllerian bodies [44–46]. In this association, fungi, as well as bacteria and nematodes, are transgenerationally transmitted by the foundress queen who transfers these organisms to a pile of parenchyma known as "patch" [25, 35, 47–50].

Several observations provide evidence of the importance of these patches for the *Azteca-Cecropia* association (Fig. 1). First, it was observed in 180 *Cecropia* saplings that the *Azteca* queens form the first patch before they start to lay their eggs [47]. Second, *Azteca* workers deposit plant tissue, ant faeces and ant corpses onto patches and constantly shape and manipulate them [24, 32, 47, 48]. Third, patch structures were found in almost every internode of the 93 colonies investigated, even in those with brood [48]. Last, none of the *Azteca* colonies inhabiting *Cecropia* stems from this study were found without patches in their nest.

Although the patches and the fungi they contain are recognized as permanent components in the *Azteca-Cecropia* mutualism [25, 31, 47, 48], nothing is currently known about the establishment of the fungal communities during the life cycle of ant colonies, nor of the influence of the inhabiting ant species and the host-plant species.

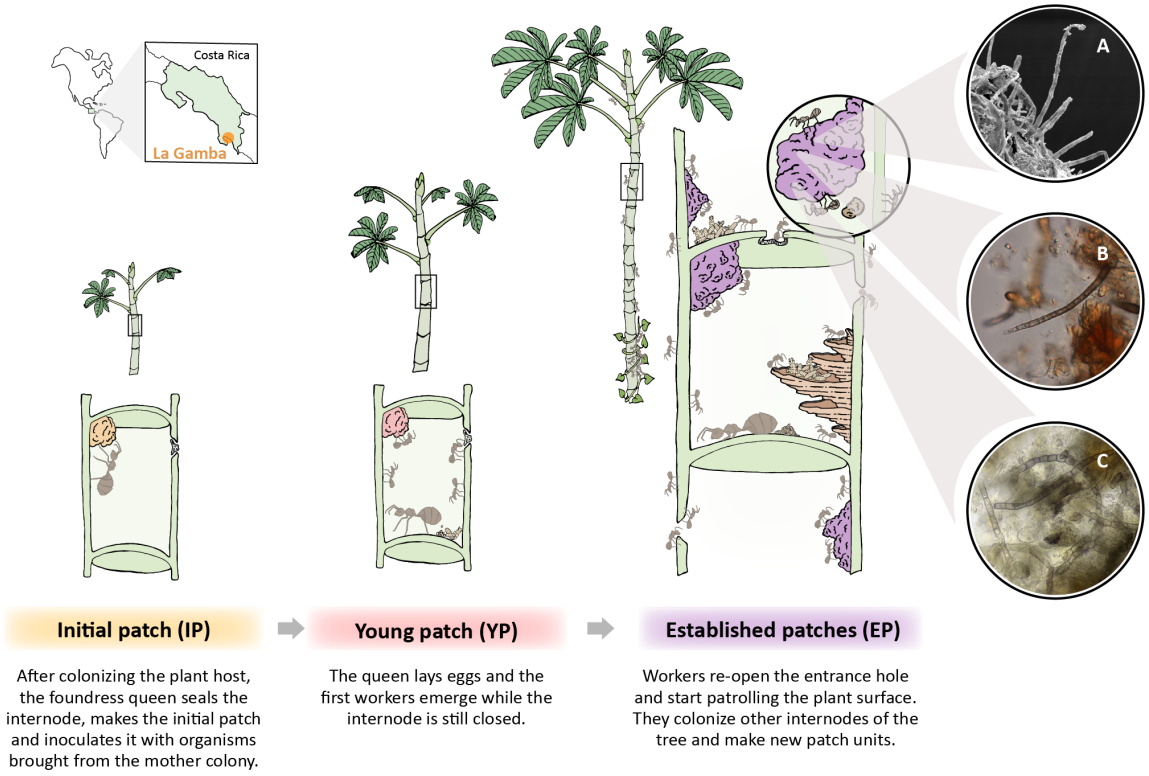

Figure 1. Graphic illustration of the *Azteca-Cecropia* association including ant-made patches from the three different ant colony development stages: initial patch (IP); young patch (YP); established patches (EP). Microscopic images of hyphae from established patches using scanning electron microscopy (A) and light microscopy (B and C). The map represents the geographic location of the sampling of this investigation (La Gamba, Puntarenas, Costa Rica).

By analysing amplicon sequence data of the ITS2 region, we investigated the fungal communities inhabiting patches of 93 colonies from three different *Azteca* species inhabiting *Cecropia* spp. Based on previous research [42, 47, 48, 51], we hypothesize that fungal diversity increases during ant colony development due to the increasing foraging and patrolling activity while the colony grows. This leads to the incorporation of spores or hyphal fragments from the environment into the patches. As ants are known to produce specific gland secretions that inhibit the germination of fungal spores and the growth of fungal hyphae [52–54], we expect a similar fungal community in patches from established colonies of the same ant species. And finally, we expect that the ant species plays a more significant role in influencing the composition of fungal patch communities than the plant species, given their evident dominance within the nesting environment [24, 35, 55]. Understanding the spatio-temporal dynamics of the fungal communities in the patches will help to unravel the purpose of these striking structures within the ant nests.

Results

Amplicon sequencing of the ITS2 region from 93 *Azteca* ant colonies (Additional File 1: Table S1) yielded 1749 amplicon sequence variants (ASVs), of which 1280 ASVs (= 86.93% of total reads) were assigned to the kingdom Fungi, and more specifically, to 26 different fungal classes. Relative read abundance of each fungal taxon will be from now on referred to as relative abundance.

a) Influence of the ant colony development on the fungal patch diversity

In the ant species *A. alfari* and *A. constructor*, we detected a significantly higher fungal alpha diversity in established patches than in the initial patches (p = 0.0008, and p = 0.0227, respectively) (Fig. 2A; Additional File 2: Tables S1-S2). Since *A. xanthochroa* colonies were only found at the initial stage, diversity comparisons could not be performed with this ant species. In initial patches of 40 *Azteca* spp. colonies, on average 4 ± 2 ASVs out of 31 ± 14 fungal ASVs accounted for at least 90% of total reads (Additional File 3: Table S1). Fungal communities of initial patches were dominated by ASVs assigned to classes Sordariomycetes (58.3% mean relative abundance), Ustilaginomycetes (20.8% mean relative abundance), Eurotiomycetes (8.9% mean relative abundance), and Dothideomycetes (4.9% mean relative abundance), except for three patches that were dominated by ASVs assigned to Mucoromycetes (Fig. 2C). These five classes represented 98.3% of total reads in all initial patches collected.

Young patches from 15 *A. alfari* colonies were significantly more diverse than initial patches and less diverse than established patches (Fig 2.A; Additional File 2: Table S1). In young colonies, classes Agaricomycetes and Leotiomycetes, which were not abundant in initial patches, increased to 8.3% and 5.7% mean relative abundance, respectively (Fig. 2C). Young patches from two *A. constructor* colonies showed a contrasting pattern: they harboured communities of slightly lower diversity than initial patches (Fig. 2A; Additional File 2: Table S2). This finding is most likely due to the notably low number of young *A. constructor* colonies included in the study.

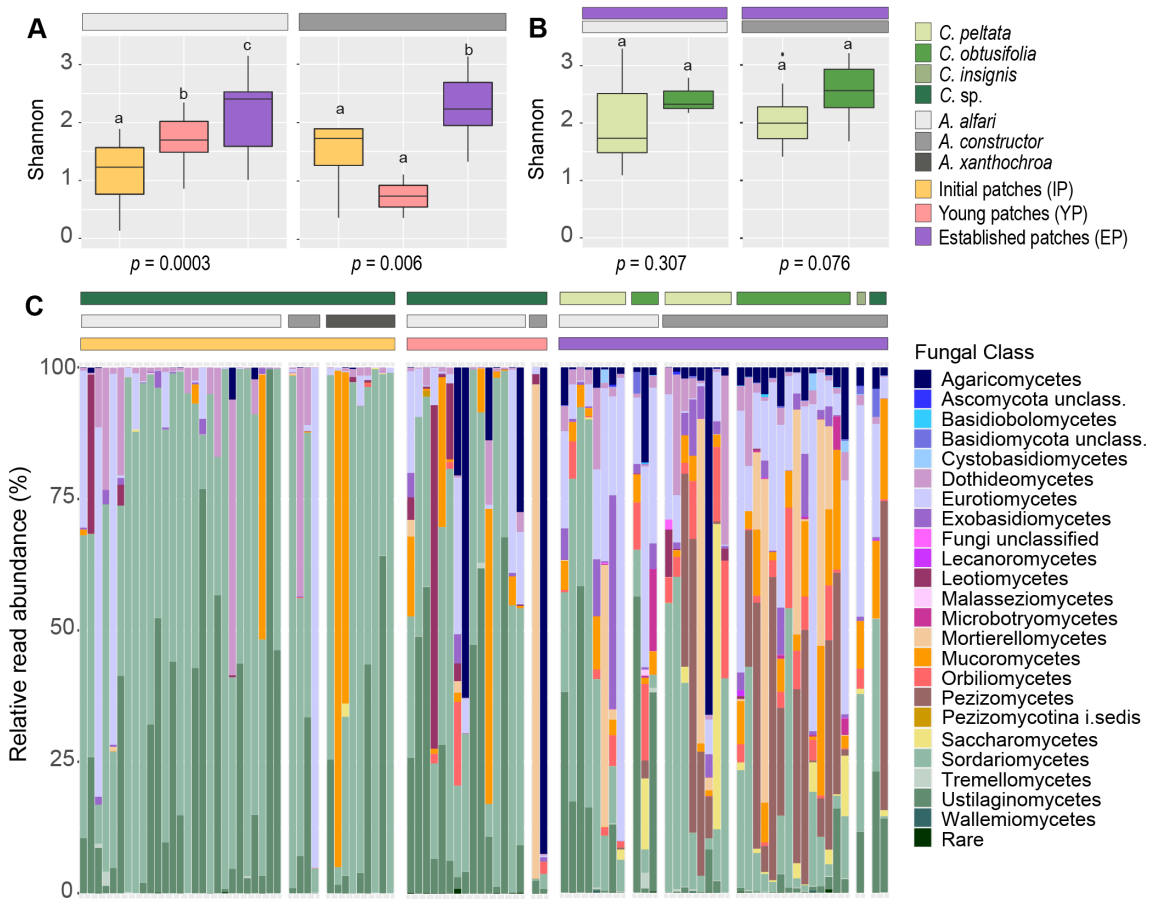

Figure 2. Diversity and taxonomic overview of fungal communities inhabiting ant-built patches. (A) Alpha diversity metrics (Shannon Index) of each ant species at different ant colony development stages (*A. alfari*: IP *n* = 27, YP *n* = 15, EP *n* = 12; *A. constructor*: IP *n* = 4, YP *n* = 2, EP *n* = 24). (B) Alpha diversity metrics (Shannon Index) of established colonies of each ant species inhabiting different plant species (*A. alfari*: *C. peltata n* = 8, *C. obstusifolia n* = 3; *A. constructor*: *C. peltata n* = 8, *C. obstusifolia n* = 14). In both cases (A, B), statistical comparisons ($p < 0.05$) by Kruskal-Wallis and Wilcoxon post-hoc tests are shown. (C) Relative read abundances (%) of abundant fungal classes (>0.5%) per ant colony, grouped by colony developmental stage, ant and plant species. Low abundant taxa (<0.5%) merged as "Rare".

The taxonomic composition of fungal patch communities from 36 established colonies revealed a high heterogeneity (Fig. 2C). Generally, established patches consisted of a few read-abundant ASVs and a high diversity of low abundant ASVs. On average, 15 ± 11 ASVs out of 189 ± 77 fungal ASVs accounted for at least 90% of total reads (Additional File 3: Table S1). In this ant colony developmental stage, we detected 11 different classes with more than 2.5% mean relative abundance, where Sordariomycetes, Eurotiomycetes and Pezizomycetes showed the highest relative

abundance (20.4%, 19% and 14.3%, respectively). In established patches, alpha diversity of fungal communities in each ant species did not vary between plant species (Fig. 2B; Additional File 2: Table S3-S4).

b) Effect of ant and plant species on the fungal community composition

To evaluate if the fungal community composition was significantly influenced by the ant or plant species, we performed beta diversity analyses based on Bray-Curtis distances among colonies (Fig. 3; Additional File 4). For initial patches, the PERMANOVA test showed no correlation between the fungal community variation and the ant species (p = 0.197). When comparing fungal community composition from established patches, we could detect a significant influence by the ant species (p = 0.001), but not by the plant species in neither *A. alfari* nor *A. constructor* colonies (p = 0.333 and p = 0.337, respectively). Since the sample size was notably unbalanced in most statistical analyses, additional PERMDISP and MiRKAT tests were performed in this study to provide sufficient statistical robustness (Additional File 4).

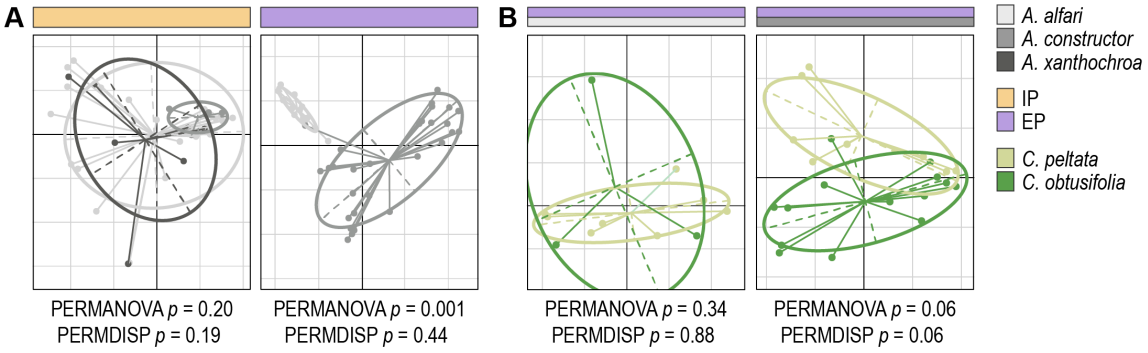

Figure 3. Beta diversity analysis of fungal community composition inhabiting ant-made patches represented by Principal Coordinate Analysis (PCoA) ordination using a Bray-Curtis dissimilarity distance matrix. (A) Comparison of different ant species per ant colony developmental stage (*A. alfari*: IP n = 27, EP n = 12; *A. constructor*: IP n = 4, EP n = 24; *A. xanthochroa*: IP n = 9). (B) Comparison of different plant species per ant species in established patches (*A. alfari*: *C. peltata* n = 8, *C. obstusifolia* n = 3; *A. constructor*: *C. peltata* n = 8, *C. obstusifolia* n = 14). Statistical analyses (p < 0.05) by PERMANOVA and PERMDISP tests are shown.

As established patches showed the most diverse and distinct fungal communities, we used this developmental stage for further analysis at lower taxonomic levels. When looking at the 30 most abundant ASVs, we observed that certain ASVs were highly abundant and common particularly in colonies of one ant species (Fig. 4). The most abundant ASV from *A. constructor* (ASV_37) was assigned to unclassified Pyronemataceae (Pezizomycetes, 19.8% mean relative abundance), yet, this ASV, and the family it belonged to, was present at only very low frequencies in patches from *A. alfari* (0.3% mean relative abundance) (adjusted $p < 0.0001$). Contrarily, ASV_02 belonging to the genus *Moesziomyces* (Ustilaginomycetes, Ustilaginaceae) was significantly more abundant in *A. alfari* (14.6% mean relative abundance) than in *A. constructor* (0.7% mean relative abundance) (adjusted $p = 0.0012$). Moreover, the second and third most abundant ASVs (ASV_03 and ASV_12), which belonged to two separate clusters of the Cyphellophoraceae family (Eurotiomycetes, Additional File 5: Fig. S1) [25, 47, 56–59], were significantly more predominant in one of the two ant species (adjusted $p = 0.0003$, and, adjusted $p < 0.0001$, respectively).

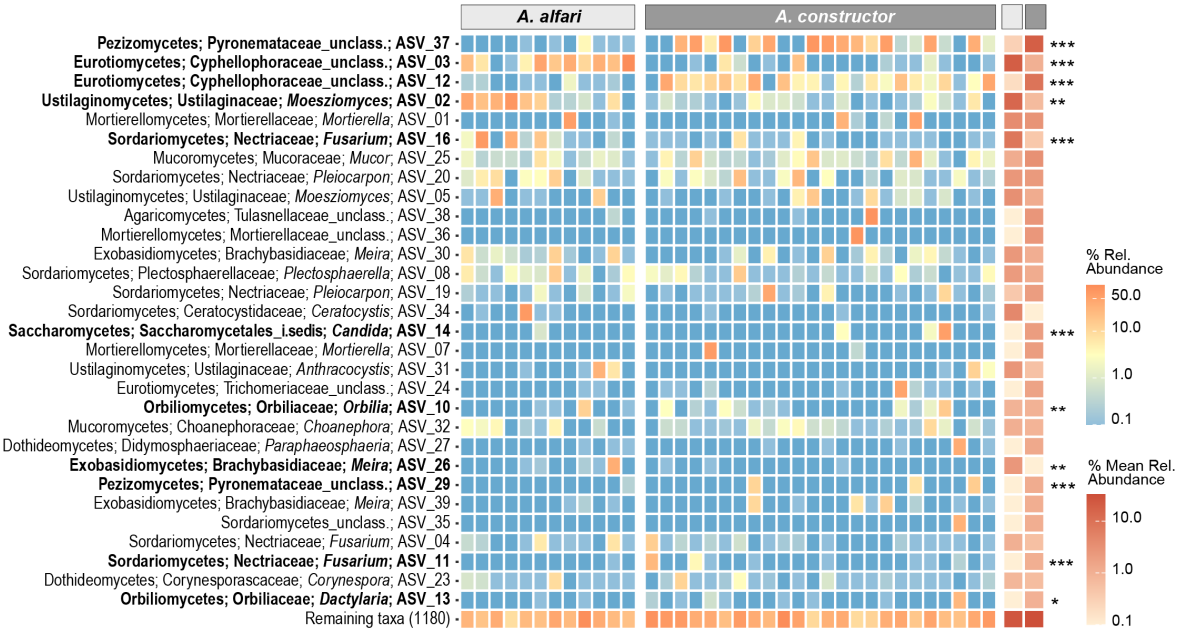

Figure 4. Heatmap depicting relative read abundances of the 30 most abundant fungal ASVs in patches from established ant colonies. Relative abundances of ASVs are shown per individual ant colony of each ant species (left, blue-orange) and as the average over all ant colonies per ant species (right, beige-terracotta). Relative abundances of ASVs between ant species are statistically compared by using DESeq2 analysis (adjusted *p* values: * < 0.05, ** <0.01 and *** <0.001). ASVs with significant different rel. abundances between ant species are depicted in bold.

c) Frequent fungal taxa in the patches and their dynamics over ant colony age

To determine which fungal taxa are widely distributed in *Azteca-Cecropia* patches and how they change with ant colony age, we first searched for frequent fungal ASVs among all established colonies in each ant species. Frequent ASVs were defined as those that were present in at least half of the samples of each ant species with a mean relative abundance of at least 0.05%. Only 13 and 14 ASVs were detected as frequent in *A. alfari* and *A. constructor* colonies, respectively, from which 7 ASVs were frequent in both ant species (Fig. 5).

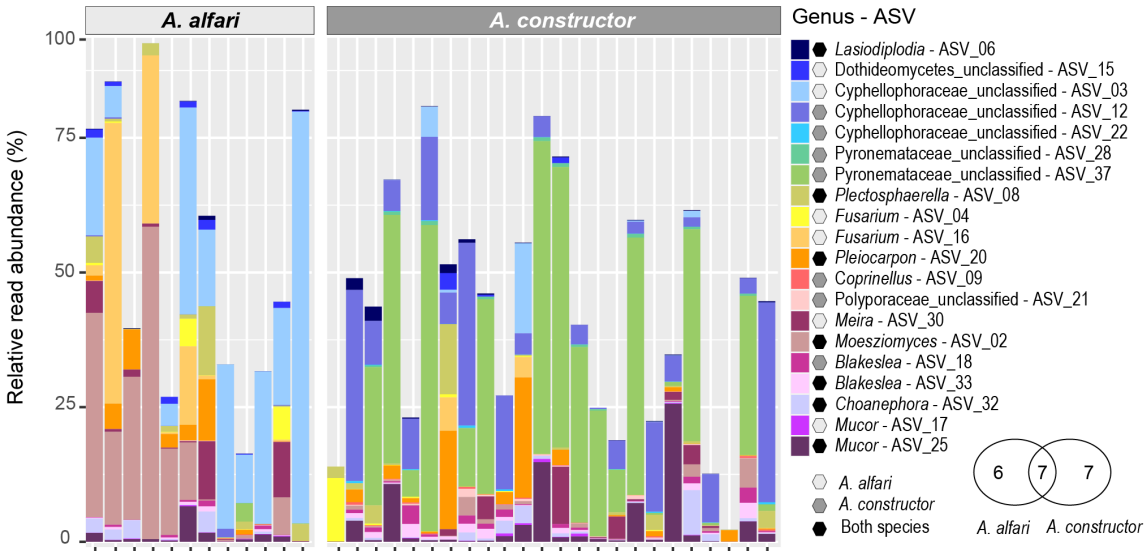

Figure 5. Taxonomic distribution of frequent fungal ASVs (present in more than 50% of colonies per ant species with a mean relative read abundance of >0.05%) in proportion to the overall fungal diversity (100%) detected in patches from each established colony. ASVs that were defined as frequent only in *A. alfari* are indicated with light grey hexagons, ASVs defined as frequent only in *A. constructor* with dark grey hexagons and ASVs defined as frequent in both ant species with black hexagons. Venn diagram shows number of frequent ASVs in either one or both ant species.

Frequent ASVs accounted for a mean relative abundance of 54.96% in patches of *A. alfari* colonies and 39.76% in patches of *A. constructor* colonies. Amongst others, ASVs belonging to the genus *Fusarium* (ASV_04 and ASV_16, Sordariomycetes, Nectriaceae) were present in both ant species, but they were only defined as frequent in *A. alfari* patches. After defining the frequent ASVs, we investigated if the relative abundance of the genera they belong to varied among patch types (developmental stages and tree sections) in all *Azteca* sp.- *Cecropia* colonies jointly (Fig. 6).

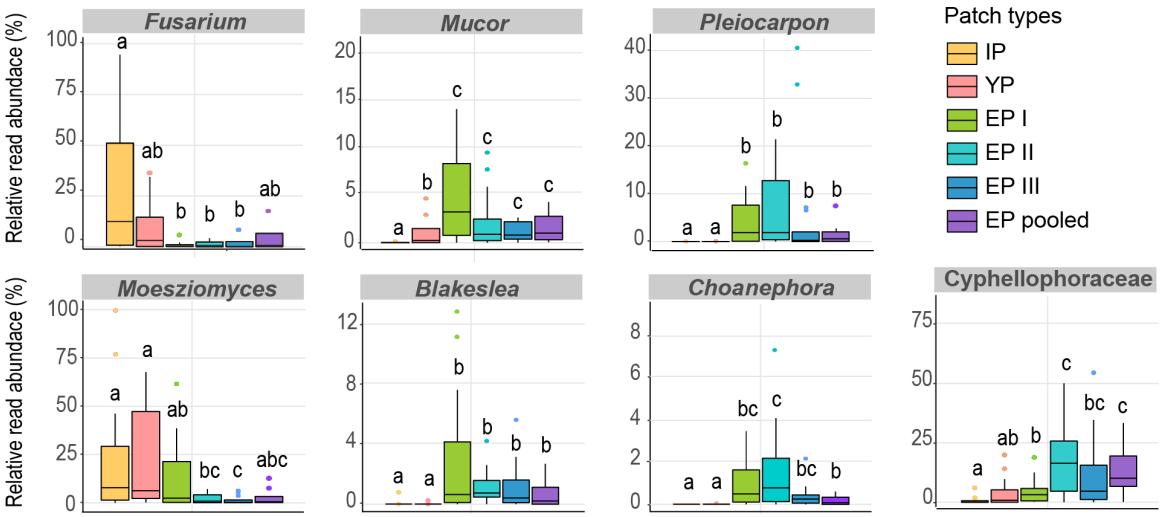

Figure 6. Relative read abundance (%) of selected genera encompassing frequent ASVs from patches of *Azteca* spp. Comparisons are made among ant colony development stages and tree sections within established colonies (initial patches, IP n = 40; young patches, YP n = 17; upper internode patches, EP I n = 9; intermediate internode patches, EP II n = 10; lower internode patches, EP III n = 6; all internode patches, EP pooled n = 11). Statistical comparisons are calculated by Kruskal-Wallis and Wilcoxon post-hoc test ($p < 0.05$).

While ASVs belonging to *Fusarium* (Sordariomycetes, Nectriaceae) were predominant in initial patches, their relative abundance significantly decreased in patches of established colonies (EP I, EP II and EP III). *Moesziomyces* ASVs (Ustilaginomycetes, Ustilaginaceae) were notably abundant in initial patches, young patches and patches from the upper part of the tree in established colonies (EP I). *Mucor* (Mucoromycetes, Mucoraceae) and *Blakeslea* ASVs (Mucoromycetes, Choanephoraceae) presented an especially high relative abundance in upper internodes patches compared to patches from other tree sections and earlier colony developmental stages. Other ASVs belonging to Cyphellophoraceae family (Eurotiomycetes) considerably increased in relative abundance in patches from several established colonies, especially in the middle and most active part of the tree (EP II, 16.2% mean relative abundance) where brood and queen are typically found. Similarly, *Pleiocarpon* (Sordariomycetes, Nectriaceae), and *Choanephora* (Mucoromycetes, Choanephoraceae) ASVs were significantly more abundant in established than in initial patches.

Discussion

a) The fungal diversity in the patches increases with the ant colony development

We showed that the fungal communities become more complex as the ant colony grows, indicated by a significant increase in alpha diversity from initial to established patches. This may be due to two factors (Fig. 7): changes in the patch substrate during colony development and an increasing transfer of fungal spores from the environment. First, after entering the domatia, the founding queen makes the initial patch by scratching parenchyma tissue from the inner domatia wall and inoculates it with patch particles she brought from the mother colony [47]. The cellulose-dominated substrate appears to cause a bottleneck in the early establishment of the fungal patch community. This phenomenon has already been observed in the bacterial community of the same patches [48] and was explained by the N-deficiency of the parenchyma which favours the growth of organisms that are adapted to the low nitrogen content [60]. As the colony develops, ant workers make new patch structures in almost every internode they colonize.

Additionally, ant workers diversify the substrate by adding different plant material such as trichomes and by depositing their faeces and the carcasses of dead nestmates and insect prey onto those patches [25, 32]. The subsequent creation of more diverse micro-niches in the patches of established colonies enhance the development of a more complex community. Second, the vertical transmission of microorganisms by the founding queen is followed by an environmental acquisition through: (i) ant-workers patrolling and foraging on the host-plant surface [43, 51]; (ii) opportunistic patch visitors such as dipteran larvae and mites [61, 62]; and, (iii) the air flow via the domatium entrance. While some fungi may indeed find a suitable niche in the patch environment, others may be inhibited by the high volatile concentration [63] or the fungicidal gland secretions [52–54] and remain as spores in the so-called microbial seed bank [64]. It is important to note that the widely used DNA-based identification approaches, such as the one used in this study, include both the active and the dormant fungal communities inhabiting the patches [65].

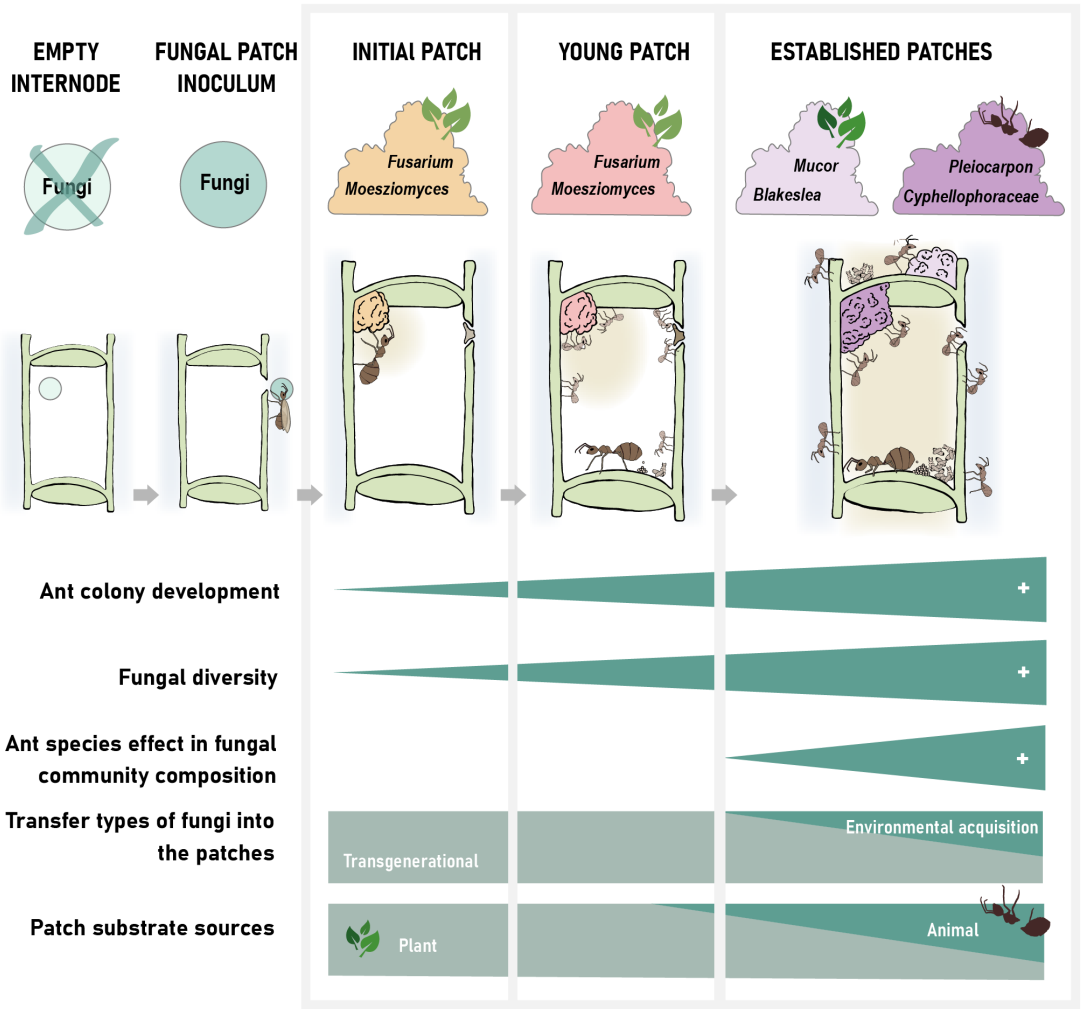

Figure 7. Conceptual illustration showing the successional progression of the fungal communities inhabiting ant-made patches from the *Azteca-Cecropia* association driven by the ant species, the diversification of substrates and the transfer types of fungi.

b) Frequent fungal genera differ between initial and established patches

The change in relative abundances of the most frequent fungal genera across the different stages of the ant colony development indicates a successional progression within the fungal patch community over time (Fig. 7). ASVs belonging to the ubiquitous and fast-growing genera *Fusarium* (Sordariomycetes, Nectriaceae) [66] and *Moesziomyces* (Ustilaginomycetes, Ustilaginaceae) [67] were dominant in initial and young patches of all *Azteca* sp.- *Cecropia* sp. colonies investigated. Given their typical saprotrophic feeding strategy [67–69], members of these groups may be able to initiate organic matter decomposition processes in cellulose-dominated patches of

early stages of the ant colony. Apart from the *Azteca-Cecropia* complex, *Fusarium* was detected in domatia of the myrmecophytic plants *Acacia drepanolobium* from Africa [37] and *Myrmecodia beccari* from Australia [36]. However, the authors did not distinguish between initial and established patches.

In the upper and younger internodes of established colonies, cellulose is also the dominant substrate, but in contrast to the initial patch where the foundress queen is the only ant in the internode, there are many more ants around. The secretions and behaviour of the ant workers may cause a shift in fungal taxa as a significantly lower relative abundance of *Fusarium* sp. ASVs and a higher relative abundance of *Mucor* sp. ASVs (Mucoromycetes, Mucoraceae) and *Blakeslea* sp. (Mucoromycetes, Choanephoraceae) ASVs were detected.

In the middle and the basal internodes of established colonies, carcasses of dead nestmates are additionally added to the patches and probably used as substrate. The most prevalent ASVs in this tree section belong to Cyphellophoraceae (Eurotiomycetes, Chaetothyriales) and the genera *Pleiocarpon* (Sordariomycetes, Nectriaceae) and *Choanephora* (Mucoromycetes, Choanephoraceae). While *Pleiocarpon* and *Choanephora* have never been found in any ant-plant-association investigated so far, Cyphellophoraceae are known from many other ant-plant associations all over the tropics worldwide [24, 25, 27, 31, 36, 37]. The finding that Cyphellophoraceae is most abundant in established colonies, particularly in the stem regions of the nurseries [42], suggests a steady and direct ecological relationship between this particular group of fungi and the ant colony.

c) Chaetothyriales fungi and their potential ecological roles in the patches

Microscopic examination of many ant-plant associations and subsequent cultivation identified Chaetothyriales as the most conspicuous and abundant fungal inhabitants of the domatia [25, 27, 30, 70]. The Chaetothyriales ITS sequences from the data set in the present study cluster in a monophyletic clade of uniquely domatia-inhabiting Chaetothyriales from Africa, Asia and the Americas. Their frequent and exclusive occurrence in geographically distant ant-colonized domatia of ant-plant mutualisms studied so far worldwide [27, 30, 31, 70], as well as their reduced genome size

compared to free-living Chaetothyriales strains [33], indicates an evolutionary advantage of vertical transmission and strongly suggests a mutualistic association with the ants [31]. Since the genomes of ant-associated Cyphellophoraceae lack genes for cellulose-active enzymes and other important polysaccharide lyase families [33], they are not major polysaccharide degraders as previously thought [32]. Their low abundance in the early stages of the colony could be explained by the fact that they need to rely on cross-feeding interactions with the fungal and bacterial network in the patches when cellulose is the main substrate. Such microbial network is still not developed in freshly made patches.

Until now, the roles of ant-associated Cyphellophoraceae have been related with secondary nutrition for the ant larvae [71], nutrient recycling [32], putative antimicrobial effects [33], and bio-filtration of the domatia air to remove toxic substances [63] that are produced by ants for communication [72] and diseases control [73]. Despite the efforts of many authors, an in-depth understanding of the ecological functions of Chaetothyriales as well as of the entire fungal community in the nests of ant-plant mutualisms remains elusive. Isolating and physiologically characterizing them will be a crucial step in the understanding of their ecology and activity in this specific environment.

d) The ant species plays an important role in shaping the fungal patch communities

Despite the observed high inter-colony heterogeneity, the fungal community composition in patches of established colonies is significantly influenced by the *Azteca* species (Fig. 7). Although most fungal ASVs were found in both ant species, several prevalent ASVs showed higher relative abundance in patches of either *A. alfari* or *A. constructor*. In fact, both ant species differ in their behaviour and the patches they build, thus creating different habitats [51]. *A. constructor* workers are more aggressive towards intruders than *A. alfari* and patrol the plant surfaces of *Cecropia* more often [51], which could increase the transfer of spores into the patches. *A. alfari* forms flat, dry and crumbly patches, whereas *A. constructor* forms larger, three-dimensional and moist patches that reach anoxic conditions [60]. Although both ant species co-occur in the same geographical area, they seem to successfully develop in distinct environments and plant species. While established *A. constructor* colonies

are regularly observed inhabiting *C. obtusifolia* in shady, humid and steep locations close to streams and surrounded by dense vegetation, established *A. alfari* colonies are more often associated with *C. peltata* in hot, dry and open areas such as river banks or road sites [51]. Despite the trend of finding more regularly each *Azteca* species in a particular *Cecropia* species, the fungal community composition in each ant species was not significantly affected by the plant species. These findings combined with the observed ability of the ant colony to modulate its nesting space [32, 35, 43, 47], suggest a pivotal role of the ants in influencing the microbial community in the patches.

e) Open questions and hypothesis of the potential ant-plant-fungi interactions

After disentangling the dynamics and drivers of fungal communities inhabiting *Azteca-Cecropia* patches, the next questions are: To what extent are the ants actively shaping the fungal communities in the patches? Do these communities provide a benefit to the ant colony, and if so, how? So far, we detected differential read abundances of frequent fungal groups among ant colony developmental stages and tree sections. However, whether such differences are related to the capability of the ant colony to promote or inhibit the growth of fungi remains unknown. Leaf-cutter ants and fungus-growing termites cultivate specific fungal symbionts in their nests while detecting and eliminating adverse fungal species [15, 74–77]. This does not seem to be the case with *Azteca* ants. Our finding of high heterogeneity in established colonies suggests that *Azteca* ants are either flexible or incapable of controlling which organisms are present in their patches. Several scenarios could explain why efficient screening has not evolved: 1) the *Azteca* ants are not affected by the presence of commensals in the patches as long as the beneficial fungi like Chaetothyriales can develop, 2) the ant colony is not adapted to a single fungus but to a fungal network, or 3) the patches provide a highly complex repertoire of niches that overcome the screening capabilities of the ants.

Compared to leaf-cutter ants and termites, ambrosia beetles are known to promote the growth of their diverse fungal partners by the colonization of ethanol-rich decaying trees [16, 78]. Similarly, *Azteca* ants could select for certain functionalities or metabolisms by modulating the addition of substrate to the patches, by altering the

ventilation in the domatia by enlarging the entrance holes, or by volatiles producing that they usually use for pathogen defence. However, such behaviours could still allow the growth of commensal or even harmful fungi that manage to adapt to these environmental conditions.

Azteca ants receive nutrient-rich food (Müllerian bodies) provided by *Cecropia* and honeydew produced by scale insects [34, 44, 46, 79]. Therefore, we would expect fungi to be used as a substitute food source for ant larvae only when food bodies are scarce or when additional nutrients are not available in the regular food sources, as it has been shown in previous studies of other ant-plant mutualisms [47, 71]. Determining whether the *Azteca-Cecropia* association is indeed a "primitive" farming system, as recently suggested by Biedermann and Vega (2020) for ant-plant associations in general [9], requires a more comprehensive understanding of the ecological interactions among the organisms co-occurring in the *Azteca-Cecropia* ecosystem.

Conclusions

The fungal communities in the *Azteca-Cecropia* association are characterized by a large diversity and high heterogeneity among colonies. A reason for this diversity is the combination of different vectors and modes of transmission affecting the fungal community: (i) vertical transmission of fungi from the queen's mother colony; (ii) environmental acquisition of fungi from the plant surface through patrolling and foraging by the ant workers; and, (iii) environmental acquisition of fungi through other arthropods such as flies and mites living in the patches of established ant colonies. Despite the high heterogeneity between colonies, the ant species significantly influences and shapes the fungal community in the patches. The ant colony seems to act as a keystone for the organisms co-habiting within the nest [48, 60], whereas the plant-host only provides the patch environment. Certainly, not all fungi in this association are symbionts, and even fewer are mutualists. A key aspect of future studies must be the development of a method to distinguish which groups are present

as spores and which are present as mycelium. This would provide important information about which fungi are directly associated with the ant colony.

However, it is still a difficult task to elucidate their ecological relationships. What Six and Klepzig [80] pointed out for the bark beetle-fungus mutualism, that it is "notoriously difficult to manipulate in controlled experiments", also applies to the *Azteca-Cecropia*-fungi association and leads to a lack of understanding of their interactions. Not only greenhouse experiments, but also field experiments have failed, as ants abandon the manipulated domatia [24]. At the moment, instead of controlled experiments, we can only rely on careful observation and molecular analysis to elucidate the role of the fungal community in the patches of ant-plant associations.

Methods

a) Study site and sample collection

Samples were collected in the conservation zone ACOSA (*Área de Conservación Osa)* near the Tropical Field Station La Gamba in Puntarenas, Costa Rica (08°42'03"N, 083°12'06"W, 70 m a.s.l.). For this investigation, 93 *Azteca* ant colonies (*A. alfari*, *A. constructor* or *A. xanthochroa)* inhabiting 68 *Cecropia* trees from three species (*C. peltata, C. obtusifolia* or *C. insignis*) were sampled next to roads, creeks, lowland forests and pastures. Identification of ant species was performed based on the morphology of the ant colony and queen following the *Azteca* species descriptions [38, 81]. *Cecropia* species were identified by leaf characteristics [82].

After transversally opening *Cecropia* stems, ant-built patch samples were collected from the colonized internodes (domatia) by removing the whole patch material found in the stem with a dental probe. Immediately after, the patch material was transferred into RNA-later solution until further processing. Patch samples were classified in three categories based on the developmental stage of ant colonies (Fig. 1). Initial (IP) and young (YP) patches were regularly analysed individually, as these colonies only contained a single patch. Patches stemming from domatia of the same established ant colony (EP) were generally pooled. The patches from two colonies of the same

ant species were pooled in eight samples due to an insufficient amount of patch material (*A. alfari* IP, n = 1; *A. alfari* YP, n = 3; *A. alfari* EP, n = 1; *A. constructor* YP, n = 1; *A. constructor* EP, n = 1; *A. xanthochroa* IP, n = 1) [83]. To investigate the fungal community variation within an established ant colony, tree stems from 17 established colonies were divided in three transverse sections based on the characteristics of domatia and then, its patch material was collected separately (Additional File 6).

In the area of sampling, the abundance of the different *Azteca* and *Cecropia* species was notably uneven. For instance, *A. xanthochroa* colonies were only detected in an initial developmental stage and *Cecropia insignis* plants were rarely found. Since the ant species was only confirmed after collecting the plant, we were unable to obtain the same number of samples per each individual group. Additionally, we were only able to identify the plant species in established ant colonies since the distinctive leaf characteristics were not visible in younger plants. In Additional File 1, an overview of the number of colonies collected per ant species, plant species and ant colony developmental stages is provided.

b) Molecular analysis

In total, 120 patch samples stored in RNA-later solution were washed twice with a phosphate buffer (pH 8.0) by centrifuging the patch material for 1 min at 14 000 rpm. DNA was extracted from patch samples with an adapted phenol-chloroform extraction protocol with three rounds of mechanical lysis via bead beating (30 s at 6.5 m s-1) [84].

To identify the most suitable amplification and sequencing method for this environmental sample type, we evaluated the performance of six primer pairs by amplifying either ITS1, ITS2 or the full-length ITS1-5.8S-ITS2 region of 6 patch samples (Additional File 7) [65, 85–94]. Based on the results obtained, the primer pair ITS3mix1-5/ITS4ngsUni targeting the ITS2 region was selected for investigating the fungal communities in this study. For generating ITS amplicon libraries, a two-step PCR protocol for highly multiplexed amplicon sequencing was followed in 120 patch samples [95]. The PCR protocol and programs used are detailed in Additional File 8. Library preparation and MiSeq Illumina sequencing was performed by the Joint

Microbiome Facility (JMF, University of Vienna, Austria). For <sequencing, we selected a 2 x 300 bp cycles paired-end mode using the MiSeq v3 Reagent kit (Illumina).

c) Sequence processing and analysis

Amplicon sequence data were processed as described in Pjevac et al. (2021) [95]. Briefly, ASVs were inferred using the DADA2 R package version 1.2.0 [96] with R v4.1.1 [97] by applying trimming at 220/230 nucleotides with allowed expected errors of 2/4. Singletons were removed from the count table. ASVs were taxonomically classified using a modified version of the UNITE v8.2 database covering eukaryotes [83, 90]. Detailed information about the sequences modified or added to the UNITE database can be found at Additional File 9 [25, 27, 30, 83, 98–103].

Downstream analyses were performed in R v4.1.2 [97] and RStudio 2021.09.1 [104]. To analyse the fungal diversity and community composition in individual ant colonies, patch samples of the 17 established colonies that were sequenced separately by tree sections were merged by adding up read counts using ampvis2 v2.7.11 R package [91]. We calculated alpha and beta-diversity analysis of fungal communities by using the R packages ampvis2 v2.7.11 [91], vegan v2.6-4 [93] and GUniFrac v1.4 [105]. For both diversity metrics, we first rarefied the read counts using the minimum read count per sample that was higher than 2000 reads. Alpha diversity metrics were analysed calculating the Shannon index and the difference between groups was tested for statistical significance by Kruskal-Wallis test and post-hoc pair-wise Wilcoxon analysis using a p value of 0.05. The beta diversity was visualized by PCoA using Bray-Curtis distances and statistically compared using PERMANOVA [106] and MiRKAT [107] tests with a p value of 0.05. Since the sample size design was notably unbalanced in most beta diversity comparisons, additional PERMDISP test [108] was performed to evaluate the heterogeneity of dispersions [109].

To inspect the fungal community composition at high taxonomic resolution (genus level), we identified the 30 most abundant ASVs and the frequent ASVs from patch samples of established colonies. Discriminative ASVs between ant species were obtained with the DESEq2 v1.34.0 R package [110] (adjusted $p < 0.05$). Furthermore,

we defined frequent ASVs per each ant species when: (i) they were present in at least half of the colonies of that ant species, and (ii) resulted in a mean relative read abundance higher than 0.05% for such ant species. For improving legibility and accessibility, representative ASVs (abundant and frequent ASVs, and Chaetothyriales ASVs) were renamed using number digits, listed and detailed in Additional File 10. To investigate the abundance dynamics of frequent genera among different patch types (ant colony developmental stages and tree sections), we used the unmerged patch samples from established colonies and analysed their relative abundance from all *Azteca* sp. colonies jointly. Statistical comparisons of relative abundance in each ant colony stage and tree section were calculated by Kruskal-Wallis and post-hoc Wilcoxon test ($p < .05$).

In order to enable a comparison with the previous studies [25, 27, 47], the ASV sequences of Chaetothyriales were aligned to a representative ITS matrix of GenBank sequences of Trichomeriaceae and Cyphellophoraceae from domatia including sequences obtained from Cecropia by Nepel et al. (2016) and Mayer et al. (2018) [25, 47]. Details about the methodology followed for constructing such phylogenetic tree can be found in Additional File 5 [25, 47, 56–59].

Declarations

Ethics approval and consent to participate: This research was conducted under the permission of the Costa Rican authorities who provided the following research permits "R-046-2015-OT-CONAGEBIO", "SINAC INV-ACOSA-001-16" and "INV-ACOSA-013-18". In accordance to these research permits, we followed the national laws of Costa Rica "Ley de Conservación de la Vida Silvestre Nº 7317", "Ley de Biodiversidad Nº 7788" and "Ley Orgánica del Ambiente Nº 7554" and applied the General Protocol "MINAE-SINAC-P-001" for the use of the Protected Wildlife Areas of the National System of Conservation Areas. Benefits from this research accrue from sharing our data on public databases.

Consent for publication: Not applicable

Availability of data and materials: All data generated or analysed during this study are included in this published article, its supplementary information files and publicly available repositories. The sequence data (raw sequence reads and metadata) are accessible on NCBI under the BioProject accession number PRJNA777006 [111]. The ITS amplicon sequencing data supporting the conclusions of this article and the R code used for downstream analysis in this investigation are available in a collection (https://doi.org/10.6084/m9.figshare.c.7072553.v1) in the publicly available figshare repository [83]. Likewise, the modified UNITE v8.2 database used for the taxonomic assignment of ITS sequences in this study can be found in the same collection in the figshare repository [83].

Competing interests: All authors declare that they have no conflict of interest.

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References

1. Bar-On YM, Phillips R, Milo R. The biomass distribution on Earth. PNAS. 2018;115:6506–11.

2. Schultheiss P, Nooten SS, Wang R, Wong MKL, Brassard F, Guénard B. The abundance, biomass, and distribution of ants on Earth. PNAS. 2022;119:e2201550119.

3. Folgarait PF. Ant biodiversity and its realtionship to ecosystem function: a review. Biodivers Conserv. 1998;7:1221–44.

4. Griffiths HM, Ashton LA, Walker AE, Hasan F, Evans TA, Eggleton P, et al. Ants are the major agents of resource removal from tropical rainforests. J Anim Ecol. 2018;87:293–300.

5. Hawksworth DL, Lücking R. Fungal diversity revisited: 2.2 to 3.8 million species. Microbiol Spectr. 2017;5:79–95.

6. Wang JB, Leger RS, Wang C. Advances in Genomics of entomopathogenic fungi. Adv Genet. 2016;94:67–105.

7. Vančurová L, Malíček J, Steinová J, Škaloud P. Choosing the right life partner: ecological drivers of lichen symbiosis. Front Microbiol. 2021;12.

8. Brundrett MC, Tedersoo L. Evolutionary history of mycorrhizal symbioses and global host plant diversity. New Phytol. 2018;220:1108–15.

9. Biedermann PHW, Vega FE. Ecology and evolution of insect – fungus mutualisms. Annu Rev Entomol. 2020;65:431–55.

10. Mani S, Cao M. Nitrogen and phosphorus concentration in leaf litter and soil in Xishuangbanna tropical forests: Does precipitation limitation matter? Forests. 2019;10:11–3.

11. Santiago LS. Nutrient limitation of eco-physiological processes in tropical trees. Trees. 2015;29:1291–300.

12. Hussa EA, Goodrich-Blair H. It takes a village: ecological and fitness impacts of multipartite mutualism. Annu Rev Microbiol. 2013;67:161–78.

13. Sutherland AM, Parrella MP. Mycophagy in Coccinellidae: review and synthesis. Biol Control. 2009;51:284–93.

14. Li H, Young SE, Poulsen M, Currie CR. Symbiont-mediated digestion of plant biomass in fungus-farming insects. Annu Rev Entomol. 2021;66:297–316.

15. Sidde Gowda DK, Rajagopal D. Association of *Termitomyces* spp. with fungus growing termites. Proc Anim Sci. 1990;99:311–5.

16. Diehl JMC, Kowallik V, Keller A, Biedermann PHW. First experimental evidence for active farming in ambrosia beetles and strong heredity of garden microbiomes. Proc R Soc B. 2022;289:20221458.

17. De Fine Licht HH, Boomsma JJ. Forage collection, substrate preparation, and diet composition in fungus-growing ants. Ecol Entomol. 2010;35:259–69.

18. Conlon BH, O'Tuama D, Michelsen A, Crumière AJJ, Shik JZ. A fungal symbiont converts provisioned cellulose into edible yield for its leafcutter ant farmers. Biol Lett. 2022;18:20220022.

19. Chapela IH, Rehner SA, Schultz TR, Mueller UG. Evolutionary history of the symbiosis between fungus-growing ants and their fungi. Science. 1994;266:1691–4.

20. Khadempour L, Kyle JE, Webb-Robertson B-JM, Nicora CD, Smith FB, Smith RD, et al. From plants to ants: fungal modification of leaf lipids for nutrition and communication in the leaf-cutter ant fungal garden ecosystem. mSystems. 2021;6:e01307-20.

21. De Fine Licht HH, Boomsma JJ, Tunlid A. Symbiotic adaptations in the fungal cultivar of leaf-cutting ants. Nat Commun. 2014;5:5675.

22. da Costa RR, Hu H, Li H, Poulsen M. Symbiotic plant biomass decomposition in fungus-growing termites. Insects. 2019;10:10040087.

23. Hulcr J, Stelinski LL. The ambrosia symbiosis: from Evolutionary Ecology to practical management. Annu Rev Entomol. 2017;62:285–303.

24. Defossez E, Selosse MA, Dubois MP, Mondolot L, Faccio A, Djieto-Lordon C, et al. Ant-plants and fungi: A new threeway symbiosis. New Phytol. 2009;182:942–9.

25. Nepel M, Voglmayr H, Blatrix R, Longino JT, Fiedler K, Schönenberger J, et al. Ant-cultivated Chaetothyriales in hollow stems of myrmecophytic *Cecropia* sp. trees – diversity and patterns. Fungal Ecol. 2016;23:131–40.

26. Kokolo B, Atteke C, Ibrahim B, Blatrix R. Pattern of specificity in the tripartite symbiosis

between *Barteria* plants, ants and Chaetothyriales fungi. Symbiosis. 2016;69:169–74.

27. Vasse M, Voglmayr H, Mayer V, Gueidan C, Nepel M, Moreno L, et al. A phylogenetic perspective on the association between ants (Hymenoptera: Formicidae) and black yeasts (Ascomycota: Chaetothyriales). Proc R Soc B. 2017;284:20162519.

28. Bailey IW. Some relations between ants and fungi. Ecology. 1920;1:174–89.

29. Miehe H. Über die javanische Myrmecodia und die Beziehung zu ihren Ameisen. Biologisches Zentralblatt; 1911.

30. Voglmayr H, Mayer V, Maschwitz U, Moog J, Djieto-Lordon C, Blatrix R. The diversity of ant-associated black yeasts: insights into a newly discovered world of symbiotic interactions. Fungal Biol. 2011;115:1077–91.

31. Mayer VE, Voglmayr H, Blatrix R, Orivel J, Leroy C. Fungi as mutualistic partners in ant-plant interactions. Front Fungal Biol. 2023;4 October:1213997.

32. Defossez E, Djiéto-Lordon C, Mckey D, Selosse MA, Blatrix R. Plant-ants feed their host plant, but above all a fungal symbiont to recycle nitrogen. Proc R Soc B. 2011;278:1419–26.

33. Moreno LF, Mayer V, Voglmayr H, Blatrix R, Benjamin Stielow J, Teixeira MM, et al. Genomic analysis of ant domatia-associated melanized fungi (Chaetothyriales, Ascomycota). Mycol Prog. 2019;18:541–52.

34. González-Teuber M, Heil M. Comparative anatomy and physiology of myrmecophytes: ecological and evolutionary perspectives. Res Reports Biodivers Stud. 2015;4:21–32.

35. Lucas J, Madden AA, Penick CA, Epps MJ, Marting PR, Stevens JL, et al. *Azteca* ants maintain unique microbiomes across functionally distinct nest chambers. Proc R Soc B. 2019;286:20191026.

36. Greenfield MJ, Lach L, Congdon BC, Anslan S, Tedersoo L, Field M, et al. Consistent patterns of fungal communities within ant-plants across a large geographic range strongly suggest a multipartite mutualism. Mycol Prog. 2021;20:681–99.

37. Baker CCM, Martins DJ, Pelaez JN, Billen JPJ, Pringle A, Frederickson ME, et al. Distinctive fungal communities in an obligate African ant-plant mutualism. Proc R Soc B. 2017;284:20162501.

38. Longino JT. Taxonomy of the *Cecropia* -inhabiting *Azteca* ants. J Nat Hist. 1991;25:1571.

39. Oliveira KN, Coley PD, Kursar TA, Kaminski LA, Moreira MZ, Campos RI. The effect of symbiotic ant colonies on plant growth: A test using an *Azteca-Cecropia* system. PLoS One. 2015;10:e0120351.

40. Janzen DH. Allelopathy by myrmecophytes: The ant *Azteca* as an allelopathic agent of *Cecropia*. Ecology. 1969;50:147–53.

41. Schupp EW. *Azteca* protection of *Cecropia*: ant occupation benefits juvenile trees. Oecologia. 1986;70:379–85.

42. Marting PR, Kallman NM, Wcislo WT, Pratt SC. Ant-plant sociometry in the *Azteca-Cecropia* mutualism. Sci Rep. 2018;8:17968.

43. Marting PR, Wcislo WT, Pratt SC. Colony personality and plant health in the *Azteca-Cecropia* mutualism. Behav Ecol. 2018;29:264–71.

44. Rickson FR. Anatomical development of the leaf trichilium and Müllerian bodies of

Cecropia peltata L. Am J Bot. 1976;63:1266–71.

45. Müller F. Ueber das Haarkissen am Blattstiel der Imbauba (*Cecropia*), das Gemüsebeet der Imbauba-Ameise. Jenaische Zeitschrift fur Medizin und Naturwiss. 1876;10:281–6.

46. Bischof S, Umhang M, Eicke S, Streb S, Qi W, Zeeman SC. *Cecropia peltata* accumulates starch or soluble glycogen by differentially regulating starch biosynthetic genes. Plant Cell. 2013;25:1400–15.

47. Mayer VE, Nepel M, Blatrix R, Oberhauser FB, Fiedler K, Schönenberger J, et al. Transmission of fungal partners to incipient *Cecropia*-tree ant colonies. PLoS One. 2018;13:e0192207.

48. Nepel M, Mayer VE, Barrajon-Santos V, Woebken D. Bacterial diversity in arboreal ant nesting spaces is linked to colony developmental stage. Commun Biol. 2023;6:1217.

49. Sudhaus W. The guild of saprobiontic nematodes associated with ants (Formicoidea). Ecol Montenegrina. 2016;7:600–13.

50. Maschwitz U, Fiala B, Dumpert K, Hashim R bin, Sudhaus W. Nematode associates and bacteria in ant-tree symbioses. Symbiosis. 2016;69:1–7.

51. Longino JT. *Azteca* ants in *Cecropia* trees: taxonomy, colony structure, and behaviour. In: Cutler C, Huxley D, editors. Ant-plant interactions. Oxford University Press; 1991. p. 271–88.

52. Tragust S, Mitteregger B, Barone V, Konrad M, Ugelvig L V., Cremer S. Ants disinfect fungus-exposed brood by oral uptake and spread of their poison. Curr Biol. 2013;23:76–82.

53. Poulsen M, Bot ANM, Nielsen MG, Boomsma JJ. Experimental evidence for the costs and hygienic significance of the antibiotic metapleural gland secretion in leaf-cutting ants. Behav Ecol Sociobiol. 2002;52:151–7.

54. Yek SH, Nash DR, Jensen AB, Boomsma JJ. Regulation and specificity of antifungal metapleural gland secretion in leaf-cutting ants. Proc R Soc B. 2012;279:4215–22.

55. Lucas J, Bill B, Stevenson B, Kaspari M. The microbiome of the ant-built home: the microbial communities of a tropical arboreal ant and its nest. Ecosphere. 2017;8:e01639.

56. Hall T. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser. 1999;41:95–8.

57. Katoh K, Rozewicki J, Yamada KD. MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. Brief Bioinform. 2019;20:1160–6.

58. Silvestro D, Michalak I. RaxmlGUI: A graphical front-end for RAxML. Org Divers Evol. 2012;12:335–7.

59. Stamatakis A. RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics. 2006;22:2688–90.

60. Nepel M, Pfeifer J, Oberhauser FB, Richter A, Woebken D, Mayer VE. Nitrogen fixation by diverse diazotrophic communities can support population growth of arboreal ants. BMC Biol. 2022;20:135.

61. Kluth S, Kruess A, Tscharntke T. Insects as vectors of plant pathogens: Mutualistic and antagonistic interactions. Oecologia. 2002;133:193–9.

62. Hofstetter RW, Moser JC. The role of mites in insect-fungus associations. Annu Rev

Entomol. 2014;59:537–57.

63. Mayer VE, de Hoog S, Cristescu SM, Vera L, Prenafeta-Boldú FX. Volatile organic compounds in the *Azteca/Cecropia* ant-plant symbiosis and the role of black fungi. J Fungi. 2021;7:836.

64. Lennon JT, Jones SE. Microbial seed banks: The ecological and evolutionary implications of dormancy. Nat Rev Microbiol. 2011;9:119–30.

65. Nilsson RH, Anslan S, Bahram M, Wurzbacher C, Baldrian P, Tedersoo L. Mycobiome diversity: high-throughput sequencing and identification of fungi. Nat Rev Microbiol. 2019;17:95–109.

66. Abdel-Azeem AM, Abdel-Azeem MA, Darwish AG, Nafady NA, Ibrahim NA. *Fusarium*: biodiversity, ecological significances, and industrial applications. In: Recent advancement in white biotechnology through fungi. Fungal Biology. Springer; 2019. p. 201–61.

67. Kruse J, Doehlemann G, Kemen E, Thines M. Asexual and sexual morphs of *Moesziomyces* revisited. IMA Fungus. 2017;8:117–29.

68. De Fine Licht HH, Biedermann PHW. Patterns of functional enzyme activity in fungus farming ambrosia beetles. Front Zool. 2012;9:13.

69. Sakai K, Yamaguchi A, Tsutsumi S, Kawai Y, Tsuzuki S, Suzuki H, et al. Characterization of FsXEG12A from the cellulose-degrading ectosymbiotic fungus *Fusarium* spp. strain EI cultured by the ambrosia beetle. AMB Express. 2020;10.

70. Quan Y, da Silva NM, de Souza Lima BJF, de Hoog S, Vicente VA, Mayer V, et al. Black fungi and ants: a genomic comparison of species inhabiting carton nests versus domatia. IMA Fungus. 2022;13:4.

71. Blatrix R, Djiéto-Lordon C, Mondolot L, la Fisca P, Voglmayr H, Mckey D. Plant-ants use symbiotic fungi as a food source: new insight into the nutritional ecology of ant-plant interactions. Proc R Soc B. 2012;279:3940–7.

72. Jackson BD, Morgan ED. Insect chemical communication: Pheromones and exocrine glands of ants. Chemoecology. 1993;4:125–44.

73. Yek SH, Mueller UG. The metapleural gland of ants. Biol Rev. 2011;86:774–91.

74. Goes AC, Barcoto MO, Kooij PW, Bueno OC, Rodrigues A. How do leaf-cutting ants recognize antagonistic microbes in their fungal crops? Front Ecol Evol. 2020;8:95.

75. Katariya L, Ramesh PB, Gopalappa T, Desireddy S, Bessière JM, Borges RM. Fungus-farming termites selectively bury weedy fungi that smell different from crop fungi. J Chem Ecol. 2017;43:986–95.

76. Mighell K, Van Bael SA. Selective elimination of microfungi in leaf-cutting ant gardens. Fungal Ecol. 2016;24:15–20.

77. Weber NA. Gardening ants, the attines. American Philosophical Society; 1972.

78. Ranger CM, Biedermann PHW, Phuntumart V, Beligala GU, Ghosh S, Palmquist DE, et al. Symbiont selection via alcohol benefits fungus farming by ambrosia beetles. PNAS. 2018;115:4447–52.

79. Pringle EG, Dirzo R, Gordon DM. Indirect benefits of symbiotic coccoids for an ant-defended myrmecophytic tree. Ecology. 2011;92:37–46.

80. Six DL, Klepzig KD. Context dependency in bark beetle-fungus mutualisms revisited: Assessing potential shifts in interaction outcomes against varied genetic, ecological, and evolutionary backgrounds. Front Microbiol. 2021;12.

81. Longino JT. A taxonomic review of the genus *Azteca* (Hymenoptera: Formicidae) in Costa Rica and a global revision of the aurita group. Zootaxa. 2007;1491:1–63.

82. Berg CC, Roselli PF, Davidson DW. *Cecropia*. Flora Neotrop. 2005;:1–230.

83. Barrajon-Santos V, Nepel M, Hausmann B, Voglmayr H, Woebken D, Mayer VE. Dynamics and drivers of fungal communities in a multipartite ant-plant association. figshare. Collection. 2024. https://doi.org/10.6084/m9.figshare.c.7072553.

84. Angel R, Claus P, Conrad R. Methanogenic archaea are globally ubiquitous in aerated soils and become active under wet anoxic conditions. ISME J. 2012;6:847–62.

85. Tedersoo L, Bahram M, Zinger L, Nilsson RH, Kennedy PG, Yang T, et al. Best practices in metabarcoding of fungi: From experimental design to results. Mol Ecol. 2022;31:2769–95.

86. White TJ, Bruns T, Lee S, Taylor J. Amplification and Direct Sequencing of Fungal Ribosomal Rna Genes for Phylogenetics. In: PCR Protocols. 1990. p. 315–22.

87. Tedersoo L, Lindahl B. Fungal identification biases in microbiome projects. Environ Microbiol Rep. 2016;8:774–9.

88. Tedersoo L, Anslan S. Towards PacBio-based pan-eukaryote metabarcoding using full-length ITS sequences. Environ Microbiol Rep. 2019;11:659–68.

89. Rognes T, Flouri T, Nichols B, Quince C, Mahé F. VSEARCH: A versatile open source tool for metagenomics. PeerJ. 2016;2016:1–22.

90. Abarenkov K, Henrik Nilsson R, Larsson K, Alexander IJ, Eberhardt U, Erland S, et al. The UNITE database for molecular identification of fungi – recent updates and future perspectives. New Phytol. 2010;186:281–5.

91. Andersen KS, Kirkegaard RH, Karst SM, Albertsen M. ampvis2: an R package to analyse and visualise 16S rRNA amplicon data. bioRxiv. 2018;:299537.

92. Kassambara A. Rstatix: Pipe-Friendly Framework for Basic Statistical Tests. 2021. https://cran.r-project.org/web/packages/rstatix/index.html.

93. Oksanen J, Simpson GL, Blanchet FG, Kindt R, Legendre P, Minchin PR, et al. Vegan: Community Ecology package. 2022. https://cran.r-project.org/web/packages/vegan/index.html.

94. Tedersoo L, Anslan S, Bahram M, Põlme S, Riit T, Liiv I, et al. Shotgun metagenomes and multiple primer pair-barcode combinations of amplicons reveal biases in metabarcoding analyses of fungi. MycoKeys. 2015;10:1–43.

95. Pjevac P, Hausmann B, Schwarz J, Kohl G, Herbold CW, Loy A, et al. An economical and flexible dual barcoding, two-step PCR approach for highly multiplexed amplicon sequencing. Front Microbiol. 2021;12:669776.

96. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods. 2016;13:581–3.

97. R Core Team. R: A Language and environment for statistical computing. 2022. http://www.r-project.org.

98. Crous PW, Schubert K, Braun U, De Hoog GS, Hocking AD, Shin HD, et al. Opportunistic, human-pathogenic species in the Herpotrichiellaceae are phenotypically similar to saprobic or phytopathogenic species in the Venturiaceae. Stud Mycol. 2007;58:185–217.

99. Attili-Angelis D, Duarte APM, Pagnocca FC, Nagamoto NS, De Vries M, Stielow JB, et al. Novel Phialophora species from leaf-cutting ants (tribe Attini). Fungal Divers. 2014;65:65–75.

100. Nepel M, Voglmayr H, Schönenberger J, Mayer VE. High diversity and low specificity of chaetothyrialean fungi in carton galleries in a neotropical ant-plant association. PLoS One. 2014;9:e112756.

101. Mayer VE, Voglmayr H. Mycelial carton galleries of *Azteca brevis* (Formicidae) as a multi-species network. Proc R Soc B. 2009;276:3265–73.

102. Ruiz-González MX, Malé PJG, Leroy C, Dejean A, Gryta H, Jargeat P, et al. Specific, non-nutritional association between an ascomycete fungus and *Allomerus* plant-ants. Biol Lett. 2011;7:475–9.

103. Schlick-Steiner BC, Steiner FM, Konrad H, Seifert B, Christian E, Moder K, et al. Specificity and transmission mosaic of ant nest-wall fungi. PNAS. 2008;105:940–3.

104. RStudio Team. RStudio: Integrated Development Environment for R. 2022. http://www.rstudio.com/.

105. Chen J, Bittinger K, Charlson ES, Hoffmann C, Lewis J, Wu GD, et al. Associating microbiome composition with environmental covariates using generalized UniFrac distances. Bioinformatics. 2012;28:2106–13.

106. Anderson MJ. A new method for non-parametric multivariate analysis of variance. Austral Ecol. 2001;:32–46.

107. Wilson N, Zhao N, Zhan X, Koh H, Fu W, Chen J, et al. MiRKAT: kernel machine regression-based global association tests for the microbiome. Bioinformatics. 2021;37:1595–7.

108. Anderson MJ, Ellingsen KE, McArdle BH. Multivariate dispersion as a measure of beta diversity. Ecol Lett. 2006;9:683–93.

109. Anderson MJ, Walsh DC. PERMANOVA, ANOSIM, and the Mantel test in the face of heterogeneous dispersions: What null hypothesis are you testing? Ecol Monogr. 2013;83.

110. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014;15:550.

111. Barrajon-Santos V, Nepel M, Hausmann B, Voglmayr H, Woebken D, Mayer VE. Sequencing microbial communities in the ant-plant *Azteca-Cecropia* association. NCBI. PRJNA777006. 2024. https://www.ncbi.nlm.nih.gov/bioproject/PRJNA777006.

Chapter II

Hidden guests of ant-plant mutualisms: unravelling the nematode dynamics in the nest of the *Azteca-Cecropia* complex

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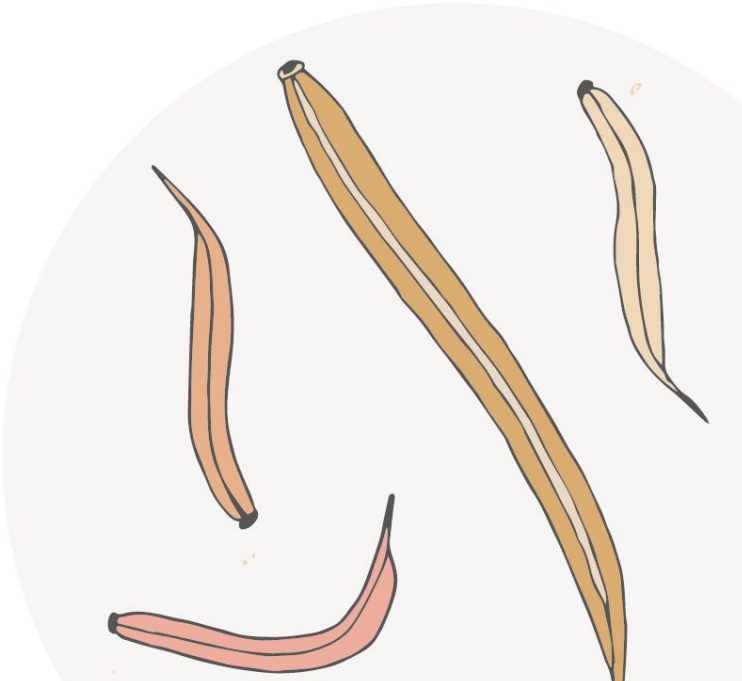

Abstract

1. In tropical ant-plant mutualisms worldwide, ants are known to host a wide variety of organisms that cohabit and continuously interact within their nesting space. These interactions are particularly prevalent in well-defined organic matter piles, which are commonly referred to as “patches”. By morphological identification methods, rhabditid nematodes have been observed attached to the bodies of the alate ant queens and on the surface of the patches. However, the dynamics of how the nematode community establishes and develops during ant colony growth and the extent of consistency across closely related ant-plant species remain unclear.

2. By combining amplicon sequence data of the partial 18S rRNA gene with morphology-based quantification, we investigated the nematode communities of ant-made patches in 65 colonies of the *Azteca-Cecropia* complex, one of the most prominent and abundant ant-plant mutualisms in the Tropics of America.

3. The diversity and composition of nematode communities in patches made by ant queens at colony foundation (initial patches) were found to be consistent when compared with those of older colonies (established patches). In addition to the previously detected bacterivorous Rhabditida nematodes, two further nematode orders (Tylenchida and Dorylaimida) with diverse feeding strategies were identified. Surprisingly, closely related ant species host distinct nematode communities in their patches.

4. The results of our study strongly support a transmission of nematodes from mother to daughter colonies and among patches within the same colony. Although the host plant provides the environment for the assembly of nematode patch communities, the ant colony appears to be the main driver of the nematode diversity in these patches.

5. Based on our findings, we suggest that nematodes could play a beneficial role in the patches by providing a diverse set of ecosystem services, thereby contributing to the overall stability and functioning of the patch-inhabiting microbiota.

Keywords: 18S rRNA gene-based metabarcoding, ant-plant mutualisms, *Azteca-Cecropia*, insect-nematode interactions, nematode community dynamics

Introduction

Nematodes are the most abundant and ubiquitous metazoans on Earth [1]. They are distributed across all trophic levels, and thus, they are often classified based on their feeding strategy (i.e. herbivores, bacterivores, fungivores and omnivores) [2]. Due to their high adaptability and their notable phenotypic plasticity, many species are involved in complex ecological networks that often include symbiotic associations with bacteria, plants and other animals [3, 4]. It is estimated that nematodes are associated with 40,000-500,000 species of insects worldwide, many of which display a social behaviour such as bees, termites, and ants [5]. Entomophilic non-parasitic nematodes are often characterized as bacteria-feeding opportunists [2]. These bacterivorous nematodes have frequently evolved the ability to utilise insects as bio-vehicles in order to access bacteria-rich environments [6–8]. This dispersal strategy, known as phoresy, is characterized by the development of highly resilient "dauerlarvae", which exhibit a nictitating behaviour (standing on the tail and waving) and a specialized cuticle-attachment mechanism [6, 9].

While the association is clearly beneficial for the nematodes, the effects recorded so far on the fitness and development of their insect partners range from mutually beneficial to detrimental [10–12]. For example, in dung beetles (Coleoptera, Scarabaeoidea), the selective grazing activity within the brood balls of transgenerationally inherited and sexually transmitted *Diplogastrellus* (Rhabditida, Diplogastridae) nematodes promotes the growth of beetle offspring [11]. On the contrary, in burying beetles, the presence of phoretic nematodes in the bodies of mating females leads to a significant reduction in brood size, larval survival, and larval mass [10]. Moreover, recent research on a fungus-growing termite (Blattodea, Isoptera) suggests a commensal relationship with nematodes [7, 13]. Their presence in the termite nest appears to be harmless to the termites, yet nematodes are rare in the chambers with brood or are even suppressed by the termites' grooming activity.

Ants have been associated with nematodes for at least 20-30 million years [14]. As for other insects, most ant-associated nematodes are non-parasitic saprobiontic bacterivores, mainly belonging to the families Rhabditidae, Diplogastridae and

Panagrolaimidae of the order Rhabditida [8, 15]. Non-parasitic ant-nematode relationships are particularly prominent and frequent in tropical ant-plant mutualisms worldwide [8, 16]. In such ant-plant associations, the ants protect their host plant from intruders in exchange for a nesting space in hollow structures provided by the host-plant (domatia) and food resources such as extrafloral nectar or food bodies [17–20]. In the domatia, large masses of nematodes seem to coexist with fungi and bacteria that inhabit the so-called "patches" [8, 16, 21–25]. These are well-defined areas found in almost all ant-plant mutualisms known to date, in which the ants accumulate organic matter from different sources [26, 27]. Several researchers have proposed that the ant-associated nematodes may be transmitted from mother to daughter colonies in ant-plant mutualisms, as they are regularly detected in the infrabuccal pockets and attached to the bodies of alate queens [16, 21, 28]. Moreover, they are consistently found in the initial patch that a queen makes during her claustral colony founding [28].

However, while the dynamics and composition of the patch communities with respect to fungi and bacteria have been extensively studied [24, 25, 29, 30], no research has been conducted regarding the occurrence and composition of the nematode community in relation to: (i) the development of the ant colony, and, (ii) the species of their ant-plant partners. To better understand the nature of the nematode-ant-plant association, we investigated the composition and the dynamics of nematode communities inhabiting ant-made patches in one of the most prominent and abundant ant-plant mutualisms in the Tropics of America: the *Azteca-Cecropia* complex (Figure 1). By analysing amplicon sequence data of the partial 18S rRNA gene, we analysed patches from 65 colonies of three different *Azteca* species (Formicidae, Dolichoderinae) inhabiting *Cecropia* spp. (Urticaceae). In this study, we hypothesize: (i) Patches of established colonies host a greater diversity of nematodes than the initial patch made by the foundress queen after plant colonisation, as seen in the fungal and bacterial patch communities [24, 25]; (ii) the nematodes are transmitted from mother to daughter colonies and among patches of the same ant colony; and, (iii) similar nematode communities are found between closely related ant and plant species. Our study on the nematode community composition associated with an ant-plant association is an invaluable foundation to further understand the role of nematodes in this striking ecosystem and its effect on the ant colony fitness and development.

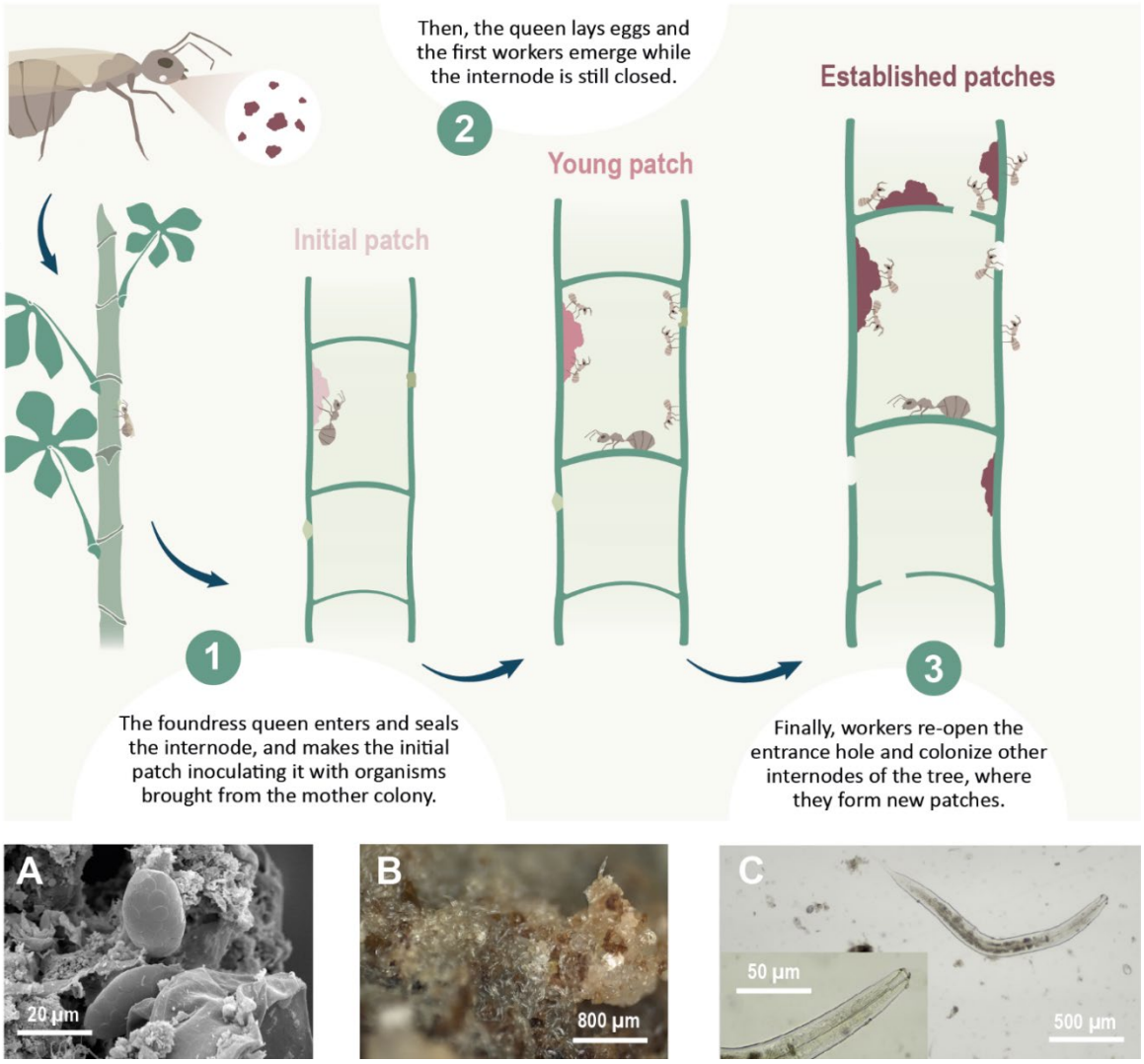

Figure 1. Graphic illustration of the *Azteca-Cecropia* association including the ant-made patches from the different ant colony development stages: initial patch (IP); young patch (YP); established patches (EP). The shades of brown are representative of age; thus, the older the ant colony, the darker the patches. (A-C) Microscopic images of nematodes with their respective scales from: (A) nematode eggs in a patch sample, (B) nematodes on the surface of a patch, and, (C) morphological characteristics of a bacterivorous nematode from the patches (Photos made by: Veronika E. Mayer, co-author).

Materials and Methods

a) Patch samples collection

Samples were collected in wet lowland forests and pastures in the conservation zone ACOSA (*Área de Coservación Osa*) in Puntarenas, Costa Rica in close vicinity to the Tropical Field Station La Gamba (08°42'03"N, 83°12'06"W, 70 m a.s.l.). For this investigation, we sampled 55 *Cecropia* trees (*C. peltata, C. obtusifolia and C. insignis*) colonized by 68 *Azteca* colonies. The *Cecropia* species were determined based on the leaf characteristics when the leaves were properly developed [31]. If the identification of the plant species was not possible, we labelled it as "*Cecropia* sp.". After transversally opening colonized *Cecropia* stems, we collected ant-built patch samples from the nesting space of *Azteca* spp. colonies using a dental probe. Subsequently, the ant species (*A. alfari*, *A. constructor* and *A. xanthochroa*) was identified through the morphology of the ant queen following J. Longino *Azteca* species description [32, 33] and by the characteristics of the ant nest [30]. Patch samples were classified in three categories depending on the developmental stage of the ant colony (Figure 1). In the initial and young developmental stages, ant colonies are spatially limited to a single plant internode and maintain only one patch. These patches were analysed separately. In established ant colonies, in which ants maintain multiple patches throughout the nesting space, patches of a single ant colony were either combined in the field or separately sampled and sequenced by tree sections and afterwards bioinformatically pooled.

In the area of sampling, the abundance of the different *Azteca* and *Cecropia* species was notably uneven: only one *Cecropia* specimen was identified as *C. insignis*; from *A. xanthochroa,* only foundress queens with initial patches were found in the study area; and all young ant colonies were identified as *A. alfari* colonies. In consequence, we were unable to obtain the same number of samples per ant and plant species and per ant colony developmental stage (Supporting Information S1).

b) Molecular analysis

For metabarcoding analysis, patch samples from 68 ant colonies stored in RNA*later* solution (1:4) were washed twice with a phosphate buffer by centrifugation at 14 000

rpm for 1 min. DNA extraction was performed following an adapted phenol-chloroform based extraction protocol (Griffiths et al., 2000; Henckel et al., 1999) using three rounds of mechanical lysis via bead beating (30 s at 6.5 m s-1) [34]. Amplicon libraries targeting the partial 18S rRNA gene were generated by a three-step PCR approach for highly multiplexed amplicon sequencing composed by: a) a semi-nested PCR protocol using metazoan selective primers (NemF and 18Sr2b) first, followed by amplification with NF1 and 18Sr2b primers [35, 36]; b) a barcoding PCR step [37]. Detailed information about the primer sequences, PCR protocol and programs used in this study is provided in Supporting Information S2. Library preparation and MiSeq Illumina sequencing (JMF-2010-2) was performed by the Joint Microbiome Facility (JMF, University of Vienna, Austria) using a 2 x 300 bp cycles paired-end mode and the MiSeq v3 Reagent kit (Illumina).

c) Sequence processing and analysis

Amplicon sequence data were processed as described in Pjevac et al. (2021) following the recent recommendations for metabarcoding studies of nematodes communities [38]. First, amplicon sequence variants (ASVs) were inferred using the DADA2 R package version 1.2.0 [39] with R v4.1.1 [40] by applying trimming at 220/230 nt with allowed expected errors of 2/4. Second, ASVs were taxonomically classified using RDP classifier [41] and the SILVA v.138 SSU database [42]. Third, the taxonomic classification of the 50 most abundant ASVs was revised by BLASTN [43] using the nucleotide collection (nt) database (updated on the 11th of October 2023). In the count and taxonomy tables, we performed a filtering process where we discarded: (i) singleton ASVs, (ii) ASVs not classified as phylum Nematoda, and, (iii) samples with less than 2000 reads. As a result, 65 samples out of 68 were kept for downstream analyses (Supporting Information S1).

Downstream analyses were performed in R v4.1.2 and RStudio 2021.09.1 [44]. When several patch samples were sequenced from the same established ant colony, their sequence data were merged by adding up read counts using the R packages ampvis2 v2.7.11 [45]. The nematode alpha and beta-diversity metrics in individual ant colonies were analysed using the R packages ampvis2 v2.7.11, vegan v2.6-4 [46] and GUniFrac v1.4 [47]. For both diversity metrics, we first rarefied the read counts using

the minimum read count per sample that was higher than 2000 reads. Alpha diversity was calculated using Shannon index and the differences between groups were statistically tested with *p* value of 0.05 by using: (i) Wilcoxon test when comparing two groups; and (ii) Kruskal-Wallis and post-hoc pairwise Wilcoxon test when comparing three groups. The beta diversity was visualized by PCoA using Bray-Curtis distances and categorical variables were statistically tested using PERMANOVA test ($p < 0.05$) [48]. Since the sample size design was notably unbalanced in most beta diversity comparisons, additional PERMDISP tests [49] were performed to evaluate the heterogeneity of dispersions [50]. Relative read abundances of ASVs belonging to frequent nematode groups were statistically compared between *A. alfari* and *A. constructor* colonies using Kruskal-Wallis and post-hoc Wilcoxon test ($p < 0.05$).

For analysing the nematode diversity and community composition among different tree sections of established ant colonies, we used the separately sequenced patch samples per colony. The alpha and beta diversity indexes were calculated and statistically tested as described above.

d) Nematode counts

To evaluate if the relative read abundances of different nematode genera based on 18S rRNA gene metabarcoding correspond to their relative abundances [51, 52], we additionally performed morphological identification and manual quantification of nematodes in homogenized patch samples from four ant colonies (two established *A. alfari* and two established *A. constructor* colonies). First, alive nematodes were extracted from the patch matrix by centrifugation at 1800g for 4 min using a highly dense non-ionic solution (Nycodenz 1.16 g/mL 1x PBS). Subsequently, the supernatant, where most of the nematodes accumulated, was transferred to a new 2 mL tube. The nematodes were pelleted by centrifugation (18000g for 3 min) and washed with 0.2 µm filtered H_2O three times, each time followed by centrifugation. The pellet was stored in RNA-*later*. The nematode suspensions of the four established ant colonies were subsampled and specimens were manually classified to the respective species based on species-specific morphological characteristics and counted using a microscope (Zeiss Axioplan 100 x Plan-Neofluar).

Results

Amplicon sequencing of the partial 18S rRNA gene from 65 *Azteca* spp. ant colonies (Supporting Information S1) resulted in 1.61 x 10^6 total reads and 200 ASVs. For downstream analysis, we used 108 ASVs (90% of total reads) which were assigned to the phylum Nematoda. The non-Nematoda ASVs were mostly assigned to the phylum Arthropoda (29 ASVs and 9% of the total reads) or to other kingdoms of Eukarya (61 ASVs and 1% of the total reads).

a) Nematodes diversity and community composition remain similar as the colony grows

The alpha diversity of the nematode communities (Figure 2A) was stable across the different developmental stages of the ant colonies, both in *A. alfari* and *A. constructor* colonies (p = 0.18, x^2 = 3.40; and p = 0.19, x^2 = 1.68, respectively). Similarly, the nematode community composition (Figure 2A) did not significantly correlate with the ant colony developmental stages in *A. constructor* patches (p = 0.36, F = 1.05), while it was slightly correlated in *A. alfari* colonies (p = 0.04, F = 1.86). Furthermore, no significant differences were detected in the alpha and beta diversity analyses when comparing tree sections of established patches (p = 0.19, x^2 = 3.29; and p = 0.51, F = 0.91, respectively; Supporting Information S3).

b) The patches are inhabited by nematodes belonging to the Rhabditida, Tylenchida and Dorylaimida orders

The most prominent taxonomic order was Rhabditida with a mean relative read abundance of 93 ± 21 % and 93 ± 15 % in *A. alfari* and *A. constructor* colonies, respectively (Figure 2B). ASVs from this order were found in every ant colony and mostly assigned to the genera *Sclerorhabditis*, *Diploscapter* and unclassified Rhabditida. Following Rhabditida, ASVs assigned to the order Tylenchida (genera *Aphelenchoides* and *Anguina*) were detected in 12 out of 36 *A. alfari* colonies with an average 7 ± 21 % mean rel. read abundance and in 13 out of 23 *A. constructor* colonies with an average 2 ± 9 % mean rel. read abundance (Figure 2B). This order exhibited a sporadic dominance in certain initial and young patches, but was generally rare in established patches (0.5 % mean relative abundance).

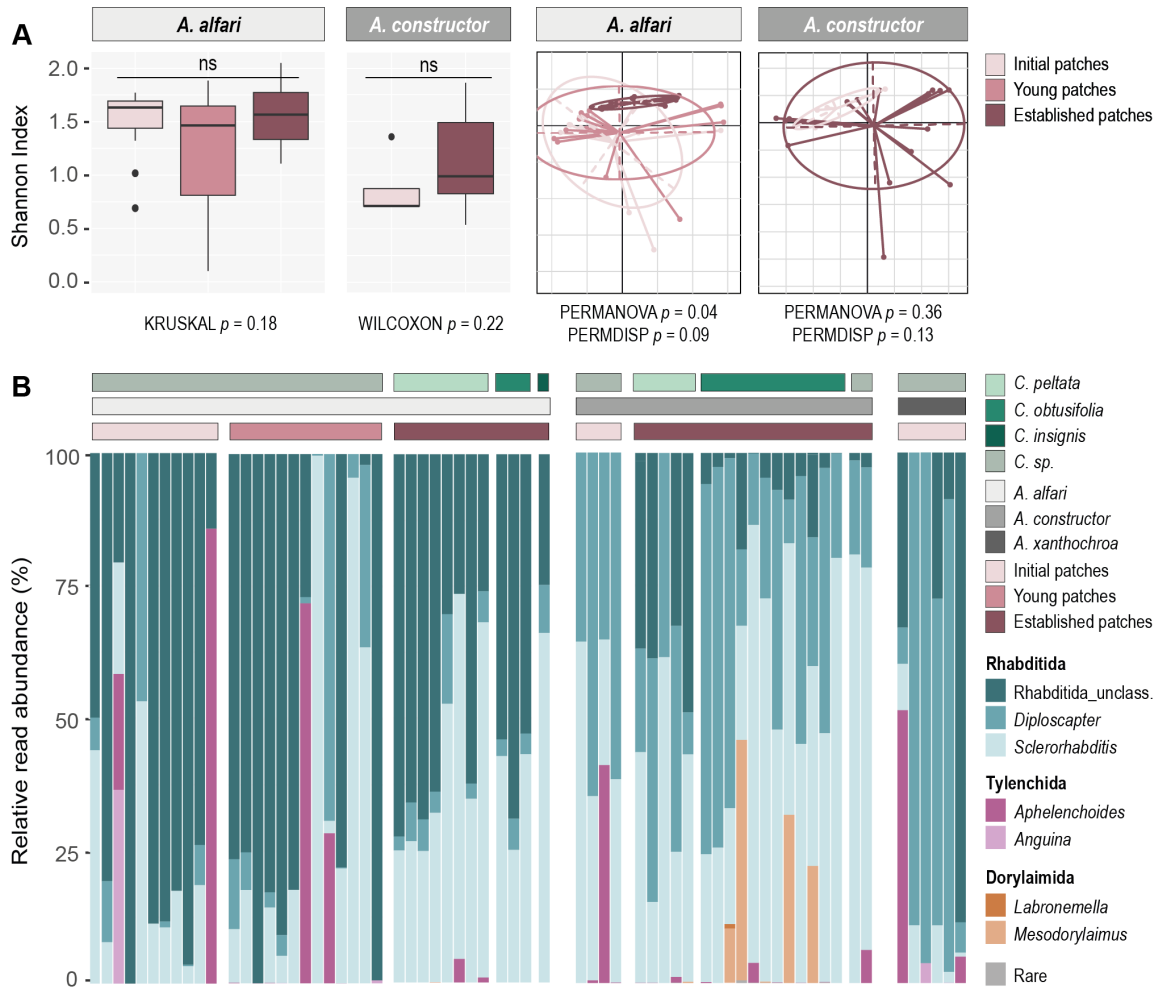

Figure 2. Comparison of nematode communities inhabiting ant-built patches among different ant colony development stages. (A) Alpha diversity (Shannon Index) and beta diversity (PCoA showing Bray-Curtis dissimilarity) metrics of each ant species. Statistical significance ($p < 0.05$) is calculated by Kruskal-Wallis or Wilcoxon test and by Permanova and Permdisp tests, respectively. (B) Relative read abundances (%) of abundant genera (>0.5%) per ant colony, grouped by Cecropia plant species, Azteca ant species and colony developmental stages. Low abundant taxa (<0.5%) are merged as "Rare".

Moreover, ASVs assigned to the taxonomic order Dorylaimida (mainly genus *Mesodorylaimus*) were detected only in *A. constructor* established colonies (6 out of 19) mostly nesting in *C. obstusifolia* trees (Figure 2B). Dorylaimida accounted for 18 ± 18 % rel. read abundance in these colonies.

c) Each ant species maintains a distinct nematode community

In order to evaluate whether the diversity or community composition of nematodes inhabiting the patches exhibits a significant correlation with the ant or plant species, we conducted alpha and beta diversity analyses across colonies. The Shannon Index showed that the nematode communities in *A. alfari* colonies exhibited a significantly higher diversity than those in *A. constructor* colonies in both, the initial (p = 0.037, x^2 = 4.36) and the established (p = 0.005, x^2 = 8.06) patches (Figure 3A).

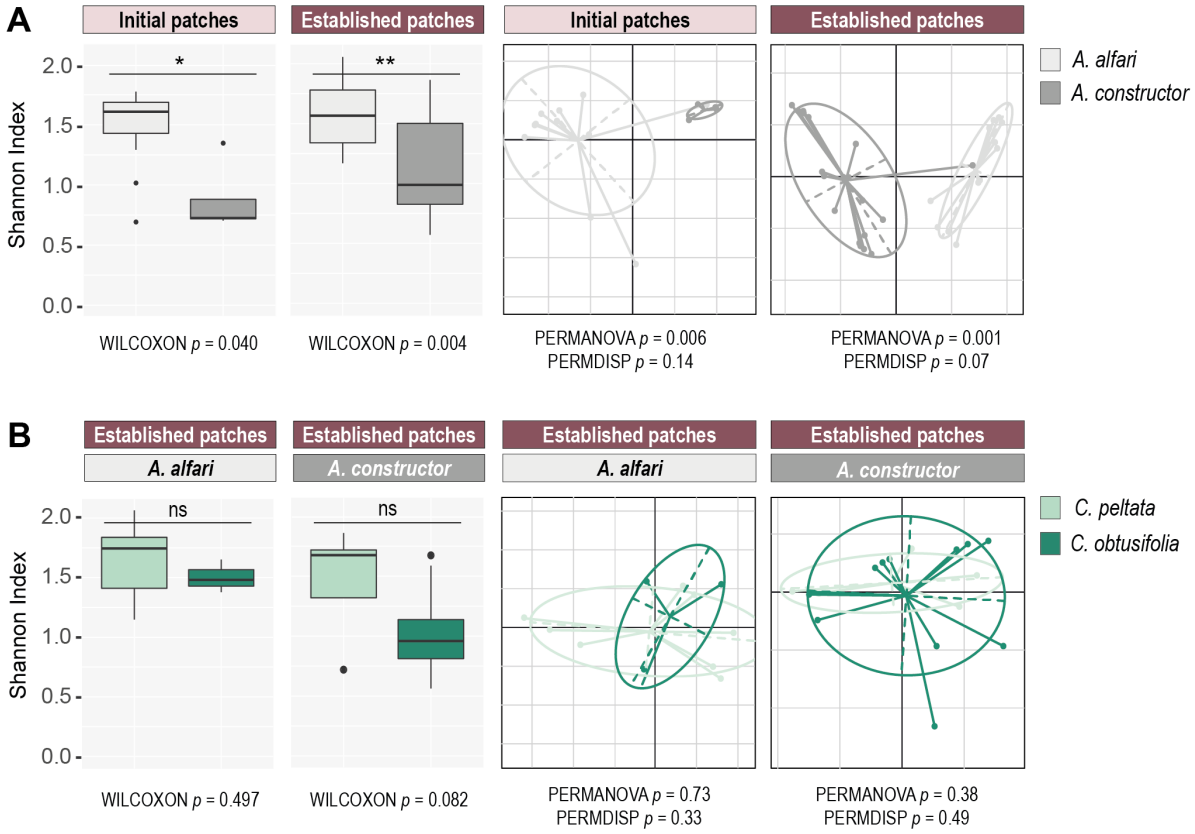

Figure 3. Comparison of nematode communities inhabiting ant-built patches between the different ant and plant species. (A) Alpha diversity (Shannon Index) and beta diversity (PCoA showing Bray-Curtis dissimilarity) metrics of initial and established patches between ant species. (B) Alpha diversity (Shannon Index) and beta diversity (PCoA showing Bray-Curtis dissimilarity) metrics of established patches between plant species. Statistical significance ($p < 0.05$) is calculated by Wilcoxon test and by Permanova and Permdisp tests, respectively.

Furthermore, the Bray-Curtis dissimilarity and the Permanova test revealed statistically significant differences in the nematode communities between *A. alfari* and *A. constructor* in both, the initial (p = 0.006, F = 4.65) and the established (p = 0.001, F = 12.71) patches (Figure 3A). In contrast, the nematode alpha and beta diversity in established patches of each ant species did not vary between plant species (*C. peltata* and *C. obstusifolia*) (Figure 3B). Given the notably unbalanced sample size between the tested groups (Supporting Information S1), an additional PERMDISP test was performed in beta diversity analyses to ensure the statistical robustness (Figure 3).

d) The same rhabditid groups occur in both ant species with significantly different relative abundances

To further evaluate the effect of the ant species on the nematode communities, we compared the relative abundances of the three most abundant nematode groups (>10 % mean rel. abundance) in the established patches of each ant species based on sequence analysis and morphological identification (Figure 4).

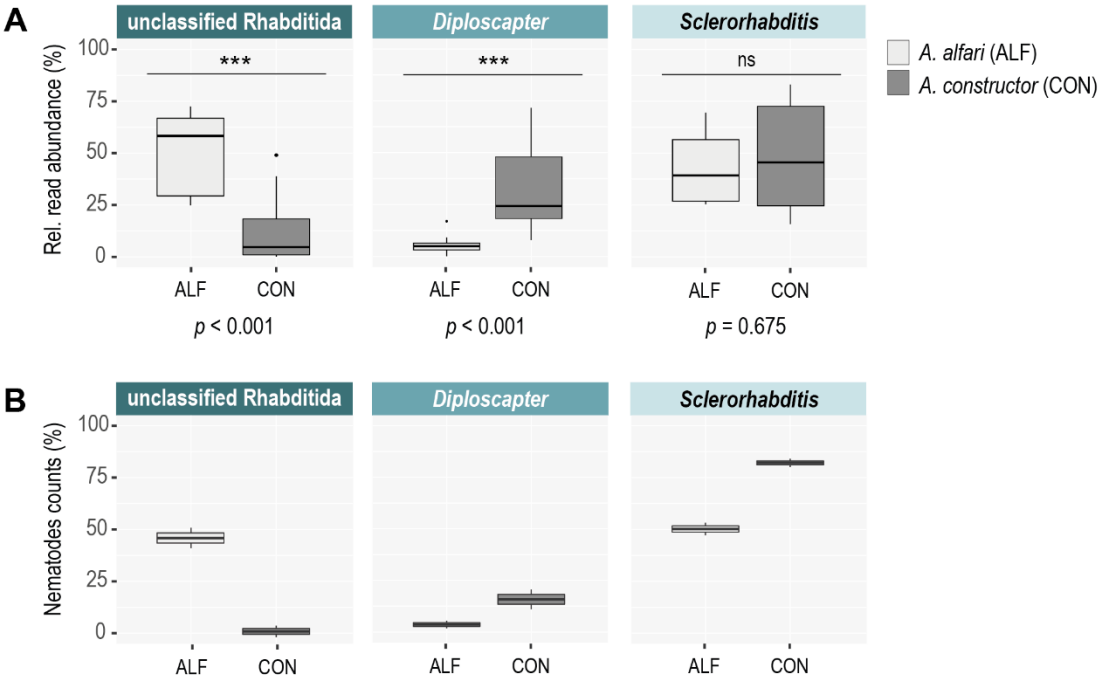

Figure 4. Relative abundance (%) of the most abundant nematode groups from the order Rhabditida in established patch samples of A. alfari (ALF) and A. constructor (CON) colonies based on: (A) 18S rRNA sequence reads; and, (B) nematodes counts. The unclassified group ("unclass.") might be composed of several genera. Statistical significance ($p < 0.05$) is calculated by Wilcoxon test.

By morphological identification, we detected an undescribed rhabditid nematode of a notably small body size, which probably matches the closely related ASVs assigned to unclassified Rhabditida in the sequence data (Supporting information S4). Both, relative read abundance and morphological nematode counts indicated that this unclassified Rhabditida group was significantly less abundant in *A. constructor* colonies than in *A. alfari* colonies ($p < 0.001$) (Figure 4). Instead, *Diploscapter* was significantly more abundant in the established patches of *A. constructor* colonies compared to *A. alfari* colonies ($p < 0.001$) (Figure 4).

Discussion

a) The nematode inhabitants of the *Azteca-Cecropia* patches

Three distinct orders of nematodes (Rhabditida, Tylenchida and Dorylaimida) exhibiting diverse putative feeding strategies were identified in the ant-made patches of the *Azteca-Cecropia* mutualism. As observed in other ant-plant mutualisms and social insect nests [8, 16, 21], bacteria-grazing rhabditid nematodes were by far the most abundant and frequent group among the 65 colonies investigated. In addition to the dominant Rhabditida, also members of the orders Tylenchida (e.g. *Aphelenchoides*) and Dorylaimida (e.g. *Mesodorylaimus*) appeared sporadically in the patches. While nematodes of the genus *Mesodorylaimus* are typically omnivorous [53] and in some cases predators of other nematodes [54, 55], *Aphelenchoides* species are known to feed either on fungi or on plant tissue [56]. The latest genus was also isolated from the nest of a fungus-growing termite [13] and from the oviduct of carpenter bees [57].

b) Transmission and development of the patch nematodes communities

The homogeneity of the nematode diversity and community composition between the initial and established patches shown in this study provides key information about the transmission and spatio-temporal dynamics of the nematode patch communities in relation to the growth of the ant colonies. The results obtained for the *Azteca-Cecropia* ant-plant mutualism provide substantial support of the hypothesis that the

transmission of rhabditid nematodes occurs from mother to daughter colonies [16, 21, 28], rather than being recruited from the environment. As shown in previous studies, alate *Azteca* queens transport rhabditid nematodes either attached to the body [21] or in the infrabuccal pocket where they store patch particles they had collected in the mother colony [28]. The nematodes are then transferred to the initial patch which the foundress makes immediately after the colonization of the *Cecropia* host tree [28]. Once the first workers emerge, they colonize other internodes of the tree where they build new patch structures [25]. Since the nematode community composition of freshly made patches in recently grown plant internodes is similar to the patches in the older ones (Supporting information S3), we can also infer a transmission of rhabditid nematodes within the ant nest during ant colony growth.

In contrast to rhabditids, the tylenchids and dorylaimids were not widespread among the different ant colony developmental stages. The *Mesodorylaimus* ASVs (Dorylaimidae, Dorylaimida) were found to be dominant only in established patches from *A. constructor* colonies inhabiting large *Cecropia obtusifolia* trees and thus, they might be rather transmitted via patrolling of ant workers or patch visitors. Similar to human-made compost piles [58, 59], *Mesodorylaimus* appeared only after the fast-growing bacterivorous nematodes had reached dominance within the patches during the earlier phase of patch maturation. The *Aphelenchoides* ASVs (Aphelenchoididae, Tylenchida) were relatively abundant in certain initial and young patches of all three *Azteca* species. This suggests that they may have been introduced by the queen from the surrounding environment during the plant colonization.

c) Influence of the *Azteca* ant and *Cecropia* plant species on nematode communities

The bacteria-grazing rhabditid nematodes detected in the patches belong to the genera *Diploscapter* and *Sclerorhabditis* (both with 0.5-1 mm in length) and to an unclassified Rhabditida group characterised by a notably small body size. All three rhabditid groups were jointly present in 29 out of the 31 established colonies investigated. However, it appears that each ant species promotes the development of specific rhabditid nematode communities, despite their coexistence in the same geographical area. The differences between the relative abundances of *Diploscapter* and the unclassified Rhabditida group in *A. alfari* and *A. constructor*, characterized

by differing body sizes, might result from the unique patch habitat that each ant species creates [30] and which could influence whether the habitat is suitable for a particular nematode species [60]. Nematodes need water films for movement and feeding [61, 62]. Furthermore, in soil, the distribution of nematode size has been shown to correlate with the distribution of aggregate and pore size, as larger nematodes require larger inter-aggregate spaces than smaller nematodes [61, 63]. In fact, *A. constructor* forms relatively big, three dimensional and very moist structures, whereas *A. alfari* patches are thin, dry and sandy [25, 64]. The low moisture level and physical structure in *A. alfari* patches may drive the community towards smaller nematodes as has been shown in soils under drought stress [65]. Thus, the ant species may be a significant driver of the nematode diversity within these patches, as has recently been shown for the fungal and bacterial communities in the patches of the *Azteca-Cecropia* association [24, 25, 30].

d) Ant-plant-nematode relationships: parasitism, commensalism or mutualism?

Fossil records indicate that nematodes and ants coexist for at least 40 million years [14], one of the fossils is particularly interesting in the context of the present work: In 20-30 million years old Dominican amber, dauer juveniles of a diplogastrid nematode occur next to a fossil *Azteca* ant [66]. This is the first documentation of Rhabditida nematodes and *Azteca* ants in the same environment.

Ant-nematode associations are widespread in saprobiontic environments (Wahab 1962; Köhler 2012). The occurrence of nematode dauer larvae in the postpharyngeal glands of ants was already described in the 19th century (Janet 1893, 1894) and meanwhile known from many ant species. Nematodes found in ant nests are mainly found in the detritus made by the ants [8]. In ant-plant mutualisms, rhabditid nematodes were originally thought to act as facultative parasites of ants since some damage to the postpharyngeal glands of alates was documented [66]. However, recent investigations have proposed a harmless or rather mutualistic association [8, 16, 21, 22]. The findings from our large-scale study supports that the nematodes are not detrimental since (i) all colonies investigated so far had healthy ant workers and brood as well as healthy host trees although big masses of nematodes have been observed actively moving on the surface of the patches in every colony (Supporting

Information S5), even in those close to the brood [16, 22]; and, (ii) the identified nematodes do not belong to the typical parasitic groups of nematodes found in the tropical rainforest of Costa Rica [67].

For the nematodes, the association is clearly beneficial. They are transported to suitable habitats with the ants as vectors, are well protected against predators inside the ant nests, and due to the deposition of ant waste onto the patches the bacterial growth remains stable and with that also the food supply. Whether, or to what extent the presence of these nematodes is beneficial to the ant colony remains to be determined. Multiple investigations have proposed that nematodes could be fed to the ant larvae when their regular food resources are scarce [16, 21]. Until now, such feeding behaviour has never been recorded and all manipulative experiments with these arboreal ants have failed [25]; therefore, the hypothesis of nematodes as a food source for ant larvae still needs to be tested.

From an ecological perspective, nematodes could contribute to the overall stability and functioning of the patch-inhabiting microbiota. As it has been repeatedly demonstrated in forest and agricultural soils [1, 68–70], nematodes provide a vast variety of ecosystem services. First, the bacterial-grazing feeding behaviour could control the biomass of bacteria and thus, contribute to maintaining a balanced food web in the patches [71]. Second, their activity could enhance organic matter transformation processes in the patches [72]. Recent investigations in the *Azteca-Cecropia* mutualism have measured high rates of atmospheric nitrogen fixation which were attributed to bacteria inhabiting the patches [30]. By feeding on bacteria and by excreting the excess N in the form of ammonia, bacterivorous nematodes could increase nitrogen mineralization in the patches [73]. In pine forest soils for example, it has been shown that bacterivorous and predatory nematodes contribute 8–19% of nitrogen mineralization under field conditions [74]. Last, nematodes could act as engineers of the patch structure at the micro-scale as they probably aerate and mix organic matter while moving through the surface of the patches (Supporting Information S5). Investigating whether nematodes contribute to these ecosystem services within the patches will provide valuable insights into the extensive yet unexplored interactions between ant-plant-nematode associations.

Conclusions

The 18S rRNA gene metabarcoding approach used in this study has provided valuable semiquantitative information on the taxonomic diversity and community composition of nematodes in the ant-made patches of the *Azteca-Cecropia* mutualism. The findings from this study support the hypothesized transmission from mother to daughter colonies and among patches of the same ant colony. By creating and maintaining a unique environment within their nest, each *Azteca* ant species favours the development of distinct Rhabditida groups in the patches. Based on our findings, we put forward the hypothesis that the nematodes may play an important role in maintaining the overall stability and functionality of the patches through the provision of different ecosystem services. Future research in this model system should aim for: (i) the use of the classical nematodes counts by morphological identification methods to obtain the absolute abundance of each nematode group among patches from different ant colony developmental stages and ant species; and, (ii) the optimization of an *in vivo* isotope-labelled nematode assay to elucidate whether the ants use the biomass-rich nematode resource as additional food for the ant colony.

Declarations

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Conflict of interest: All authors declare that they have no conflict of interest.

Author contributions: V.E.M., M.N. and D.W. designed the research. V.B.S., V.E.M. and M.N. performed the research. V.B.S. and B.H. contributed with analytical tools. V.B.S. and B.H. processed and analysed the data; V.B.S analysed and interpreted the results. V.E.M. and D.W. provided significant intellectual contribution. V.B.S wrote the original draft of the manuscript. All authors contributed to the final version and approved it for publication.

Statement on inclusion: This research was conducted under the permission of the Costa Rican authorities who provided the following research permits "R-046-2015-OT-CONAGEBIO", "SINAC INV-ACOSA-001-16" and "INV-ACOSA-013-18". In accordance to these research permits, we followed the national laws of Costa Rica "Ley de Conservación de la Vida Silvestre Nº 7317", "Ley de Biodiversidad Nº 7788" and "Ley Orgánica del Ambiente Nº 7554" and applied the General Protocol "MINAE-SINAC-P-001" for the use of the Protected Wildlife Areas of the National System of Conservation Areas. Benefits from this research accrue from sharing all our data (i.e. metadata, sequencing data, taxonomic tables and R scripts) on public databases.

Data availability statement: All sequence data (raw sequence reads and metadata) are accessible on NCBI under the BioProject accession number PRJNA777006. The 18S rRNA amplicon sequencing dataset and the R script obtained in this investigation will be uploaded at figshare upon acceptance (https://doi.org/10.6084/m9.figshare.c.7499025).

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Supporting Information

Supporting Information S1: Overview of samples used in this study

Table S1.1 Overview of sampled ant colonies (in total n=65) per *Azteca* species and colony development stage.

	A. alfari	*A. constructor*	*A. xanthochroa*	Sum
Initial patches (IP)	11	4	6	21
Young patches (YP)	13	0	0	13
Established patches (EP)	12	19	0	31
Sum	36	23	6	65

Table S1.2 Overview of sampled established ant colonies (in total n=31) per *Azteca* species and *Cecropia* species.

	A. alfari	*A. constructor*	Sum
C. peltata	8	5	13
C. obtusifolia	3	12	15
C. insignis	1	0	1
C. sp	0	2	2
Sum	12	19	31

Supporting Information S2: PCR protocol and program used in this study

Methods S2.1

In the first step of the semi-nested PCR, 4 µL of DNA (2 ng/µL) were added to a 50 µL PCR reaction per patch sample. After 15 cycles of amplification, the PCR product was normalized with a SEQPREP Normalization Plate Kit 96 (Thermo Fisher Scientific, A1051001). In the second step of the semi-nested PCR, 4 µL of a 1:10 dilution of the PCR product was added to a 50 µL PCR reaction. The reactions were amplified for another 15 cycles and subsequently, normalized as described above. In the last barcoding PCR step, 10 µL of the PCR product from the first step PCR were added to a 50 µL PCR reaction. After 8 cycles of amplification, PCR products were normalized again as described above and subsequently, all samples were purified by the innuPREP PCR pure Kit (Analytic Jena, 845-KS-5010250).

Table S2.2 Primer sequences used in this study. Primers N1F and 18Sr2b had been modified by adding a 16 nt head sequence (H) for the subsequent barcoding PCR step.

Primer	Sequence	Reference
NemF	5'-GGGGAAGTATGGTTGCAAA-3'	Sapkota & Nicolaisen, 2015
(H)-N1F	5'- GCTATGCGCGAGCTGC GGTGGTGCATGGCCGTTCTTAGTT-3'	modified after Porazinska et al., 2009
(H)-18Sr2b	5'- TAGCGCACACCTGGTA TACAAAGGGCAGGGACGTAAT-3'	modified after Porazinska et al., 2009

Table S2.3 PCR cycle programs used for the amplification of partial 18S rRNA gene.

PCR program	Cycles	T (°C)	Time	PCR program	Cycles	T (°C)	Time
1st step semi-nested PCR	1x	95	3 min	2nd step semi-nested PCR	1x	95	3 min
	15x	95	30 sec		15x	95	30 sec
		53	30 sec			58	30 sec
		72	1 min			72	1 min
	1x	72	7 min		1x	72	7 min
		4	∞			4	∞

PCR program	Cycles	T (°C)	Time
3° step PCR (Barcoding)	1x	94	4 min
	7x	94	30 sec
		52	30 sec
		72	1 min
	1x	72	7 min
		4	∞

Supporting Information S3: Comparison of nematodes communities among tree sections

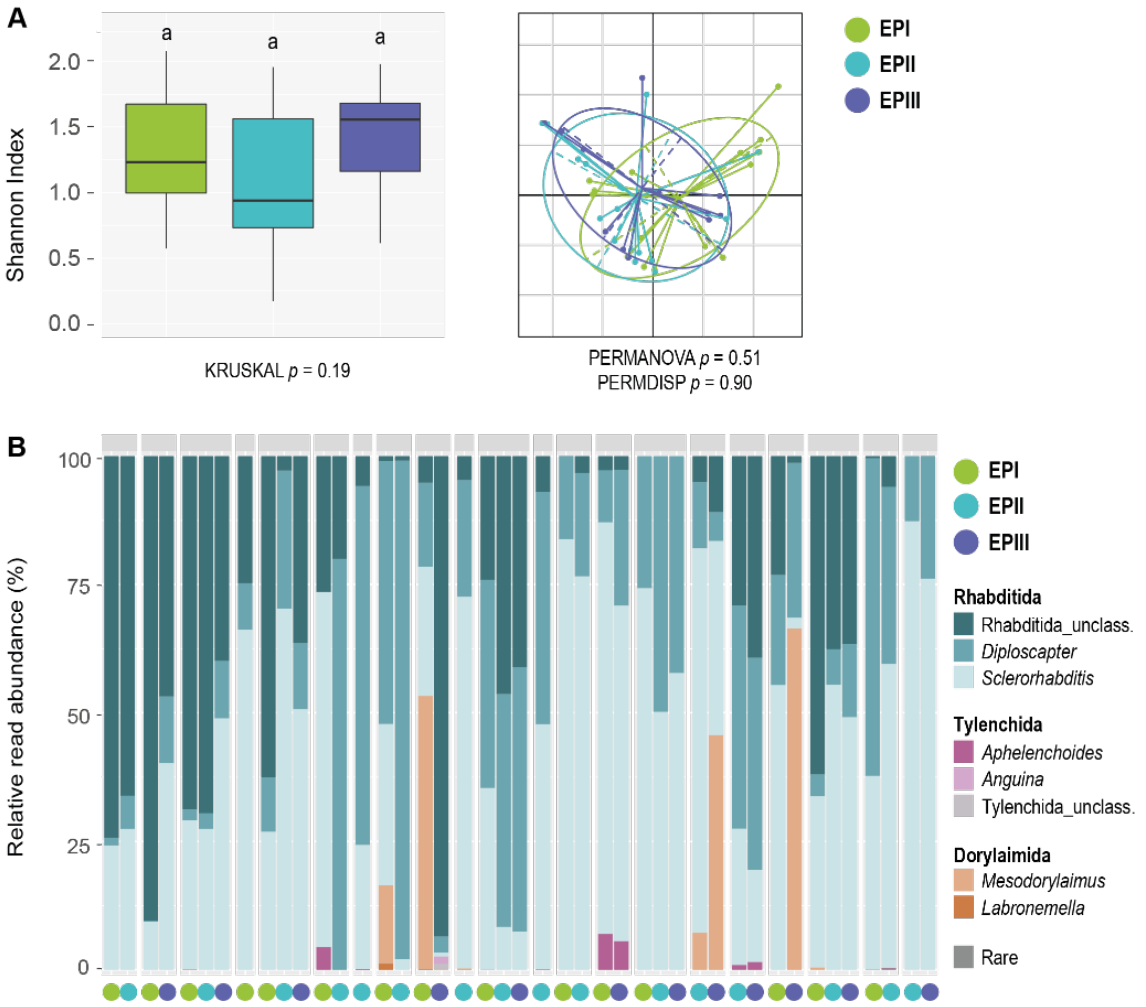

Figure S3.1 Comparison of nematode communities in established patches among tree sections (EPI: apical and younger internodes of the tree; EPII: intermediate internodes of the tree where most brood and pupae are found; EPIII: lower and older internodes of the tree. (A) Alpha diversity (Shannon Index) and beta diversity (PCoA showing Bray-Curtis dissimilarity) metrics. Statistical significance ($p < 0.05$) is calculated by Kruskal-Wallis and post-hoc Wilcoxon tests and by Permanova and Permdisp tests, respectively. (B) Relative read abundances (%) of abundant genera (>0.5%) per tree section in each ant colony. Low abundant taxa (<0.5%) are merged as "Rare".

Supporting Information S4: Similarity analysis of unclassified Rhabditida ASVs

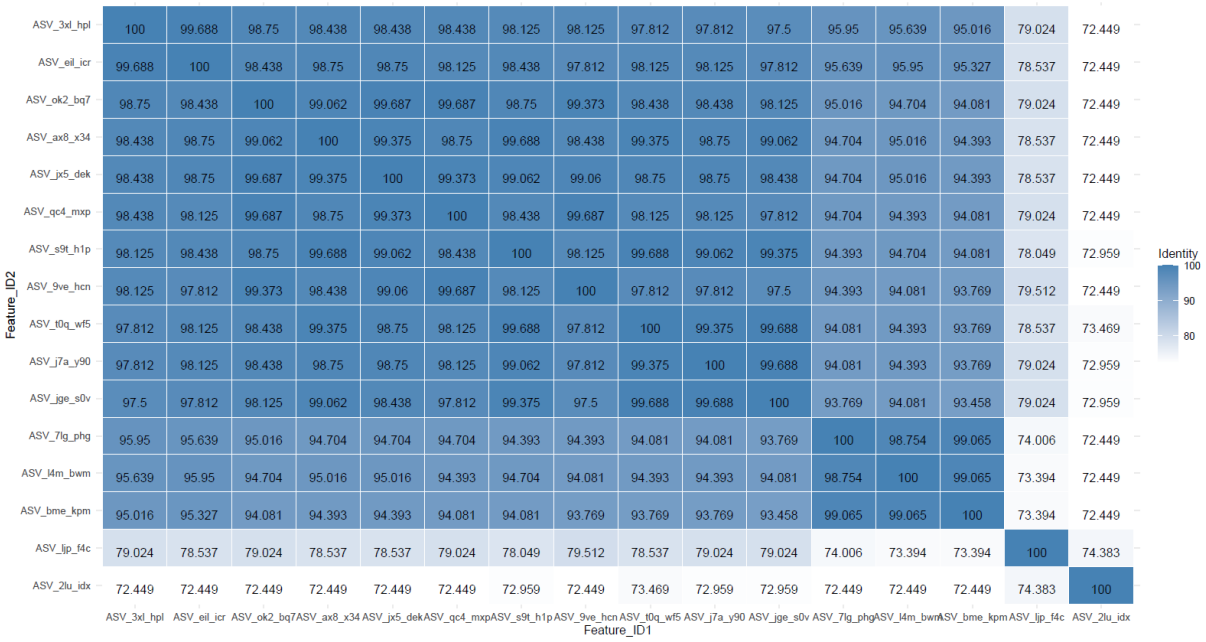

Figure S4.1 Similarity matrix of the 16 most abundant unclassified Rhabditida ASVs.

Supporting Information S5: Video of nematodes on the patches

The Supporting Information S5 includes a video showing the notably high biomass of nematodes moving on the patches of an *Azteca constructor* colony. The video was made by Veronika E. Mayer (co-author) with a high-resolution camera fixed on a stereomicroscope. This video (in ".avi" format) can be found at the figshare public repository (https://doi.org/10.6084/m9.figshare.c.7499025) upon acceptance of the manuscript in a scientific journal.

References

1. Yeates GW. Nematodes in ecological webs. Encycl Life Sci. 2010; June. https://doi.org/10.1002/9780470015902.a0021913.

2. Bongers T, Bongers M. Functional diversity of nematodes. Appl Soil Ecol. 1998;10:239–51.

3. Topalović O, Geisen S. Nematodes as suppressors and facilitators of plant performance. New Phytol. 2023;238:2305–12.

4. Tarasco E, Fanelli E, Salvemini C, El-Khoury Y, Troccoli A, Vovlas A, et al. Entomopathogenic nematodes and their symbiotic bacteria: from genes to field uses. Front Insect Sci. 2023;3 August:1–13.

5. Giblin-Davis RM, Kanzaki N, Davies KA. Nematodes that ride insects: Unforeseen consequences of arriving species. Florida Entomol. 2013;96:770–80.

6. Borges RM. Phoresy involving insects as riders or rides: Life history, embarkation, and disembarkation. Ann Entomol Soc Am. 2022;115:219–31.

7. Foley JR, Chouvenc T, Giblin-davis RM, Su N, Kanzaki N. Phoresy and within-colony transmission of nematodes associated with alates of subterranean termites (Isoptera: Rhinotermitidae). Environ Entomol. 2018;47 August:1107–16.

8. Sudhaus W. The guild of saprobiontic nematodes associated with ants (Formicoidea). Ecol Montenegrina. 2016;7:600–13.

9. Brown FD, D'Anna I, Sommer RJ. Host-finding behaviour in the nematode *Pristionchus pacificus*. Proc R Soc B. 2011;278:3260–9.

10. Wang Y, Rozen DE. Fitness costs of phoretic nematodes in the burying beetle, *Nicrophorus vespilloides*. Ecol Evol. 2019;9:26–35.

11. Ledón-Rettig CC, Moczek AP, Ragsdale EJ. *Diplogastrellus* nematodes are sexually transmitted mutualists that alter the bacterial and fungal communities of their beetle host. PNAS. 2018;115:10696–701.

12. Van Goor J, Piatscheck F, Houston DD, Nason JD. Differential effects of nematode infection on pollinating and non-pollinating fig wasps: Can shared antagonism provide net benefits to a mutualism? J Anim Ecol. 2021;90:1764–75.

13. Kanzaki N, Liang WR, Chiu CI, Yang CT, Hsueh YP, Li HF. Nematode-free agricultural system of a fungus-growing termite. Sci Rep. 2019;9:1–10.

14. Poinar G. The evolutionary history of nematodes: As revealed in stone, amber and mummies. Brill; 2011.

15. Mahboob M, Tahseen Q. Diversity, prevalence and microhabitat specificity of nematodes (Rhabditidae Örley, 1880 and Diplogastridae Micoletzky, 1922) associated with insects: an overview. Int J Pest Manag. 2021;0:1–42.

16. Maschwitz U, Fiala B, Dumpert K, Hashim R bin, Sudhaus W. Nematode associates and bacteria in ant-tree symbioses. Symbiosis. 2016;69:1–7.

17. González-Teuber M, Heil M. Comparative anatomy and physiology of myrmecophytes: ecological and evolutionary perspectives. Res Reports Biodivers Stud. 2015;4:21–32.

18. Fischer RC, Richter A, Wanek W, Mayer V. Plants feed ants: Food bodies of myrmecophytic *Piper* and their significance for the interaction with *Pheidole bicornis* ants. Oecologia. 2002;133:186–92.

19. Heil M, McKey D. Protective Ant-plant Interactions as Model Systems in Ecological and Evolutionary Research. Annu Rev Ecol Evol Syst. 2003;34:425–53.

20. Michelangeli FA. Ant protection against herbivory in three species of *Tococa* (Melastomataceae) occupying different environments. Biotropica. 2003;35:181–8.

21. Morera J, Mora-Pineda G, Esquivel A, Hanson P. Detection, ultrastructure and phylogeny of *Sclerorhabditis neotropicalis* (Nematoda: Rhabditidae) nematodes associated with the *Azteca* ant-*Cecropia* tree symbiosis. Rev Biol Trop. 2018;66:368–80.

22. Esquivel A, Abolafia J, Hanson P, Pinto A. A new species of nematode, *Sclerorhabditis neotropicalis* sp.n. (Rhabditida), associated with *Azteca* ants in *Cecropia obtusifolia*. Nematropica. 2012;42:163–9.

23. Blatrix R, Bouamer S, Morand S, Selosse MA. Ant-plant mutualisms should be viewed as symbiotic communities. Plant Signal Behav. 2009;4:554–6.

24. Nepel M, Mayer VE, Barrajon-Santos V, Woebken D. Bacterial diversity in arboreal ant nesting spaces is linked to colony developmental stage. Commun Biol. 2023;6:1217.

25. Barrajon-Santos V, Nepel M, Hausmann B, Voglmayr H, Woebken D, Mayer VE. Dynamics and drivers of fungal communities in a multipartite ant-plant association. BMC Biol. 2024;22:112.

26. Defossez E, Selosse MA, Dubois MP, Mondolot L, Faccio A, Djieto-Lordon C, et al. Ant-plants and fungi: A new threeway symbiosis. New Phytol. 2009;182:942–9.

27. Mayer VE, Voglmayr H, Blatrix R, Orivel J, Leroy C. Fungi as mutualistic partners in ant-plant interactions. Front Fungal Biol. 2023;4 October:1–14.

28. Mayer VE, Nepel M, Blatrix R, Oberhauser FB, Fiedler K, Schönenberger J, et al. Transmission of fungal partners to incipient *Cecropia*-tree ant colonies. PLoS One. 2018;13:e0192207.

29. Lucas J, Bill B, Stevenson B, Kaspari M. The microbiome of the ant-built home: the microbial communities of a tropical arboreal ant and its nest. Ecosphere. 2017;8:e01639.

30. Nepel M, Pfeifer J, Oberhauser FB, Richter A, Woebken D, Mayer VE. Nitrogen fixation by diverse diazotrophic communities can support population growth of arboreal ants. BMC Biol. 2022;20:135.

31. Berg CC, Roselli PF, Davidson DW. *Cecropia*. Flora Neotrop. 2005;:1–230.

32. Longino JT. Taxonomy of the *Cecropia* -inhabiting *Azteca* ants. J Nat Hist. 1991;25:1571–602.

33. Longino JT. A taxonomic review of the genus *Azteca* (Hymenoptera: Formicidae) in Costa Rica and a global revision of the aurita group. Zootaxa. 2007;1491:1–63.

34. Angel R, Claus P, Conrad R. Methanogenic archaea are globally ubiquitous in aerated soils and become active under wet anoxic conditions. ISME J. 2012;6:847–62.

35. Sapkota R, Nicolaisen M. High-throughput sequencing of nematode communities from total soil DNA extractions. BMC Ecol. 2015;15:1–8.

36. Ahmed M, Back MA, Prior T, Karssen G, Lawson R, Adams I, et al. Metabarcoding of soil nematodes: the importance of taxonomic coverage and availability of reference sequences in choosing suitable marker(s). Metabarcoding and Metagenomics. 2019;3:77–99.

37. Pjevac P, Hausmann B, Schwarz J, Kohl G, Herbold CW, Loy A, et al. An economical and flexible dual barcoding, two-step PCR approach for highly multiplexed amplicon sequencing. Front Microbiol. 2021;12:669776.

38. Macheriotou L, Guilini K, Bezerra TN, Tytgat B, Nguyen DT, Phuong Nguyen TX, et al. Metabarcoding free-living marine nematodes using curated 18S and CO1 reference sequence databases for species-level taxonomic assignments. Ecol Evol. 2019;9:1211–26.

39. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods. 2016;13:581–3.

40. R Core Team. R: A Language and environment for statistical computing. 2022. http://www.r-project.org.

41. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol. 2007;73:5261–7.

42. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. Nucleic Acids Res. 2012;41:590–6.

43. Zhang Z, Schwartz S, Wagner L, Miller W. A greedy algorithm for aligning DNA sequences. J Comput Biol. 2000;7:203–14.

44. RStudio Team. RStudio: Integrated Development Environment for R. 2022. http://www.rstudio.com/.

45. Andersen KS, Kirkegaard RH, Karst SM, Albertsen M. ampvis2: an R package to analyse and visualise 16S rRNA amplicon data. bioRxiv. 2018;:299537.

46. Oksanen J, Simpson GL, Blanchet FG, Kindt R, Legendre P, Minchin PR, et al. Vegan: Community Ecology package. 2022. https://cran.r-project.org/web/packages/vegan/index.html.

47. Chen J, Bittinger K, Charlson ES, Hoffmann C, Lewis J, Wu GD, et al. Associating

microbiome composition with environmental covariates using generalized UniFrac distances. Bioinformatics. 2012;28:2106–13.

48. Anderson MJ. A new method for non-parametric multivariate analysis of variance. Austral Ecol. 2001;:32–46.

49. Anderson MJ, Ellingsen KE, McArdle BH. Multivariate dispersion as a measure of beta diversity. Ecol Lett. 2006;9:683–93.

50. Anderson MJ, Walsh DC. PERMANOVA, ANOSIM, and the Mantel test in the face of heterogeneous dispersions: What null hypothesis are you testing? Ecol Monogr. 2013;83:557–74.

51. Du XF, Li Y Bin, Han X, Ahmad W, Li Q. Using high-throughput sequencing quantitatively to investigate soil nematode community composition in a steppe-forest ecotone. Appl Soil Ecol. 2020;152.

52. Schenk J, Kleinbölting N, Traunspurger W. Comparison of morphological, DNA barcoding, and metabarcoding characterizations of freshwater nematode communities. Ecol Evol. 2020;10:2885–99.

53. Yeates GW, Bongers T, De Goede RGM, Freckman DW, Georgieva SS. Feeding habits in soil nematode families and genera - An outline for soil ecologists. J Nematol. 1993;25:315–31.

54. Bilgrami AL. Biological Control Potentials Of Predatory Nematodes. In: Integrated Management and Biocontrol of Vegetable and Grain Crops Nematodes. 2008. p. 3–28.

55. Cabos RYM, Wang KH, Sipes BS, Heller WP, Matsumoto TK. Detection of plant-parasitic nematode DNA in the gut of predatory and omnivorous nematodes. Nematropica. 2013;43:44–8.

56. Ruess L, Garcia Zapata EJ, Dighton J. Food preferences of a fungal-feeding *Aphelenchoides* species. Nematology. 2000;2:223–30.

57. Kanzaki N. Description of *Aphelenchoides xylocopae* n. sp. (Nematoda: Aphelenchoididae), the first observed association between nematodes and carpenter bees. Nematology. 2006;8:555–62.

58. Zapałowska A, Skwiercz A, Tereba A, Puchalski C, Malewski T. Next-Generation sequencing for evaluating the soil nematode diversity and its role in composting processes. Int J Mol Sci. 2023;24.

59. Steel H, Moens T, Vandecasteele B, Hendrickx F, De Neve S, Neher DA, et al. Factors influencing the nematode community during composting and nematode-based criteria for compost maturity. Ecol Indic. 2018;85 June 2017:409–21.

60. Bongers T. The maturity index, the evolution of nematode life history traits, adaptive radiation and cp-scaling. Plant Soil. 1999;212:13–22.

61. Neher DA. Ecology of plant and free-living nematodes in natural and agricultural soil. Annu Rev Phytopathol. 2010;48:371–94.

62. Zheng L, Wu S, Lu L, Li T, Liu Z, Li X, et al. Unraveling the interaction effects of soil temperature and moisture on soil nematode community: A laboratory study. Eur J Soil Biol. 2023;118 August:103537.

63. Bongers T, Ferris H. Nematode community structure as a bioindicator in environmental monitoring. Trends Ecol Evol. 1999;14:224–8.

64. Longino JT. Azteca ants in Cecropia trees: taxonomy, colony structure, and behaviour. In: Cutler C, Huxley D, editors. Ant-plant interactions. Oxford University Press; 1991. p. 271–88.

65. Lu L, Li G, He N, Li H, Liu T, Li X, et al. Drought shifts soil nematodes to smaller size across biological scales. Soil Biol Biochem. 2023;184 June:109099.

66. Poinar G. Nematode parasites and associates of ants: Past and present. Psyche (London). 2012;2012.

67. Powers TO, Neher DA, Mullin P, Esquivel A, Giblin-Davis RM, Kanzaki N, et al. Tropical nematode diversity: Vertical stratification of nematode communities in a Costa Rican humid lowland rainforest. Mol Ecol. 2009;18:985–96.

68. Gebremikael MT, Steel H, Buchan D, Bert W, De Neve S. Nematodes enhance plant growth and nutrient uptake under C and N-rich conditions. Sci Rep. 2016;6:32862.

69. Jiang Y, Liu M, Zhang J, Chen Y, Chen X, Chen L, et al. Nematode grazing promotes bacterial community dynamics in soil at the aggregate level. ISME J. 2017;11:2705–17.

70. Porazinska DL, Giblin-Davis RM, Powers TO, Thomas WK. Nematode spatial and ecological patterns from tropical and temperate rainforests. PLoS One. 2012;7:1–11.

71. Morise H, Miyazaki E, Yoshimitsu S, Eki T. Profiling nematode communities in unmanaged flowerbed and agricultural field soils in Japan by DNA barcode sequencing. PLoS One. 2012;7.

72. Ferris H. Contribution of nematodes to the structure and function of the soil food web. J Nematol. 2010;42:63–7.

73. Ferris H, Venette RC, Scow KM. Soil management to enhance bacterivore and fungivore nematode populations and their nitrogen mineralisation function. Appl Soil Ecol. 2004;25:19–35.

74. Neher DA, Weicht TR, Barbercheck ME. Linking invertebrate communities to decomposition rate and nitrogen availability in pine forest soils. Appl Soil Ecol. 2012;54:14–23.

Chapter III

Composting in an ant-plant nest? Activity and metabolic potential of ant-associated microbial communities for degrading polysaccharide-rich substrates

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Abstract

The establishment and maintenance of an efficient microbiome throughout the lives of many animals are essential for their survival and proper development. Tropical arboreal ants, particularly those living in mutualism with plants, are known to host highly diverse and complex microbial communities within their nests, specifically in ant-built piles known as patches. These patches and the organisms inhabiting them appear to be crucial for the development and survival of ant colonies, yet their specific roles within the ant-plant complex remain largely unknown. While ants consistently supply these microorganisms with a wide variety of plant- and insect-based waste, the ability of these communities to efficiently metabolize such substrates has not been demonstrated. In this study, we investigated the potential of patch microbial communities to metabolize the ant waste in one of the most prominent ant-plant mutualisms in the Tropics of America, the *Azteca-Cecropia* complex. By performing isotope-based activity assays with patch samples, we demonstrated that the patch microbial communities can degrade the cellulose and chitin contained in the deposited substrates. Furthermore, through metagenomic analysis, we further revealed that a rich and diverse genetic repertoire involved in polysaccharide breakdown is widely distributed among the reconstructed 214 MAGs representing the bacterial microbiome of ant-made patches. Our findings suggest that *Azteca* ants have engineered a microbial-driven waste recycling system analogous to human compost piles.

Keywords: Ant-plant mutualism, *Azteca*, *Cecropia*, cellulose, chitin, compost, ant-made patches, organic matter degradation, carbon cycle, metagenomics

Background

Cellulose and chitin are considered the most abundant biopolymers on Earth [1, 2]. Their crystalized forms constitute the structural base of many organisms. Cellulose is the primary component of plant cell walls [3], whereas chitin is an essential part of the fungal cell wall and the exoskeleton of many arthropods [2]. Despite their widespread availability as nutritional source, the stable and strong crystalline fibres of these biopolymers are recalcitrant to degradation [4, 5]. Many animals have no or limited ability to digest cellulose and chitin and have therefore evolved a symbiotic relationship with cellulolytic or chitinolytic microorganisms in order to utilise these substances for their nutritional requirements [6–10]. Microbes, mainly from the bacterial and fungal kingdoms, carry out the breakdown of polysaccharide chains mainly through the secretion of catalytic enzymes which cleave the long polymer chains into smaller, more easily assimilable oligomers [11]. Since this process often relies on the coordinated activity of a complex microbial community [12–14], the underlying mechanisms of efficient polysaccharide degradation in animal-microbe associations remain poorly understood.

The establishment and maintenance of an efficient microbiome throughout the life of many animals is essential for host health, including nutrition, development, behaviour, immune system function [15–17]. For instance, ruminants are known to fully depend on the by-products of plant digestion that is carried out by their complex ruminal microbiota [18]. Similarly, a specialised chitinolytic microbiome that is likely involved in the breakdown of the recalcitrant chitin-rich cuticles of ants have been identified in the gut of convergent myrmecophagous mammals [9]. Since research has predominantly focused on animal-associated endosymbiotic microbiomes with chitinolytic and cellulolytic properties [9, 19–22], much less is known about animal-associated microbial polysaccharide degradation processes occurring outside the animals' bodies. A well-documented example is observed in the leaf-cutting ants, which rely on cultivated fungal garden to degrade the cellulose from the harvested leaves and utilise the nutrient-rich tips of the hyphae (gongylidia) as primary nutrition source [23]. In addition, the ants transfer the remaining recalcitrant organic matter

from the fungal gardens to refuse piles outside the ant nest, where it gets further digested by a complex microbial community [24, 25].

New insights from tropical ant-plant mutualisms have shown that many other ants maintain complex microbial communities within their nests in cavities (so-called domatia) inside their living host plants [26–28]. These communities are predominantly located in organic matter piles (referred to as patches) inside the nesting chambers by depositing plant- and insect-based waste material [27, 29–31]. Unlike leaf-cutter ants, arboreal ants living in mutualism with plants are usually supplied with food resources by their host plant (e.g., food bodies and/or extrafloral nectaries) and by scale insects they often herd (i.e., honeydew) [32, 33]. Although the purpose of the patch-associated communities is less apparent than in the fungal gardens, their ability to metabolise the constant supply of waste material has been hypothesised [26, 27, 34, 35]. Such utilization and transformation of organic matter was hypothetically attributed to an abundant and recurrent group of fungi from the order Chaetothyriales associated with ant-plant mutualisms worldwide [30, 35–37]. However, comparative genomics of free-living Chaetothyriales species and the domatia-associated monophyletic clade recently showed how the latter lost important genes involved in cellulose degradation processes [38]. In consequence, it remains unclear whether other patch-inhabiting microorganisms are capable of degrading the cellulose and chitin found in the plant- and insect- waste material, and if so, what are their genetic mechanisms for such degradation process?

To address this research gap, we selected the *Azteca-Cecropia* complex as a model system, as it is one of the most prominent ant-plant mutualisms in the Tropics of America. By combining bacterial metagenomics from patches of ten *Azteca-Cecropia* colonies with in-situ activity assays from six patch samples, this study seeks to explore the potential of patch communities to digest and recycle complex polysaccharides present in ant waste. Recent studies in this model system have shown that patch communities are vertically transmitted, as the ant queen collects a patch inoculum from the mother colony and deposits it in the first patch she creates before laying eggs [12]. In addition, the ant workers seem to build new patch structures in almost internode they colonize by adding patch particles from older patches [26, 27]. Based

on these findings, we hypothesised that: (i) the microbial patch communities are able to break-down cellulose- and chitin-rich substrates; (ii) the inherited patch microbiome probably holds an abundant and diverse set of genes involved in the degradation of cellulose and chitin; and (iii) the degradation process within these patches is likely driven by a highly adapted and complex consortium of heterotrophic microorganisms. The findings gathered in this investigation sheds light on the underlying mechanisms of recalcitrant polysaccharide degradation in an animal-microbe association occurring outside the host's bodies.

Material and Methods

a) Study site and sample collection

Cecropia trees colonized by arboreal *Azteca* ants were collected in the vicinity of roads, creeks, lowland forests, and pastures in the conservation zone ACOSA (*Área de Conservación Osa)* near the Tropical Field Station La Gamba in Puntarenas, Costa Rica (08°42'03"N, 083°12'06"W, 70 m a.s.l.). The ant and plant species were morphologically identified as described in Barrajon-Santos et al. (2024) [26]. After sampling, stems were opened transversely and ant-built patches found along the ant nesting space (domatia) were collected and transferred to a 2 mL tube, one for each ant colony. In total, 16 ant colonies were analysed, from which six colonies (three per ant species) were used for activity assays and 10 (seven *A. constructor* and three *A. alfari* colonies) were used for metagenomics.

b) Activity assays using isotope-labelled substrates

Fresh patch samples were transported to the University of Vienna (Austria). After 48h from sampling, parallel activity assays per ant colony were performed by incubating homogenized patch samples with ^{13}C-labelled chitin and ^{13}C-labelled cellulose to assess chitin and cellulose degradation activity, respectively. For these assays, triplicates à 30mg FW were placed into 2mL screw-cap GC vials. ^{13}C-labelled cellulose (Sigma Aldrich, 97 at%) and ^{13}C-labelled chitin (IsoLife, from *Aspergillus* sp., U^{13}C, >98 at%) were diluted with unlabelled cellulose or chitin, respectively, to reach 10 at%

^{13}C. Samples assigned for cellulose or chitin treatments were amended with ^{13}C cellulose or ^{13}C chitin by pipetting 20µL of a 2% suspension onto the patch material. 20µL of distilled water was used as control. After substrate addition, each GC vial was placed into a 53 mL gas vial plugged with a butyl septum (Figure 1). The samples were incubated at 25 °C for 72h. To analyse the respiration rates of patch-inhabiting organisms during the incubation period, headspace samples of 15mL from each incubation flask were taken with gas tight syringes at the start of the incubation (T0), after 24h, 48h and 72h. Subsequently, total CO_2 and $^{13}CO_2$ respiration rates were analysed using a GasBench II system coupled to a Delta V Advantage IRMS (Thermo Scientific) to define the proportion of CO_2 respired from isotope-labelled substrates.

c) Sample processing for metagenome sequencing

Prior to DNA extraction, a protocol for the enrichment of a "bacterial fraction" was optimised and performed as described in Supplementary Material 1 to reduce the amount of eukaryotic DNA in the patch samples to a level that allows efficient metagenome sequencing of the bacterial communities. DNA was extracted from the completely processed "bacterial fractions" in eight patch samples (three *A. alfari* and five *A. constructor* colonies) using an adapted phenol-chloroform based DNA extraction protocol as described in Barrajon-Santos et al., 2024 [26]. These DNA samples were then used for short-read Illumina metagenome sequencing as described in the section below. The DNA yield and fragment size from the "bacterial fractions" of these eight samples was insufficient for long-read metagenome sequencing using Oxford Nanopore technology (ONT). Therefore, of two patch samples belonging to *A. constructor* colonies, DNA was additionally extracted from an earlier step in the sample preparation workflow referred to as "organic matter fraction" (extractions performed using the MPBio Fast SPIN extraction kit (MP Biomedicals)).

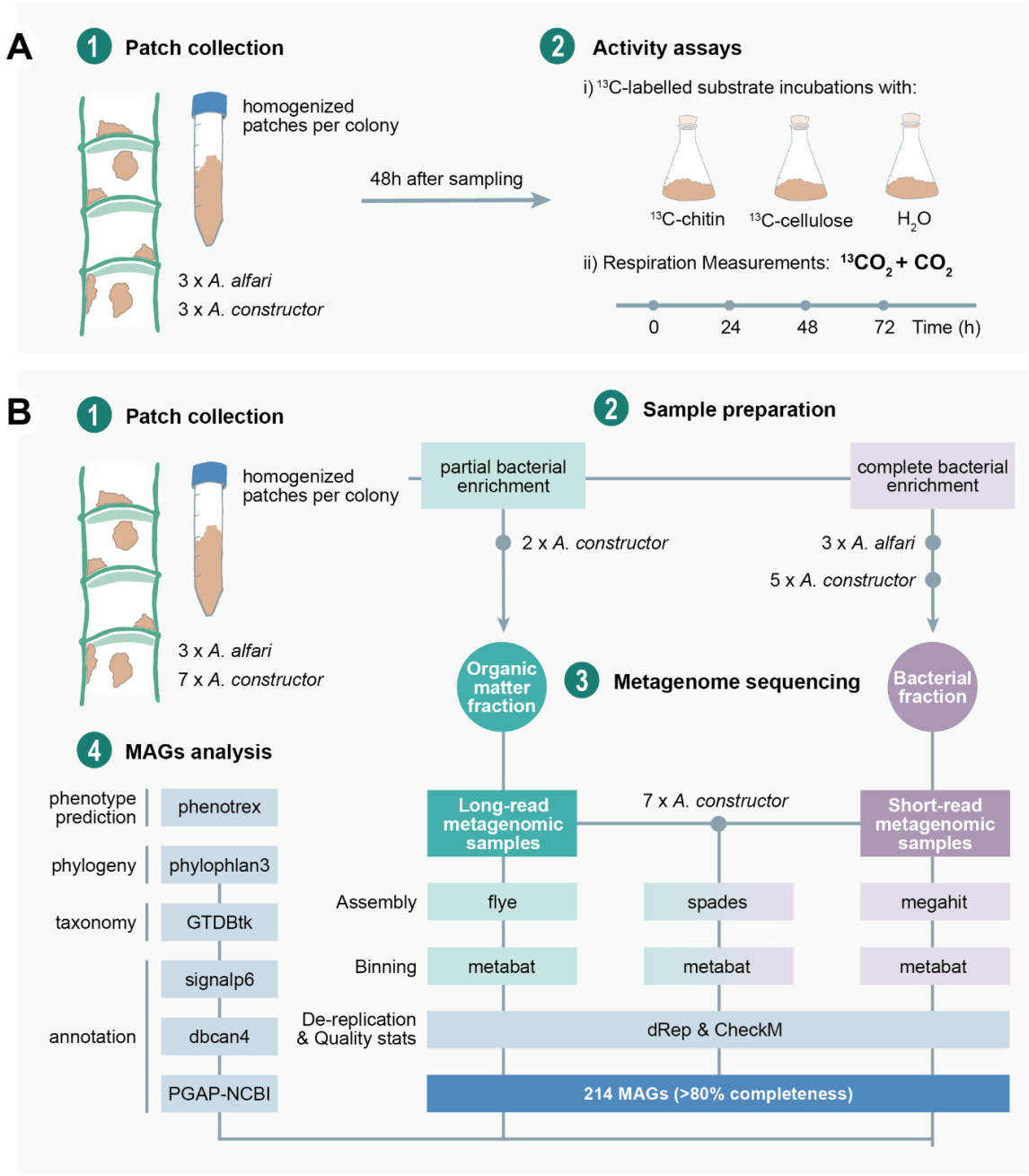

Figure 1. Methodological workflow of this investigation. (A) Polysaccharides degradation activity assays performed in patch samples from 6 *Azteca* colonies using ^{13}C-labelled chitin and ^{13}C-labelled cellulose as substrates. (B) Overview of sample preparation for metagenome sequencing (patches from 10 *Azteca* colonies) and analysis of metagenomic reads and generated MAGs.

d) Metagenome sequencing

DNA library preparation and sequencing were performed at the Joint Microbiome Facility (JMF, University of Vienna, Austria). For Illumina sequencing, the NEBNext® Ultra™ II FS DNA Library Prep Kit (New England Biolabs) and a 1/2 Illumina NovaSeq SP Lane (2x 100 bp) was used. For ONT sequencing, DNA was prepared for sequencing using a native barcoding sequencing kit (SQK-NBD112.24, Oxford Nanopore Technologies). Finally, libraries were split, and about 10fmol were loaded on two R10.4 flowcells (FLO-PRO112, Oxford Nanopore Technologies) and sequenced on a Promethion P24 (Oxford Nanopore Technologies) for 72h. The DNA sequencing was carried out using Minknow (v. 22.05.7, Oxford Nanopore Technologies).

e) Reconstruction of metagenome-assembled genomes (MAGs)

We reconstructed MAGs from the ten metagenome libraries following three different methods (Figure 1). First, Illumina-sequenced reads were trimmed with cutadapt (v. 3.1) [39] by keeping sequences with adaptors found in both ends, read length > 80bp and phred score > 20, assembled by megahit (v. 1.1.2) [40] and filtered with seqtk (v. 1.3, https://github.com/lh3/seqtk) by keeping contigs of 1kB or longer. Second, Oxford Nanopore-sequenced reads were basecalled by Guppy (v. 6.1.15) using super accuracy mode [41] and assembled by flye (v. 2.9) [42]. Contigs were polished with medaka (v. 1.6.1, https://github.com/nanoporetech/medaka). Third, reads from *A. constructor* samples (n=5 for Illumina and n=2 for Nanopore) were used for a hybrid assembly with SPAdes (v. 3.15.5) and the "-meta" flag [43].

Finally, reads were mapped to the resulting contigs in the three assembling approaches separately using minimap2 (v. 2.22) [44], and read mappings were converted using samtools (v. 1.11) [45] for binning with metabat2 (v. 2.15) [46]. The binning output from the three assembly approaches were concatenated and dereplicated by dRep (v. 3.4.0) [47] using a 95% ANI cut-off. Standard MAG statistics were computed with QUAST (v. 5.0.2) [48]. The completeness and contamination levels of the resulting MAGs was evaluated by CheckM v.1.2.0 [49] (Supplementary Material 2).

f) Read-based taxonomic classification

Short- (Illumina-based) and long-read (Oxford Nanopore-based) metagenomic reads were taxonomically classified by sourmash (v. 4.6.1) using the k31 bacterial and archaeal reference databases from GenBank (March 2022) recommended for species-level matching [50, 51]. Additionally, 16S rRNA gene sequences from Illumina short-reads were reconstructed and taxonomically classified by phyloflash (v. 3.4.1) [52] using the SILVA 138.1 SSU Ref NR99 database [53]. The taxonomic overview of the ten metagenome libraries was compared with the 16S rRNA gene amplicon sequences from 38 patch samples (22 *A. constructor* and 16 *A. alfari* colonies) that were previously analysed [27].

g) Annotation, taxonomy and phylogeny of MAGs

MAGs with a completeness ratio higher than 80% (= 214 MAGs) were submitted to NCBI for running the prokaryotic genome annotation pipeline (PGAP) [54]. Subsequently, annotation of potential carbohydrate active enzyme (CAZy) genes (excluding pseudogenes) was performed using the predicted gene sequences from PGAP and the dbCAN4 (v. 4.0.0) tool [55]. For downstream analysis, we included CAZymes detected by HMMER against dbCAN HMMdb and any of the other two methods (Diamond against CAZyDB or HMMER against dbCAN-sub HMMdb) [56–58]. Moreover, signal peptides were predicted in the gene sequences with the signalP6 (v. 6.0h) in a “--fast” mode [59].

Based on a literature search including 14 research articles/reviews, 49 CAZymes were placed in the following substrate categories: chitin, cellulose/hemicellulose, glycogen/starch or oligosaccharides (Supplementary Material 3). From those, we further categorised CAZymes families based on their EC (enzyme commission) numbers in the CAZy database (http://www.cazy.org) [60]. As an example, if a CAZy family was associated with the EC 3.2.1.14, EC 3.5.1.41 or EC 1.14.99.53, this family presents characterized enzymes with chitinase activity, whereas if it contained the EC 3.2.1.4 they have enzymes with endo-cellulase activity.

The selected 214 MAGs were taxonomically classified by GTDBtk (v. 2.1.0) [61]. Additionally, a strain-level phylogenetic tree was reconstructed using clade-specific markers with PhyloPhlAn (v. 3.1.1) in a "--diversity high" and a "--fast" modes [62].

h) Phenotype trait prediction of MAGs

To predict the cellulose degradation trait on the MAGs, we trained and optimised two novel prediction models as described in Supplementary material 4. Briefly, 187 publicly available genomes of bacterial strains in which cellulose degradation capability was experimentally tested were collected to train the model. In these genomes, genes were predicted using Prodigal (v. 2.6.3) [63] and annotated in each model as follows: (i) with HMMer (v. 3.3.2) using the COG-based (Cluster of Orthologous Groups) Eggnog database (v. 5.0) [64]; or, (ii) through BLAST using the CAZy database (version updated on July 2023) [60]. Then, the prediction model was built using the phenotrex tool (v. 0.6.0, https://github.com/univieCUBE/phenotrex) [65]. Finally, the 214 MAGs were tested for cellulose degradation using both novel prediction models (COG and CAZy).

Results

a) Patches of *Azteca* species show chitin and cellulose degradation activity

To determine if the microbial communities inhabiting the ant-built patches of *A. alfari* and *A. constructor* are capable of degrading polysaccharides generally found in the ant deposits, we performed cellulose- and chitin-degradation assays using ^{13}C-labelled chitin or ^{13}C-labelled cellulose. During the 72h incubation period, all patch samples exhibited chitin degradation activity and five out of six patch samples showed cellulose degradation activity. In most patches of both ant species $^{13}CO_2$ was already detectable at the first timepoint of the incubation (24h) and the peak of cellulose and chitin respiration rate (15,359 and 81,985 mean µmol $^{13}CO_2$ h^{-1} g^{-1} dw, respectively) was reached between 48h and 72h (Figure 2).

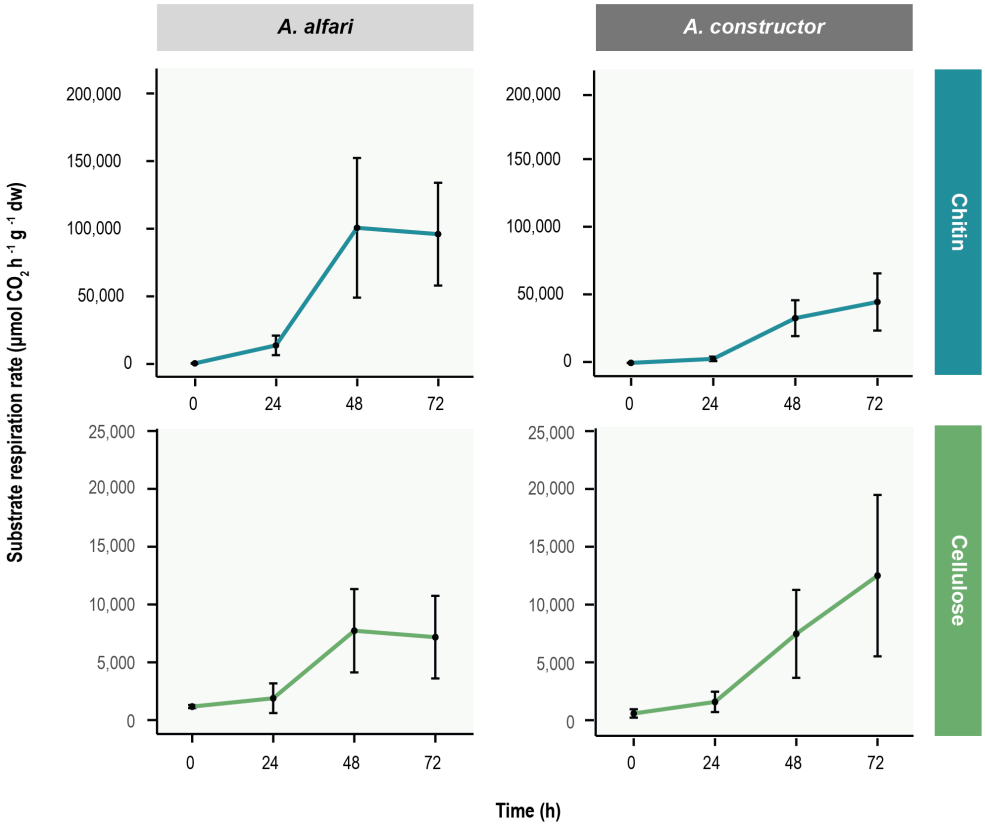

Figure 2. Isotope-based substrate respiration measurements in incubations of patch samples with: (blue) ^{13}C-labelled chitin substrate, and (green) ^{13}C-labelled cellulose substrate (in triplicates per ant species and substrate).

b) The 214 MAGs are representative of the main bacterial phyla inhabiting the *Azteca* patches: Pseudomonadota, Actinomycetota and Bacteroidota.

Short read metagenome sequencing of patches from 10 *Azteca* colonies resulted in 6.9-11.9 Gbp trimmed short-read sequence data from eight samples (three *A. alfari* and five *A. constructor*) and 1.4-38.78 Gbp of trimmed long-read sequence data from two *A. constructor* samples (Supplementary Material 5).

The taxonomic overview of the metagenomic libraries containing all sequence reads or only reconstructed 16S rRNA genes showed a high diversity of bacteria, which were comparable in composition with a recent 16S rRNA gene metabarcoding study (Figure 3A). The majority of the metagenomic reads were assigned to the phyla Pseudomonadota (mean ± SD: 83.6 ± 3.7 %; e.g. Hyphomicrobiales, Burkholderiales and Enterobacteriales) and Actinomycetota (7.1 ± 2.3 %; e.g. Micrococcales). The phylum Bacteroidota showed high relative read abundances in every patch sample

analysed with 16S rRNA gene metabarcoding (23.7 ± 7.9 %; e.g. Chitinophagales), whereas the relative abundances of metagenomic reads and 16S rRNA extracted from the metagenomic libraries assigned to this phylum were relatively low (4.6 ± 2.7 %; Figure 3A).

The most abundant phyla in the metagenomic reads were well represented among the 214 MAGs with a completeness higher than 80% (Figure 3B, C). In fact, 91 and 53 MAGs were classified as Pseudomonadota and Actinomycetota, respectively. The remaining MAGs represented eight additional phyla, like Bacteroidota with 37 MAGs. Based on the MIMAG standards [66], 162 MAGs out of the 214 qualified for "high quality" based on completeness (> 90 %) and contamination (< 5 %) rates (Figure 3C) and from those, 90 MAGs also presented the required rRNA/tRNA gene content (Supplementary Material 2).

c) The frequent glycoside hydrolases in the patches are related with the breakdown of chitin and cellulose/hemicellulose

To investigate the genetic repertoire that drives polysaccharide breakdown in the bacterial microbiome of the patches, we examined the CAZy domain types and families found in the 214 reconstructed MAGs. A total of putative 25,648 CAZymes domains encoding 135 glycoside hydrolases (GHs), 59 glycosyltransferases (GTs), 47 carbohydrate-binding modules (CBMs), 30 polysaccharide lyases (PLs), 18 carbohydrate esterases (CEs) and nine auxiliary activities (AAs) families were annotated, of which 9,127 (36%) presented a signal peptide (i.e., CAZy-signalp) in the same genes (Supplementary Material 6).

From the 298 CAZy families found in the patches, 49 families representing 36 % of the total CAZy domain abundance were categorised into the following substrate types: chitin, cellulose/hemicellulose, glycogen/starch or oligosaccharides (Figure 4). Among those, the most frequent glycoside hydrolases were GH5 and GH78 from the cellulose/hemicellulose degradation processes (present in 113 and 76 MAGs, respectively) and GH18 from the chitin pathway (present in 74 MAGs). MAGs containing these families, had 2 to 3 copies on average, of which 58% were linked to a signal peptide.

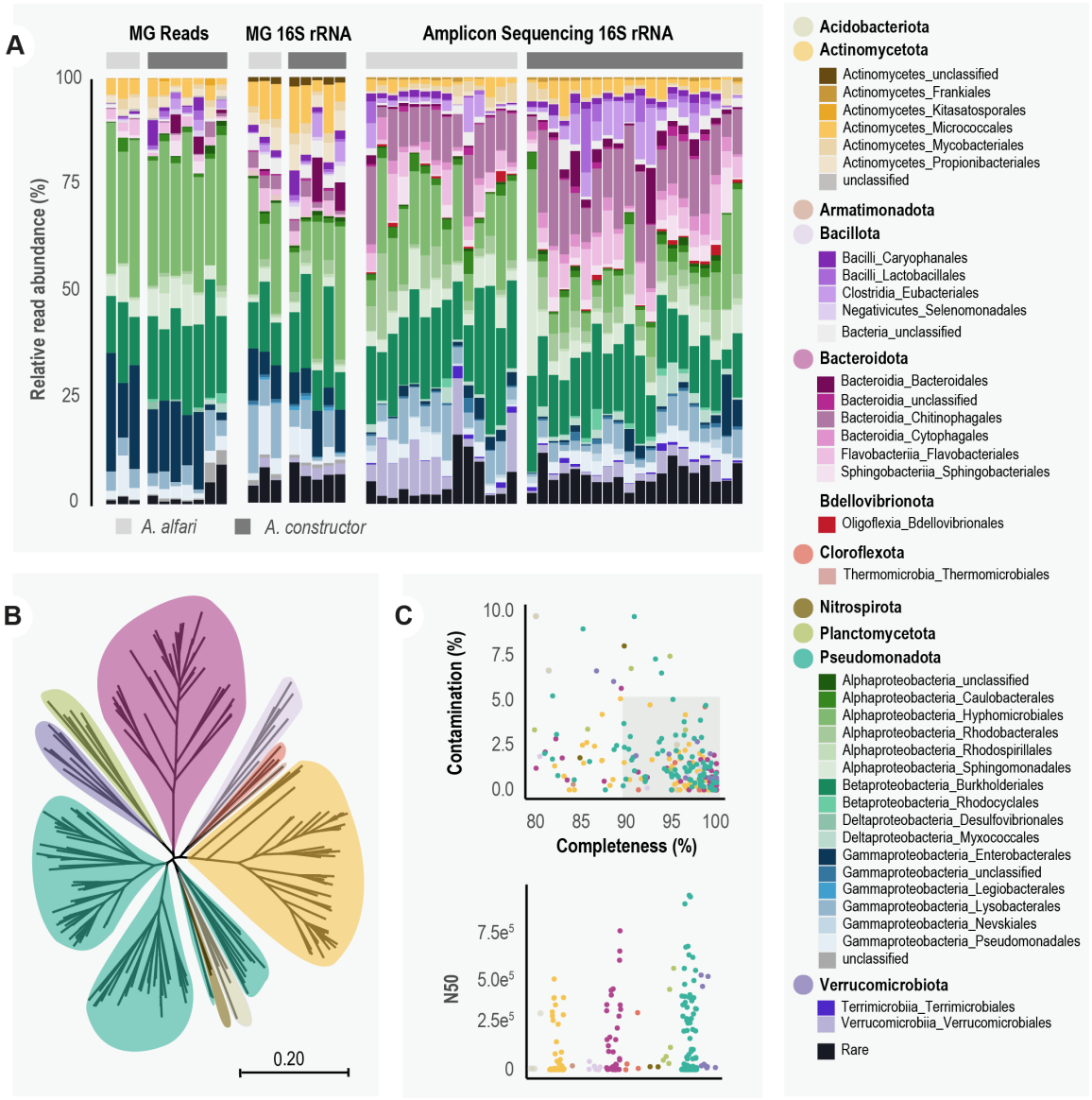

Figure 3. Taxonomic and phylogenetic affiliation of bacterial communities inhabiting the patches. (A) Relative read abundances of the taxonomic orders derived from: metagenomic reads (MG Reads), 16S rRNA gene reconstructed from metagenomic reads (MG 16S rRNA) and amplicon sequences of 16S rRNA genes from a previous metabarcoding study [27]. (B) Reconstructed phylogenetic tree containing the selected 214 MAGs assembled from patch samples of 10 *Azteca* colonies. (C) Quality statistics overview of the selected 214 MAGs including completeness and contamination rates (upper graph) and N50 values (lower graph).

Other frequent CAZy families were GH13 and CE4, which are involved in the degradation of glycogen/starch and in the deacetylation of chitin to chitosan, respectively [4, 21]. While GH13 was present in 192 MAGs with an average of 7 copies per MAG, only 17% of the genes showed a secretion signal. On average, three CE4 copies were found in 164 MAGs, with a signal detected in 28% of these genes.

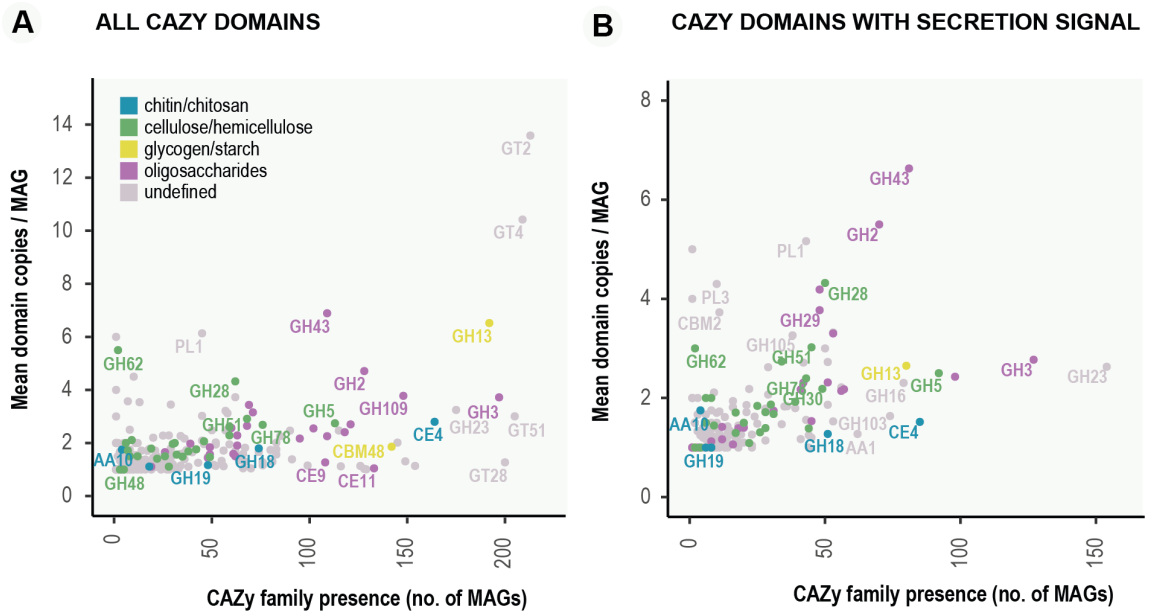

Figure 4. Genetic repertoire associated with the carbohydrate breakdown found in the patch microbiome which is represented by the reconstructed 214 MAGs. (A) Displaying the presence of CAZy families in MAGs. (B) Displaying the presence of CAZy families in which a linked signal peptide has been detected in the same genes. X-axis denotes in how many MAGs the CAZy family was detected at least once. Y-axis denotes the average CAZy domain copy number per MAG when the CAZy family was detected. The colour legend relates the CAZy families with their respective substrates. The families where no substrate was assigned were merged into the "undefined" category.

d) The machinery for polysaccharide degradation is widespread in the bacterial microbiome of patches

To explore the potential polysaccharide utilization mechanisms of the different bacterial taxa within the patches, we compared the abundance and frequency of the different categorised CAZy families among the 214 MAGs (Figure 5). Polysaccharide-cleaving domains were widely detected in the MAGs representing the bacterial community inhabiting the patches (Figure 5A). However, the abundance of categorised CAZy families linked to a secretion signal notably varied between taxa (Figure 5B).

On the one hand, MAGs belonging to the phyla Pseudomonadota (Polyangiaceae, MAGs 073-075; Caulobacteraceae, MAGs 107-110), Verrucomicrobiota (unclassified Pedosphaerales, MAG 167; Opitutaceae, MAGs 168-171) and Bacteroidota (Dysgonomonadaceae, MAGs 179-182; Chitinophagaceae, MAGs 188-196 and 205-207), contained the highest proportion of domains belonging to categorised CAZy families (2 to 183 domains per MAG) which were often linked to a signal peptide (25 to 154 domains per MAG). In addition, these MAGs presented the highest abundance of CAZy-signalp domains associated with the degradation of cellulose/hemicellulose (4 to 67 domains per MAG) and oligosaccharides (10 to 113 domains per MAG). Particularly, MAGs belonging to Bacteroidota were the most versatile in potential substrate utilization, as they also hold CAZy families related to chitin and glycogen/starch degradation.

On the other hand, MAGs from Bacillota (Lachnospiraceae, MAGs 001-003), Chloroflexota (unclassified Thermomicrobiales, MAGs 009-011), Actinomycetota (Propionibacteriaceae, MAGs 019-031), and some alphaproteobacteria within the Pseudomonadota (Rhizobiaceae, MAGs 092-094) featured a wide repertoire of different domains related to the break-down of oligosaccharides (16 to 65 CAZy domains per MAG) and glycogen/starch (5 to 22 CAZy domains per MAG). However, a low proportion of domains belonging to categorised CAZy families in these MAGs were linked to a secretion signal (0 to 4 and 0 to 2 domains per MAG, respectively).

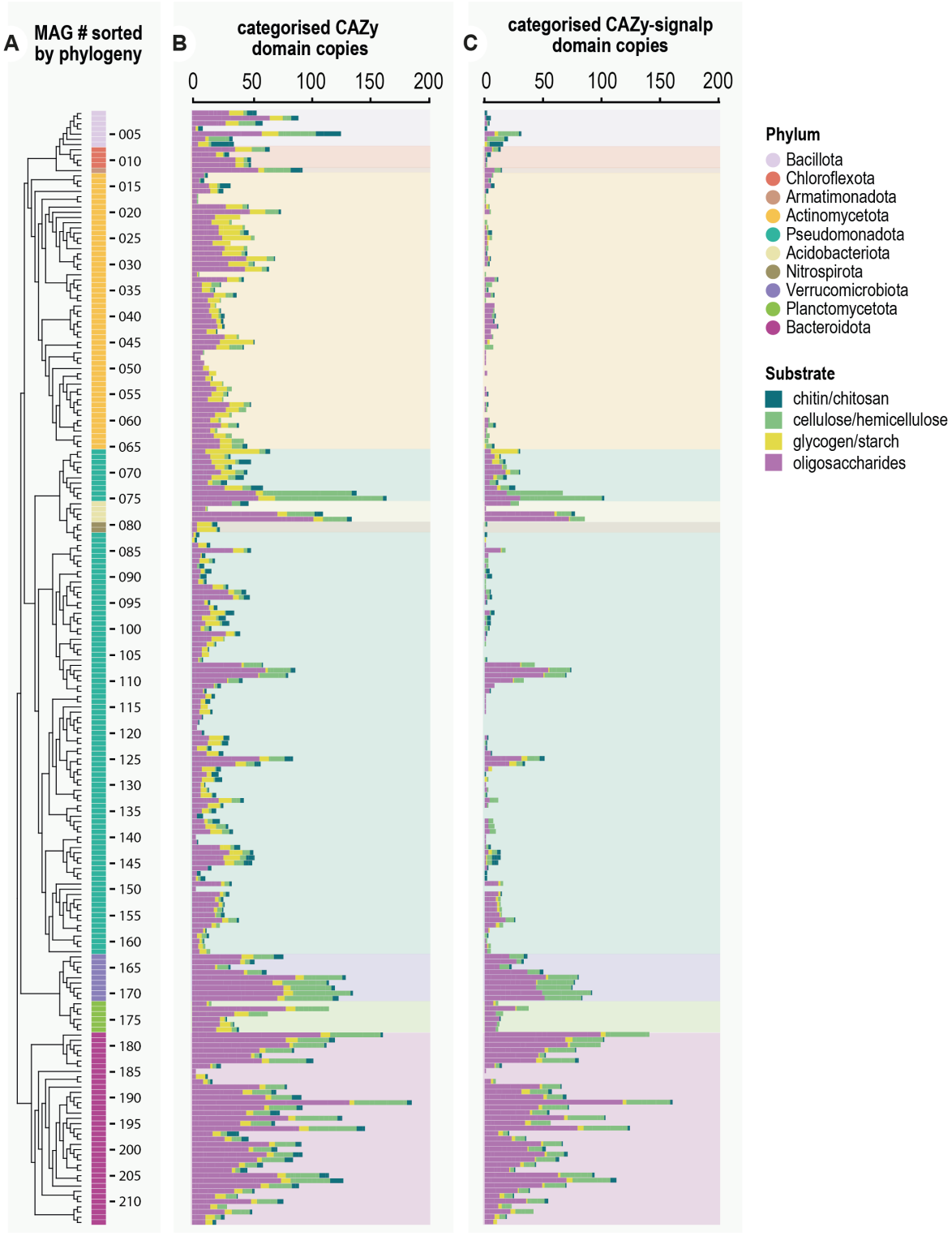

Figure 5. Description of the genetic mechanisms of polysaccharide breakdown found in each MAG reconstructed from the ant-made patches. (A) Taxonomic and phylogenetic assignment of the 214 MAGs. (B) Barplot showing the abundance of CAZy domain copies found in each MAG that were categorized into substrate types. (C) Barplot showing the abundance of CAZy domains linked to a signal peptide (CAZy-signalp) in the same gene sequence.

e) Nearly half of the reconstructed MAGs representing the bacterial microbiome of patches hold genes coding for chitinases or cellulases

To determine which MAGs have the potential to degrade crystalline chitin or cellulose, we looked at the abundance and diversity of CAZy-signalp domains that are described as chitinases (EC 3.2.1.14, EC 1.14.99.53 and EC 3.5.1.41) or cellulases (EC 3.2.1.4) in the CAZy database (Figure 6). While 109 and 126 out of the 214 MAGs contained at least one gene encoding for enzymes with potential chitinase or cellulase activity, respectively, a distinct composition and abundance of chitinase- and cellulase-CAZy families were detected among taxa. Firstly, MAGs from Deltaproteobacteria (Haliangiaceae, MAG 071; unclassified Myxococcales, MAG 073; Polyangiaceae, MAG 073) and Betaproteobacteria (Comamonadaceae, MAGs 125-126) within Pseudomonadota and from Bacteriodota (Paludibacteraceae, MAGs 183-184; Chitinophagaceae, MAGs 188-194, 200-202 and 205-206) contained a wide repertoire of different domains related to the breakdown of both biopolymers. Particularly, the Bacteriodota MAGs mostly conserved the same type of CAZy families (GH18, CE4, GH5 and GH51). Secondly, MAGs from Verrucomicrobiota (Opitutaceae, MAGs 169-171) and from Planctomycetota (Tepidisphaeraceae, MAG 174; and, unclassified Pirellulales, MAG 177) contained a notably higher abundance of cellulase domains than those related to chitin. In addition, these MAGs resulted positive in both prediction models for cellulose degradation. Finally, certain MAGs from Gammaproteobacteria within Pseudomonadota (Enterobacteriaceae, MAGs 144-145) and from Bacillota (Lachnospiraceae MAGs 001-003; and unclassified Caryophanales, MAG 007) presented a rich and highly diverse repertoire of chitinase families.

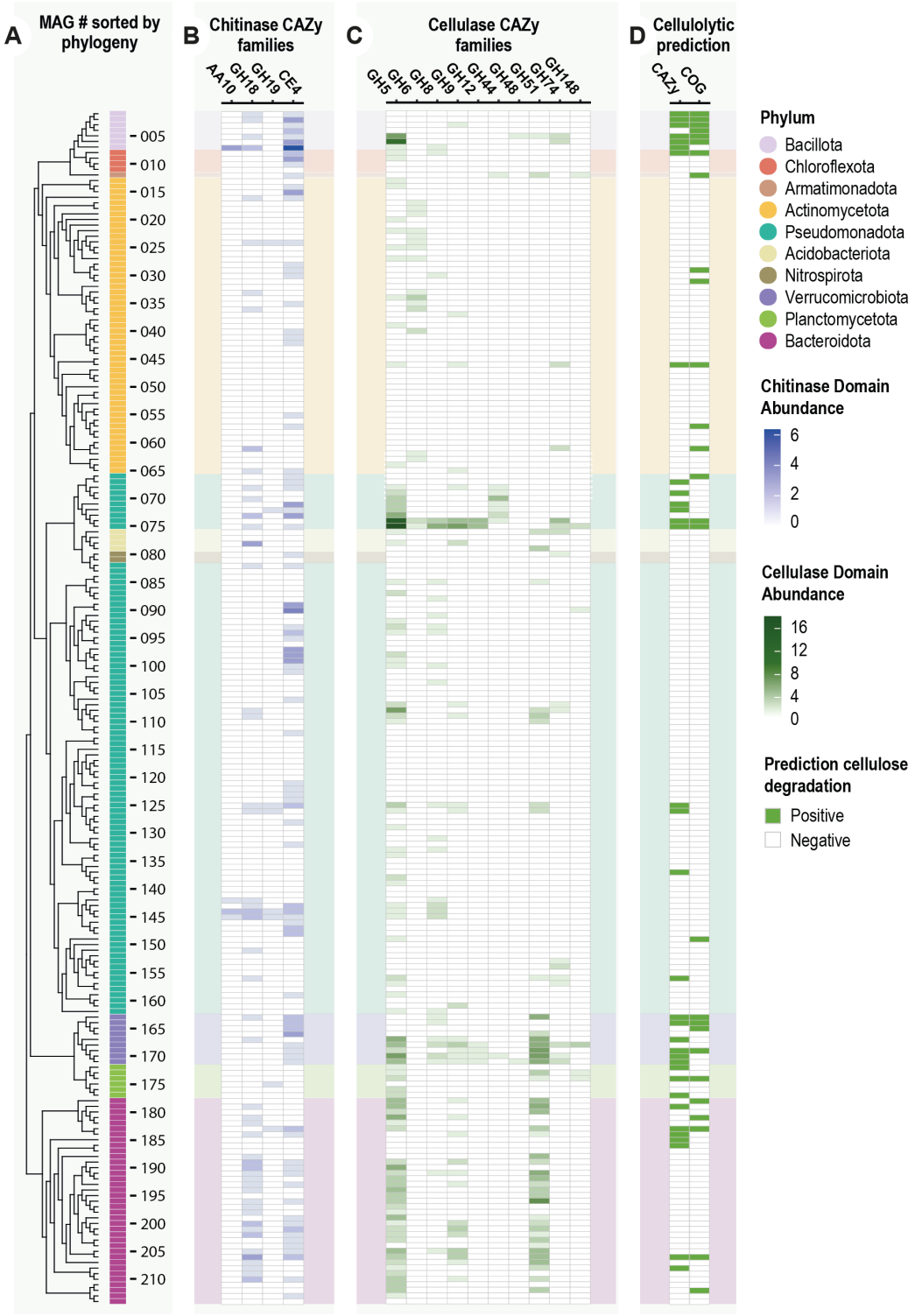

Figure 6. Cellulase and chitinase gene content in the 214 MAGs reconstructed from the patches. (A) Taxonomic and phylogenetic assignment. (B) Heatmap showing the abundance of CAZy-signalp domains categorized as chitinases per MAG (C) Heatmap showing the abundance of CAZy-signalp domains categorized as cellulases per MAG. (D) Potential cellulolytic activity predicted in the MAGs by two novel machine learning- based prediction models (COG and CAZy).

Discussion

The *Azteca* ants inhabiting the stem of *Cecropia* nourish the complex microbial community that they maintain in patches with a wide variety of plant- and animal-based substrates. In particular, parenchyma tissue, Müllerian food bodies, leaflets of mosses and chopped dead nestmates are deposited on the patches. In addition, ant workers daily excrete onto the patches pellets regurgitated from the infrabuccal pocket —a filtration device in the oral cavity of all ants— as well as liquid ant faeces (Supplementary material S7; [67, 68]). Our data confirms the hypothesized ability of the patch communities to degrade recalcitrant organic matter such as cellulose-rich plant material and chitin-rich exoskeletons of ants. First, the isotope-based activity assays demonstrated that cellulose and chitin are metabolised in the patches. And second, our results from the metagenomic analysis showed a widespread distribution of diverse polysaccharide-cleaving genes among the MAGs representing the bacterial microbiome in the patches, including those genes related with the breakdown of crystalline chitin and cellulose.

a) Genetic mechanisms of polysaccharide-degrading bacteria in the patches

Previous metabarcoding analyses revealed a diverse bacterial and fungal community composition in ant-made patches and further suggested that both microbial groups could participate in organic matter degradation processes [26, 27]. Nevertheless, the notable fungal diversity within these patches [26], in combination with the large size of fungal genomes [69] and the limited availability of reference genomes, present major challenges for conducting fungal metagenomic analyses [70]. Thus, this study focuses on the bacterial communities, exploring their genetic potential and mechanisms of polysaccharide degradation.

The CAZyme repertoire, we identified in the 214 bacterial MAGs, effectively covers the wide range of substrates deposited by the ants on the patches. Therefore, one can expect that bacteria contribute considerably to polysaccharide breakdown in the patches as it occurs in human composting [71–74]. Firstly, GH18, the most prevalent bacterial endo-chitinase [75], was detected in 35% of the MAGs, suggesting its potential involvement in the breakdown of chitin from dead ant bodies. As observed

in the Lachnospiraceae and Chitinophagaceae MAGs in the patches, multiple copies of GH18 are frequently found in the genomes of chitinolytic bacteria, which is thought to enhance substrate utilization efficiency [75, 76]. Additionally, an alternative chitin degradation pathway, involving its deacetylation to chitosan, may be facilitated in the patches by carbohydrate esterases such as CE4 [77].

Secondly, the frequent glycoside hydrolases in the bacterial microbiome of patches like GH5, GH51 and GH78 are likely involved in the breakdown of plant material. This is supported by the observation that parenchyma tissue is scraped off the inner walls of domatia by the ants and deposited on the patches [34]. In particular, GH5 is also one of the most abundant cellulases in the metagenomes from the fungal gardens of *Atta* leaf-cutter ants [78] and from the human compost piles [71, 73, 74]. Due to the high poly-specificity of the protein families involved in plant fibre degradation, the specific biochemical activities and the type of cleavage linkages of these glycoside hydrolases are difficult to predict solely using annotation-based genomic methods [55]. To address this limitation, we developed and implemented a novel machine learning-based prediction model for cellulose degradation. By integrating this approach with the annotation-based results, we more accurately identified 34 out of the 214 MAGs from distinct taxonomic phyla (e.g. Pseudomonadota, Verrucomicrobiota, Bacillota and Bacteroidota), as potential candidates for the breakdown of crystalline cellulose in the patches. In particular, some of these MAGs belonged to the same families (e.g. Comamonadaceae and Chitinophagaceae) as the most abundant and frequent ASVs previously detected by metabarcoding in the patches of well-established *Azteca* spp. colonies [27].

Finally, another abundant compound in the patches is the glycogen or phyto-glycogen originated from the haemolymph of dead ant bodies or from the Müllerian food bodies, respectively [79–81]. However, this homoglycan also serves as a primary storage compound in bacteria [82, 83]. While bacterial glycogen will be likely metabolised intracellularly, the breakdown of ant- and plant-based glycogen requires extracellular enzymatic activity. The multiple GH13 domains linked to a secretion signal and found in MAGs from Chitinophagaceae (Bacteroidota) and Anaeromyxobacteraceae (Pseudomonadota) are likely involved in the extracellular degradation process.

b) Substrate utilization dynamics among bacteria inhabiting the patches

The vertical transmission of patch communities from mother to daughter colonies and the constant supply of complex biopolymers on the patches [12, 41] likely drive a natural selection pressure favouring heterotrophic nutrition. As nearly 50% of the MAGs contained at least one CAZy-signalp domain related with the breakdown of crystalline cellulose or chitin, a strong adaptation to this polysaccharide-rich environment was confirmed. These results align with the highly efficient cellulolytic and chitinolytic gut microbiomes of herbivorous and myrmecophagous mammals, respectively [9, 21, 84].

The distinct composition and abundance of CAZy families in the MAGs provided valuable insights into the substrate utilization dynamics of the bacterial community in the patches. Based on their genetic repertoire, bacteria from the families Comamonadaceae, Paludibacteraceae and Chitinophagaceae are likely acting as generalist degraders. Their genomes showed the largest CAZyme-signalp domain profiles which were generally associated with all categorised substrates. Despite their apparently versatile metabolism, these MAGs shared a rather conservative genetic repertoire of polysaccharide-cleaving glycoside hydrolases, mainly GH5, GH51, GH18 and GH13. In contrast to the generalists, the presence of MAGs predominantly containing cellulase-CAZy domains (e.g., Opitutaceae, Tepidisphaeraceae, and unclassified Pirellulales) or chitinase-CAZy domains (e.g., Enterobacteraceae and Lachnospiraceae) suggests that specialist degraders also play an important role in the digestion of patch deposits. Finally, by cross-feeding on cellodextrins and N-acetyl chito-oligosaccharides, or by utilizing the glucose and GlcNAc monomers generated from the polysaccharide breakdown, those bacteria containing only CAZy domains related with oligosaccharide metabolism could act as beneficiaries [12, 85, 86]. The detection of diverse genetic mechanisms linked to the breakdown of complex polysaccharides suggests that different bacterial taxa might be acting at different stages in the degradation process or under different substrate concentrations. Overall, this study indicates that a complex and highly adapted heterotrophic community efficiently convert plant- and insect-based waste material into more stable nutrient forms.

c) Concluding remarks: The practice of composting by *Azteca* ants

Previous investigations of the *Azteca-Cecropia* model have provided evidence of the importance of patches for the development and survival of the ant colony [34, 87]. However, unlike the fungal gardens in farming insects [88–91], the purpose of patches is less apparent in ant-plant mutualisms as these arboreal ants are usually supplied with food resources from their host plant and exudates from the scale insects. In this study, we demonstrated that the microbial communities within the patches are able to fully metabolise the cellulose and chitin deposited on the patches. Through metagenomic analysis, we further revealed that the bacterial microbiome in the patches contains a rich and diverse genetic repertoire responsible for polysaccharide degradation. Overall, the polysaccharide degradation processes occurring in the patches seem to be comparable with the human compost piles [71, 73, 74]. In agriculture, composting is a process of decomposition of organic waste mediated by microorganisms under controlled conditions [92–94]. Based on this definition, *Azteca* ants practise the art of composting by accumulating their waste into piles within their nest and by actively adding a microbial inoculum from mature patches that likely accelerates and improves the degradation process [72]. While our findings showed that *Azteca* ants have indeed engineered a system that facilitates the microbial degradation of plant- and insect-based waste material, future research is necessary to uncover how the recycled nutrients are re-assimilated by the ant-plant complex.

Declarations

Author Contributions: V.E.M., M.N. and D.W. designed this study. V.B.S., V.E.M. and M.N. conducted fieldwork and collected the samples. V.B.S., J.S., D.Z. and T.R. contributed with analytical tools. J.S. performed sample preparation and sequencing. V.B.S. and J.S. processed the metagenomics data. D.W., V.E.M., M.P. and T.R. provided significant intellectual contribution. V.B.S analysed the data and wrote the original draft of the manuscript. All authors contributed to the final version of the manuscript and approved it for publication.

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Competing interests: All authors declare that they have no conflict of interest.

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Availability of data and materials: All data generated or analysed during this study are included in this published article, its supplementary information files and publicly available repositories. The sequence data (raw sequence reads and metadata) are accessible on NCBI under the BioProject accession number PRJNA777006. Supplementary data not included will be uploaded at figshare upon acceptance (https://doi.org/ 10.6084/m9.figshare.c.7499031) .

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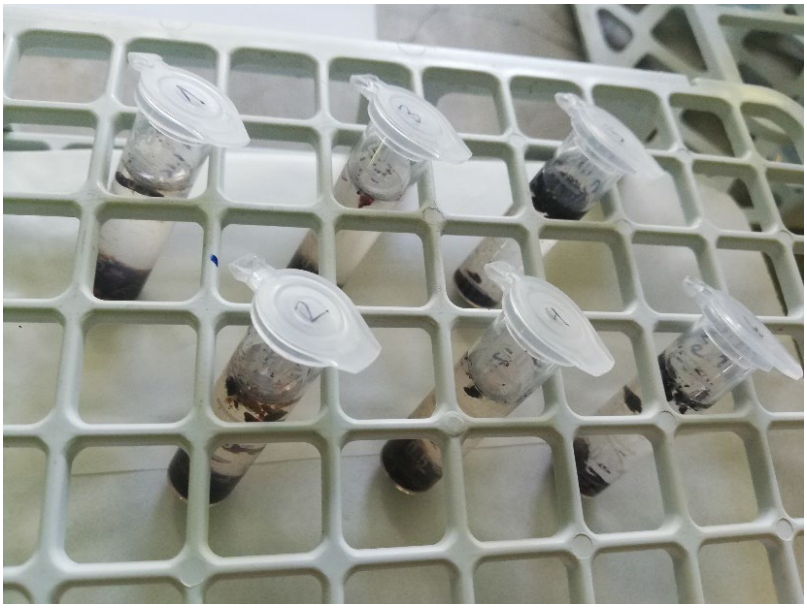
Supplementary Material

Supplementary material S1: Detailed information about the bacterial enrichment protocol used for samples preparation of metagenome sequencing

The ant-made patches from the *Azteca-Cecropia* association are inhabited by a wide variety of organisms including fungi, nematodes and bacteria [26–28, 95]. In consequence, we could expect that a notable amount of eukaryotic DNA would be extracted along with the prokaryotic DNA when preparing the patch samples for bacterial metagenome sequencing. Since a high proportion of eukaryotic DNA could drastically affect the performance of sequencing, we optimized and applied a bacterial DNA enrichment protocol. By the use of this method, we aimed to physically separate, prior to TNA extraction, most bacterial cells in the patches from the eukaryotic organisms and the organic matter present in the patch matrix. The protocol consisted of two main steps, a nematode removal step based on centrifugation and a bacterial enrichment step based on density gradient centrifugation and filtration [96, 97]. The complete bacterial enrichment protocol was applied in eight patch samples used in shotgun Illumina metagenome sequencing, whereas a partial bacterial enrichment protocol (up to step 1) was followed in two patch samples used in Oxford Nanopore metagenome sequencing.

1. Removal of nematodes

To isolate the nematodes from the patch matrix, we adapted a centrifugal flotation method described in Bezooijen (2006) [98] for its use in patches. In this method, samples containing nematodes are suspended in an extraction fluid such as sugar, MgSO4 or $ZnSO_4$. By centrifugation at low speed (1800 g 4 min), alive nematodes containing air in their digestive system float while the other sample particles with a higher specific gravity than the fluid settle into the pellet. Since some patch particles remained in the supernatant when using sucrose or MgSO4, we instead used a non-ionic iodinated gradient medium (Nycodenz) which performed the best at a concentration of 1.16 g/mL (Supplementary Figure S1.1).



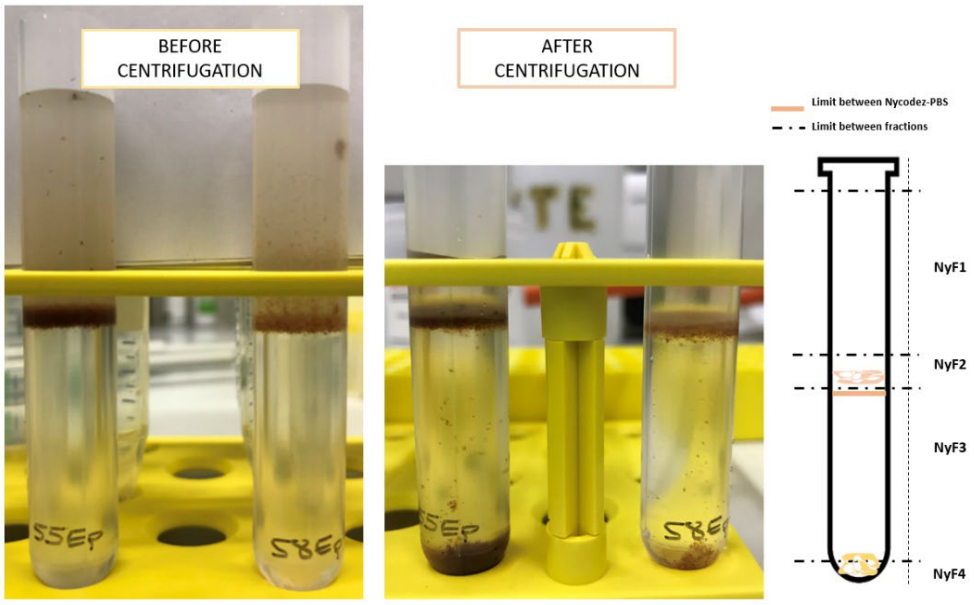
Supplementary Figure S1.1 Evaluation of performance of nematodes removal method using patch samples resuspended in three extraction fluids at two different concentrations. From left to right: (i) Nycodenz, (ii) sucrose and (iii) MgSO4. In the upper tubes, the concentration of each extraction fluid was 1.16 g/mL , whereas in the lower tubes, a concentration of 1.22 g/mL was used. (Photo by: Veronica Barrajon-Santos).

After centrifugation, the supernatant containing nematodes in the 10 patch samples was transferred to a new tube. Since the supernatant could still contain bacteria, both fractions (supernatant and pellet) were separately washed with distilled H_2O by three consecutive centrifugation steps at 18000 g for 3 min. The supernatant from these centrifugation steps was discarded, and the pellets (nematode fraction and organic matter fraction) were stored in 1 mL RNA later for later use. The two patch samples used in Oxford Nanopore metagenome sequencing were processed until here. The organic matter fraction in this two samples was then used for TNA extraction.

2. Density gradient centrifugation

The organic matter fractions from the 8 patch samples of Illumina sequencing were further processed by performing an adapted density gradient centrifugation protocol described in Eichorst et al., 2015 [97]. Briefly, we washed the samples in RNA later with 1x PBS by three consecutive centrifugation steps (14000 rcf 2 min 4 °C). Then, the resulting pellet was resuspended in 1mL 1xPBS and transferred to 15mL tubes where additional 4 mL of 1xPBS were added. Subsequently, a cell detachment step was performed in each sample by incubating the tubes in an ultra-sonication bath 5 times during 15s.

After, we added 6 mL of Nycodenz solution (1.42 g/mL in 1xPBS) into the tubes and centrifuged them at 10000 g 4°C for 90 min with a swing-out rotor on a Beckman Ultracentrifuge (rotor SWT14i). After centrifugation, the upper aqueous phase (NyF1) was transferred to a new tube (Nycodenz fraction) for later use (Supplementary Figure 2).



Supplementary Figure S1.2 Visualization of the organic matter fraction from patch samples before and after the density gradient centrifugation process. On the left, a graphical illustration of the gradient-based phases obtained in the tubes after centrifugation. (Photo by: Veronica Barrajon-Santos)

3. Filtration

Finally, the Nycodenz fraction and the nematodes fractions were jointly filtered using 5 µM filter to separate the bacteria (passing through the pore) from the fungi and nematodes (staying on the filter) that remained in these fractions (Supplementary Figure S1.3). The bacterial fraction of each sample was then concentrated by filtering through a 2 µM filter. The filters from the 8 patch samples used in Illumina sequencing were stored in RNA later for their use in TNA extraction.

Supplemetary Figure S1.3 Filtration devise used for filtrating the bacterial enrichment fractions of patch samples. (Photo by: Veronica Barrajon-Santos).

Supplementary material S2: Quality stats and taxonomic assignment of the 214 MAGs

The supplementary material S2 shows the supplementary table S2.1 with detailed information from the 214 reconstructed MAGs in this study, including their quality stats (e.g., completeness, contamination, N50, genome size, fragmentation) and their taxonomic assignment. This table (in ".xlsx" format) can be found at the figshare public repository upon acceptance of the manuscript (https://doi.org/ 10.6084/m9.figshare.c.7499031).

Supplementary material S3: Detailed list of CAZymes categorised into substrates

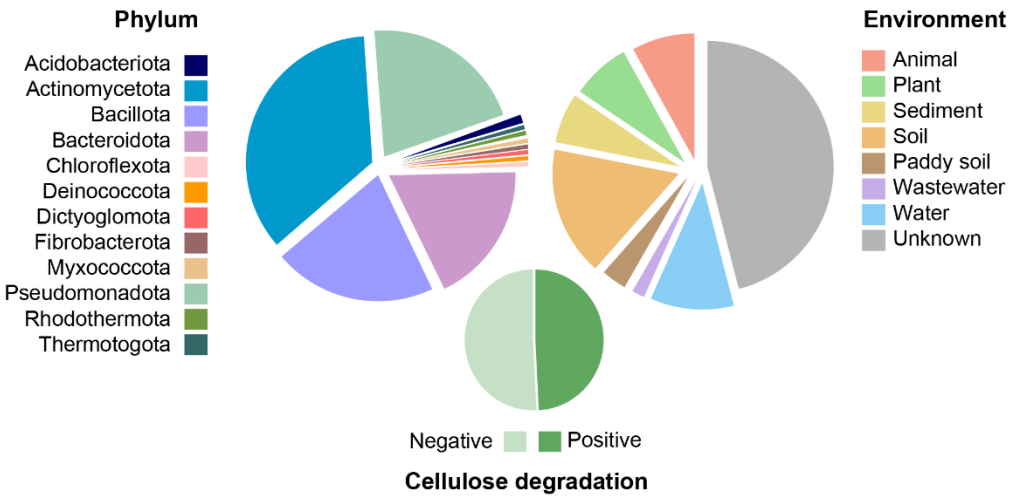
The supplementary material S3 shows the supplementary table S3.1 which contains detailed information about the categorised CAZy families into the following substrates: (i) chitin/chitosan, (ii) cellulose/hemicellulose, (iii) glycogen/ starch, and, (iv) oligosaccharides. Moreover, the citations of the 14 scientific articles from where the category information was collected are facilitated. This table (in ".xlsx" format) can be found at the figshare public repository (https://doi.org/ 10.6084/m9.figshare.c.7499031) upon acceptance.

Supplementary material S4: Training process of the two novel cellulose prediction models used in this investigation

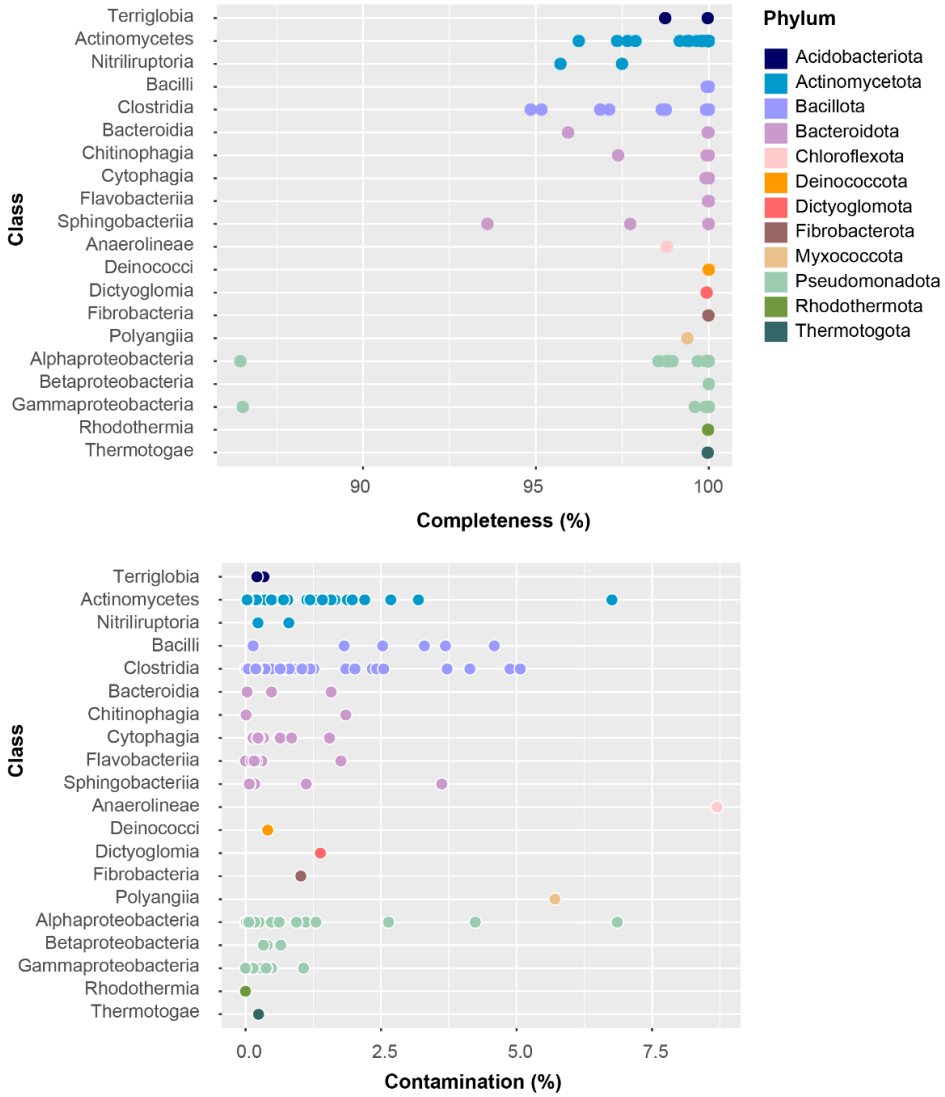
1. Genome collection and preparation for the model

The cellulose prediction models were trained using a collection of publicly available genomes from 187 isolated bacterial strains in which cellulose degradation capability was experimentally tested (Supplementary Table S4.1 and Supplementary Figure S4.2). In Supplementary table S4.1, detailed information about the 187 strains (cellulolytic activity, literature references) and their genomes (Refseq ID, quality stats such as completeness, contamination, N50, genome size and fragmentation) is shown. This table (in ".xlsx" format) can be found at the figshare public repository (https://doi.org/10.6084/m9.figshare.c.7499031) upon acceptance of the manuscript.

First, their genomic information —including genome sequence, genome access number, quality stat and taxonomic assignment— was obtained from NCBI RefSeq DB (Supplementary Figure S4.3) [99]. Second, gene prediction was performed using Prodigal (v. 2.6.3) [63]. Third, the predicted gene sequences were annotated using two different approaches: (i) with HMMer (v. 3.3.2) using the Eggnog database (v. 5.0) [64]; or, (ii) through BLAST using the CAZy database (version updated on July 2023) [60].



Supplementary Figure S4.2. Overview of the genome collection used for training the cellulose prediction models. The description of the 187 bacterial strains is shown as follows: (i) the taxonomic phyla to which they belong (left panel), (ii) their experimentally characterized cellulose degradation ability (middle panel), and (iii) the environmental origins of the strains (right panel).



Supplementary Figure S4.3. Quality stats of the 187 genomes used for training the prediction models. The first panel shows completeness rate (%) and the second, contamination (%).

2. Training process of cellulose prediction model

Each annotation output was used to construct one of the two cellulose prediction models using phenotrex (v. 0.6.0, https://github.com/univieCUBE/phenotrex), a the python-based program [65]. For determining the performance of each model, the nested cross-validation feature available in phenotrex was used to conduct a randomised 5-fold cross validation with 10 repeats [65, 100]. Moreover, three rounds of manual validation were performed in each model by splitting the genomes based on taxonomic phyla and then, grouping the phyla aiming for a 80/20 ratio in training/validation sets (Supplementary Table S4.4) [101]. Confusion matrices were built for each validation step to calculate the percentage of training data that was correctly predicted when used as testing data (Supplementary Table S4.5) [65].

Supplementary Table S4.4. Performance metrics of the two novel prediction models (COG and CAZy) using Phenotrex, including: BA (balanced accuracy), F1, P (precision), R (recall) and S (specificity). In addition, the performance metrics resulted from the four validations steps are shown. In Test1 a random selection of genomes was used as testing data (20%) while the rest was used as training data (80%). In Tests 2-4, the genomes belonging to the mentioned phyla were used as testing data (approx. 20%) and the rest as training data (80%).

Prediction Method	Phenotrex Metrics	Training Model	Test 1 random	Test 2 Bacillota	Test 3 Bacteroidota	Test 4 Pseudomonadota
COG	BA	0.74		0.70	0.80	0.76
	F1	0.71		0.60	0.81	0.77
	P	0.79		0.73	0.83	0.79
	R	0.66		0.53	0.80	0.76
	S	0.82		0.87	0.80	0.75
CAZy	BA	0.78	0.81	0.69	0.81	0.76
	F1	0.77	0.80	0.58	0.82	0.78
	P	0.82	0.85	0.73	0.84	0.79
	R	0.73	0.77	0.49	0.81	0.78
	S	0.83	0.86	0.88	0.81	0.74

Supplementary Table S4.5. Confusion matrices resulted from each validation step showing the percentage of training data that was correctly predicted when used as testing data.

Prediction Method	Test 1 random	Test 2 Bacillota (%)	Test 3 Bacteroidota (%)	Test 4 Pseudomonadota(%)
COG		88	44	80
CAZy	66	79	55	82

Prediction model testing

The 214 bacterial MAGs from this study were analysed using the same workflow as the used for the genome collection. The annotation output was used to run each prediction model separately.

Supplementary material S5: Sequencing performance of the 10 patch samples.

Supplementary Table S5.1. Metagenomic data (Gbp) obtained in each metagenomic sample before and after qc and filtering.

Sample	Ant species	Dataset	Data before qc (Gbp)	Data after qc (Gbp)	Data after filtering (%)
JMF-2106-05-0001	*A. constructor*	Illumina	8.276370664	6.923830413	83.65780961
JMF-2106-05-0002	*A. constructor*	Illumina	11.18812815	10.28542164	91.93156806
JMF-2106-05-0003	*A. constructor*	Illumina	15.44318502	13.95299048	90.35047148
JMF-2106-05-0004	*A. alfari*	Illumina	12.03984943	11.03477451	91.65209729
JMF-2106-05-0005	*A. alfari*	Illumina	11.91489304	10.20142414	85.6190996
JMF-2106-05-0006	*A. constructor*	Illumina	13.26132788	10.83476209	81.70193957
JMF-2106-05-0007	*A. constructor*	Illumina	17.50241625	15.40118901	87.99464477
JMF-2106-05-0008	*A. constructor*	Illumina	13.40892625	11.85379865	88.40229585
JMF-2111-01-0001	*A. constructor*	ONT	1.402430692	NA	NA
JMF-2111-01-0005	*A. constructor*	ONT	38.78229923	NA	NA

Supplementary Table S5.2. Metagenomic data (Gbp) of the assemblies that mapped into the individual metagenomic samples.

Sample	Used for hybrid assembly	Mapped data hybrid 0001	Mapped data hybrid 0005	Mapped data ONT assembly
JMF-2106-05-0001	yes	57.28	59.91	NA
JMF-2106-05-0002	yes	67.87	70.24	NA
JMF-2106-05-0003	yes	84.27	84.9	NA
JMF-2106-05-0004	no	NA	NA	NA
JMF-2106-05-0005	no	NA	NA	NA
JMF-2106-05-0006	yes	72.66	74.32	NA
JMF-2106-05-0007	yes	82.09	82.86	NA
JMF-2106-05-0008	yes	NA	NA	NA
JMF-2111-01-0001	yes	NA	NA	22.67
JMF-2111-01-0005	yes	NA	NA	81.02

Supplementary material S6: CAZy family types detected among the 214 MAGs

Supplementary Figure S6.1. Description of the CAZy family types found in each MAG reconstructed from the ant-made patches. (A) Taxonomic and phylogenetic assignment of the 214 MAGs. (B) Barplot showing the abundance of CAZy domain copies per CAZy family type found in each MAG. (C) Barplot the abundance of CAZy domain types linked to a signal peptide (CAZy-signalp) in the same gene sequence.

Supplementary material S7: Video showing *Azteca* ants regurgitation and defecation behaviours

The Supplementary material S7 includes a video showing an *Azteca constructor* worker regurgitating a pellet from the infrabuccal pocket and, immediately after, defecating liquids on the patches. The video was made by Veronika E. Mayer (co-author) with a high-resolution camera fixed on a stereomicroscope. This video (in ".asf" format) can be found at the figshare public repository upon acceptance (https://doi.org/ 10.6084/m9.figshare.c.7499031).

References

1. Klemm D, Heublein B, Fink HP, Bohn A. Cellulose: Fascinating biopolymer and sustainable raw material. Angew Chemie - Int Ed. 2005;44:3358–93.

2. Moussian B. Chitin : Structure , Chemistry and Biology. In: Targeting Chitin-containing Organisms. Springer Singapore; 2019. p. 5–18.

3. Rongpipi S, Ye D, Gomez ED, Gomez EW. Progress and opportunities in the characterization of cellulose – an important regulator of cell wall growth and mechanics. Front Plant Sci. 2019;9 March:1894.

4. Varlamov VP, Il'ina A V., Shagdarova BT, Lunkov AP, Mysyakina IS. Chitin/chitosan and its derivatives: fundamental problems and practical approaches. Biochem. 2020;85:154–76.

5. McCann MC, Carpita NC. Biomass recalcitrance: A multi-scale, multi-factor, and conversion-specific property. J Exp Bot. 2015;66:4109–18.

6. Conlon BH, O'Tuama D, Michelsen A, Crumière AJJ, Shik JZ. A fungal symbiont converts provisioned cellulose into edible yield for its leafcutter ant farmers. Biol Lett. 2022;18:20220022.

7. Russell JB, Muck RE, Weimer PJ. Quantitative analysis of cellulose degradation and growth of cellulolytic bacteria in the rumen. FEMS Microbiol Ecol. 2009;67:183–97.

8. Brune A. Symbiotic digestion of lignocellulose in termite guts. Nat Rev Microbiol. 2014;12:168–80.

9. Teullet S, Tilak M-K, Magdeleine A, Schaub R, Weyer NM, Panaino W, et al. Metagenomics uncovers dietary adaptations for chitin digestion in the gut microbiota of convergent myrmecophagous mammals. mSystems. 2023;8.

10. Bouchebti S, Cohen TM, Bodner L, Levin E. Chitin digestion in a eusocial insect: the digestive role of larvae in hornet colonies. Entomol Gen. 2023;43:491–4.

11. Datta R. Enzymatic degradation of cellulose in soil: A review. Heliyon. 2024;10:e24022.

12. Cui J, Mai G, Wang Z, Liu Q, Zhou Y, Ma Y, et al. Metagenomic insights into a cellulose-rich niche reveal microbial cooperation in cellulose degradation. Front Microbiol. 2019;10 MAR:1–12.

13. Bao Y, Dolfing J, Wang B, Chen R, Huang M, Li Z, et al. Bacterial communities involved directly or indirectly in the anaerobic degradation of cellulose. Biol Fertil Soils. 2019;55:201–11.

14. Stubbusch AK, Keegstra JM, Schwartzman J, Pontrelli S, Clerc EE, Stocker R, et al. Polysaccharide breakdown products drive degradation-dispersal cycles of foraging bacteria through changes in metabolism and motility. Elife. 2024;13:1–44.

15. Jarett JK, Kingsbury DD, Dahlhausen KE, Ganz HH. Best practices for microbiome study design in companion animal research. Front Vet Sci. 2021;8 April:1–15.

16. de Jonge N, Carlsen B, Christensen MH, Pertoldi C, Nielsen JL. The gut microbiome of 54 mammalian species. Front Microbiol. 2022;13 June:886252.

17. Peixoto RS, Harkins DM, Nelson KE. Advances in microbiome research for animal health. Annu Rev Anim Biosci. 2021;9:289–311.

18. Kruger Ben Shabat S, Sasson G, Doron-Faigenboim A, Durman T, Yaacoby S, Berg Miller ME, et al. Specific microbiome-dependent mechanisms underlie the energy harvest efficiency of ruminants. ISME J. 2016;10:2958–72.

19. Marynowska M, Sillam-Dussès D, Untereiner B, Klimek D, Goux X, Gawron P, et al. A holobiont approach towards polysaccharide degradation by the highly compartmentalised gut system of the soil-feeding higher termite *Labiotermes labralis*. BMC Genomics. 2023;24:1–20.

20. Liu N, Li H, Chevrette MG, Zhang L, Cao L, Zhou H, et al. Functional metagenomics reveals abundant polysaccharide- degrading gene clusters and cellobiose utilization pathways within gut microbiota of a wood-feeding higher termite. ISME J. 2019;13:104–17.

21. Cabral L, Persinoti GF, Paixão DAA, Martins MP, Morais MAB, Chinaglia M, et al. Gut microbiome of the largest living rodent harbors unprecedented enzymatic systems to degrade plant polysaccharides. Nat Commun. 2022;13:1–16.

22. Wertz JT, Béchade B. Symbiont-mediated degradation of dietary carbon sources in social herbivorous insects. Adv In Insect Phys. 2020;58:63–109.

23. De Fine Licht HH, Boomsma JJ, Tunlid A. Symbiotic adaptations in the fungal cultivar of leaf-cutting ants. Nat Commun. 2014;5:5675.

24. Lewin GR, Johnson AL, Moreira Soto RD, Perry K, Book AJ, Horn HA, et al. Cellulose-enriched microbial communities from leaf-cutter ant (*Atta colombica*) refuse dumps vary in taxonomic composition and degradation ability. PLoS One. 2016;11:1–22.

25. Scott JJ, Budsberg KJ, Suen G, Wixon DL, Balser TC, Currie CR. Microbial community structure of leaf-cutter ant fungus gardens and refuse dumps. PLoS One. 2010;5.

26. Barrajon-Santos V, Nepel M, Hausmann B, Voglmayr H, Woebken D, Mayer VE. Dynamics and drivers of fungal communities in a multipartite ant-plant association. BMC Biol. 2024;22:112.

27. Nepel M, Mayer VE, Barrajon-Santos V, Woebken D. Bacterial diversity in arboreal ant

nesting spaces is linked to colony developmental stage. Commun Biol. 2023;6:1217.

28. Morera J, Mora-Pineda G, Esquivel A, Hanson P. Detection, ultrastructure and phylogeny of *Sclerorhabditis neotropicalis* (Nematoda: Rhabditidae) nematodes associated with the *Azteca* ant-*Cecropia* tree symbiosis. Rev Biol Trop. 2018;66:368–80.

29. Heil M, Rattke J, Boland W. Postsecretory hydrolysis of nectar sucrose and specialization in ant/plant mutualism. Science. 2005;308:560–3.

30. Nepel M, Voglmayr H, Blatrix R, Longino JT, Fiedler K, Schönenberger J, et al. Ant-cultivated Chaetothyriales in hollow stems of myrmecophytic *Cecropia* sp. trees – diversity and patterns. Fungal Ecol. 2016;23:131–40.

31. Greenfield MJ, Lach L, Congdon BC, Anslan S, Tedersoo L, Field M, et al. Consistent patterns of fungal communities within ant-plants across a large geographic range strongly suggest a multipartite mutualism. Mycol Prog. 2021;20:681–99.

32. Ueda S, Quek SP, Itioka T, Inamori K, Sato Y, Murase K, et al. An ancient tripartite symbiosis of plants, ants and scale insects. Proc R Soc B. 2008;275:2319–26.

33. Mayer VE, Frederickson ME, Mckey D, Blatrix R. Current issues in the evolutionary ecology of ant – plant symbioses. 2014;:749–64.

34. Mayer VE, Nepel M, Blatrix R, Oberhauser FB, Fiedler K, Schönenberger J, et al. Transmission of fungal partners to incipient *Cecropia*-tree ant colonies. PLoS One. 2018;13:e0192207.

35. Mayer VE, Voglmayr H, Blatrix R, Orivel J, Leroy C. Fungi as mutualistic partners in ant-plant interactions. Front Fungal Biol. 2023;4 October:1213997.

36. Vasse M, Voglmayr H, Mayer V, Gueidan C, Nepel M, Moreno L, et al. A phylogenetic perspective on the association between ants (Hymenoptera: Formicidae) and black yeasts (Ascomycota: Chaetothyriales). Proc R Soc B. 2017;284:20162519.

37. Voglmayr H, Mayer V, Maschwitz U, Moog J, Djieto-Lordon C, Blatrix R. The diversity of ant-associated black yeasts: insights into a newly discovered world of symbiotic interactions. Fungal Biol. 2011;115:1077–91.

38. Moreno LF, Mayer V, Voglmayr H, Blatrix R, Benjamin Stielow J, Teixeira MM, et al. Genomic analysis of ant domatia-associated melanized fungi (Chaetothyriales, Ascomycota). Mycol Prog. 2019;18:541–52.

39. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet J. 2011;17:10–2.

40. Li D, Liu CM, Luo R, Sadakane K, Lam TW. MEGAHIT: An ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. Bioinformatics. 2015;31:1674–6.

41. Wick RR, Judd LM, Holt KE. Performance of neural network basecalling tools for Oxford Nanopore sequencing. Genome Biol. 2019;20:1–10.

42. Kolmogorov M, Yuan J, Lin Y, Pevzner PA. Assembly of long, error-prone reads using repeat graphs. Nat Biotechnol. 2019;37:540–6.

43. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, et al. SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol. 2012;19:455–77.

44. Li H. Minimap2: Pairwise alignment for nucleotide sequences. Bioinformatics. 2018;34:3094–100.

45. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics. 2009;25:2078–9.

46. Kang DD, Li F, Kirton E, Thomas A, Egan R, An H, et al. MetaBAT 2: An adaptive binning algorithm for robust and efficient genome reconstruction from metagenome assemblies. PeerJ. 2019;7:e7359.

47. Olm MR, Brown CT, Brooks B, Banfield JF. DRep: A tool for fast and accurate genomic comparisons that enables improved genome recovery from metagenomes through de-replication. ISME J. 2017;11:2864–8.

48. Gurevich A, Saveliev V, Vyahhi N, Tesler G. QUAST: quality assessment tool for genome assemblies. Bioinformatics. 2013;29:1072–5.

49. Parks DH, Imelfort M, Skennerton CT, Hugenholtz P, Tyson GW. CheckM: Assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. Genome Res. 2015;25:1043–55.

50. Portik DM, Brown CT, Pierce-Ward NT. Evaluation of taxonomic classification and profiling methods for long-read shotgun metagenomic sequencing datasets. BMC Bioinformatics. 2022;23:541.

51. Pierce NT, Brown CT, Reiter T, Brooks P. Large-scale sequence comparisons with sourmash. F1000Research. 2022;8.

52. Gruber-Vodicka HR, Seah BKB, Pruesse E. phyloFlash: Rapid small-subunit rRNA profiling and targeted assembly from metagenomes. mSystems. 2020;5:10–1128.

53. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. Nucleic Acids Res. 2012;41:590–6.

54. Tatusova T, Dicuccio M, Badretdin A, Chetvernin V, Nawrocki EP, Zaslavsky L, et al. NCBI prokaryotic genome annotation pipeline. Nucleic Acids Res. 2016;44:6614–24.

55. Zheng J, Ge Q, Yan Y, Zhang X, Huang L, Yin Y. dbCAN3 : automated carbohydrate-active enzyme and substrate annotation. Nucleic Acids Res. 2023;51:W115–21.

56. Drula E, Garron ML, Dogan S, Lombard V, Henrissat B, Terrapon N. The carbohydrate-active enzyme database: Functions and literature. Nucleic Acids Res. 2022;50:D571–7.

57. Buchfink B, Xie C, Huson DH. Fast and sensitive protein alignment using DIAMOND. Nat

Methods. 2014;12:59–60.

58. Zheng J, Hu B, Zhang X, Ge Q, Yan Y, Akresi J, et al. dbCAN-seq update: CAZyme gene clusters and substrates in microbiomes. Nucleic Acids Res. 2023;51:D557–63.

59. Teufel F, Almagro Armenteros JJ, Johansen AR, Gíslason MH, Pihl SI, Tsirigos KD, et al. SignalP 6.0 predicts all five types of signal peptides using protein language models. Nat Biotechnol. 2022;40:1023–5.

60. Cantarel BI, Coutinho PM, Rancurel C, Bernard T, Lombard V, Henrissat B. The Carbohydrate-Active EnZymes database (CAZy): An expert resource for glycogenomics. Nucleic Acids Res. 2009;37 SUPPL. 1:233–8.

61. Chaumeil PA, Mussig AJ, Hugenholtz P, Parks DH. GTDB-Tk: A toolkit to classify genomes with the genome taxonomy database. Bioinformatics. 2020;36:1925–7.

62. Asnicar F, Thomas AM, Beghini F, Mengoni C, Manara S, Manghi P, et al. Precise phylogenetic analysis of microbial isolates and genomes from metagenomes using PhyloPhlAn 3.0. Nat Commun. 2020;11:1–10.

63. Hyatt D, Chen GL, LoCascio PF, Land ML, Larimer FW, Hauser LJ. Prodigal: Prokaryotic gene recognition and translation initiation site identification. BMC Bioinformatics. 2010;11.

64. Huerta-Cepas J, Szklarczyk D, Heller D, Hernández-Plaza A, Forslund SK, Cook H, et al. EggNOG 5.0: A hierarchical, functionally and phylogenetically annotated orthology resource based on 5090 organisms and 2502 viruses. Nucleic Acids Res. 2019;47:D309–14.

65. Feldbauer R, Schulz F, Horn M, Rattei T. Prediction of microbial phenotypes based on comparative genomics. BMC Bioinformatics. 2015;16:1–8.

66. Bowers RM, Kyrpides NC, Stepanauskas R, Harmon-Smith M, Doud D, Reddy TBK, et al. Minimum information about a single amplified genome (MISAG) and a metagenome-assembled genome (MIMAG) of bacteria and archaea. Nat Biotechnol. 2017;35:725–31.

67. Little AEF, Murakami T, Mueller UG, Currie CR. The infrabuccal pellet piles of fungus-growing ants. Naturwissenschaften. 2003;90:558–62.

68. Wang C, Cocco A, Lin CC, Billen J. Morphology and ultrastructure of the infrabuccal pocket in *Strumigenys* ants. Arthropod Struct Dev. 2022;68:101154.

69. Mohanta TK, Bae H. The diversity of fungal genome. Biol Proced Online. 2015;17:1–9.

70. Renzi S, Nenciarini S, Bacci G, Cavalieri D. Yeast metagenomics: analytical challenges in the analysis of the eukaryotic microbiome. Microbiome Res Reports. 2024;3:1–16.

71. Wang C, Dong D, Wang H, Müller K, Qin Y, Wang H, et al. Metagenomic analysis of microbial consortia enriched from compost: New insights into the role of Actinobacteria in lignocellulose decomposition. Biotechnol Biofuels. 2016;9:1–17.

72. Aguilar-Paredes A, Valdés G, Araneda N, Valdebenito E, Hansen F, Nuti M. Microbial community in the composting process and its positive impact on the soil biota in sustainable agriculture. Agronomy. 2023;13.

73. Braga LPP, Pereira RV, Martins LF, Maria L, Moura S, Sanchez FB, et al. Genome-resolved metagenome and metatranscriptome analyses of thermophilic composting reveal key bacterial players and their metabolic interactions. BMC Genomics. 2021;22:652.

74. Santos-Pereira C, Sousa J, Costa ÂMA, Santos AO, Rito T, Soares P, et al. Functional and sequence-based metagenomics to uncover carbohydrate-degrading enzymes from composting samples. Appl Microbiol Biotechnol. 2023;107:5379–401.

75. Beier S, Bertilsson S. Bacterial chitin degradation-mechanisms and ecophysiological strategies. Front Microbiol. 2013;4 JUN:1–12.

76. Itoh T, Kimoto H. Bacterial chitinase system as a model of chitin biodegradation. In: Targeting Chitin-containing Organisms. Springer Singapore; 2019. p. 131–51.

77. Aragunde H, Biarnés X, Planas A. Substrate recognition and specificity of chitin deacetylases and related family 4 carbohydrate esterases. Int J Mol Sci. 2018;19:1–30.

78. Aylward FO, Burnum KE, Scott JJ, Suen G, Tringe SG, Adams SM, et al. Metagenomic and metaproteomic insights into bacterial communities in leaf-cutter ant fungus gardens. ISME J. 2012;6:1688–701.

79. Rickson FR. Glycogen plastids in Müllerian body cells of *Cecropia peltata* - A higher green plant. Science. 1971;173:344–7.

80. Bischof S, Umhang M, Eicke S, Streb S, Qi W, Zeeman SC. *Cecropia peltata* accumulates starch or soluble glycogen by differentially regulating starch biosynthetic genes. Plant Cell. 2013;25:1400–15.

81. Steele JE. Glycogen phosphorylase in insects. Insect Biochem. 1982;12:131–47.

82. Wilkinson JF. The problem of energy-storage compounds in bacteria. Exp Cell Res. 1959;7 SUPPL.:111–30.

83. Wilson WA, Roach PJ, Montero M, Baroja-Fernández E, Muñoz FJ, Eydallin G, et al. Regulation of glycogen metabolism in yeast and bacteria. FEMS Microbiol Rev. 2010;34:952–85.

84. Gharechahi J, Vahidi MF, Bahram M, Han J-L, Ding X-Z, Salekdeh GH. Metagenomic analysis reveals a dynamic microbiome with diversified adaptive functions to utilize high lignocellulosic forages in the cattle rumen. ISME J. 2021;15:1108–1120.

85. Pollak S, Gralka M, Sato Y, Schwartzman J, Lu L, Cordero OX. Public good exploitation in natural bacterioplankton communities. 2021; July:1–10.

86. Pontrelli S, Szabo R, Pollak S, Schwartzman J, Ledezma-tejeida D, Cordero OX, et al. Metabolic cross-feeding structures the assembly of polysaccharide degrading communities. 2022;3076 February:1–12.

87. Nepel M, Pfeifer J, Oberhauser FB, Richter A, Woebken D, Mayer VE. Nitrogen fixation by diverse diazotrophic communities can support population growth of arboreal ants. BMC Biol. 2022;20:135.

88. Pinto-tomás AA, Anderson MA, Suen G, Stevenson DM, Chu FST, Cleland WW, et al. Symbiotic nitrogen fixation in the fungus gardens of leaf-cutter ants. Science. 2009;5956:1120–3.

89. Biedermann PHW, Vega FE. Ecology and evolution of insect – fungus mutualisms. Annu Rev Entomol. 2020;65:431–55.

90. Mueller UG, Gerardo NM, Aanen DK, Six DL, Schultz TR. The evolution of agriculture in insects. Annu Rev Ecol Evol Syst. 2005;36:563–95.

91. Diehl JMC, Kowallik V, Keller A, Biedermann PHW. First experimental evidence for active farming in ambrosia beetles and strong heredity of garden microbiomes. Proc R Soc B. 2022;289:20221458.

92. Smith JL, Collins HP. Management of organisms and their processes in soils. In: Soil Microbiology, Ecology and Biochemistry: Third Edition. Third Edit. Elsevier Inc.; 2007. p. 471–502.

93. Diaz LF, De Bertoldi M, Bidlingmaier W. Compost science and technology. Elsevier; 2011.

94. Meghvansi MK, Chaudhary KK, Khan MH, Siddiqui S, Varma A. Biology of composts. 2020.

95. Maschwitz U, Fiala B, Dumpert K, Hashim R bin, Sudhaus W. Nematode associates and bacteria in ant-tree symbioses. Symbiosis. 2016;69:1–7.

96. Rickwood D, Ford T, Graham J. Nycodenz: A new nonionic iodinated gradient medium. Anal Biochem. 1982;123:23–31.

97. Eichorst SA, Strasser F, Woyke T, Schintlmeister A, Wagner M, Woebken D. Advancements in the application of NanoSIMS and Raman microspectroscopy to investigate the activity of microbial cells in soils. FEMS Microbiol Ecol. 2015;91:fiv106.

98. Van Bezooijen J. Methods and Techniques for Nematology. Wageningen Univ. 2006;20.

99. O'Leary NA, Wright MW, Brister JR, Ciufo S, Haddad D, McVeigh R, et al. Reference sequence (RefSeq) database at NCBI: Current status, taxonomic expansion, and functional annotation. Nucleic Acids Res. 2016;44:D733–45.

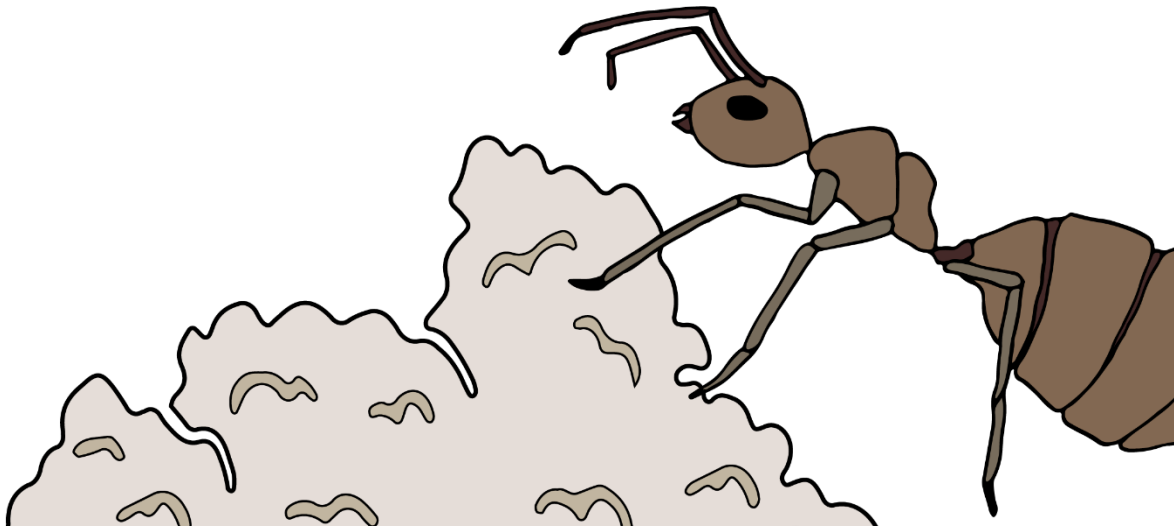
100. Bates S, Hastie T, Tibshirani R. Cross-validation : what does it estimate and how well does it do it ? J Am Stat Assoc. 2022;119:1434–45.

101. Brown OM, Curtis AB, Goodwin JA. Principles for evaluation AI/ML model performance and robustness. Lexington, yMassachusetts; 2021.

General Discussion

Re-defining the origins of composting: arboreal ants make compost piles within their nests using inherited inoculants

Veronica Barrajon-Santos



Azteca ants make compost piles in their nest

Ants inhabit nearly every terrestrial ecosystem on Earth, from the Arctic tundra to the Sahara Desert [1]. This ubiquity is often credited to their complex eusocial organization, which allows them to comprehend and subsequently shape their environment according to their needs [1]. Ants are extraordinarily diverse and abundant in tropical regions [2], where they act as key ecosystem engineers [3]. Operating from the forest floor up to the canopy, many tropical ant species mobilize vast amounts of nutrient resources from their surroundings to their nests [3]. Once inside the nest, such organic matter is either utilized by the ant colony for their nutrition [1], or instead, processed through the activity of microorganisms [4]. While tropical ants are known to play a vital role in the redistribution and transformation of nutrients in rainforests [3, 5], only a few ant genera apart from *Atta* and *Acromyrmex* have been attributed with the ability to modulate complex microbial-driven organic matter decomposition processes [6–8]. Overall, this PhD thesis suggests that tropical ants inhabiting plants have engineered a nutrient recycling system in which plant- and insect-derived waste material is efficiently degraded through the action of an ant-inherited consortium of organisms.

By the use of cost- and time- efficient molecular techniques and bioinformatic analyses, in Chapter I and II, I first conducted an in-depth and comprehensive examination of the fungal and nematodes communities found in the patches of the *Azteca-Cecropia* mutualism. Chapter I demonstrates that *Azteca* patches harbour a diverse range of fungi and thus, supports the findings in other ant-plant associations [9, 10]. Chaetothyriales, thought to be the major fungal group in these patches are indeed prominent in the ITS amplicon data [11, 12]. Nevertheless, other genera such as *Fusarium*, *Mucor*, *Mortierella, Moesziomyces* and *Pleiocarpon* also appeared with high relative read abundance. Remarkably, the first three genera are among the most common fungi isolated from the compost piles made by humans, which suggests their potential involvement in similar processes within the patches [13].

Furthermore, Chapter II highlights the presence of three different groups of bacterivorous Rhabditida nematodes in the patches, the previously described genera *Sclerorhabditis* and *Diploscapter* along with an unclassified group that earlier studies

have just briefly mentioned [14–16]. In addition to Rhabditida, occasional occurrences of Tylenchida (e.g., *Aphelenchoides*) and Dorylaimida (e.g., *Mesodorylaimus*) nematodes, likely belonging to groups with distinct feeding strategies, were detected in the 18S rRNA amplicon data.

Following the thorough dissection of the patch communities in the *Azteca-Cecropia* association, in Chapter III, I investigated the ability of bacterial patch communities to metabolize the recalcitrant cellulose and chitin found in the deposited organic matter. By metagenomics analysis, this chapter confirms the high and consistent diversity of bacterial communities in the patches that was previously described in Nepel et al. (2023) by 16S rRNA amplicon sequencing. Compared to the taxonomy-based metabarcoding analysis, the 214 MAGs reconstructed from metagenomics, which represent the bacterial microbiome in patches, also revealed their genetic mechanisms related to complex polysaccharide degradation processes. From these results, potential bacterial players in the decomposition of organic matter in the patches (e.g., Chitinophagaceae, Comamonadaceae, Opitutaceae, and Lachnospiraceae) were suggested. Given that this thesis does not address the potential mechanisms of fungi to degrade the substrates, future research should aim to isolate more fungal strains in the patches —apart from Chaetothyriales— and perform genomic analyses and activity assays with them [17, 18].

Overall, using a broad definition of composting as "the biological process of organic waste decomposition mediated by microorganisms under controlled conditions", I conclude from this thesis that *Azteca* ants build compost piles within their nests.

How is the art of composting by *Azteca* ants?

The practice of composting takes on diverse forms among closely related *Azteca* species, each following its own recipe. Chapter I describes how the patches made by *A. alfari* and *A. constructor* are morphologically different despite the co-occurrence of these ant species in the same geographical area and in association with the same *Cecropia* species. In addition, Chapter I and II showed that the patches of each ant species harbour unique fungal and nematodes communities, respectively, as it was

previously observed in the bacterial communities [19]. Therefore, these results suggest that each *Azteca* species, with its unique way of making the patch and generally building their nest along with their noticeable different behaviour [20, 21], somehow shapes the communities inhabiting the patches (Chapter I and II) [19].

Despite the significant taxonomic variation observed, the next question was whether the overall activity of the patches remained consistent, implying functional redundancy. For instance, the predominant rhabditid groups in each ant species were different, but they all were morphologically identified as bacterivorous nematodes (Chapter II). Moreover, the patches of each ant species harbour different microbial communities (Chapter I; [19]); and yet, they similarly showed nitrogen fixation activity [22] and the ability to break down cellulose and chitin (Chapter III). This highlights the limitations of relying solely on DNA-based taxonomic analysis and underscores the importance of combining it with other techniques whenever possible such as the morphologically-based identification of nematodes and the activity measurements coupled with bacterial metagenomics that were conducted in Chapter II and III of this thesis, respectively.

In addition to the shared functional processes identified in the patches of both *Azteca* species, these structures also seem to follow rather similar dynamics as the ant colony grows. Before leaving the mother colony, an alate queen seems to collect an inoculum of patch organisms, which she later adds to the initial patch while building it within the recently colonized *Cecropia* sapling [23]. Although the patch inoculum is likely to be a fraction of the community from the established patches of the mother colony, these communities are exposed to a drastic substrate bottleneck at the colony founding stage, where only parenchyma tissue is available [19]. At this stage, generalist bacteria (e.g., Enterobacterales) and fast-growing fungi (e.g., *Fusarium* and *Moesziomyces*) seem to be predominant (Chapter I; [19]), whereas the nematode biomass is rather low (personal field observations).

As the ant colony grows, the *Azteca* workers make patch structures in almost every internode they colonize and their overall microbial communities get more diverse (Chapter I, [19]). Multiple findings could explain such diversification process. For instance, the substrate sources get also notably diversified, which opens new niches

for the development of other microbial community members within the patches (Chapter I, [19]). At this stage, the biomass of nematodes increases (personal field observations) and slow-growing organisms like Chaetothyriales become prominent (Chapter I), which probably fosters new biotic interactions. Moreover, in contrast to the founding colony stage, which occurs in a sealed plant internode [23], the domatia of established colonies have open entrance holes [24], and the workers continuously forage on the tree surface to protect the plant against herbivores and competitors [25]. This is probably an entry point of new bacteria and fungi from the surrounding environment. Finally, in some highly developed colonies and big trees, specially from *A. constructor*, an additional group of nematodes often described as nematode predators, was detected (Chapter II). Remarkably, such transition from bacteria-feeding nematodes to predators is well-described in composting by humans as an indicator of ongoing maturation [26, 27].

Overall, the results gathered about the community dynamics of the *Azteca* patches suggest an ecological succession over time, similar to the processes observed in the compost piles made by humans [26, 28, 29]. To build on this finding, future research could examine the activity dynamics of organic matter decomposition processes in patches along the ant colony development. This could be achieved by comparing activity measurements and bacterial metatranscriptomics —focused on gene expression related to cellulose and chitin degradation— between initial and established colonies.

Why did compost making evolve in ant-plant mutualisms?

The tropical forests are known to be limited in available nutrient sources, specially nitrogen and phosphorous [30–33]. Despite this limitation, *Azteca* ants manage to grow colonies up to approx. 15,000 workers without foraging outside the host tree [34, 35]. Until now, it is known that the ant colony is nutritionally sustained by the *Cecropia* host plant via the provided food bodies and the honeydew of hemipterans. However, several questions remain: can the *Cecropia* plant assimilate enough nutrients to maintain itself and the whole ant colony? Otherwise, are the nutrients

recycled in the patches supporting the development of the ant population, or could they even support the plant? Based on findings gathered in this PhD thesis along with the extensive research previously performed in this model system [15, 20–23, 34, 36, 37], I here summarize the nutrient fluxes, including the potential nutrient sources and sinks, along with the trophic relationships that are so far known from an established *Azteca* colony inhabiting a *Cecropia* plant. This summary, recently highlighted as necessary by Ješovnik A. and Schultz T. (2022) [38], aims to provide clearer insights into why patch-making evolved in ant-plant mutualisms.

From *Cecropia* to the *Azteca* ants. The *Cecropia* plant acquires carbon by photosynthesis performed in the leaves and additional mineral nutrients (e.g., N and P) by uptake through the roots. These nutrients are partially used for its own structural growth and some are accumulated in the Müllerian food bodies, primarily as glycogen [36]. The nutrients stored on the *Cecropia* leaves are gradually redistributed during senescence [39]. When a *Cecropia* plant is colonized by *Azteca* ants, the ant workers collect the food bodies to feed their larvae [40]. Additionally, they tend scale insects as "cattle" to obtain carbohydrate-rich honeydew derived from plant sap which serves as nutrition for the workers [20]. Overall, the ant worker caste represents a temporal sink of nutrients for the duration of each individual's life.

From the ant-plant to the patch organisms. The ants are constantly supplying the patches with plant- and animal-derived organic matter (Chapter III). From the plant, the ants deposit the cellulose-rich parenchyma tissue located on the inner wall of internodes [41] as well as trichomes and some food bodies. Moreover, they deposit chitin-rich substrates such as dead nestmates and other insect bodies. Finally, ants have been recorded secreting pellets through regurgitation and liquids by defecation (Chapter III).

Among the patch organisms. The diverse trophic interactions among patch inhabitants, combined with their specific metabolic capabilities, create highly complex nutrient fluxes within the patches. In the patch ecosystem, most nutrients originate from plant- and ant-derived waste, except for nitrogen, which is also supplied by the nitrogen fixation activity of a diverse diazotrophic community [22]. Recalcitrant cellulose and chitin from the substrates are then broken down by a complex microbial

consortium into to easily assimilable oligomers (Chapter III). Beyond the degraders, these oligomers are likely used by others microorganisms which benefit from such extracellular cellulase and chitinase activity (Chapter III). As the bacterial biomass increases, rhabditid nematodes found in the patches feed on bacteria (Chapter II), assimilating their nutrients and excreting large amounts of ammonia [42]. Like bacteria, fungi and nematodes found in the patches act as temporal sinks of nutrients until they are predated or die. Finally, this decomposition process likely results in the accumulation of mineral nutrients, as it was shown in compost made by humans [28].

From the patches to… Despite the efforts of many scientists, this part of the nutrient cycles in the *Azteca-Cecropia* complex remains unclear. This is largely due to the fact that ants in obligate associations with plants are notoriously difficult to manipulate in controlled experiments. Both greenhouse and field experiments with *Azteca* ants have failed, mostly because they respond immediately to the manipulation of their domatia, in some cases even abandoning the affected internodes and migrating to other parts of the tree. (Chapter I, [43]). By relying mostly on molecular techniques, this PhD thesis has made a significant contribution to the understanding of these striking structures, allowing more precise hypotheses about their purposes.

What is the potential purpose of composting for the *Azteca* ants?

The findings gathered in this thesis in relation to the development of patches suggest that the ant colony —first, the ant queen and then, the ant workers— initiate and shape most processes occurring in the patches. While these findings imply that *Azteca* ants have indeed engineered a system that facilitates microbial-driven degradation of plant- and insect-derived waste material, future research is still necessary to uncover whether and how the recycled nutrients are re-assimilated by the ant-plant complex. By relating this particular association with other ant-plant mutualisms worldwide or even with other types of ants, here I formulate some hypotheses about the potential purposes of patches.

Patches as nutrition for the ant colony. *Azteca* ants are known to receive nutritious food rewards from the host *Cecropia* plant. However, several questions have been

previously asked in this regard: is the *Cecropia* plant continuously producing food bodies even when its own access to nutrients is limited? how do the first workers develop at the beginning of colony foundation without the access to food bodies? and, are the nutrient demands of the whole ant colony during its entire lifetime fully covered by the host plant provided nutrients? For the first question, several studies showed that Müllerian food bodies production indeed varies depending on the environmental conditions. For instance, the production rates are positively correlated with the availability of nitrogen for the plant [44, 45] and such production drastically decreases during periods of drought [46]. For the second question, a recent investigation experimentally showed how ^{15}N-labelled amino acids added to the initial patch of founding colonies was later detected in the larvae, which suggests that larvae, at least at this initial stage, take up nutrients from patch material [23]. Finally, when it comes to the third question of whether the host plant fully meets the nutritional demands of the ant colony, a clear answer is still lacking in the *Azteca-Cecropia* mutualism. A study in other ant-plant mutualisms (e.g., *Petalomyrmex phylax* associated with *Leonardoxa africana*, and *Tetraponera aethiops* with *Barteria fistulosa*) showed how the ant larvae —under experimental conditions where the ant workers had no access to food bodies— were fed by the ant workers with either patch material or Chaetothyriales cultures after the patch was removed [47]. As the cultivation of fungi for nutritional purposes has evolved in several eusocial insects (e.g., leafcutter ants, fungus-growing termites and ambrosia beetles) [48–51], it would be likely that fungi growing in the patches are used to feed larvae at the beginning of colony foundation and at a later stage when food bodies are scarce.

Patches as nutrition for the *Cecropia* plant. Some findings could support the hypothesis that *Cecropia* plants nutritionally benefit from the patches. Colonized *Cecropia* plants grow bigger than uncolonized ones and also have a higher nitrogen content [52]. This is often related with the protective behaviour of the ants against herbivores and competitors [53, 54]. However, it is also possible that nutrients, especially nitrogen from mature patch material, somehow gets assimilated by the *Cecropia* plant when it gets spread on the young internodes by *Azteca* workers (Chapter III). This hypothesis is supported by the fact that myrmecotrophy —plants feeding on ant waste— has already been identified in other ant-plant mutualisms (e.g.

Leonardoxa Africana inhabited by *Petalomyrmex phylax* , myrmecophytic *Piper* or *Caularthron bilamellatum* associated with *Azteca* cf *velox*) [55–61]. Particularly for the *Azteca-Cecropia* association, a previous investigation using carbon and nitrogen isotopes postulated that 93% of the nitrogen in the *Cecropia* plant is coming from the patches made by *Azteca* [62]. While a potential contribution of patches to plant growth is possible, it is unlikely that a ground-rooting plant in a secondary habitat gains 93% of its total N from less than two grams of fresh weight patch material that is often collected from an entire *Azteca* colony (personal field observations). To clarify whether and how *Cecropia* assimilates nutrients from the ant-made patches, a deeper investigation focusing on the mechanisms of the plant to uptake nutrients from patches is needed.

Patches as immune system of the colony. *Azteca* ants dispose all types of compostable material in their patches, including numerous dead nestmates. As known for composting by humans [63–65], introducing ant-derived waste could significantly increase the risk of contamination by pathogens, thereby boosting the spread of diseases within the colony. However, Chapter I showed that entomopathogenic fungi appeared to be absent from the *Azteca-Cecropia* patches (Chapter I). This prompts the question: have the ants found a way to suppress entomopathogens in their compost? Fungus-growing ants are known to maintain a close symbiotic association with *Pseudonocardia* strains for their protection against fungal pathogens [66, 67]. Similarly, eight Actinobacteria strains recently isolated from three ant-plant mutualisms have shown antifungal activity [68]. Based on these findings, one could hypothesize that the microbial communities inhabiting the patches of *Azteca-Cecropia* indeed offer immunity to the ants by synthesising antibiotics in the patches. A further look into the secondary metabolite biosynthesis gene clusters in 214 bacterial MAGs obtained in Chapter III —especially in the genomes belonging to Actinobacteria such as *Nocardioides*— could provide valuable insights about their capability of protecting the ant colony against entomopathogens.

Concluding remarks

This thesis is a further proof of the ability of eusocial insects to comprehend and shape their environment according to their needs. By using a holistic approach, this study emphasizes the remarkably complex biodiversity and biotic interactions within the *Azteca-Cecropia* mutualism. First, a detailed examination of the ant-made patches provided an in-depth and comprehensive understanding of the microbial and nematodes communities involved in this multipartite association. Second, by combining activity assays with bacterial metagenomics, this research demonstrated how *Azteca* ants have engineered a system similar to composting by humans, where plant- and insect-derived waste is decomposed by microorganisms. Lastly, based on the findings of this thesis and the extensive research on this model system, I discussed the potential purposes of the ant-made patches and suggested key directions for future investigations.

Under the assumption that following hypothesis are confirmed: (i) closely related ant species follow their own “composting recipe", leading to distinct communities in their patches which are still functionally similar; (ii) the ant queens transfer the patch microbiome from mother to daughter colonies, which likely results in a high adaptation of these organisms to the domatia; (iii) the ants maintain these organisms physically enclosed within patches inside the nest, enhancing their separation from free-living strains; (iv) the ants somehow control the nutrition and surrounding environmental conditions of these communities; and, (v) the colony gains direct or indirect benefits from the organic matter recycling process; one could speculate that the composting consortium maintained by *Azteca* ants across generations is considered a form of domestication. As recently investigated in the fungal crop (*Leucoagaricus gongylophorus*) of leafcutter ants [69], a deep exploration into the genomes of organisms inhabiting the patches and a subsequent comparison with closely related free-living strains, could allow the identification of genomic signatures often related with domestication. Moreover, a broad and thorough comparative study of patches in functionally diverse ant-plant mutualisms following the same methodology of this PhD thesis could also shed light into the origin, evolution and purposes of patch making in these exceptional ants.

References

1. Hölldobler B, Wilson EO. The Ants. Harvard University Press; 1990.

2. Schultheiss P, Nooten SS, Wang R, Wong MKL, Brassard F, Guénard B. The abundance, biomass, and distribution of ants on Earth. PNAS. 2022;119:e2201550119.

3. Griffiths HM, Ashton LA, Walker AE, Hasan F, Evans TA, Eggleton P, et al. Ants are the major agents of resource removal from tropical rainforests. J Anim Ecol. 2018;87:293–300.

4. Conlon BH, O'Tuama D, Michelsen A, Crumière AJJ, Shik JZ. A fungal symbiont converts provisioned cellulose into edible yield for its leafcutter ant farmers. Biol Lett. 2022;18:20220022.

5. Nascimento DL, Chiapini M, Vidal-Torrado P, Phillips JD, Ladeira FSB, Machado DFT, et al. The underestimated role of leaf-cutting ants in soil and geomorphological development in neotropical America. Earth-Science Rev. 2024;248 August 2023:104650.

6. Li H, Young SE, Poulsen M, Currie CR. Symbiont-mediated digestion of plant biomass in fungus-farming insects. Annu Rev Entomol. 2021;66:297–316.

7. Moreira-Soto RD, Sanchez E, Currie CR, Pinto-Tomás AA. Ultrastructural and microbial analyses of cellulose degradation in leaf-cutter ant colonies. Microbiol (United Kingdom). 2017;163:1578–89.

8. Lewin GR, Johnson AL, Moreira Soto RD, Perry K, Book AJ, Horn HA, et al. Cellulose-enriched microbial communities from leaf-cutter ant (*Atta colombica*) refuse dumps vary in taxonomic composition and degradation ability. PLoS One. 2016;11:1–22.

9. Baker CCM, Martins DJ, Pelaez JN, Billen JPJ, Pringle A, Frederickson ME, et al. Distinctive fungal communities in an obligate African ant-plant mutualism. Proc R Soc B. 2017;284:20162501.

10. Greenfield MJ, Lach L, Congdon BC, Anslan S, Tedersoo L, Field M, et al. Consistent patterns of fungal communities within ant-plants across a large geographic range strongly suggest a multipartite mutualism. Mycol Prog. 2021;20:681–99.

11. Nepel M, Voglmayr H, Blatrix R, Longino JT, Fiedler K, Schönenberger J, et al. Ant-cultivated Chaetothyriales in hollow stems of myrmecophytic *Cecropia* sp. trees – diversity and patterns. Fungal Ecol. 2016;23:131–40.

12. Mayer VE, Voglmayr H, Blatrix R, Orivel J, Leroy C. Fungi as mutualistic partners in ant-plant interactions. Front Fungal Biol. 2023;4 October:1213997.

13. Wright C, Gryganskyi AP, Bonito G. Fungi in Composting. In: Fungal Applications in Sustainable Environmental Biotechnology. Springer International Publishing; 2016. p. 3–28.

14. Sudhaus W. Evolution of insect parasitism in rhabditid and diplogastrid nematodes. Adv Arachnol Dev Biol. 2008;12:143–61.

15. Maschwitz U, Fiala B, Dumpert K, Hashim R bin, Sudhaus W. Nematode associates and bacteria in ant-tree symbioses. Symbiosis. 2016;69:1–7.

16. Morera J, Mora-Pineda G, Esquivel A, Hanson P. Detection, ultrastructure and phylogeny of *Sclerorhabditis neotropicalis* (Nematoda: Rhabditidae) nematodes associated with the *Azteca* ant-*Cecropia* tree symbiosis. Rev Biol Trop. 2018;66:368–80.

17. Moreno LF, Mayer V, Voglmayr H, Blatrix R, Benjamin Stielow J, Teixeira MM, et al.

Genomic analysis of ant domatia-associated melanized fungi (Chaetothyriales, Ascomycota). Mycol Prog. 2019;18:541–52.

18. Teixeira MM, Moreno LF, Stielow BJ, Muszewska A, Hainaut M, Gonzaga L, et al. Exploring the genomic diversity of black yeasts and relatives (Chaetothyriales, Ascomycota). Stud Mycol. 2017;86:1–28.

19. Nepel M, Mayer VE, Barrajon-Santos V, Woebken D. Bacterial diversity in arboreal ant nesting spaces is linked to colony developmental stage. Commun Biol. 2023;6:1217.

20. Longino JT. A taxonomic review of the genus *Azteca* (Hymenoptera: Formicidae) in Costa Rica and a global revision of the aurita group. Zootaxa. 2007;1491:1–63.

21. Longino JT. *Azteca* ants in *Cecropia* trees: taxonomy, colony structure, and behaviour. In: Cutler C, Huxley D, editors. Ant-plant interactions. Oxford University Press; 1991. p. 271–88.

22. Nepel M, Pfeifer J, Oberhauser FB, Richter A, Woebken D, Mayer VE. Nitrogen fixation by diverse diazotrophic communities can support population growth of arboreal ants. BMC Biol. 2022;20:135.

23. Mayer VE, Nepel M, Blatrix R, Oberhauser FB, Fiedler K, Schönenberger J, et al. Transmission of fungal partners to incipient *Cecropia*-tree ant colonies. PLoS One. 2018;13:e0192207.

24. Longino JT. Geographic Variation and Community Structure in an Ant-Plant Mutualism: *Azteca* and *Cecropia* in Costa Rica. Biotropica. 1989;21:126.

25. Marting PR, Kallman NM, Wcislo WT, Pratt SC. Ant-plant sociometry in the *Azteca-Cecropia* mutualism. Sci Rep. 2018;8:17968.

26. Zapałowska A, Skwiercz A, Tereba A, Puchalski C, Malewski T. Next-Generation sequencing for evaluating the soil nematode diversity and its role in composting processes. Int J Mol Sci. 2023;24.

27. Steel H, Moens T, Vandecasteele B, Hendrickx F, De Neve S, Neher DA, et al. Factors influencing the nematode community during composting and nematode-based criteria for compost maturity. Ecol Indic. 2018;85 June 2017:409–21.

28. Meghvansi MK, Chaudhary KK, Khan MH, Siddiqui S, Varma A. Biology of composts. 2020.

29. Wang X, He X, Liang J. Succession of microbial community during the co-composting of food waste digestate and garden waste. Int J Environ Res Public Health. 2022;19.

30. Mani S, Cao M. Nitrogen and phosphorus concentration in leaf litter and soil in Xishuangbanna tropical forests: Does precipitation limitation matter? Forests. 2019;10:11–3.

31. Santiago LS. Nutrient limitation of eco-physiological processes in tropical trees. Trees. 2015;29:1291–300.

32. Camenzind T, Hättenschwiler S, Treseder KK, Lehmann A, Rillig MC. Nutrient limitation of soil microbial processes in tropical forests. Ecol Monogr. 2018;88:4–21.

33. Dalling JW, Heineman K, Lopez OR, Wright SJ, Turner BL. Nutrient availability in tropical rain forests: the paradigm of phosphorus limitation. In: Goldstein G, Santiago L, editors. Tropical Tree Physiology. Springer; 2016. p. 261–73.

34. Marting PR. Collective Personality in the *Azteca-Cecropia* Mutualism. 2018.

35. Vasconcelos HL, Casimiro AB. Influence of *Azteca alfari* ants on the exploitation of *Cecropia* trees by a leaf-cutting ant. Biotropica. 1997;29:84–92.

36. Bischof S, Umhang M, Eicke S, Streb S, Qi W, Zeeman SC. *Cecropia peltata* accumulates starch or soluble glycogen by differentially regulating starch biosynthetic genes. Plant Cell. 2013;25:1400–15.

37. Choe JC, Perlman DL. Social conflict and cooperation among founding queens in ants.pdf. In: The Evolution of Social Behaviour in Insects and Arachnids. Cambridge University Press; 1997. p. 392–406.

38. Ješovnik A, Schultz TR. Agricultural and proto-agricultural symbioses in ants. In: Schultz TR, Gawne R, Peregrine PN, editors. The Convergent Evolution of Agriculture in Humans and Insects. The MIT Press; 2022. p. 143–59.

39. Hazlett DL, Gessel SP, Walker RB. Growth response of *Cecorpia obtusifolia* to greenhouse fertilization. Turrialba. 1982;33:271–6.

40. Rickson FR. Anatomical development of the leaf trichilium and Müllerian bodies of *Cecropia peltata* L. Am J Bot. 1976;63:1266–71.

41. Valverde JP, Hanson P. Parenchyma: A neglected plant tissue in the *Cecropia*/ant mutualism. Symbiosis. 2011;55:47–51.

42. Ferris H, Venette RC, Scow KM. Soil management to enhance bacterivore and fungivore nematode populations and their nitrogen mineralisation function. Appl Soil Ecol. 2004;25.

43. Defossez E, Selosse MA, Dubois MP, Mondolot L, Faccio A, Djieto-Lordon C, et al. Ant-plants and fungi: A new threeway symbiosis. New Phytol. 2009;182:942–9.

44. Folgarait PF, Johnson HL, Davidson DW. Responses of *Cecropia* to Experimental Removal of Mullerian Bodies. Funct Ecol. 1994;8:22–8.

45. Folgarait PJ, Davidson DW, Davidson W. Myrmecophytic *Cecropia*: Antiherbivore defenses under different nutrient treatments. Oecologia. 1995;104:189–206.

46. Riha KA. Herbivory and alkaloid concentration in *Cecropia obtusifolia* (Cecropiaceae) trees with and without ants. Digital Commons University of South Florida. 2010; May.

47. Blatrix R, Djiéto-Lordon C, Mondolot L, la Fisca P, Voglmayr H, Mckey D. Plant-ants use symbiotic fungi as a food source: new insight into the nutritional ecology of ant-plant interactions. Proc R Soc B. 2012;279:3940–7.

48. Vanderpool D, Bracewell RR, McCutcheon JP. Know your farmer: Ancient origins and multiple independent domestications of ambrosia beetle fungal cultivars. Mol Ecol. 2018;27:2077–94.

49. Biedermann PHW, Vega FE. Ecology and evolution of insect – fungus mutualisms. Annu Rev Entomol. 2020;65:431–55.

50. Mueller UG, Gerardo NM, Aanen DK, Six DL, Schultz TR. The evolution of agriculture in insects. Annu Rev Ecol Evol Syst. 2005;36:563–95.

51. Schultz TR, Sosa-Calvo J, Kweskin MP, Lloyd MW, Dentinger B, Kooij PW, et al. The coevolution of fungus-ant agriculture. Science. 2024;386:105–10.

52. Oliveira KN, Coley PD, Kursar TA, Kaminski LA, Moreira MZ, Campos RI. The effect of symbiotic ant colonies on plant growth: A test using an *Azteca-Cecropia* system. PLoS One. 2015;10:e0120351.

53. Rocha CFD, Bergallo HG. Bigger ant colonies reduce herbivory and herbivore residence time on leaves of an ant-plant: *Azteca muelleri* vs. *Coelomera ruficornis* on *Cecropia pachystachya*. Oecologia. 1992;91:249–52.

54. Janzen DH. Allelopathy by myrmecophytes: The ant *Azteca* as an allelopathic agent of *Cecropia*. Ecology. 1969;50:147–53.

55. Defossez E, Djiéto-Lordon C, Mckey D, Selosse MA, Blatrix R. Plant-ants feed their host plant, but above all a fungal symbiont to recycle nitrogen. Proc R Soc B. 2011;278:1419–26.

56. Gegenbauer C, Bellaire A, Schintlmeister A, Schmid MC, Kubicek M, Voglmayr H, et al. Exo- and endophytic fungi enable rapid transfer of nutrients from ant waste to orchid tissue. New Phytol. 2023;238:2210–23.

57. Bazile V, Moran JA, Le Moguédec G, Marshall DJ, Gaume L. A carnivorous plant fed by its ant symbiont: A unique multi-faceted nutritional mutualism. PLoS One. 2012;7.

58. Beattie A. Myrmecotrophy: Plants fed by ants. Trends Ecol Evol. 1989;4:172–6.

59. Solano PJ, Dejean A. Ant-fed plants: Comparison between three geophytic myrmecophytes. Biol J Linn Soc. 2004;83:433–9.

60. Fischer RC, Richter A, Wanek W, Mayer V. Plants feed ants: Food bodies of myrmecophytic *Piper* and their significance for the interaction with *Pheidole bicornis* ants. Oecologia. 2002;133:186–92.

61. Fischer RC, Wanek W, Richter A, Mayer V. Do ants feed plants? A 15N labelling study of nitrogen fluxes from ants to plants in the mutualism of *Pheidole* and *Piper*. J Ecol. 2003;91:126–34.

62. Sagers CL, Ginger SM, Evans RD. Carbon and nitrogen isotopes trace nutrient exchange in an ant-plant mutualism. Oecologia. 2000;123:582–6.

63. Werner KA, Castro-Herrera D, Yimer F, Tadesse M, Kim DG, Prost K, et al. Microbial risk assessment of mature compost from human excreta, cattle manure, organic waste, and biochar. Sustain. 2023;15.

64. Kelova ME, Eich-Greatorex S, Krogstad T. Human excreta as a resource in agriculture – Evaluating the fertilizer potential of different composting and fermentation-derived products. Resour Conserv Recycl. 2021;175 June:105748.

65. Harder R, Wielemaker R, Larsen TA, Zeeman G, Öberg G. Recycling nutrients contained in human excreta to agriculture: Pathways, processes, and products. Crit Rev Environ Sci Technol. 2019;49:695–743.

66. Goldstein SL, Klassen JL. *Pseudonocardia* symbionts of fungus-growing ants and the evolution of defensive secondary metabolism. Front Microbiol. 2020;11 December:1–8.

67. Poulsen M, Cafaro M, Boomsma JJ, Currie CR. Specificity of the mutualistic association between actinomycete bacteria and two sympatric species of *Acromyrmex* leaf-cutting ants. Mol Ecol. 2005;14:3597–604.

68. Hanshew AS, McDonald BR, Díaz Díaz C, Djiéto-Lordon C, Blatrix R, Currie CR. Characterization of Actinobacteria associated with three ant–plant mutualisms. Microb Ecol. 2015;69:192–203.

69. Leal-dutra CA, Vizueta J, Baril T, Kooij PW, Rødsgaard-jørgensen A. Genomic signatures of domestication in a fungus obligately farmed by leafcutter ants. Mol Biol Evol. 2024;41.

Appendix

Summary

Ants thrive in nearly every terrestrial ecosystem on Earth, from the Arctic tundra to the Sahara Desert. Their success is attributed to their extraordinary eusocial organization and remarkable ability to adapt and shape their environment, often fostering complex biotic interactions. Tropical arboreal ants, particularly those living in obligate association with plants, are a perfect example of such sophisticated capability of adaptation and habitat shaping. In this mutually beneficial relationship, the ants protect their host plant from herbivores and competitors in exchange for nutrient-rich food resources and a nesting space within specialized plant cavities. Worldwide, these plant-nesting ants accumulate plant- and animal-derived organic waste in dark-coloured piles, known as "patches", within their nest. As regular inhabitants of the patches, a complex and diverse community of organisms, including bacteria, fungi, and nematodes have been identified. While patch making appear to be an essential behaviour for the survival and successful development of the ant colonies, the overall significance of patches and their inhabitants in the ant-plant mutualism remains unclear.

To better understand the functional role of patches within the ant-plant complex, I selected the *Azteca-Cecropia* association as model system, one of the most prominent and ubiquitous ant-plant mutualisms in the Tropics of America. In this particular association, previous studies have shown that ant queens transfer an inoculum of patch organisms across generations, and that, a highly diverse bacterial community —capable of fixing atmospheric nitrogen— has been identified within the patches. To provide a comprehensive overview of the communities that regularly inhabit these structures, this PhD thesis investigates the diversity and dynamics of fungal and nematode communities of patches at different ant colony developmental stages and among closely related ant species. By the use of ITS2-based metabarcoding analysis, this study elucidated that a complex fungal community in the patches changes and gets highly diverse as the ant colony grows, probably due to a substrate diversification and an introduction of new organisms from the environment.

In contrast, the 18S rRNA gene amplicon analysis revealed that nematode diversity —with a predominance of bacterivorous rhabditids— remains rather consistent as the colony grows. The results indicate that the plant seems to provide the environment and an important part of the substrate for the creation of patches, whereas the ant colony appears to be the main driver shaping the patch communities.

Following the thorough dissection of the communities associated with the *Azteca-Cecropia* mutualism, the activity and metabolic potential of patch microbial communities for degrading the polysaccharide-rich substrates was investigated. By conducting isotope-based activity assays, this thesis demonstrated that patch communities are able to metabolize the recalcitrant cellulose and chitin found in the deposited organic matter. Then, bacterial metagenomic analysis resulting in the reconstruction of 214 metagenome assembled genomes (MAGs) revealed that a rich and diverse genetic repertoire involved in polysaccharide breakdown is widely distributed within the bacterial microbiome. From these results, potential bacterial players in the decomposition of organic matter in the patches were suggested and their potential substrate utilization mechanisms were discussed.

The findings gathered in this thesis shows how *Azteca* ants have engineered a system similar to composting by humans, in which organic waste is transformed into more assimilable and stable nutrient forms by the action of microorganisms under controlled conditions. Overall, this thesis represents a significant step forward in understanding the fundamentals of ant-made patches.

Zusammenfassung

Ameisen kommen in fast allen terrestrischen Ökosystemen der Erde vor, von der arktischen Tundra bis hin zu Wüsten. Ihr Erfolg basiert auf ihrer Anpassungsfähigkeit, ihrer außergewöhnliche eusoziale Organisation und ihrer bemerkenswerten Fähigkeit, ihre Umwelt zu gestalten. Häufig gehen sie dabei komplexe biotische Interaktionen mit anderen Organismen ein. Tropische baumlebende Ameisen, auch solche, die in einer obligaten „Wohngemeinschaft" mit Pflanzen leben, sind ein Beispiel dafür, wie Ameisen ihren Lebensraum gestalten. In einer solchen - für beide Seiten vorteilhaften- Beziehung schützen die Ameisen ihre Wirtspflanze vor Pflanzenfressern und konkurrenzierenden Pflanzen, und erhalten im Gegenzug Nahrung und einen Nistplatz in hohlen Strukturen der Pflanzen, sogenannten Domatien. Im Nest im Inneren der Wirtspflanzen häufen die Ameisen pflanzliche und tierische organische Abfälle in Häufchen, sogenannten „patches", an. Diese werden von einer komplexen und vielfältigen Organismengemeinschaft bewohnt, bestehend aus Bakterien, Pilzen und Nematoden. Obwohl die Bildung der patches eine wichtige Voraussetzung für die erfolgreiche Entwicklung der Kolonien zu sein scheint, ist die tatsächliche Funktion der patches und ihrer Bewohner im Ameisen-Pflanzen-Komplex noch unklar.

Um zur Klärung der Funktion der patches beizutragen wurde die *Azteca-Cecropia*-Assoziation als Modellsystem gewählt. Sie ist eine der bekanntesten und am häufigsten vorkommenden Ameisen-Pflanzen-Wechselbeziehungen in den amerikanischen Tropen und besiedelt erfolgreich Straßen- und Feldränder und Lichtungen in Primärwäldern und ist eine der ersten Pflanzen bei Sukzessionen an gestörten Stellen. Frühere Studien haben gezeigt, dass die *Azteca* Königinnen ein Inokulum mit patch-bewohnenden Organismen über Generationen hinweg weitergeben. Außerdem wurde innerhalb der patches eine äußerst vielfältige Bakteriengemeinschaft identifiziert; es wurde sogar nachgewiesen, dass manche Bakterien atmosphärischen Stickstoff fixieren. Um einen umfassenden Überblick über die Lebensgemeinschaften in den patches zu erhalten, werden in der hier vorliegenden Doktorarbeit die Vielfalt und Dynamik der Pilze und Nematoden in den patches in drei Entwicklungsstadien der Ameisenkolonien bei zwei eng verwandten Ameisenarten untersucht.

Mithilfe der ITS2-basierten Metabarcoding-Analyse konnte in der hier vorliegenden Arbeit festgestellt werden, dass in den patches eine Pilzgemeinschaft lebt, die sich mit dem Wachstum des Ameisenvolkes verändert und an Diversität zunimmt. Dies beruht wahrscheinlich einerseits auf einer Diversifizierung des Substrats der patches (anfangs vor allem Pflanzengewebe der Wirtspflanze, später dann Exoskelette von toten Nestgenossinnen), zusätzlich wird aber auch das Einschleppen von Sporen und Hyphenfragmenten aus der Umgebung eine Rolle spielen. Im Gegensatz dazu ist die Vielfalt der Nematoden über die gesamte Entwicklung der Ameisenkolonien hin konstant. Dominant sind bakterienfressende Rhabditiden, wie mit 18S rRNA Gen-Amplikonanalyse gezeigt werden konnte. Zusammenfassend kann aus der Dynamik der Lebensgemeinschaft aus Bakterien, Pilzen und Nematoden während der Entwicklung der Ameisenkolonien geschlossen werden, dass die Wirtspflanze offenbar die Umgebung für die patches bereitstellt, aber die Ameisenkolonie die Zusammensetzung der Gemeinschaft in den patches maßgeblich beeinflußt.

Mit isotopenbasierten Aktivitätstests konnte in dieser Arbeit nachgewiesen werden, dass die patch-Gemeinschaften in der Lage sind, schwer abbaubare Substrate wie Zellulose und Chitin zu verarbeiten. Daher wurde das metabolische Potenzial für den Abbau Polysaccharid-reicher Substrate in den patches bei Bakterien untersucht. Eine Metagenom-Analyse, die zur Rekonstruktion von 214 MAGs („aus Metagenomen assemblierte Genome“) führte, zeigte, dass im bakteriellen Mikrobiom ein vielfältiges genetisches Repertoire zum Abbau von Polysacchariden weit verbreitet ist. Basierend auf diesen Ergebnissen wurden Bakteriengruppen vorgeschlagen, die beim Abbau von Zellulose und Chitin in den patches eine zentrale Rolle spielen, und ihre möglichen physiologischen Mechanismen zur Substratverwertung diskutiert.

In Summe zeigen die Ergebnisse der vorliegenden Arbeit, dass die patches der im Stamm von *Cecropia* Bäumen wohnenden *Azteca* Ameisen schwer abbaubare organische Abfälle mit Hilfe von Mikroorganismen und Nematoden unter kontrollierten Bedingungen in leichter assimilierbare und stabilere Nährstoffformen umgewandelt werden können. Damit ähneln die von Ameisen gebildeten patches der Art, wie Menschen ihre organischen Abfälle kompostieren.

Resumen

Las hormigas habitan casi todos los ecosistemas terrestres de la Tierra, desde la tundra ártica hasta el desierto del Sahara. Su éxito está atribuido a su extraordinaria organización social que les proporciona una notable capacidad para adaptar su entorno y para desarrollar complejas interacciones bióticas. Las hormigas arbóreas tropicales, y en particular aquellas que viven en asociación con plantas, son un ejemplo perfecto de la sofisticada capacidad de las hormigas para adaptarse y para modelar su hábitat. En esta relación mutualista, las hormigas protegen la planta huésped de herbívoros y competidores a cambio de recursos alimenticios ricos en nutrientes y de un espacio para la formación del hormiguero en cavidades especializadas de la planta. En el hormiguero, estas hormigas además acumulan residuos orgánicos de origen vegetal y animal en pilas, definidas como parches, donde se ha identificado una gran variedad de organismos tales como bacterias, hongos, nemátodos y otros visitantes ocasionales. Aunque la formación de parches parece ser un comportamiento esencial para la supervivencia y el adecuado desarrollo de las colonias de hormigas, el propósito general de los parches y sus habitantes en este complejo ecosistema de hormiga-planta aún se desconoce.

Para profundizar en la comprensión del papel funcional de los parches dentro del sistema hormiga-planta, se utilizó la asociación *Azteca-Cecropia* como sistema modelo, uno de los mutualismos hormiga-planta más destacados y abundantes en el trópico de América. En esta asociación en particular, estudios previos han demostrado que las hormigas reina transfieren un inóculo de organismos procedente de los parches a través de generaciones. Asimismo, en estas estructuras se han identificado comunidades bacterianas muy diversas, de las cuales una población es capaz de fijar nitrógeno atmosférico. Con el objetivo de esclarecer la composición de las comunidades que habitan los parches, la presente tesis doctoral ha examinado en profundidad la biodiversidad y las dinámicas de las comunidades de hongos y nemátodos en parches provenientes de colonias con diferentes niveles de desarrollo y de especies de hormigas filogenéticamente cercanas. Por una parte, a través de la secuenciación del espaciador transcrito interno 2 (ITS2, por sus siglas en inglés), este estudio ha demostrado que la compleja comunidad fúngica de los parches cambia y

se diversifica a medida que la colonia de hormigas crece, probablemente debido a una diversificación de sustratos depositados por las hormigas y la incorporación de nuevos organismos del entorno. Por otra parte, el análisis de la secuenciación del gene ribosomal 18S (18S rRNA, por sus siglas en inglés) ha revelado que la diversidad de nemátodos, especialmente bacterívoros, se mantiene relativamente constante a medida que crece la colonia, lo que contribuye a la hipótesis de transmisión de organismos desde las colonias madre a las colonias hija. Aunque la planta ofrece el entorno y parte del sustrato para la formación de los parches, la colonia de hormigas parece ser el principal factor que define sus comunidades.

Tras la minuciosa disección de las comunidades asociadas al mutualismo *Azteca-Cecropia*, posteriormente se procedió a investigar la actividad y el potencial metabólico de las comunidades microbianas de los parches para descomponer los sustratos ricos en polisacáridos que depositan las hormigas. Mediante la realización de ensayos de actividad basados en isótopos, esta tesis ha demostrado que las comunidades del parche son capaces de metabolizar la celulosa y quitina que se encuentran en la materia orgánica depositada. Posteriormente, el análisis metagenómico bacteriano, que resultó en la reconstrucción de 214 genomas ensamblados de metagenomas (MAGs, por sus siglas en inglés), ha mostrado que un diverso y extenso repertorio genético relacionado con la degradación de polisacáridos está ampliamente distribuido en el microbioma bacteriano de los parches. A partir de estos resultados, esta tesis sugiere varios grupos de bacterias que posiblemente sean responsables de la descomposición de la materia orgánica en los parches, incluyendo sus posibles mecanismos de utilización del sustrato. Los hallazgos recogidos en esta tesis doctoral muestran cómo las hormigas *Azteca* han diseñado un sistema similar a las pilas de compost hechas por humanos, en el que los residuos orgánicos se transforman en formas más asimilables y estables de nutrientes por la acción de microorganismos bajo condiciones controladas. En conclusión, esta tesis representa un avance significativo en la comprensión de los principios detrás de la creación de parches por las hormigas.

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