Decoding symbioses in grain pest beetles

Ecology, evolutionary history, and specificity of symbiont-mediated nutrient supplementation in Silvanidae and Bostrichidae (Coleoptera)

Dissertation

Zur Erlangung des Grades

Doktor der Naturwissenschaften

Am Fachbereich Biologie
Der Johannes Gutenberg-Universität Mainz

Julian Simon Thilo Kiefer
geb. am 18.06.1989 in Freiburg im Breisgau

Mainz, 2022

Dekan: Prof. Dr. Eckhard Thines

1. Gutachten: Prof. Dr. Martin Kaltenpoth

2. Gutachten: Prof. Dr. Susanne Foitzik

Tag der mündlichen Prüfung: 20.01.2023

„Do. Or do not. There is no try. “

Master Yoda, *Star Wars: The Empire Strikes Back*

Summary

Each individual organism interacts with at least one or more other species. When these relationships between two (or more) species are close over the long term, they are called symbioses. Symbiosis is considered a key driver of evolution, as it drives the development of morphological adaptations and new capabilities. A particular form of symbiosis is found between insects, especially beetles, where the symbiosis with bacteria has enabled them to access new habitats and food sources that they would not have had access to without their symbiotic partners. One example of challenging environments are grain storages which are by nature very dry habitats and at the same time the grain only offers a diet poor in nutrients - and yet beetles of multiple families, including Silvanidae and Bostrichidae have independently invaded this habitat. At the same time, beetles from both families are associated with symbionts of the same clade of Flavobacteria, which made it possible for them to spread around the world as pests in grain and wood.

In my work, I used various molecular biology methods as well as experimental approaches to study the endosymbiont of the sawtoothed grain beetle *Oryzaephilus surinamensis* from the family Silvanidae. Via the shikimate pathway, the symbiont *Shikimatogenerans silvanidophilus* (Bacteria: Bacteroidota) provides precursors for the amino acid tyrosine, which the host requires for its cuticle synthesis. After contact with glyphosate, which specifically inhibits an enzyme of the shikimate pathway, *Shikimatogenerans* can no longer supply its host with this required nutrient. As a result, the symbiont titer decreases, or the symbiont is completely lost. The same effect is observed when the host has direct access to tyrosine through its diet. In addition to *Shikimatogenerans*, *O. surinamensis* is also infected with the Wolbachia strain *w*Sur. *Wolbachia* is commonly known to manipulate the reproduction of its host. In *O. surinamensis*, *w*Sur causes cytoplasmic incompatibility that results in the development of less viable embryos derived from spermatozoa of *w*Sur-negative ♂- and oocytes of *w*Sur-positive ♀-beetles. The genome of *w*Sur also contains homologues of a gene responsible for male-killing in various insects. However, in grain storage populations of *O. surinamensis*, the gene sequence underwent a frameshift mutation. This mutation causes a premature translation stop and the loss of the protein's functional domain, possibly contributing to the widespread colonisation of grain stores.

My analysis of 29 species from the family Bostrichidae shows that the entire family is consistently associated with *Shikimatogenerans bostrichidophilus*. In addition, only species of the genera Dinoderinae and Lyctinae are associated with *Bostrichicola ureolyticus* (Bacteria: Bacteroidota). While *Shikimatogenerans* provides the host with the precursor to tyrosine via the shikimate pathway, *Bostrichicola* supports its host by providing additional amino acids as well as recycling nitrogen. A comparison of host and symbiont phylogenies reveals a high degree of co-cladogenesis. One peculiarity stood out in this study: the endosymbiont *S. bostrichidophilus* of the large grain borer *Prostephanus truncatus* is divided into three strains in its host. The genes for the shikimate pathway and also the ribosomal proteins are thereby encoded by the three different strains, which complement each other but also exhibit a considerable degree of redundancy in encoded functions.

The results of this work highlight the importance of symbioses, in which bacterial symbionts support the biosynthesis of the cuticle in insects by supplying tyrosine. Furthermore, it provides insight into the evolutionary processes of these communities, which have existed for millions of years and have led to mutual dependence between symbiont and host. As a result, contact with substances such as glyphosate could cause lasting damage to the symbiosis and even results in its collapse – an alarming scenario in view of the recent decline in insect populations.

Zusammenfassung

Jeder einzelne Organismus interagiert mit mindestens einer oder mehreren anderen Arten. Wenn diese Beziehungen zwischen zwei (oder mehreren) Arten langfristig eng sind, wird sie als Symbiosen bezeichnet. Die Symbiose gilt als eine der wichtigsten Triebkräfte der Evolution, da sie die Entwicklung morphologische Anpassungen und neuer Fähigkeiten vorantreibt. Eine besondere Form der Symbiose findet sich zwischen Insekten, insbesondere Käfern, wo die Symbiose mit Bakterien ihnen den Zugang zu neuen Lebensräumen und Nahrungsquellen ermöglicht hat, zu denen sie ohne ihre symbiotischen Partner keinen Zugang gehabt hätten. Von Menschen eingerichtete Getreidelager sind von Natur aus sehr trockene Lebensräume und gleichzeitig bietet das Getreide auch nur eine sehr einseitige Nahrungsgrundlage – und dennoch wurden sie unter anderem von Käfern der Familie Silvanidae und Bostrichidae unabhängig voneinander für sich als Habitat erschlossen. Voraussetzung dafür war, dass Käfer beider Familien mit Symbionten aus der gleichen Gruppe der Flavobakterien eine Verbindung eingegangen sind, die es ihnen erst ermöglichte, sich weltweit als Schädlinge in Getreide und Holz zu verbreiten.

In meiner Arbeit habe ich mit verschiedenen molekularbiologischen Methoden sowie experimentellen Ansätzen den Endosymbionten des Getreideplattkäfers *Oryzaephilus surinamensis* aus der Familie der Silvanidae untersucht. Über den Shikimatweg liefert der Symbiont *Shikimatogenerans silvanidophilus* (Bacteria: Bacteroidota) Vorstufen für die Aminosäure Tyrosin, welche der Wirt für seine Kutikulasynthese benötigt. Nach Kontakt mit Glyphosat, welches gezielt ein Enzym des Shikimatwegs inhibiert, kann *Shikimatogenerans* seinen Wirt nicht mehr mit diesem benötigten Nährstoff versorgen. Infolgedessen sinkt der Symbiontentiter ab oder der Symbiont geht vollständig verloren. Der gleiche Effekt ist zu beobachten, wenn der Wirt direkten Zugang zu Tyrosin über seine Nahrung hat. Neben *Shikimatogenerans* ist *O. surinamensis* auch mit dem *Wolbachia*-Stamm *w*Sur infiziert. *Wolbachia* ist allgemein dafür bekannt die Reproduktion seines Wirts zu manipulieren. In *O. surinamensis* verursacht *w*Sur zytoplasmatische Inkompatibilität, die zur Entwicklung weniger lebensfähiger Embryonen führt, die aus den Spermien von *w*Sur negativen ♂- und Eizellen von *w*Sur positiven ♀-Käfern hervorgegangen sind. Des Weiteren codiert das Genom von *w*Sur für ein Gen, welches für Male-Killing in diversen

Insekten verantwortlich ist. In Getreidelager-Populationen von *O. surinamensis* erfuhr die Gensequenz allerdings eine Frameshift-Mutation. Durch diese Mutation bricht die Translation zum Protein frühzeitig ab, dieses verliert seine funktionale Domäne und hat damit möglicherweise zur weit verbreiteten Besiedlung der Getreidelager beigetragen.

Meine Analyse von 29 Spezies aus der Familie der Bostrichidae konnte aufzeigen, dass die gesamte Familie durchweg mit *S. bostrichidophilus* assoziiert ist. Zusätzlich besitzen nur Spezies der Genera Dinoderinae und Lyctinae den zweiten Endosymbionten *Bostrichicola ureolyticus* (Bacteria: Bacteroidota). Während *Shikimatogenerans* den Wirt auch hier über den Shikimatweg mit der Vorstufe zu Tyrosin versorgt, unterstützt *Bostrichicola* zusätzlich seinen Wirt, in dem er weitere Aminosäuren bereitstellen sowie Stickstoff recyceln kann. Eine Gegenüberstellung der Phylogenie von Wirt und Symbiont zeigt zudem ein hohes Maß an Co-Kladogenese. Eine Besonderheit ist bei dieser Untersuchung aufgefallen: der Endosymbiont *S. bostrichidophilus* des großen Kornbohrers *Prostephanus truncatus* ist in seinem Wirt in drei Stämme aufgeteilt. Die Gene für den Shikimatweg und auch die ribosomalen Proteine werden dabei von den drei verschiedenen Stämmen kodiert, die sich gegenseitig ergänzen, aber auch ein beträchtliches Maß an Redundanz in den kodierten Funktionen aufweisen.

Die Ergebnisse dieser Arbeit unterstreichen die Bedeutung von Symbiosen, bei denen bakterielle Symbionten den Aufbau der Kutikula bei Insekten durch die Versorgung von Tyrosin unterstützten. Des Weiteren gibt sie Einsicht in die evolutionären Prozesse dieser seit Millionen von Jahren bestehende Lebensgemeinschaften, welche zu einer gegenseitigen Abhängigkeit zwischen Symbiont und Wirt geführt hat. Dies hat dazu geführt, dass der Kontakt zu Substanzen wie z.B. Glyphosat die Symbiose nachhaltig schädigen und gar zum Zusammenbruch bringen könnte - mit Hinblick auf den jüngsten Rückgang der Insektenpopulationen ein alarmierendes Szenario.

Contents

List of publications

Kiefer JST, Batsukh S, Bauer E, Hirota B, Weiss B, Wierz JC, Fukatsu T, Kaltenpoth M, Engl T (2021): Inhibition of a nutritional endosymbiont by glyphosate abolishes mutualistic benefit on cuticle synthesis in *Oryzaephilus surinamensis*. *Commun. Biol.* 4, 554 (Chapter 2)

Kiefer JST, Schmidt G, Krüsemer R, Kaltenpoth M, Engl T (submitted): *Wolbachia* causes cytoplasmic incompatibility, but not male-killing in a grain pest beetle. (Chapter 3)

Kiefer JST, Bauer E, Okude G, Fukatsu T, Kaltenpoth M, Engl T (in preparation): Co-speciation and functional complementarity of dual Bacteroidota symbionts in powderpost beetles (Coleoptera: Bostrichidae). (Chapter 4)

Kiefer JST, Bauer E, Kaltenpoth M, Engl T (in preparation): Loss of genomic cohesion: Symbiont lineage splitting in the large grain borer *Prostephanus truncatus* (Coleoptera: Bostrichidae). (Chapter 5)

Chapter 1

General Introduction

1.1 Symbioses

No organism on this planet lives in isolation[1]. Instead, every single organism one can imagine interacts with at least one or multiple other species. If these interactions between two or more species are close over long-term, they are called symbioses[2]. Symbiosis is a central driver of evolution[3,4]. In these partnerships, one of the partners can benefit while the other is not affected (commensalism) or suffers (parasitism), or both partners benefit (mutualism)[5]. If one of the partners provides for example access to additional pathways or metabolites, the partner may gain new abilities, whereas the other may lose some. and access to otherwise unavailable ecological niches[6].

One of the most prominent examples of symbiosis is the origin of the eukaryotic cell, which arose probably around 2 billion years ago after different prokaryotic lineages fused during an endosymbiotic event[7–10]. From then until today, many symbioses have evolved, some of which can even be seen with the bare eye (Figure 1). As best known is probably the pollinator-flower-symbiosis, where the flower offers the pollinator nectar and in return, is fertilized through pollen transport by the pollinator[11]. Another example of two organisms benefiting the other is the symbiotic relationship between a sea anemone and an anemonefish, where the anemone provides the anemonefish with protection and shelter, while the anemonefish provides the anemone nutrients in the form of waste while also defending against potential predators[12]. Another example is the symbiosis between aphids and ants in which aphids offers honeydew to the ants. In return, the ants protect aphids from natural enemies such as ladybird beetles[13,14]. However, the majority of symbiotic interactions of eukaryotes occur on a microscopic level with bacteria, fungi, and viruses.

1.2 Symbioses in insects

Insects are one of the most successful animal classes on Earth[15]. They are present in every terrestrial and limnic environment, even in the Arctic and Antarctic[16]. However, they are not solely responsible for their success. Several hundred million years ago some insect families formed

partnerships with another, highly successful kingdom: bacteria[1,17]. The symbionts provided new capabilities to their hosts, allowing the insects to acquire new abilities and evolve novel life strategies[1,18–23]. Today, numerous symbioses between an insect host and a bacterial symbiont are known. In some cases, the insect host benefits from secondary metabolites produced by the symbiont[24], which provide context-dependent protection against natural enemies. Some examples of defensive symbioses can be found in aphids[25], the European beewolf *Philanthus triangulum* (Hymenoptera: Crabronidae)[26], the darkling beetle *Lagria villosa* (Coleoptera: Tenebrionidae)[27], or the *Paederus* beetles (Coleoptera: Staphylinidae)[28,29]. Also, the bacterial symbiont can help their insect host with the digestion of plant material by delivering digestive enzymes, e.g. in the tortoise leaf beetles (Chrysomelidae: Cassidinae)[30].

Figure 1: Examples of symbiosis. Pollinators like the European honeybee *Apis mellifera* **(A)** and the blue-chinned sapphire *Chlorestes notata* **(B)** fertilize the plant through pollen transport in exchange for food in the form of nectar offered by the flower. **(C)** Lichens are a symbiotic association of a fungus and a photobiont (algae or cyanobacteria). While the fungus provides a suitable habitat, the partner provides photosynthetically fixed carbon in return. **(D)** A common remora *Remora remora* attached to a green sea turtle *Chelonia mydas*. While remoras use their host as a taxi to travel through the sea and eat food that falls out, they rid the host of parasites in return. **(E)** A sea anemone and its resident anemonefish *Amphiprion ocellaris*. While the anemone offers the fish protection and shelter, the fish provides the anemone nutrients in the form of waste while also defending against potential predators in return.

The mechanisms for acquiring symbionts or passing them on to the progeny to keep the symbiont in the population are very diverse[31]. The symbionts have to be acquired *de novo* from the environment[32,33], horizontally from the outside[34], or are transmitted vertically from the mother to the offspring. The latter can also be variously shaped, as a layer of bacteria on the eggs[27], special egg caplets which contain the symbiont cells[35], to cells of the symbiont already present within the developing egg[36]. When the bacterial symbionts are transmitted vertically from infected mothers to the offspring, the persistence across host generations of the symbiont population relies on the survival and successful reproduction of infected females. This dependency has driven the development of numerous transmission strategies. One of these strategies is to infect the eggs and manipulate the reproduction of the host to increase the number of progeny that can pass the manipulator by avoiding the production of useless males (from the symbiont's perspective)[37]. One popular example of a reproductive manipulator is *Wolbachia*, which represents the most prevalent symbiotic bacterial genus, associated with over 60% of insect species. In infected insects, *Wolbachia* often manipulates host reproduction to enhance their transmission. The mechanisms of reproductive manipulation caused by *Wolbachia* include cytoplasmic incompatibility (CI), parthenogenesis, male-killing, or feminisation[37,38], and all lead to a higher proportion of female individuals in the population. This increases the fitness of the symbiont that is predominantly transmitted maternally. Thereby, they can rapidly sweep through uninfected populations and then maintain a high prevalence within a population. However, while *Wolbachia* infection can be detrimental to the species/population level, the individual's fitness is enhanced as compared to uninfected individuals in the same population[39]. Furthermore, *Wolbachia* can even evolve into a mutualist and enhance its host's fitness by supplementing dietary limited nutrients, such as B-vitamins like riboflavin[40–42].

The most widespread type of symbiotic interaction is nutritional[1]. At the origin of the symbiosis, the microorganisms mostly gain the imminent advantage of a stable environment and the provision of nutrients, while the insect host benefits from the more specialized metabolic capabilities of the symbiont - giving both of them a competitive advantage or the ability to expand to a previously inaccessible ecological niche[1,19,20]. Specifically, insects often benefit from the metabolic capabilities of microorganisms that they lack themselves, for example, the synthesis of essential amino acids and B-vitamins, which affect the host at several biological levels: physiology, ecology, and evolution[17,43]. This is especially important when the insect host feeds on

nutritionally unbalanced diets. For example, the endosymbiont *Buchnera aphidicola*[44] in the plant sap-sucking aphids, and *Wigglesworthia*[45] in the vertebrate blood-sucking tsetse flies deliver limiting essential nutrients to complement the host's diet. After a certain period, a dependency has evolved between the partners in many symbioses, as a result of which one can no longer survive without the other[46]. In addition, since they are morphologically and functionally adapted to their respective food source, an insect cannot simply switch the food source, i.e. the host plant[30].

Figure 2: The sawtoothed grain beetle *Oryzaephilus surinamensis* on oat flakes.

1.3 Ecology of grain pest beetles

Already one century ago, entomologists described symbiotic organs in numerous species of invertebrates, the so-called bacteriome[47]. This specialised organ is made up mainly of cells called bacteriocytes which house the bacteria intracellularly. Among others, they found bacteriomes in beetle species of various families, several of which are known as grain pests[47–49]. These beetles appear to benefit from an excess of food but face the challenge of unbalanced diets and low

humidity, especially nowadays maintained in storage facilities to control mold growth, but also insect infestation. Several beetle groups nevertheless managed to invade the same ecological niche of stored grain and dried plant products independently. Examples are weevils of the genus *Sitophilus*, several silvanid beetles including the sawtoothed grain beetle *Oryzaephilus surinamensis* (Figure 2, Chapter 2) as well as several bostrichid beetles (Figure 3, Chapter 4).

The symbiont *Nardonella* (γ-proteobacteria), which is associated with multiple weevils of the families Curculionidae and Brentidae[50] encodes a complete tyrosine biosynthetic pathway while lacking pathways for all other essential and non-essential amino acids and vitamins[46]. Experimental suppression of *Nardonella* results in beetles with soft and less melanised cuticles, revealing their role in cuticle sclerotisation and melanisation[46,51]. Similar effects have been observed in *Sitophilus* spp. (Curculionidae, Dryophthorinae) and in *O. surinamensis* (Silvanidae), with both harbouring symbionts that are phylogenetically distinct from *Nardonella*[36,52,53]. In addition, several Bostrichidae[36] harbour symbionts that are closely related to that of *O. surinamensis*[36], suggesting an involvement in cuticle tanning. In contrast to many other nutritional endosymbionts, the symbionts of *Sitophilus* and of *O. surinamensis* can be completely removed by treating the beetles with antibiotics or heat without interrupting the host life cycle[36,46,52,54]. The loss of a nutritional endosymbiont usually has severe effects on its host, e.g. by arresting development and reproduction and/or causing high mortality[46]. In contrast, the experimentally symbiont-deprived (aposymbiotic) *O. surinamensis* beetles were viable, able to reproduce, and could be maintained in stable aposymbiotic populations under laboratory conditions, allowing disentangling the effect of symbiont loss from direct antibiotic treatment[36]. In comparison to symbiont-containing control beetles, aposymbiotic individuals of *O. surinamensis* exhibited a thinner and noticeably lighter cuticle, which resulted in a significant fitness decrease due to higher mortality[55], especially under desiccation stress in stored grain products, the contemporary predominating habitat of the beetle[36].

1.4 Cuticle formation

Among the holometabolous insects, beetles (Coleoptera) are the most speciose of all insect orders. Their most outstanding characteristic is that their front pair of wings are hardened into protective wing-cases, called elytrae[56]. The elytrae guarantee the beetles protection against predators and pathogens and provide a higher desiccation resistance[57]. Beetles develop their full imaginal cuticle

during metamorphosis and the first days as an imago[58]. The cuticle of insects is primarily composed of chitin fibrils enveloped in protein chains rich in aromatic amino acids[57,59–61]. In addition, insects modify the native cuticle through the integration of phenolic compounds derived from the aromatic amino acid tyrosine in two processes called tanning (melanisation) and hardening (sclerotisation)[61], which lead to a cuticle that is darker, harder, and less permeable to water. However, in many herbivorous diets, nitrogenous compounds including tyrosine and phenylalanine from which tyrosine could be derived by insects, are limited. Thus, the thick cuticle comes with a cost as it requires a very high tyrosine investment during development[57,62]. Plants, fungi, and bacteria can synthesise precursors of tyrosine via the shikimate pathway[63]. Since beetles lack this pathway, they have to either ingest the aromatic amino acid via a food source or have it supplied by symbiotic microorganisms.

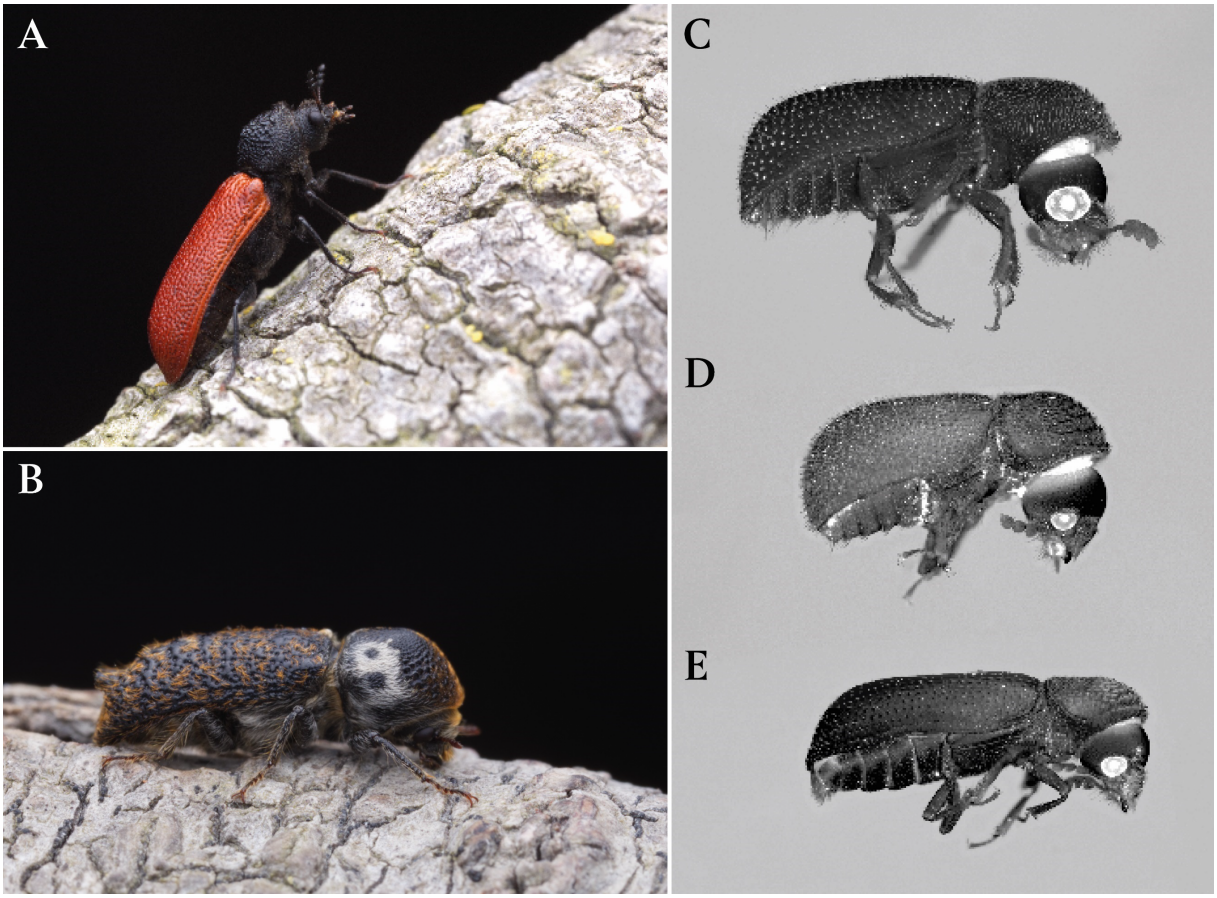


Figure 3: Beetles of the Bostrichidae family. Images of *Bostrichus capucinus* **(A)** and *Amphicerus bimaculatus* **(B)** were taken by Thomas Hörren in the wild. Autofluorescence microscopy images of *Prostephanus truncatus* **(C)**, *Rhyzopertha dominica* **(D)**, and *Dinoderus porcellus* **(E)**.

Like all holometabolous insects, beetles undergo a stepwise development, meaning from the embryo in the egg to the larva, to pupation, and finally to the adult animal. For the animal, this means that the anatomy of internal and external morphological organisations is remodelled fundamentally[64]. For some insect species, this also means that the individual life stages of an animal feed on different food sources because the morphology of e.g. the mouthparts is also changing[65,66]. Thus, the larval stage of the butterfly - the caterpillar - feeds on leaves, while the adult butterfly feeds on nectar. Further, in some species, food intake is limited to the larval stage, while the adult animal is only devoted to reproduction and thus to the survival of the species. The morphological reorganisation during metamorphosis requires a high investment in nutrients. As a consequence, young imagines initially have few resources left and have to take them up again first - or have them provided by symbionts.

As beetles are not able to feed during metamorphosis, they are reliant on stored nutrients acquired during their larval development for metamorphosis and cuticle formation. In the case of tyrosine, the amount an insect can store is limited, because the amino acid is toxic in high concentrations[68]. In this situation, harbouring a bacterial endosymbiont that can synthesize tyrosine or its precursors' chorismate or prephenate[57,63] in the moment of demand represents a strategy to cope with the storage problem[36,46,52,53].

1.5 Bacteroidota symbionts

Beetles of the families Silvanidae and Bostrichidae harbour abdominal bacteriomes, housing their bacterial endosymbionts[47]. The paired bacteriomes are located between the gut, fat body, and reproductive organs, but without direct connection to any of these tissues[36,69]. Despite the phylogenetic distance of Bostrichidae and Silvanidae of about 105 - 175 Mya[87,88], both beetle families are associated with symbionts belonging to the class Flavobacteria in the phylum Bacteroidota[36]. In addition, they are not the only ones, as phylogenetic analyses of insect-bacteria symbioses have shown in recent years (Figure 4). Accordingly, the Bacteroidota symbionts of the silvanid *O. surinamensis* and the bostrichids such as the lesser grain borer *Rhyzopertha dominica* are derived from the same ancestor as *Blattabacterium, Uzinura, Walczuchella,* and *Sulcia muelleri*. They diverged about 409 Mya ago[36] and evolved different functional specialisations[36,69–71]. *Uzinura* (endosymbiont of armoured scales), *Walczuchella* (endosymbiont of giant scales), *Blattabacterium* (endosymbiont of roaches and primitive termite), and *Sulcia* (endosymbiont of numerous sap-

feeding hemipterans) are known to supplement their host's diet with essential amino acids and vitamins[71–74]. In addition, there is evidence for nitrogen recycling in *Blattabacterium*[75–77]. As *Blattabacterium* still retains one of the largest genomes, it can be argued that urea catabolism appears to be an ancestral capability of Bacteroidota symbionts.

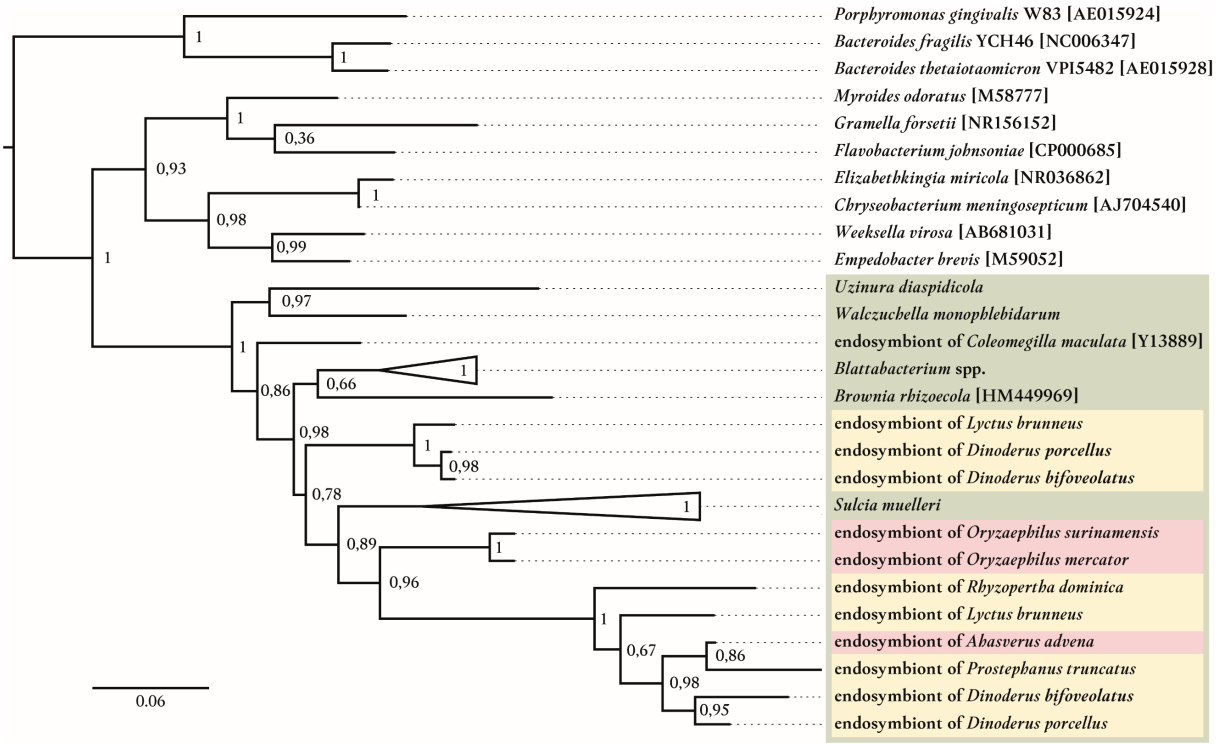


Figure 4: Phylogenetic placement of intracellular symbionts in silvanid (red) and bostrichid (yellow) beetles within the Bacteroidota, and their close association within the clade of exclusively insect-associated endosymbionts (green), based on 16S rRNA sequences. Node numbers represent local support values. RefSeq assembly accession in square brackets.

Since then, the ancestor of Bacteroidota symbionts has been associated with various insect families: scale insects[71,72,78], cockroaches and termits[75], Auchenorrhyncha e.g. cicadas, leafhoppers, planthoppers and splittlebugs[79–81] as well as the silvanid[36,52], bostrichid[36,82] and nosodendrid beetles[69]. Bacteroidota symbionts also show a divergent specialisation in different insect orders: *Uzinura diaspidicola* in armoured scale insects[71], *Walczuchella monophlebidarum* in scale insects[72], *Blattabacterium* spp. in cockroaches, *Sulcia muelleri* in the Auchenorrhyncha, and now *Shikimatogenerans* sp. and *Bostrichicola* sp. in beetles (Chapter 2 and 4). The repeated independent acquisitions of symbionts from this specific clade of Bacteroidota bacteria indicate that these bacteria were once highly capable of infecting and establishing in insects, akin to the α-proteobacterium *Wolbachia* (Chapter 3) or the γ-proteobacterium *Sodalis*[83,84]. This hypothesis is

supported by the observation that a basal clade of Bacteroidota endosymbionts is described as male-killing endosymbionts in different ladybird beetles[85–87].

Hirota et al. (2017) and Engl et al. (2018) provided evidence that the sawtoothed grain beetle *O. surinamensis* harbours a Bacteroidota endosymbiont which is somehow involved in the beetle's cuticle biosynthesis. Still, what the symbiont delivers to the host remained unknown. Also, the full metabolic potential beyond supporting cuticle synthesis of the endosymbiont remained hidden. In addition, Okude et al. (2017) and Engl et al. (2018) showed that beetles of the Bostrichidae family are associated with close related Bacteroidota endosymbionts, but the evolutionary origins and ancestral ecological contexts of these symbioses were unknown. Still, it remained elusive how exactly these symbioses between beetles of the Silvanidae and Bostrichidae families and their respective symbiont (or symbionts) function in detail as the metabolic potential of these bacteria was not elucidated.

1.6 Thesis outline

In this thesis, I aimed to identify the influence of symbiosis on the physiology, ecology, and evolution of grain beetles associated with Bacteroidota symbionts using genome sequencing, fluorescence microscopy, and experimental manipulation. I set out to (i) sequence the genomes and reconstruct the metabolic potential of the Bacteroidota endosymbionts associated with representative species of the families Silvanidae and Bostrichidae, (ii) experimentally verify the function of the endosymbionts in *Oryzaephilus surinamensis*, and (iii) reconstruct the evolutionary history of the symbiotic association in the species-rich bostrichid family.

In Chapter 2, I report the genome of *Shikimatogenerans silvanidophilus* OSUR, the Bacteroidota endosymbiont of the sawtoothed grain beetle *O. surinamensis*. Further, as the genome encodes genes of the shikimate pathway, I utilized the herbicide glyphosate, a selective pharmacological inhibitor of the shikimate pathway, to block the beneficial symbiotic contribution to its host's physiology and assessed the impact of symbiont elimination on host fitness correlates. Doing so, *O. surinamensis* is introduced as a well-suited system for experimental and detailed work on symbiosis because of the amenability for manipulation of the host-symbiont association.

In addition to being associated with the Bacteroides endosymbiont, *O. surinamensis* is also regularly infected with the reproductive manipulator *Wolbachia*. Therefore, I characterized this *Wolbachia* strain and its effect on the beetle's reproduction in Chapter 3.

In Chapter 4, I examined the evolution of symbiosis in the beetle family Bostrichidae. Genome reconstruction revealed a high degree of co-cladogenesis between host and symbiont phylogenies and gave insight into the dual symbiosis within the genera *Dinoderus* and *Lyctus* with the intracellular symbionts *Shikimatogenerans bostrichidophilus* and *Bostrichicola ureolyticus*.

The sequencing of the large grain borer *Prostephanus truncatus* surprisingly revealed that in this particular bostrichid, of the Bacteroidota endosymbiont *Shikimatogenerans bostrichidophilus* has diverged into three strains with partially complementary metabolic capabilities. Utilizing different microscopy approaches, I aimed at verifying the genomic predictions by localising the individual genes and genomes in the bacteriome in Chapter 5.

I conclude by discussing all the above-mentioned results in Chapter 6.

1.7 References

1. Moran, N. A. Symbiosis. *Curr. Biol.* **16**, R866–R871 (2006).
2. Bary, A. de. *Die Erscheinung der Symbiose*. *Die Erscheinung der Symbiose* (De Gruyter, 1879).
3. Margulis, L. & Fester, R. *Symbiosis as a source of evolutionary innovation: speciation and morphogenesis.* (MIT Press, Cambridge, 1991).
4. Margulis, L. & Bermudes, D. Symbiosis as a mechanism of evolution: status of cell symbiosis theory. *Symbiosis* **1**, 101–124 (1985).
5. Sapp, J. *Evolution by association: a history of symbiosis*. (Oxford University Press, 1994).
6. Heckman, D. S. *et al.* Molecular evidence for the early colonization of land by fungi and plants. *Science (80-.).* **293**, 1129–1133 (2001).
7. Archibald, J. M. Endosymbiosis and eukaryotic cell evolution. *Curr. Biol.* **25**, R911–R921 (2015).
8. Cavalier-Smith, T. Origin of mitochondria by intracellular enslavement of a photosynthetic purple bacterium. *Proc. R. Soc. B Biol. Sci.* **273**, 1943–1952 (2006).
9. Sadava, D., Hillis, D. M., Heller, H. C. & Hacker, S. D. *Purves Biologie*. (Springer Berlin Heidelberg, Berlin, Heidelberg, 2019).
10. Alberts, B. *et al. Molekularbiologie der Zelle*. (John Wiley & Sons, Weinheim, 2011).
11. Barth, F. Insects and flowers. The biology of a partnership. (1985).
12. Mariscal, R. N. The symbiosis between tropical sea anemones and fishes: a review. in *The Galápagos* 157–171 (University of California Press, 1966).
13. Depa, Ł., Kaszyca-Taszakowska, N., Taszakowski, A. & Kanturski, M. Ant-induced evolutionary patterns in aphids. *Biol. Rev. Camb. Philos. Soc.* **95**, 1574–1589 (2020).
14. Douglas, A. E. Phloem-sap feeding by animals: problems and solutions. *J. Exp. Bot.* **57**, 747–754 (2006).
15. Stork, N. E. How many species of insects and other terrestrial arthropods are there on Earth? *https://doi.org/10.1146/annurev-ento-020117-043348* **63**, 31–45 (2018).
16. Gillespie, M. A. K. *et al.* Status and trends of terrestrial arthropod abundance and diversity in the North Atlantic region of the Arctic. *Ambio* **49**, 718–731 (2020).
17. Douglas, A. E. The microbial dimension in insect nutritional ecology. *Funct. Ecol.* **23**, 38–47 (2009).
18. van den Bosch, T. J. M. M. & Welte, C. U. Detoxifying symbionts in agriculturally important pest insects. *Microb. Biotechnol.* **10**, 531–540 (2017).
19. Lemoine, M. M., Engl, T. & Kaltenpoth, M. Microbial symbionts expanding or constraining abiotic niche space in insects. *Curr. Opin. Insect Sci.* **39**, 14–20 (2020).
20. Feldhaar, H. Bacterial symbionts as mediators of ecologically important traits of insect hosts. *Ecol. Entomol.* **36**, 533–543 (2011).
21. Moran, N. A. & Telang, A. Bacteriocyte-associated symbionts of insects. *Bioscience* **48**, 295–304 (1998).
22. Oliver, K. M. & Martinez, A. J. How resident microbes modulate ecologically-important traits of insects. *Curr. Opin. Insect Sci.* **4**, 1–7 (2014).
23. Douglas, A. E. Symbiosis as a general principle in eukaryotic evolution. *Cold Spring Harb. Perspect. Biol.* **6**, (2014).
24. Klassen, J. L. Microbial secondary metabolites and their impacts on insect symbioses. *Current Opinion in Insect Science* vol. 4 15–22 (2014).
25. Douglas, A. E. Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria *Buchnera*. *Annu. Rev. Entomol.* **43**, 17–37 (1998).
26. Kaltenpoth, M., Göttler, W., Herzner, G. & Strohm, E. Symbiotic bacteria protect wasp larvae from fungal infestation. *Curr. Biol.* **15**, 475–479 (2005).
27. Flórez, L. V. *et al.* An antifungal polyketide associated with horizontally acquired genes supports symbiont-mediated defense in *Lagria villosa* beetles. *Nat. Commun.* **9**, 1–10 (2018).
28. Kellner, R. L. L. & Dettner, K. Differential efficacy of toxic pederin in deterring potential arthropod predators of *Paederus* (Coleoptera: Staphylinidae) offspring. *Oecologia* **107**, 293–300 (1996).
29. Kellner, R. L. L. Molecular identification of an endosymbiotic bacterium associated with pederin biosynthesis in *Paederus sabaeus* (Coleoptera: Staphylinidae). *Insect Biochem. Mol. Biol.* **32**, 389–395 (2002).
30. Salem, H. *et al.* Symbiont digestive range reflects host plant breadth in herbivorous beetles. *Curr. Biol.* **30**, 2875-2886.e4 (2020).
31. Salem, H., Florez, L., Gerardo, N. & Kaltenpoth, M. An out-of-body experience: the extracellular dimension for the transmission of mutualistic bacteria in insects. *Proc. R. Soc. B Biol. Sci.* **282**, 20142957 (2015).
32. Kikuchi, Y., Hosokawa, T. & Fukatsu, T. Insect-microbe mutualism without vertical transmission: a

stinkbug acquires a beneficial gut symbiont from the environment every generation. *Appl. Environ. Microbiol.* **73**, 4308–4316 (2007).
33. Kikuchi, Y., Meng, X. Y. & Fukatsu, T. Gut symbiotic bacteria of the genus *Burkholderia* in the broad-headed bugs *Riptortus clavatus* and *Leptocorisa chinensis* (Heteroptera: Alydidae). *Appl. Environ. Microbiol.* **71**, 4035–4043 (2005).
34. Onchuru, T. O., Javier Martinez, A., Ingham, C. S. & Kaltenpoth, M. Transmission of mutualistic bacteria in social and gregarious insects. *Curr. Opin. Insect Sci.* **28**, 50–58 (2018).
35. Salem, H. *et al.* Drastic genome reduction in an herbivore's pectinolytic symbiont. *Cell* **171**, 1520–1531 (2017).
36. Engl, T. *et al.* Ancient symbiosis confers desiccation resistance to stored grain pest beetles. *Mol. Ecol.* **27**, 2095–2108 (2018).
37. Doremus, M. R. & Hunter, M. S. The saboteur's tools: common mechanistic themes across manipulative symbioses. *Adv. In Insect Phys.* **58**, 317–353 (2020).
38. Werren, J. H., Baldo, L. & Clark, M. E. *Wolbachia*: master manipulators of invertebrate biology. *Nat. Rev. Microbiol.* **6**, 741–751 (2008).
39. Zug, R. & Hammerstein, P. Bad guys turned nice? A critical assessment of *Wolbachia* mutualisms in arthropod hosts. *Biol. Rev.* **90**, 89–111 (2015).
40. Hosokawa, T., Koga, R., Kikuchi, Y., Meng, X.-Y. & Fukatsu, T. *Wolbachia* as a bacteriocyte-associated nutritional mutualist. *Proc. Natl. Acad. Sci.* **107**, 769–774 (2010).
41. Moriyama, M., Nikoh, N., Hosokawa, T. & Fukatsu, T. Riboflavin provisioning underlies *Wolbachia's* fitness contribution to its insect host. *MBio* **6**, (2015).
42. Ju, J. F. *et al. Wolbachia* supplement biotin and riboflavin to enhance reproduction in planthoppers. *ISME J. 2019 143* **14**, 676–687 (2019).
43. Douglas, A. E. The B vitamin nutrition of insects: the contributions of diet, microbiome and horizontally acquired genes. *Curr. Opin. Insect Sci.* **23**, 65–69 (2017).
44. Wilson, A. C. C. *et al.* Genomic insight into the amino acid relations of the pea aphid, *Acyrthosiphon pisum*, with its symbiotic bacterium *Buchnera aphidicola*. *Insect Mol. Biol.* **19**, 249–258 (2010).
45. Snyder, A. K. & Rio, R. V. M. "*Wigglesworthia morsitans*" folate (Vitamin B_9) biosynthesis contributes to tsetse host fitness. *Appl. Environ. Microbiol.* **81**, 5375–5386 (2015).
46. Anbutsu, H. *et al.* Small genome symbiont underlies cuticle hardness in beetles. *Proc. Natl. Acad. Sci.* **114**, E8382–E8391 (2017).
47. Buchner, P. Endosymbiosis of Animals with Plant Microorganisms. *Z. Allg. Mikrobiol.* (1965).
48. Koch, A. Die Symbiose von *Oryzaephilus surinamensis* L. (Cucujidae, Coleoptera). *Zeitschrift für Morphol. und Ökologie der Tiere* **23**, 389–424 (1931).
49. Mansour, K. Memoirs: on the intracellular micro-organisms of some bostrychild beetles. *J. Cell Sci*. 77, 243–253 (1934).
50. Zhang, S. Q. et al. Evolutionary history of coleoptera revealed by extensive sampling of genes and species. *Nat. Commun*. 9, 1–11 (2018).
51. Kuriwada, T. et *al.* Biological role of *Nardonella* endosymbiont in its weevil host. *PLoS One* **5**, e13101 (2010).
52. Hirota, B. *et al.* A novel, extremely elongated, and endocellular bacterial symbiont supports cuticle formation of a grain pest beetle. *MBio* **8**, 1–16 (2017).
53. Vigneron, A. *et al.* Insects recycle endosymbionts when the benefit is over. *Curr. Biol.* **24**, 2267–2273 (2014).
54. Huger, A. Experimentelle Eliminierung der Symbionten aus den Myzetomen des Getreidekapuziners, *Rhizopertha dominica* F. *Naturwissenschaften* **41**, 170–171 (1954).
55. Kanyile, S. N., Engl, T. & Kaltenpoth, M. Nutritional symbionts enhance structural defence against predation and fungal infection in a grain pest beetle. J. Exp. Biol. 225, (2022).
56. Grimaldi, D. & Engel, M. S. Evolution of the insects. (Cambridge University Press, 2005).
57. Noh, M. Y., Muthukrishnan, S., Kramer, K. J. & Arakane, Y. Cuticle formation and pigmentation in beetles. *Curr. Opin. Insect Sci.* **17**, 1–9 (2016).
58. Hackman, R. H. Chemistry of the insect cuticle. in *The Physiology of Insecta* (ed. Rodstein, M.) 215–270 (Academic Press, New York and London, 1974).
59. Muthukrishnan, S., Merzendorfer, H., Arakane, Y. & Kramer, K. J. Chitin metabolism in insects. in *Insect Molecular Biology and Biochemistry* (ed. Gilbert, L. I.) 193–235 (Elsevier, Cambridge, 2012).
60. Kramer, K. J. & Hopkins, T. L. Tyrosine metabolism for insect cuticle tanning. *Arch. Insect Biochem. Physiol.* **6**, 279–301 (1987).
61. Hopkins, T. L. & Kramer, K. J. Insect cuticle sclerotization. *Annu. Rev. Entomol.* **37**, 273–302 (1992).

62. Brunet, P. C. J. The metabolism of the aromatic amino acids concerned in the cross-linking of insect cuticle. *Insect Biochem.* **10**, 467–500 (1980).
63. Tzin, V. & Galili, G. New Insights into the shikimate and aromatic amino acids biosynthesis pathways in plants. *Mol. Plant* **3**, 956–972 (2010).
64. Truman, J. W. & Riddiford, L. M. The origins of insect metamorphosis. *Nature* **401**, 447–452 (1999).
65. Rolff, J., Johnston, P. R. & Reynolds, S. Complete metamorphosis of insects. *Philos. Trans. R. Soc. B Biol. Sci.* **374**, 20190063 (2019).
66. Moran, N. A. Adaptation and constraint in the complex life cycles of animals. *Annu. Rev. Ecol. Syst.* **25**, 573–600 (1994).
67. Engl, T., Schmidt, T. H. P., Kanyile, S. N. & Klebsch, D. Metabolic cost of a nutritional symbiont manifests in delayed reproduction in a grain pest beetle. *Insects* **11**, 717 (2020).
68. Sterkel, M. *et al.* Tyrosine detoxification is an essential trait in the life history of blood-feeding arthropods. *Curr. Biol.* **26**, 2188–2193 (2016).
69. Hirota, B., Meng, X.-Y. & Fukatsu, T. Bacteriome-associated endosymbiotic bacteria of nosodendron tree sap beetles (Coleoptera: Nosodendridae). *Front. Microbiol.* **11**, 2556 (2020).
70. Kiefer, J. S. T. *et al.* Inhibition of a nutritional endosymbiont by glyphosate abolishes mutualistic benefit on cuticle synthesis in *Oryzaephilus surinamensis*. *Commun. Biol.* **4**, 554 (2021).
71. Sabree, Z. L., Huang, C. Y., Okusu, A., Moran, N. A. & Normark, B. B. The nutrient supplying capabilities of *Uzinura*, an endosymbiont of armoured scale insects. *Environ. Microbiol.* **15**, 1988–1999 (2013).
72. Rosas-Pérez, T., Rosenblueth, M., Rincón-Rosales, R., Mora, J. & Martínez-Romero, E. Genome sequence of "*Candidatus* Walczuchella monophlebidarum" the Flavobacterial endosymbiont of *Llaveia axin axin* (Hemiptera: Coccoidea: Monophlebidae). *Genome Biol. Evol.* **6**, 714–726 (2014).
73. Tokuda, G. *et al.* Maintenance of essential amino acid synthesis pathways in the *Blattabacterium cuenoti* symbiont of a wood-feeding cockroach. *Biol. Lett.* **9**, 20121153 (2013).
74. McCutcheon, J. P., McDonald, B. R. & Moran, N. A. Convergent evolution of metabolic roles in bacterial co-symbionts of insects. *Proc. Natl. Acad. Sci.* **106**, 15394–15399 (2009).
75. Ló Pez-Sánchez, M. J., Neef, A., Peretó, J., Patiñ O-Navarrete, R. & Pignatelli, M. Evolutionary convergence and nitrogen metabolism in *Blattabacterium* strain Bge, primary endosymbiont of the cockroach *Blattella germanica*. *PLoS Genet* **5**, 1000721 (2009).
76. Sabree, Z. L., Kambhampati, S. & Moran, N. A. Nitrogen recycling and nutritional provisioning by *Blattabacterium*, the cockroach endosymbiont. *Proc. Natl. Acad. Sci.* **106**, 19521–19526 (2009).
77. Hansen, A. K., Pers, D. & Russell, J. A. Symbiotic solutions to nitrogen limitation and amino acid imbalance in insect diets. in *Mechanisms Underlying Microbial Symbiosis* (ed. Kerry M. Oliver, J. A. R.) vol. 58 161–205 (Academic Press, Cambridge, 2020).
78. Gruwell, M. E., Hardy, N. B., Gullan, P. J. & Dittmar, K. Evolutionary relationships among primary endosymbionts of the mealybug subfamily Phenacoccinae (Hemiptera: Coccoidea: Pseudococcidae). *Appl. Environ. Microbiol.* **76**, 7521–7525 (2010).
79. Bennett, G. M. & Moran, N. A. Small, smaller, smallest: the origins and evolution of ancient dual symbioses in a phloem-feeding insect. *Genome Biol. Evol.* **5**, 1675 (2013).
80. Takiya, D. M., Tran, P. L., Dietrich, C. H. & Moran, N. A. Co-cladogenesis spanning three phyla: leafhoppers (Insecta: Hemiptera: Cicadellidae) and their dual bacterial symbionts. *Mol. Ecol.* **15**, 4175–4191 (2006).
81. Koga, R., Bennett, G. M., Cryan, J. R. & Moran, N. A. Evolutionary replacement of obligate symbionts in an ancient and diverse insect lineage. *Environ. Microbiol.* **15**, 2073–2081 (2013).
82. Okude, G. *et al.* Novel bacteriocyte-associated pleomorphic symbiont of the grain pest beetle *Rhyzopertha dominica* (Coleoptera: Bostrichidae). *Zool. Lett.* **3**, 13 (2017).
83. Dale, C., Young, S. A., Haydon, D. T. & Welburn, S. C. The insect endosymbiont *Sodalis glossinidius* utilizes a type III secretion system for cell invasion. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 1883–1888 (2001).
84. Ewald, P. W. Transmission modes and evolution of the parasitism-mutualism continuum. *Ann. N. Y. Acad. Sci.* **503**, 295–306 (1987).
85. Hurst, G. D. D. D. *et al.* Male–killing *Wolbachia* in two species of insect. *Proc. R. Soc. London. Ser. B Biol. Sci.* **266**, 735–740 (1999).
86. HURST, G. D. D. *et al. Adonia variegata* (Coleoptera: Coccinellidae) bears maternally inherited Flavobacteria that kill males only. *Parasitology* **118**, 125–134 (1999).
87. Hurst, G. D. D. *et al.* Male-killing bacterium in a fifth ladybird beetle, *Coleomegilla maculata* (Coleoptera: Coccinellidae). *Hered. 1996 772* **77**, 177–185 (1996).
88. McKenna, D. D. *et al.* The evolution and genomic basis of beetle diversity. *Proc. Natl. Acad. Sci. U. S. A.* **116**,

24729–24737 (2019).
89. Zhang, S. Q. *et al.* Evolutionary history of Coleoptera revealed by extensive sampling of genes and species. *Nat. Commun.* **9**, 1–11 (2018).

Chapter 2

Inhibition of a nutritional endosymbiont by glyphosate abolishes mutualistic benefit on cuticle synthesis in *Oryzaephilus surinamensis*

Communication Biology, 4 (1), 1-16

Julian Simon Thilo Kiefer[1], Suvdanselengee Batsukh[1], Eugen Bauer[1], Bin Hirota[2,3], Benjamin Weiss[1,5], Jürgen C. Wierz[1], Takema Fukatsu[2,3,4], Martin Kaltenpoth[1,5,6], Tobias Engl[1,5]

[1]Evolutionary Ecology, Institute of Organismic and Molecular Evolution, Johannes Gutenberg-University, Mainz, Germany; [2]Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology, Tsukuba 305-8566, Japan; [3]Department of Biological Sciences, Graduate School of Science, University of Tokyo, Tokyo 113-0033, Japan; [4]Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba 305-8571, Japan; [5]Research Group Insect Symbiosis, Max-Planck-Institute for Chemical Ecology, Jena, Germany; [6]Department of Insect Symbiosis, Max-Planck-Institute for Chemical Ecology, Jena, Germany

2.1 Abstract

Glyphosate is widely used as a herbicide, but recent studies begin to reveal its detrimental side effects on animals by targeting the shikimate pathway of associated gut microorganisms. However, its impact on nutritional endosymbionts in insects remains poorly understood. Here, we sequenced the tiny, shikimate pathway encoding symbiont genome of the sawtoothed grain beetle *Oryzaephilus surinamensis*. Decreased titers of the aromatic amino acid tyrosine in symbiont-depleted beetles support the ability to synthesize prephenate as the precursor for host tyrosine synthesis and its importance for cuticle sclerotisation and melanisation. Glyphosate exposure inhibited symbiont establishment during development and abolished the mutualistic benefit on cuticle synthesis in adults, which could be partially rescued by dietary tyrosine supplementation. Furthermore, phylogenetic analyses indicate that the shikimate pathways of many nutritional endosymbionts contain a glyphosate sensitive 5-enolpyruvylshikimate-3-phosphate synthase. These findings highlight the importance of symbiont-mediated tyrosine supplementation for cuticle biosynthesis in insects, but also paint an alarming scenario regarding the use of glyphosate in light of recent declines in insect populations.

2.2 Introduction

Glyphosate is a widely used, broad-spectrum herbicide that targets the shikimate pathway by inhibition of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)[1,2]. The shikimate pathway is present in plants, fungi, and bacteria to biosynthesize the aromatic amino acids phenylalanine, tyrosine and tryptophan and folates. As the inhibition of the EPSPS by glyphosate in susceptible plants is lethal[3], this herbicide is extensively used in agriculture in combination with genetically modified glyphosate-resistant crops to eliminate competing plants[4]. Animals, by contrast, are assumed to be unaffected by glyphosate, as they lack the shikimate pathway and meet their demands for aromatic amino acids from external sources[5].

However, animals do not live in isolation but engage in manifold mutualistic interactions with microorganisms[6]. While the microorganisms mostly gain the imminent advantage of a stable environment and the provision of basic nutrients[7], animals benefit from a more specialized metabolic capabilities of the symbiont, giving both of them a competitive advantage or the ability to expand to a certain niche[8–14]. Specifically, animals often benefit from metabolic capabilities of microorganisms that they lack themselves, for example, the synthesis of essential amino acids and (B-)vitamins, which is especially important when the insect host feeds on nutritionally unbalanced diets like plant sap or blood[14,15]. In the same way, semi-essential nutrients, i.e. nutrients like the aromatic amino acid tyrosine that can in principle be derived from the other aromatic amino acids taken up from the diet, but are in certain life stages like the metamorphosis of holometabolic insects still limited, are supplied by many nutritional endosymbionts[16–19]. Despite several studies emphasizing that glyphosate displays only "minimal toxicity" in animals via off-target activity due to the lack of the shikimate pathway in their genomes[1,20,21], recent work demonstrates that glyphosate does have a negative impact on insects, either directly or by inhibiting the EPSPS of animal-associated, mutualistic bacteria, with negative fitness consequences for the host[22,23]. In blood-feeding tsetse flies, glyphosate interferes with the biosynthesis of folate by the ɣ-proteobacterial symbiont *Wigglesworthia glossinidia*[24]. The herbicide also alters the gut microbiota of honeybees that consequently become more susceptible to opportunistic pathogens[25,26]. Since many obligate insect endosymbionts encode the shikimate pathway and supply aromatic amino acids to their host[19,27,28], glyphosate could prove highly detrimental for diverse insect hosts, with potentially severe ecological implications given the widespread nature of these mutualistic interactions in insects[29–31]. However, the impact of

glyphosate on insect-microbe associations remains poorly studied, particularly with respect to obligate nutritional endosymbionts in herbivorous insects.

The cuticle of insects is primarily composed of chitin fibrils enveloped in protein chains rich in aromatic amino acids[32–34]. Additionally, insects modify the native cuticle through the integration of phenolic compounds derived from tyrosine in two processes called melanisation and sclerotisation, which lead to a cuticle that is darker, harder, and less permeable to water[35,36]. However, in many herbivorous diets nitrogen in general, but also specifically tyrosine as well as phenylalanine and tryptophan from which it could be derived by insects are limited[37,38]. Among the holometabolous insects, beetles (Coleoptera) are distinguished by an especially strong adult cuticle, including the fully hardened forewing, i.e. the elytrae[39]. In addition, holometabolous insects develop their full imaginal cuticle during the pupal stage when the insect is undergoing metamorphosis as well during the first days as an imago[36]. As insects are not able to feed during metamorphosis, they are reliant on stored nutrients acquired during their larval development for metamorphosis and cuticle formation. In the case of tyrosine, the amount an insect can store is limited, because the amino acid is toxic in high concentrations[40]. In this situation, harbouring an endosymbiont that can synthesize tyrosine or its precursors chorismate or prephenate[5,41] in the moment of demand represents a strategy to cope with the storage problem[16,19,42,43].

We recently described a bacteriome-localised Bacteroidota endosymbiont in the sawtoothed grain beetle *Oryzaephilus surinamensis* (Coleoptera, Silvanidae)[42,43], which is a worldwide distributed pest of cereals and other stored food[44]. In contrast to many other nutritional endosymbionts, the symbiont of *O. surinamensis* can be removed by treating the beetle with antibiotics or heat without interrupting the host life cycle[42,43]. The loss of a nutritional endosymbiont usually has severe effects on its host, e.g. by arresting development and reproduction and/or causing high mortality[19]. In contrast, the experimentally symbiont-deprived (aposymbiotic) *O. surinamensis* beetles were viable, able to reproduce, and could be maintained in stable aposymbiotic populations under laboratory conditions, allowing to disentangle the effect of symbiont loss from direct antibiotic treatment[42]. In comparison to symbiont-containing control beetles, aposymbiotic individuals exhibited a 30% thinner and noticeably lighter cuticle, which resulted in a significant fitness decrease due to higher mortality, especially under desiccation stress in stored grain products, the natural habitat of the beetle[42].

In this work, we demonstrate (i) that the symbiont genome of *O. surinamensis* is extremely streamlined, providing precursors for cuticle synthesis via the shikimate pathway, (ii) that exposure to glyphosate compromises the symbiont establishment in *O. surinamensis* and induces the fitness-relevant cuticular defects of the host in a similar manner as the complete loss of the symbiont, (iii) that these phenotypic effects can be partially rescued by dietary tyrosine supplementation, and (iv) that the shikimate pathways of *O. surinamensis* as well as many other nutritional endosymbionts contain class I EPSPSs that are predicted to be glyphosate-sensitive. These results experimentally validate the functionality of the shikimate pathway in an obligate endosymbiont and more generally demonstrate the severe impact of glyphosate on organisms that are dependent on bacterial endosymbionts.

2.3 Results

2.3.1 Symbiont genome is extremely reduced and GC poor

We sequenced the metagenome of *O. surinamensis* combining short and long-read technologies (Illumina and ONT) into a hybrid assembly to gain first insights into its symbiont's metabolic capabilities. As expected, we could detect the 16S rRNA sequence of a Bacteroidota bacterium in the assembly that matched PCR-based Sanger sequences from previous studies[42,43]. In total, 13 contigs were extracted from the metagenomic assembly via taxonomic classification, GC content filtering as well as by manually searching for tRNAs and ribosomal proteins of Bacteroidota bacteria (Figure 1.1, Supplementary File 1). The longest was 74,813 kbp and the shortest 6,352 kbp in length. Together they had a length of 307,680 kbp with an average GC content of 16.2%. The draft genome encoded for 299 genes and had a coverage of 120x with short-read sequences and 61x with long-read sequences.

The phylogenetic reconstruction based on the conserved Clusters of Orthologous Group (COG) genes confirmed the placement of the endosymbiont of *O. surinamensis* in a group of insect-associated Bacteroidota bacteria and specifically the close relationship to *Blattabacterium* sp. and *Sulcia muelleri* (*S. muelleri*) that was already previously reported based on a 16S rRNA gene phylogeny (Supplementary Figure 1)[42]. The symbiont's genome encodes for 299 protein-coding sequences, 28 tRNAs and 51 ribosomal proteins (21 SSU and 30 LSU proteins). Based on a set of single-copy marker genes that are assumed to be essential, the *O. surinamensis* symbiont genome is estimated to be 66.7% complete[45]. However, this 'completeness' measure is based on essential genes in free-living bacteria and known to severely underestimate the completeness of highly eroded genomes of intracellular bacterial symbionts[46,47]. Concordantly, the estimation falls in the range of other insect-associated Bacteroidota mutualists that exhibit highly eroded, closed genomes (54.20 - 67.38% of *S. muelleri* PSPU and *W. monophlebidarum*; Supplementary Table 1 & 2). Despite the remaining gaps in the symbiont genome sequence, the 13 contigs are thus inferred to contain the complete or almost complete set of coding sequences, which is corroborated by the presence of a full complement of tRNAs, tRNA synthetases, and ribosomal proteins to a similar extent as in other Bacteroidota endosymbionts. The contigs are likely also fragments of a single chromosome, as it does not feature duplicated genes, multiple rRNA operons or variable coverage across the different contigs, which are indicative of such fragmentation reported from multiple

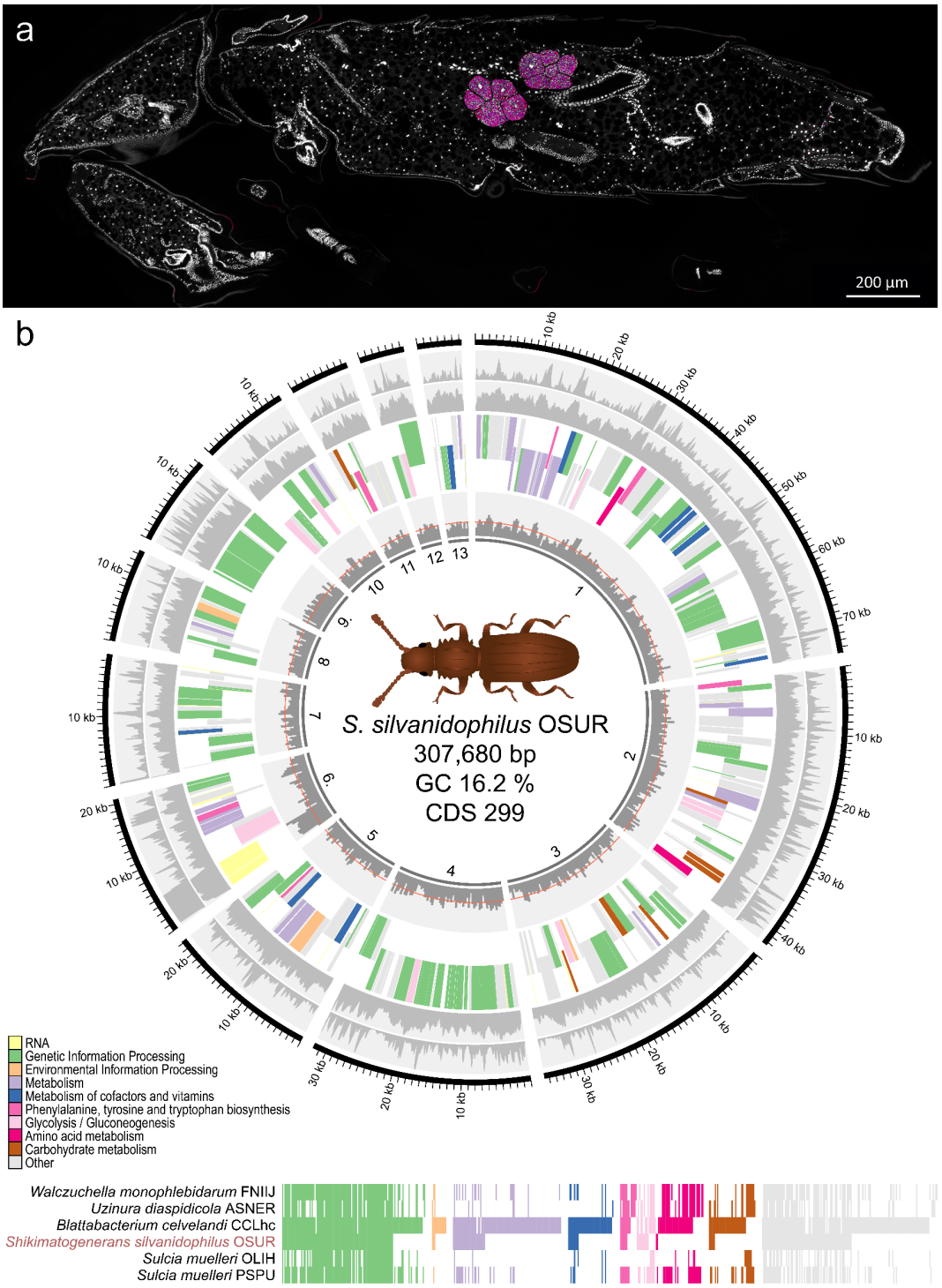


Figure 1.1: *Shikimatogenerans silvanidophilus*, the symbiont of *O. surinamensis*. **(a)** Fluorescence *in situ* hybridisation micrograph of a sagittal section of a 5-day-old *O. surinamensis* pupa stained with CFB563mod-Cy3 (magenta) and DAPI (white). **(b)** Circular representation of the draft genome of *S. silvanidophilus*. Single contigs are sorted clockwise by length. The outer grey circles denote coverage with long- and short-reads, respectively, the intermediate circles indicate annotated functional KEGG categories separated by direction of transcription (see legend for depicted categories). The inner grey circle denotes relative GC content and the average GC content of 16.2% by the red line. Below: comparison of the functional gene repertoires of Bacteroidota symbionts in insects. Box colors are based on KEGG's categories.

fragmented chromosomes of *Hodgkinia* strains of different *Magicicada* species[48–50]. As an extremely low GC content (16.2%) and long repeat sequences towards contig ends are known issues for sequencing technologies and PCRs[51–54], these features likely prevented successful amplification steps and the *in silico* assembly of the contigs into a single genome despite the high coverage (120x with short-read sequences and 61x with long-read sequences), especially as the GC content at the ends of most contigs reached even lower values (~ 4% within the last 100 bp).

We also compared the presence and arrangement of the genes between Bacteroidota endosymbionts of different host insects. A synteny plot (Supplementary Figure 2) revealed that there is no conserved arrangement of genes between the genomes of related Bacteroidota symbionts. This likely explains futile attempts to assemble the genome by mapping the assembled contigs to *Blattabacterium* or *S. muelleri* as reference genomes or to sort contigs into a single scaffold.

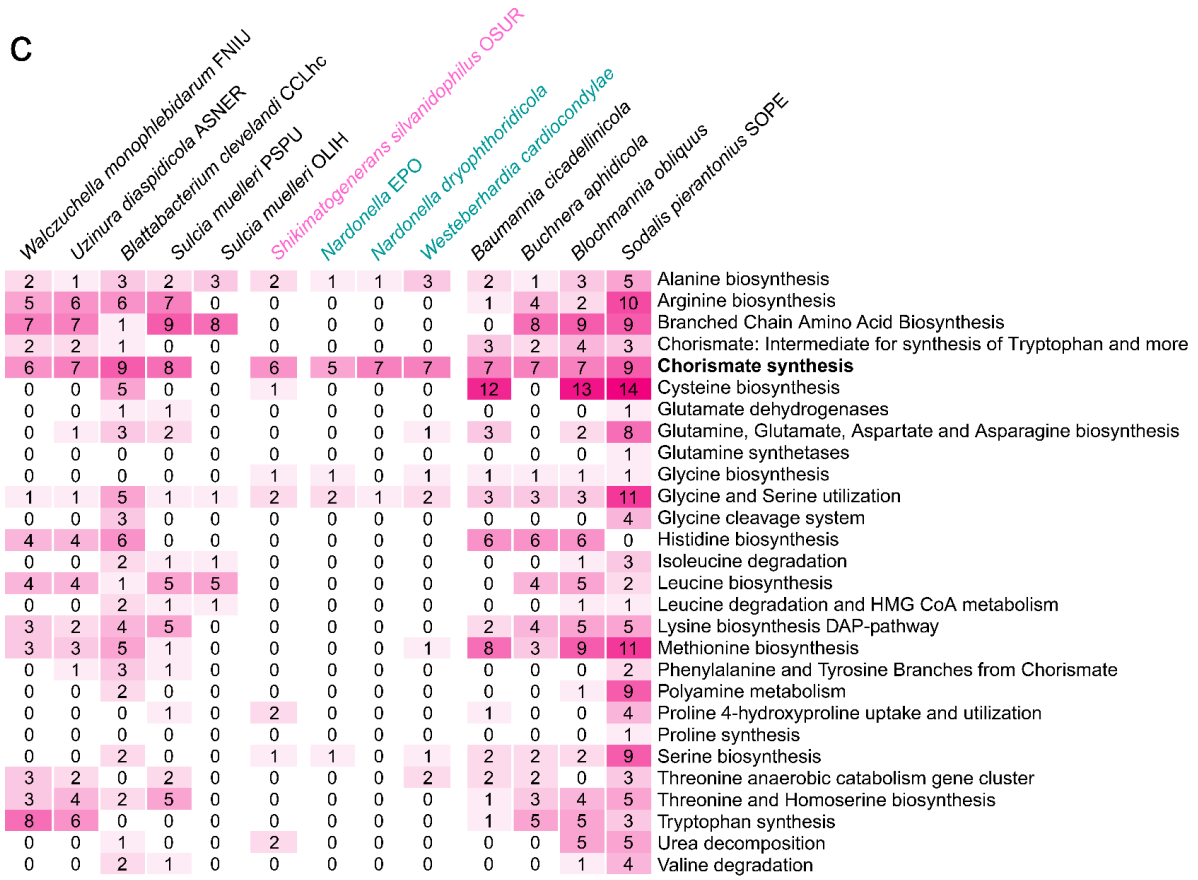


Figure 1.2. (c) Detailed comparison of the amino acid metabolism gene repertoires between the *Shikimatogenerans silvanidophilus* genome (pink), other Bacteroidota symbionts (left) and Proteobacteria symbionts (right), some of which are known to exclusively provision tyrosine precursors to their insect host (blue).

2.3.2 *Candidatus* Shikimatogenerans silvanidophilus OSUR encodes glycolysis and shikimate pathways

The metabolic repertoire of the *O. surinamensis* symbiont is highly reduced (Figure 1.2, Supplementary File 1). Apart from general genetic information processing including DNA replication and repair, transcription, and translation, it only encodes an extremely limited set of metabolic pathways including a full glycolysis pathway to process glucose-6-phosphate to erythrose 4-phosphate (E4P) and phosphoenolpyruvate (PEP). In addition, the genome encodes all the genes of the shikimate pathway except a shikimate dehydrogenase (*aroE* [EC:1.1.1.25]) that utilizes PEP and E4P to produce chorismate, as well as a chorismate mutase to catalyze the conversion of chorismate into prephenate, the precursor of the aromatic amino acids phenylalanine, tryptophan and tyrosine (Figure 2). The lack of aroE is described in other tyrosine-supplementing bacterial symbionts: *Cd* Carsonella ruddii in *Pachypsylla venusta*[55] and *Cd* Nardonella EPO in *Euscepes postfasciatus*[19]. AroD (3-dehydroquinate dehydratase [EC:4.2.1.10]) and aroE are also often found as a bifunctional enzyme aroDE (3-dehydroquinate dehydratase/shikimate dehydrogenase [EC:4.2.1.10 1.1.1.25])[56], possibly complementing the loss of one of the enzymes. Like all other Bacteroidota symbionts, the *O. surinamensis* symbiont genome encodes the bifunctional *aroG*/*pheA* gene (phospho-2-dehydro-3-deoxyheptonate aldolase/chorismate mutase [EC:2.5.1.54 5.4.99.5]). The genomic data revealed no transporter for glucose, so it remains unknown how the symbiont acquires the substrate for glycolysis from the host (Figure 2).

Whether the *O. surinamensis* symbiont can recycle nitrogen, like *Blattabacterium* sp.[57,58] remains unclear, as it encodes the urease α and γ subunits (*ureC* [EC:3.5.1.5]), but no glutamate dehydrogenase (*gdhA*) that would allow integrating the resulting ammonium into the amino acid metabolism via glutamate was detected. Instead, it encodes a proline transporter (*opuE*), proline dehydrogenase [EC:1.5.5.2] and oxidoreductase [EC:1.2.1.88] to import and convert proline into glutamate[59,60], which may then be exported to function as an amino group donor for the synthesis of the aromatic amino acids from chorismate/prephenate by the beetle itself[19]. This alternative pathway for glutamate synthesis was not described in *Blattabacterium*[61], *S. muelleri*[62], *Uzinura diaspidicola*[63] or *Walczuchella monophlebidarum*[64].

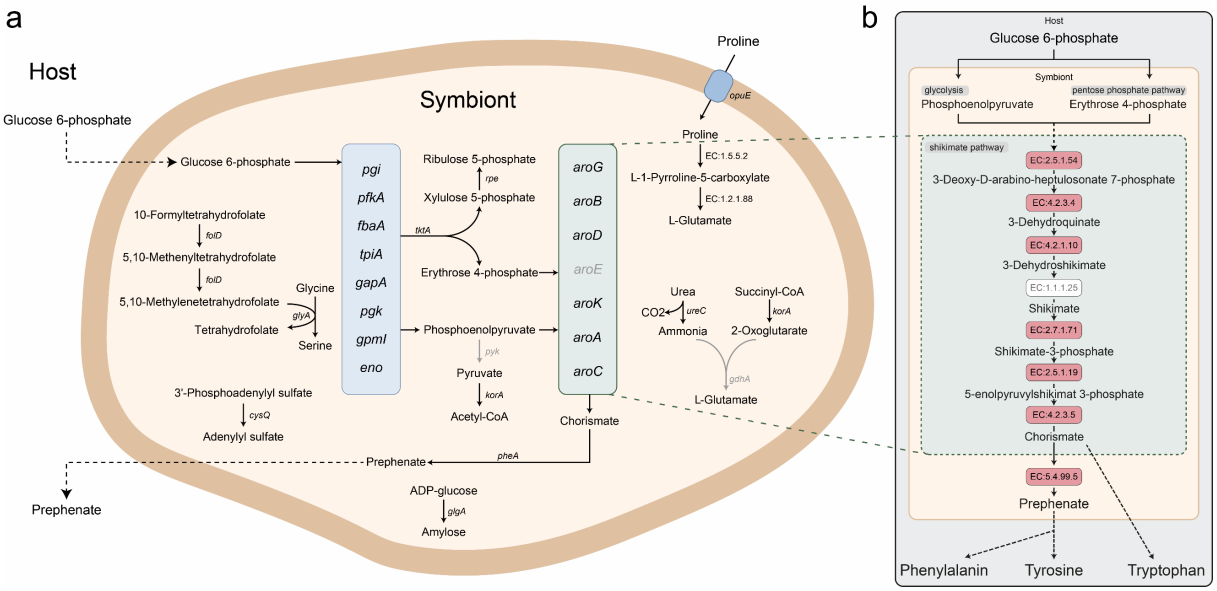


Figure 2: Metabolism of the symbiont *Shikimatogenerans silvanidophilus*. **(a)** Complete, reconstructed metabolism of *S. silvanidophilus*, as inferred from genomic data. Enzymes and arrows in grey were missing in the genome annotation. Dashed arrows indicate transport processes without annotated transporters, but which are expected to occur based on observed phenotypes. **(b)** Schematic diagram of the shikimate pathway in *S. silvanidophilus*. Dashed arrows represent multiple enzymatic steps. Red boxes: enzyme present in the genome. White box: enzymes not annotated in the genome, but reaction probably catalysed by another enzyme.

A comparison of the metabolic gene repertoires for amino acid biosynthesis revealed convergent genome erosion between the *Oryzaephilus* symbiont and ɣ-proteobacterial symbionts known to provision precursors for the host's cuticle biosynthesis (Figure 1c). We observed a gradual loss of functions across the Bacteroidota insect symbionts, with the *O. surinamensis* symbiont genome exhibiting the most strongly reduced repertoire of biosynthetic genes. A convergent reduction of metabolic functions was observed in the ɣ-proteobacterial symbionts *Westerberhardia* and *Nardonella* sp., whose sole metabolic function appears to be the provisioning of aromatic amino acid precursors that are in high demand for cuticle biosynthesis of their insect host[19,27,65].

Based on our findings, we propose the name '*Candidatus* Shikimatogenerans silvanidophilus OSUR' for the endosymbiont of *O. surinamensis*, henceforth called *S. silvanidophilus*. The genus name *Shikimatogenerans* refers to its ability to perform the shikimate pathway. Previous studies have shown that there might be other closely related Bacteroidota bacteria associated with other beetle families[42,66,67]. Thus, we propose *silvanidophilus* as species name to indicate that this symbiont is associated with a silvanid beetle. As the same studies also revealed that *O. mercator*

has a similar symbiont we also propose to add OSUR to identify the strain associated with *O. surinamensis*.

2.3.3 Amino acid titers are influenced by symbiont presence

Using the genomic data as a basis, we then asked whether the symbiont-encoded pathways are indeed functional. To this end, we tested if the symbiont presence indeed influenced the titer of aromatic amino acids. This would be expected when the symbiont is delivering prephenate to the host, a precursor that can also be transformed to tyrosine or phenylalanine by the beetles themselves[17,27,68]. Due to the presence of the proline-transporter and proline-converting enzymes in the symbiont genome, an influence on proline titer and glutamic acid was also expected based on the metabolic reconstruction. After hatching from the egg, the larva of *O. surinamensis* spends weeks in multiple instars before it pupates and hatches five days later as an imago. During metamorphosis, the biosynthesis of the adult cuticle starts and continues in the first days of the imago[36]. As tyrosine is used in copious amounts in the cuticle biosynthesis[69] amino acid titers might change dynamically. Thus, we expected the major symbiont contribution of tyrosine in the pupal stage and early adulthood[19]. Accordingly, we found an overall significantly positive influence of the symbiont presence on the titer of tyrosine (FDR corrected GLMs, $p = 0.0000858$, see Figure 3 and Supplementary Figure 3 and detailed statistic results for all the following values in Supplementary Table 3 and 4). Specifically, late symbiotic pupae had a significantly higher tyrosine titer than aposymbiotic ones (Wilcox rank-sum tests with FDR corrected p-values: $p = 0.00078$), shortly before adult emergence. By contrast, there was a negative impact of symbiont presence on proline and glutamic acid levels (FDR corrected GLMs, $p = 0.0000858$), which are interconverted by the symbiont based on the genomic prediction and used to synthesize tyrosine from prephenate. Again, free proline was significantly lower in late symbiotic vs. aposymbiotic pupae (FDR corrected Wilcox rank-sum test, $p = 0.00078$) and bound proline in early adults (FDR corrected Wilcox rank-sum test, $p = 0.00078$). Free glutamic acid was significantly lower in early and late symbiotic pupae (FDR corrected Wilcox rank-sum tests, both $p = 0.00078$, Figure 3) and the elytrae of early symbiotic adults (FDR corrected Wilcox rank-sum test, $p = 0.0059$). There was no difference in the titer of bound or cuticular tyrosine in the different life stages of the beetle in direct comparison (FDR corrected Wilcox rank-sum test, $p > 0.05$) and only protein-bound glutamic acid of the full body of early symbiotic beetles was significantly higher than in aposymbiotic ones (FDR corrected Wilcox rank-sum test, $p = 0.0059$). All other

amino acids were not influenced by symbiont presence alone, although we detected several interaction effects (FDR corrected GLMs, $p > 0.005$; Supplementary Table 3).

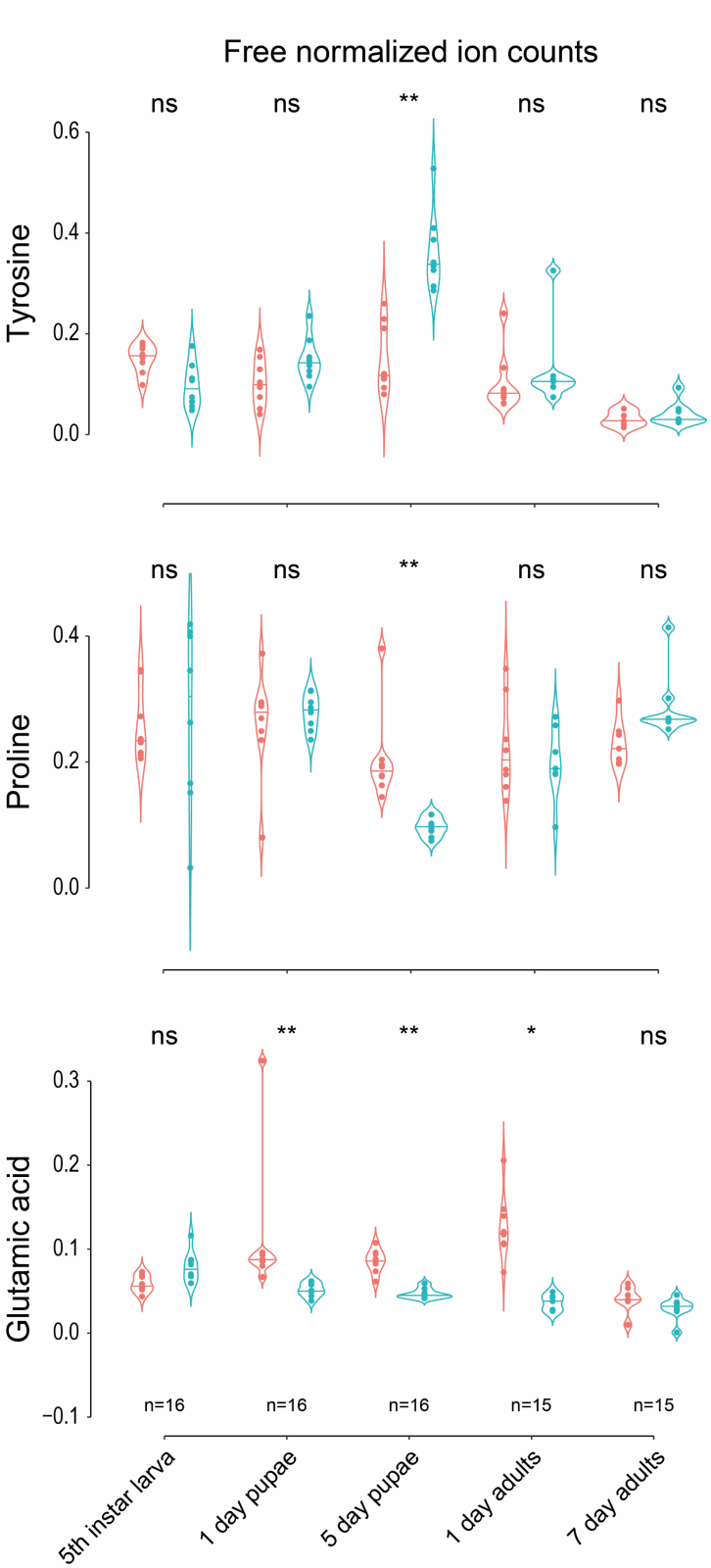


Figure 3: Comparison of titers of the three amino acid tyrosine, proline and glutamic acid that that were influenced by symbiont presence. Shown are free amino acid titers in the whole body (without elytrae in case of adults) of symbiotic and aposymbiotic *O. surinamensis* beetles. Red: aposymbiotic beetles, Blue: symbiotic beetles. The data distribution is visualized with violin plots and an additional horizontal line depicting the median. The FDR-corrected unpaired, two-sided Wilcoxon-rank-sum-tests: ns $p > 0.05$, *$0.05 < p < 0.01$, **$p < 0.01$.

2.3.4 The symbiont's shikimate pathway is sensitive to glyphosate and its inhibition results in an aposymbiotic phenotype

Next, we investigated the consequences of a pharmacological inhibition of the shikimate pathway on the symbiosis. Therefore, we made use of the pesticide glyphosate, an allosteric inhibitor of the enzyme EPSPS (5- enolpyruvoylshikimate-3-phosphate synthase) which is encoded by *aroA*[1]. We experimentally determined the impact of glyphosate exposure and aromatic amino acid supplementation during the entire larval and early adult development on phenotypic parameters that were previously demonstrated to be impacted by the symbiosis, i.e. cuticle thickness and melanisation[42,43]. As predicted, both the thickness (Kruskal-Wallis $\chi^2 = 27.8525$, df = 7, p-value = 0; Figure 4a, Supplementary Table 5) and melanisation (Kruskal-Wallis $\chi^2 = 35.967$, df = 7, p-value = 0; Figure 4b, Supplementary Table 5) of the cuticle were influenced by glyphosate exposure and by aromatic amino acid supplementation of the beetle diet. Symbiotic beetles showed a significant reduction of both cuticle traits after inhibition of the shikimate pathway with 1% glyphosate (Dunn's test: symbiotic vs. symbiotic + glyphosate: p = 0.0038 & p = 0.0049), to the same level as observed in aposymbiotic beetles (Dunn's test: aposymbiotic vs. symbiotic: p = 0.0002 & p = 0.0008; aposymbiotic vs. symbiotic + glyphosate: p = 0.6039 & p = 0.5360). A lower amount of glyphosate (0.1%) led to intermediate phenotypes that did not differ significantly from either symbiotic or aposymbiotic or 1% glyphosate treated individuals (Dunn's test: $p > 0.05$). Importantly, the thinner and less melanized cuticle of both aposymbiotic and glyphosate-exposed (1%) symbiotic beetles could be rescued to an intermediate state by adding aromatic amino acids to the diet, resulting in cuticular traits that differed neither from symbiotic nor aposymbiotic or 1% glyphosate treated beetles (Dunn's test: $p > 0.05$). By contrast, aromatic amino acid supplementation did not affect cuticular traits of symbiotic beetles that were not exposed to glyphosate (Dunn's test: $p > 0.05$). These findings support the genome-based prediction that *S. silvanidophilus* supplements precursors for tyrosine biosynthesis via the shikimate pathway.

The two plausible scenarios for the impact of glyphosate exposure on symbiont contributions are (i) a reduced chorismate biosynthesis by a stable symbiont population, or (ii) an overall decrease in symbiont titers due to glyphosate exposure. This includes either direct effects trough glyphosate toxicity, but also indirect effects via consequences of an inhibited chorismate biosynthesis like a lack of aromatic amino acids or host feedback mechanisms. Concordant with

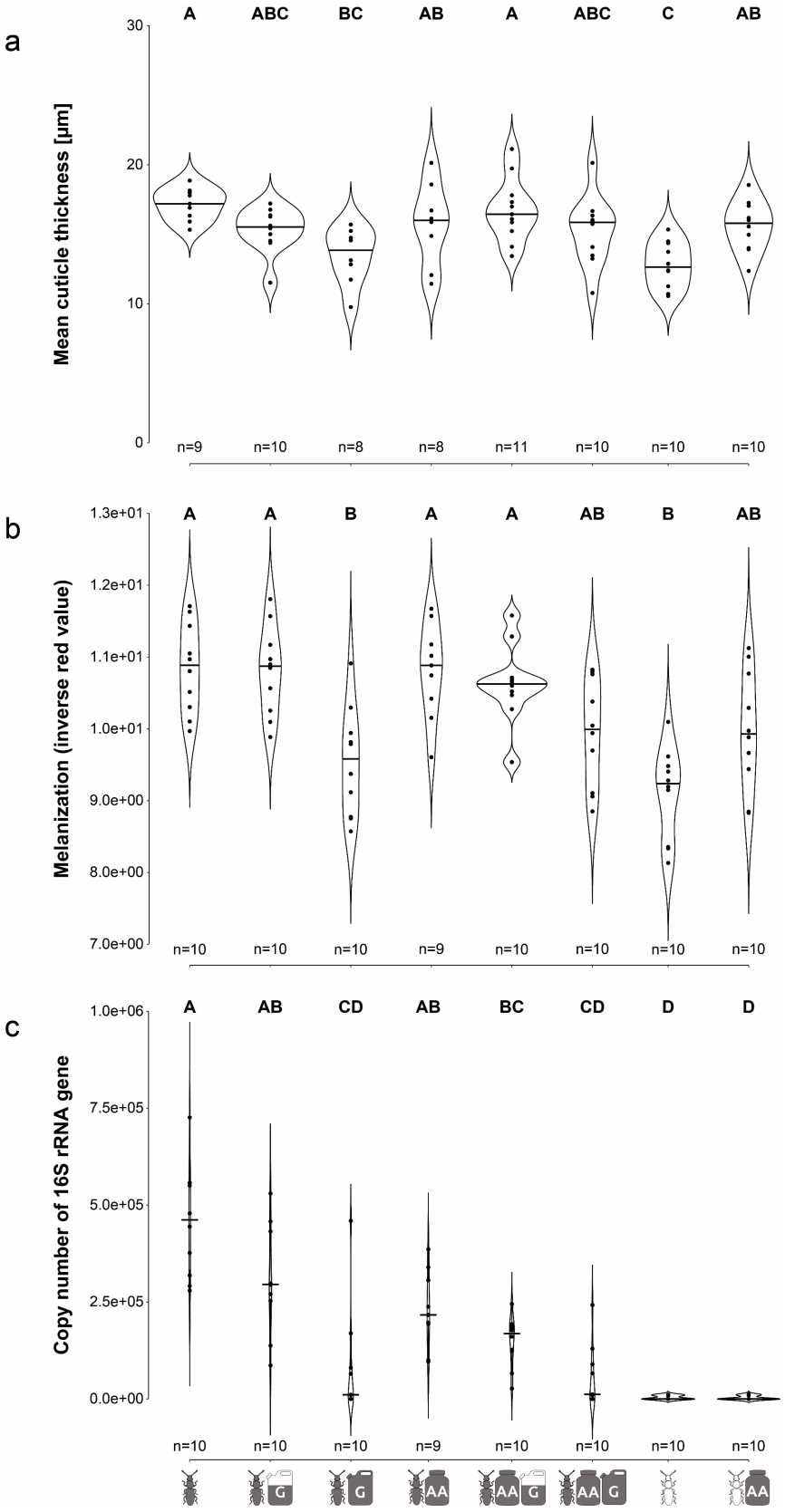


Figure 4: Effect of glyphosate exposure and aromatic amino acid supplementation on cuticle traits and symbiont titers in symbiotic and aposymbiotic beetles. Cuticle thickness (**a**), melanisation measured as thorax coloration (**b**), and symbiont titers (**c**) of aposymbiotic and symbiotic adults reared on different food compositions. Different letters indicate significant differences between experimental treatments (Dunn's Test, $\alpha \leq 0.05$).

the latter hypothesis, we detected significant differences in symbiont titers of one-week-old symbiotic adults across experimental treatments (Kruskal-Wallis $\chi^2 = 59.0049$, df = 7, p-value = 0; Figure 5c, Supplementary Table 5). The symbiont titers in symbiotic beetles exposed to high glyphosate concentrations were significantly reduced by 98% (based on medians; Dunn's test: sym vs. 1% glyphosate: $p = 0.00034$). Low glyphosate concentrations and supplementation with aromatic amino acids also reduced symbiont titers to an intermediate level that did not differ significantly from either untreated beetles nor those treated with high glyphosate amounts (Dunn's test: $p > 0.05$). In combination with low amounts of glyphosate, supplementation of aromatic amino acid reduced the symbiont titers further, leading to a significant difference to untreated beetles (Dunn's test: AA + 0.1% glyphosate vs untreated: $p = 0.028$).

Finally, we tested whether the EPSPS of other intracellular symbionts besides the one of *S. silvanidophilus* belong to glyphosate sensitive EPSPS variants by a phylogenetic analysis of their amino acid sequences[25]. The EPSPSs of all included Bacteroidota and also Proteobacteria symbionts of insects clustered with class I EPSPS and are thus predicted to be sensitive to glyphosate – in contrast to insensitive class II enzymes of several free-living bacteria[70] (Figure 5 and Supplementary Figure 4).

Figure 5: Phylogenetic classification of EPSPS enzymes. Enzymes from different Bacteroidota and γ-Proteobacteria insect symbionts as well as some free-living bacteria and plants were classified based on FastTree and maximum- likelihood analyses of amino acid sequences of EPSPSs using the Jones–Taylor–Thorton model. Enzymes of plants (green), bacterial symbionts in the honeybee gut (yellow), and obligate intracellular insect symbionts (red) are highlighted. Node values indicate FastTree support values.

2.4 Discussion

The sawtoothed grain beetle *O. surinamensis* engages in an intimate association with the Bacteroidota endosymbiont *S. silvanidophilus* that confers desiccation resistance via enhanced cuticle synthesis[42,43]. This physiological contribution represents a significant fitness benefit in dry habitats like mass grain storage[42]. Here, we demonstrated that the genome of *S. silvanidophilus* experienced a drastic erosion, resulting in a genome of 308 kbp in size and a strongly AT-biased nucleotide composition (16.2% GC), akin to what has been described for other obligate intracellular[47,71,72] or extracellular insect symbionts[73–76].

Moreover, the symbiont genome encodes only a few metabolic pathways, of which the substantial remaining ones are the glycolysis and shikimate pathways. Phosphoenolpyruvate (PEP) and Erythrose-4-phosphate (E4P) - both originating from glycolysis - are used as substrates in the shikimate pathway to synthesize chorismate, which in turn is transformed to prephenate by the symbiont and then converted to the aromatic amino acids phenylalanine, tryptophan and tyrosine by the host, as recently demonstrated in the weevil-*Nardonella* endosymbiotic system[19]. Of the seven genes in the shikimate pathway (*aroG*, *aroB*, *aroD*, *aroE*, *aroK*, *aroA*, *aroC*), the genome only lacks the gene for shikimate dehydrogenase (*aroE*), which catalyzes the reversible NADPH-linked reduction of 3-dehydroshikimate to shikimate. However, the shikimate pathways of *Nardonella* EPO, the endosymbiont of the sweetpotato weevil *Euscepes postfasciatus* (Curculionidae: Cryptorhynchinae) and *Carsonella ruddii*, the endosymbiont of the gall-forming psyllid *Pachypsylla venusta* (Aphalaridae: Pachypsyllinae) also lack *aroE*, but remain functional[19,55], suggesting that the function can be taken over by other enzymes, either from the host or the endosymbiont—that have yet to be identified.

The genome also encodes for an urease (*ureC*, Urease α and γ subunits [EC:3.5.1.5]), so *S. silvanidophilus* may be able to recycle nitrogenous waste products of its host, as has been described for *Blattabacterium* in cockroaches and *Blochmannia* in carpenter ants[61,64,77]. Alternatively, however, this enzyme could be a non-functional remnant in the eroded genome, as no glutamate dehydrogenase (*gdhA*) synthesizing glutamate from ammonium and 2-oxogluterate was detected and the symbiont genome encodes no other annotated gene that could incorporate the resulting ammonium. Instead, the genome encodes for a proline transporter (*opuE*) and the genes to convert proline into glutamic acid (EC:1.5.5.2 and EC:1.2.1.88)[78,79]. Thus, proline as one of the most

abundant amino acids in insect hemolymph might be utilized by the symbiont, among others as a source of glutamate[80]. As the genome encodes no other pathways of potential relevance to the host, we hypothesized that the symbiont's beneficial impact on host fitness results from the supplementation of the tyrosine precursor prephenate, and possibly, from additional nitrogen recycling.

The quantification of amino acid titers throughout insect development in symbiotic and aposymbiotic beetles supports the genome-based predictions of symbiont-mediated tyrosine precursor biosynthesis and proline consumption. Both tyrosine and proline concentrations revealed significant differences between symbiotic and aposymbiotic beetles in late pupae, consistent with proline consumption and tyrosine biosynthesis during metamorphosis, which coincides with the reported maximum symbiont titers in *O. surinamensis*[81]. While our results indicate that the symbionts consume proline, the dynamics of proline conversion to glutamic acid remain elusive, as glutamic acid titers were lower in symbiotic than in aposymbiotic beetles across multiple life stages. Possibly, glutamic acid is predominantly utilized by the symbiont itself. Alternatively, as more chorismate is available for tyrosine synthesis in symbiotic than in aposymbiotic beetles, the glutamic acid titers might be constantly depleted, while tyrosine may only accumulate during metamorphosis, and is directly channelled into cuticle biosynthesis in all other life stages, as tyrosine accumulation may be toxic[40]. A similar phenomenon of increased symbiont titers and intense cuticle synthesis and modification during metamorphosis and early adulthood was described in another grain pest beetle, the weevils *S. oryzae*[16]and *P. infernalis*[19], as well as the ant *Cardiocondyla obscurior*[27].

Symbiont-mediated contributions to cuticle biosynthesis evolved multiple times convergently in insects, with genomic evidence for tyrosine supplementation by symbionts in the ant genus *Cardiocondyla*, and both genomic and experimental support in carpenter ants[82,83], as well as the weevils *P. infernalis*[19] and *S. oryzae*[16,84]. The increasing number of tyrosine-supplementing symbioses provide evidence for this aromatic amino acid being a key nutrient that is limiting for many insects to produce their strongly sclerotized and melanized exoskeleton for protection against desiccation and natural enemies[16,18,19,82]. While all the previously described symbioses that exclusively provision tyrosine precursors to their hosts involve ɣ-proteobacterial symbionts, *S. silvanidophilus* belongs to the Bacteroidota, providing an example for functional convergence in

genome-eroded symbionts across different bacterial phyla. Such convergence has been previously described for obligate endosymbionts of plant sap-sucking Hemiptera and Coleoptera that provision essential amino acids and/or vitamins to their hosts[62,85,86]. Interestingly, the Bacteroidota clade containing *S. silvanidophilus* comprises exclusively insect-associated bacteria, suggesting the possibility that the ancestor was a successful symbiont or pathogen of insects, reminiscent of the widespread reproductive manipulator *Wolbachia* and the common insect associate *Sodalis*[72,87,88].

Symbioses relying on nutritional supplements derived from the shikimate pathway are prone to inhibition of the *aroA*-encoded EPSPS by the herbicide glyphosate. Glyphosate sensitivity has already been used as an experimental tool to manipulate the obligate symbiont of tsetse flies, *Wigglesworthia morsitans*: chorismate derived folate (Vitamin B9) biosynthesis by *Wigglesworthia* was inhibited by glyphosate, resulting in delayed larval development[24]. Furthermore, glyphosate exposure was found to have a detrimental effect on the honey bee gut microbiota, which translated into increased mortality of the honeybees under pathogen pressure[1,25,26,70,89]. Concordantly, our results on an intracellular beetle symbiosis show that exposure to agronomically applied[90] or previously tested glyphosate levels[24,25] decreases symbiont titers and recapitulates cuticular phenotypes of aposymbiotic beetles, indicating that the inhibition of the symbiont's shikimate pathway results in aromatic amino acid starvation of both host and symbiont. Aromatic amino acid supplementation to glyphosate-exposed beetles partially rescued the host phenotype, but not the symbiont titers, indicating that the host uses the dietary tyrosine preferentially or exclusively for its own supply.

Interestingly, aromatic amino acid supplementation to symbiotic beetles suppressed the establishment of the symbiont population in the host to a similar level as glyphosate, suggesting the either the symbionts cannot grow in the absence of self-produced precursors for aromatic amino acids or the possibility that, though speculative, the host may sanction its symbionts when the latter's nutrient supplementation is no longer needed. While this phenomenon has been documented in some dynamic partnerships of plants and their root-associated mycorrhizae and rhizobia[91,92], it seems surprising in an intimate and co-evolved mutualism that may have been expected to show little potential for conflict between the partners[72]. Conceivably, however, symbiont titers may be regulated by the host based on tyrosine or L-DOPA concentrations during

normal host development, resulting in symbiont suppression in times of high tyrosine availability.

The use of glyphosate in agriculture is currently heavily debated, based on increasing evidence for its detrimental impact on animals due to symbiont depletion[25,26,89,93] or inhibition of cuticle melanisation[22]. Our findings on the glyphosate susceptibility of a beetle via its prephenate-supplementing endosymbiont exacerbate these concerns, particularly when considering our predictions based on an EPSPS phylogeny that many insects harbour endosymbionts susceptible to glyphosate. Whether insects whose symbionts provision tyrosine or its precursors among other amino acids or vitamins[28,65,74,94] equally suffer from glyphosate exposure needs to be experimentally tested and will sharpen our predictions. However, the widespread occurrence of nutritional endosymbionts relying on shikimate pathway-derived nutrients paints an alarming picture and suggests that glyphosate application holds a tremendous risk of severe ecological impacts. Especially in light of recent declines in the number and diversity of insects[56,95,96] and its impact on higher trophic levels[97–101], the use of herbicides with potential side-effects on animals or their associated microorganisms should be carefully reconsidered.

2.5 Material & Methods

2.5.1 Insect cultures

The initial *Oryzaephilus surinamensis* culture (strain JKI) was obtained from the Julius-Kühn-Institute/Federal Research Centre for Cultivated Plants (Berlin, Germany) in 2014 and kept in culture since then. Continuous symbiotic and aposymbiotic (see below) *O. surinamensis* cultures were maintained in 1.8-L plastic containers, filled with 50 g oat flakes, at 28°C, 60% relative humidity and a day and night cycles of 16 to 8 hours. Another *O. surinamensis* population (strain OsNFRI) was obtained from the National Food Research Institute (Tsukuba, Japan) and used for genome sequencing.

2.5.2 Elimination of *O. surinamensis* symbionts

An *O. surinamensis* sub-population was treated for twelve weeks with tetracycline to eliminate their symbionts and then kept for several generations on a normal diet to exclude direct effects of tetracyclin on the host physiology[42]. Before the following experiments the aposymbiotic status of this beetle sub-population was confirmed. Therefore, 10 female adult beetles were individually separated in a single jar with oat flakes to lay eggs, as were symbiotic beetles in parallel populations. After four weeks, the adult generation was removed before their offspring finished metamorphosis and DNA of these females extracted and the symbiont titer was analyzed by quantitative PCR (see below; [42]).

2.5.3 Symbiont genome sequencing, assembly, and annotation

Total DNA was isolated from 20 pooled adult abdomina (without wings) of *O. surinamensis* JKI using the Epicentre MasterPure™ Complete DNA and RNA Purification Kit (Illumina Inc., Madison, WI, USA) including RNase digestion. Short-read library preparation and sequencing was performed at the Max-Planck-Genome-centre Cologne, Germany (SRR12881563 - SRR12881566) on a HiSeq2500 Sequencing System (Illumina Inc., Madison, WI, USA). Two further libraries library were created from *O. surinamensis* strain OsNFRI. For the first library the DNA was extracted by QIAamp DNA Mini Kit (Qiagen, Germany) from 210 bacteriomes dissected from 60 adults. The library was prepared using the Nextera XT DNA Library Preparation Kit (Illumina Inc., Madison, WI, USA) and sequenced on a MiSeq (Illumina Inc., Madison, WI, USA) of AIST (Japan). For the second library the DNA was extracted by QIAamp DNA Micro Kit (Qiagen, Germany) from 24 bacteriomes dissected from 6 adults (uncounted).

The library was prepared using the Nextera DNA Library Preparation Kit (Illumina Inc., Madison, WI, USA) and sequenced on a NovaSeq 6000 (Illumina Inc., Madison, WI, USA) of Novagen (China). Adaptor and quality trimming was performed with Trimmomatic[102]. In addition, we used two publicly available metagenome libraries of *O. surinamensis* (SRR5279855 and SRR6426882).

Long-read sequencing (SRR12881567 - SRR12881568) was performed on a MinION Mk1B Sequencing System (Oxford Nanopore Technologies (ONT), Oxford, UK). Upon receipt of flowcells, and again immediately prior to sequencing, the number of pores on flowcells was measured using the MinKNOW software (v18.12.9 and 19.05.0, ONT, Oxford, UK). Flowcells were replaced into their packaging, sealed with parafilm and tape, and stored at 4°C until use. Library preparation was performed with the Ligation Sequencing Kit (SQK-LSK109, ONT, Oxford, UK) and completed libraries were loaded on a flowcell (FLO-MIN106D, ONT, Oxford, UK) following the manufacturer's instructions.

Quality-controlled long reads were mapped using a custom-made kraken2 database containing the publicly available genomes of Bacteroidota bacteria[103,104] to filter beetle-associated sequences using the supercomputer Mogon of the Johannes Gutenberg-University (Mainz, Germany). Hybrid assembly of MinION and Illumina reads was performed using SPAdes (v3.13.0) with the default settings[105]. This resulted in ~70,000 contigs that were then binned using BusyBee Web[106], screened for GC content and taxonomic identity to Bacteroidota bacteria, and additionally checked manually for tRNAs and ribosomal proteins of Bacteroidota bacteria. In total, 13 contigs were extracted, which were then automatically annotated with RAST[107] using the app *Annotate Microbial Assembly* (RAST_SDK v0.1.1) on KBase[108]. The annotated contigs were plotted using CIRCOS[109] (v0.69-6) for the visualisation of gene locations, GC content and coverage. Additionally, the completeness of the obtained genome was assessed with the app *Assess Genome Quality with CheckM - v1.0.18* in KBase[45].

2.5.4 Phylogenetic analyses

A phylogenetic tree for placement of the intracellular symbiont of *O. surinamensis* within the Bacteroidota was reconstructed using the KBase app *Insert Set of Genomes Into Species Tree* v2.1.10 (SpeciesTreeBuilder v0.0.12) based on the FastTree2 algorithm[110], including 49 highly conserved Clusters of Orthologous Groups (COG) genes[111].

A phylogenetic tree of the *aroA* gene (which codes for the EPSPS enzyme in the shikimate pathway) from the symbiont of *O. surinamensis* to predict its sensitivity to glyphosate was performed according to Motta et al.[25]. Manually selected *aroA* sequences from plants, gut bacteria as well as several intracellular insect symbionts were obtained from Uniprot (UniProt Consortium 2019), translated and aligned using MUSCLE[112] (v3.8.425) implemented in Geneious Prime 2019 (v2019.1.3, https://www.geneious.com). Phylogenetic reconstruction was performed with FastTree[110] (v2.1.12) and PhyML[113] (v2.2.4) implemented in Geneious Prime 2019 (v2019.1.3, https://www.geneious.com) using the Jones-Taylor-Thorton model with 20 rate categories and an optimized Gamma20 likelyhood (FastTree) and 1000 bootstrap replicates (PhyML). The obtained trees were visualized using FigTree (v1.4.4, http://tree.bio.ed.ac.uk/software/figtree/).

2.5.5 Comparison of bacteria

Previously published Bacteroidota genomes were re-annotated with RAST[107,114] in KBase[108] to compare the bacteria and to estimate the genome-wide nucleotide sequence divergence level. Therefore, we identified single-copy orthologs in each genome pair using OrthoMCL[115] (v2.0) in KBase. KEGG categories were then assessed via GhostKOALA[116] (v2.2) of each gene's amino acid sequence. Heatmaps were visualized using the 'ComplexHeatmap' package in RStudio (V1.1.463 with R V3.6.3). CIRCOS[109] (v0.69-6) was used to link orthologous genes.

Genomes of Bacteroidota bacteria and other bacteria described as cuticle supplementing symbionts were compared in KBase[108] in more detail. Therefore, all genomes were re-annotated with RAST[107] and used to classify all annotated genes according to the SEED Subsystem[117] using the app *View Function Profile for Genomes* (v1.4.0, SEED Functional Group: Amino Acids and Derivatives). The resulting raw count of genes with annotation was visualized as a heatmap using the function 'heatmap.2' in the 'ggplot' package in RStudio (V1.1.463 with R V3.6.3).

2.5.6 Glyphosate and aromatic amino acid supplementation

Eight treatments were prepared to assess the supplementation of chorismate by the symbiont to the host (Table 2). For each treatment, jars were filled with 5 g finely ground oat flakes and different combinations of aromatic amino acids (1 w/w% of each L-tyrosine, L-tryptophan and L-phenylalanine; Sigma-Aldrich, Germany) and glyphosate (0.1 w/w% or 1 w/w%; Sigma-Aldrich, Germany).

Table 2: Experimental treatments for assessing impact of symbiont elimination, glyphosate exposure, and dietary aromatic amino acid supplementation on cuticle traits and symbiont titers in *O. surinamensis*.

	 SYM	 APO	 1% AROMATIC AMINO ACIDS	 0.1% GLYPHOSATE	 1% GLYPHOSATE
A	x				
B	x			x	
C	x				x
D	x		x		
E	x			x	x
F	x		x		x
G		x			
H		x	x		

The glyphosate concentration of 0.1% and 1% (or 0.059 and 0.0059 mmol/g) was chosen based on the experiment by Snyder and Rio[24] on tsetse flies. The 10 and 20 mM glyphosate added to the tsetse flies bloodmeal correspond to 0.1595 and 0.319% w/w glyphosate based on a blood density of 1.06 g/cm^3. Helander et al report 250 mg per 48 L of soil to be equivalent to the maximum recommended amount of glyphosate for agronomical applications which translates to 0.0004% based on a fertile soil density of 1.3 g/cm^3[90].

Food with the different supplements was weighed, mixed with distilled water, and dried overnight at 50°C in an incubator. Afterwards, the dry material was ground and filled into the jars, before 50 adult apo- or symbiotic *O. surinamensis* with undefined sex were added to each jar to lay eggs. After four weeks of incubation at standard conditions mentioned above, the adult beetles were removed, and the jars checked daily for adult offspring. Freshly hatched beetles were isolated in a 48-well plate with the corresponding manipulated diet and developed for seven days until cuticle biosynthesis is largely completed in symbiotic beetles[43]. Then the beetles were stored at -80°C for DNA extraction or fixated in 4% paraformaldehyde in PBS for histological analysis[118].

2.5.7 Quantitative PCR

DNA of 8 to 10 adult beetle abdomina (without wings) per treatment group (symbiotic and aposymbiotic parent generations and diet supplementation treatments), respectively, was isolated using the Epicentre MasterPure™ Complete DNA and RNA Purification Kit following the manufacturer's instruction (Illumina Inc., USA) to confirm infection status and evaluate the impact of glyphosate and amino acid addition on symbiont titer. Bacterial 16S rRNA copies were quantified via quantitative PCR (qPCR) from single adult abdomina of *O. surinamensis*. DNA was dissolved in 30 µL low TE buffer (1:10 dilution of 1x TE buffer: 10 mM Tris-HCl + 1 mM EDTA). qPCRs were carried out in 25 µl reactions using EvaGreen (Solis BioDyne, Estonia), including 0.5 µM of each primer and 1 µl template DNA. All reagents were mixed, vortexed and centrifugated in 0.1-mL reaction tubes (Biozym, 711200). To amplify a symbiont specific 16S rRNA gene fragment the forward OsurSym_fwd2 (5'-GGCAACTCTGAACTAGCTACGC-3') and reverse mod.CFB563_rev (5'-GCACCCTTTAAACCCAAT-3') primers were used[42]. qPCR was carried out in a Rotor-Gene Q thermal cycler (Qiagen, Germany).

Standard curves with defined copy numbers of the 16S rRNA gene were created by amplifying the fragment first, followed by purification and determination of the DNA concentration via NanoDrop1000 (Peqlab, Germany). After determination of the DNA concentration, a standard containing 10^{10} copies/µL was generated and 1:10 serial dilutions down to 10^{1} copies/µL. 1 µL of each standard was included in a qPCR reaction to standardize all measurements.

Influence of amino acids and glyphosate on the symbiont titer of the adult beetles was analyzed using Dunn's test from the package 'FSA' in RStudio (V 1.1.463 with R V3.6.3) with two-sided, for multiple testing corrected post-hoc tests using the Benjamini-Hochberg method[119]. Plots were visualized using 'ggplot2'.

2.5.8 Analysis of cuticle traits

First, we determined melanisation single-blinded via the inverse of digital red color values of 8 to 10 beetles from each treatment group to evaluate the impact of glyphosate, aromatic amino acids and symbiont elimination on cuticle formation. Photographs were taken with Zeiss StereoDiscovery V8 dissection stereoscope (Zeiss, Germany) under identical conditions using ZENCore software (Zeiss, Germany). Average red values were measured within a circular area covering the thorax with Natsumushi[120] and transformed into inverse red values[16,42].

Further, we measured cuticle thickness single-blinded of 8 to 10 adult beetles per treatment group, which were fixated in 4% paraformaldehyde in PBS. These beetles were embedded in epoxy resin (Epon_812 substitute; Sigma-Aldrich, Germany), and 1 µm cross-sections of the thorax next to the second pair of legs were cut on a microtome (Leica RM2245, Wetzlar, Germany) with a diamond blade and mounted on silanised glass slides with Histokitt (Roth, Germany). Images to measure cuticle diameter were taken with an AxioImager Z2 (Zeiss, Germany) at 200x magnification and differential interference contrast. Mean cuticle diameter was measured at one randomly chosen dorsal, ventral and lateral position, respectively, with the ZEN 2 Blue software distance tool (v2.0.0.0, Zeiss, Germany).

Influence of aromatic amino acids and glyphosate on the thickness and melanisation of the adult beetles was analysed by Dunn's test from the package 'FSA' in RStudio (V1.1.463 with R V3.6.3) with two-sided, for multiple testing corrected post-hoc tests using the Benjamini-Hochberg method[119]. Plots were visualized using 'ggplot2'.

2.5.9 Amino acid extraction

For both aposymbiotic and symbiotic *O. surinamensis* beetles, eight individuals for each of five different developmental stages were analysed: 5th instar larvae (of random age), one-day-old pupae, five-day-old pupae, one-day-old adults, and seven-day-old adults. Aged individuals were obtained by separating 5th instar larvae individually into a 48-well plate coated with Fluon (AGC Chemicals, UK) to avoid beetles to escape, filled with three oat flakes for provision, and daily monitoring for pupation or emergence of adults. Once they reached the desired age, individuals were frozen until further analysis. Adults had their elytrae removed to analyse them separately and both were dried overnight at 50°C.

The following extraction, derivatisation, and analysis procedure of amino acids (AAs) were adapted from Perez-Palacios et al.[121]. For the extraction of free AA in larvae, pupae, and elytra-free adults, three stainless steel beads and 500 µL 0.1 M HCl (Merck, Germany) were added to each insect sample and homogenized in a mixer mill MM200 (Retsch, Germany) at 30 Hz for 6 min. Subsequently, samples were centrifuged at 5,000 rpm for 15 minutes at 4°C using a CT15RE microcentrifuge (Himac, Japan) and the supernatant transferred to glass vials. Pellets were kept for extraction of bound AA (see below), supernatants containing free AA were mixed with 300 µL Acetonitril (Carl-Roth, Germany) for deproteinisation and 5 µL L-norleucine (2.5 µmol/mL;

Sigma, Germany), which was used as an internal standard. Following centrifugation at 10,000 rpm for 3 min, samples were transferred to new vials and dried in a Savant DNA 110 SpeedVac Concentrator (Thermo Fisher Scientific, Germany). For the extraction of protein-bound AAs, the aforementioned pellets of the remaining body were resuspended in 500 µL 0.1 M HCl and transferred to separate glass vials. Similarly, dissected elytra were homogenized as described above and transferred to separate glass vials. After these samples were dried, 12 open vials (Kimble, USA) were placed in a larger hydrolysis vial (Kimble, USA) and a mixture of 187.5 µL HPLC grade water (Carl-Roth, Germany), 62.5 µL saturated phenol solution (84 g/L, Carl-Roth, Germany) and 250 µL 12 M HCl was added for gas-phase derivatisation to the bottom of the hydrolysis vial. To ensure oxygen-free atmosphere the hydrolysis vial was three times evacuated and aerated with argon and afterwards tightly sealed. The hydrolysis was performed for 24 hours at 110°C. After equilibration to room temperature, 200 µL HCl were added to the single sample vials, the supernatant transferred to a new GC vial and the samples dried again for 10 minutes in a Speedvac evaporator (Thermo Scientific, Germany).

2.5.10 Derivatisation of amino acids

Derivatisation is needed to analyse amino acid via gas chromatography-mass spectrometry (GC-MS). N-tert-butyldimethylsilyl-N-methyltriflouroacetamid (MTBSTFA, Sigma-Aldrich, Germany) was utilized to silylate amino acids[121]: 50 µL MTBSTFA and 50 µL acetonitrile were added to all dried extracts of bound and free AA, followed by derivatisation at 100°C for 1 hour. Also, a mixture of 17 AA (each 25 µmol/L: L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine, L-valine; and 12.5 µmol/L L-cysteine, Sigma-Aldrich, Germany) was prepared and derivatised, also including L-norleucine as an internal standard to identify single amino acid derivatives. 1 µL was used for subsequent analysis.

2.5.11 Amino acid analysis with gas chromatography-mass spectrometry (GC-MS)

GC-MS analysis was performed with a Varian 240MS ion trap mass spectrometer coupled to a Varian 450 gas chromatograph (Agilent, USA), using external ionisation for all analyses and splitless injection with 280°C injector temperature. Initial temperature of the GC oven was 100°C for 2 min, followed by steady increase by 25°C per minute up to 300°C and final isothermal hold

for 5 min. The carrier gas helium had a constant flow of 1 mL/min through a DB-5ms column (30 m x 2.25 mm ID, 0.25 µm film Agilent, USA). Electron impact spectra were recorded with the ion source at 160°C and ion trap at 90°C and analyzed using the Varian MS Workstation Version 6.9.3. AA derivatives were identified using the fragmentation patterns and retention times according to Pérez-Palacios et al.[121] and the external standards. Quantification was carried out with two to four fragment ions to increase signal to noise ratio (L-alanine m/z = 158 & 232 & 260 & 428; L-arginine m/z = 199 & 442; L-aspartic acid m/z = 302 & 316 & 390 & 418; L-cystine m/z = 58 & 341 & 442; L-glutamic acid m/z = 272 & 286 & 359 & 432; glycine m/z = 189 & 218 & 246; L-histidine m/z = 196 & 280.5 & 338.5 & 440.5, L-isoleucine m/z = 200 & 274 & 302; L-leucine m/z = 200 & 274 & 302; L-lysine m/z = 198 & 272 & 300; L-methionine m/z = 218 & 292 & 320; L-norleucine m/z = 200 & 274 & 302; L-phenylalanine m/z = 234 & 392 & 308 & 336; L-proline m/z = 184 & 258 & 286; L-serine m/z = 288 & 362 & 390; L-threonine m/z = 303 & 376 & 404; L-tyrosine m/z = 302 & 364 & 438 & 466; L-valine m/z = 186 & 260 & 288).

The single amino acid measurements were first normalized by the internal standard to account for deviations in amount or concentration during the analysis procedure and further normalized by the total amount of all amino acids to account for differences in body size between life stages but also animals with and without symbionts. The single normalized amino acid measurements were transformed to approximate Gaussian distribution. The transformation was chosen using the powerTransform command from the package 'car' (alanin: negative square root, arginine: cubic root, aspartic acid: decadic logarithm, glutamic acid: square root, glycine: cubic root, isoleucine: decadic logarithm, leucine: decadic logarithm, lysine: square root, ornithine: square root, phenylalanine: square root, proline: decadic logarithm, serine: cubic root, threonine: decadic logarithm, tyrosine: square root, valine: cubic root; the amino acids cystine, histidine and methionine were not detected).

Symbiont influence on the titer of the single amino acids was analysed with separate generalized linear models with life stage (larva, two pupa stages, two adult stages), amino acid source (free amino acids in body, protein-bound amino acids in body, total amino acids in elytrae) and symbiont presence/absence as factors allowing for interactions between all of them using the command glm from the 'stats' package in R Studio (V1.1.463 with R V3.6.3). P-values were

corrected for multiple testing following the classical Bonferroni[122]. In case of significant symbiont influence, we conducted pairwise post-hoc tests between symbiotic and aposymbiotic samples for each life stage/amino acid source using unpaired Wilcoxon rank-sum tests including Bonferroni correction[122] to identify the specific life stage/amino acid source of symbiont influence. Plots were visualized using 'ggplot2'.

2.5.12 Data availability

Sequencing libraries and the assembles genome of the *Oryzaephilus surinamensis* symbiont (proposed *Candidatus* Shikimatogenerans silvanidophilus OSUR) were uploaded to the DNA Databank of Japan (accession numbers DRA010986 and DRA010987), the NCBI Sequence Read Archive (accession numbers SRR12881563 - SRR12881566) and Genbank (JADFUB000000000). Raw data of quantitative cuticle measurements are available at the data repository of the Max Planck Society 'Edmond'[123].

2.6 Data Accessibility Statement

Sequencing libraries and the assembles genome of the *Oryzaephilus surinamensis* symbiont (proposed *Candidatus* Shikimatogenerans silvanidophilus OSUR) were uploaded to the DNA Databank of Japan (accession numbers DRA010986 and DRA010987), the NCBI Sequence Read Archive (accession numbers SRR12881563–SRR12881566) and GenBank (JADFUB000000000). Raw data of quantitative cuticle measurements are available at the data repository of the Max Planck Society 'Edmond'[123].

2.7 Acknowledgments

The authors thank Dagmar Klebsch and Rebekka Janke for valuable technical assistance, Cornel Adler for the original provisioning of an *O. surinamensis* culture, John McCutcheon and Piotr Lukasik for their initial input on our genome sequencing approach, the Johannes Gutenberg University Mainz for computation time granted on the supercomputer Mogon, Christian Meesters' administrative assistance on Mogon and Minoru Moriyama's support for genome sequencing. The authors further acknowledge the financial support of the Johannes Gutenberg University Mainz (intramural funding to T.E.), a Consolidator Grant of the European Research Council (ERC CoG 819585 "SYMBeetle" to M.K.), the Japan Science and Technology Agency ERATO Grant (JPMJER1803 and JPMJER1902 to T.F.), the Japan Society for the Promotion of Science KAKENHI Grant (20J13769 to B.H.), and the Max-Planck-Society (to T.E., B.W., and M.K.). T.E. also acknowledges the stimulating International Symbiosis Society meeting 2018 in Oregon, especially inspiring presentations by Rita Rio and Joel Sachs.

2.8 Contributions

T.E. and M.K. conceived the study. J.S.T.K., E.B., B.H. and T.E. sequenced and assembled the symbiont genome. J.S.T.K. and E.B. annotated the genomes and performed symbiont genomic analysis and J.S.T.K. and T.E. performed phylogenetic analyses of the symbionts. S.B., J.C.W., and T.E. performed amino acid analysis. J.K. and B.W. performed dietary supplementation experiments and analyses. J.S.T.K. and T.E. wrote the paper, with input from M.K. and T.F. All authors read and commented on the manuscript.

2.9 References

1. Sikorski, J. A. & Gruys, K. J. Understanding glyphosate's molecular mode of action with EPSP synthase: evidence favoring an allosteric inhibitor model. *Acc. Chem. Res.* **30**, 2–8 (1997).
2. Duke, S. O. & Powles, S. B. Glyphosate: a once-in-a-century herbicide. *Pest Manag. Sci.* **64**, 319–325 (2008).
3. Siehl, D. L. Inhibitors of EPSP synthase, glutamine synthetase and histidine synthesis. in *Herbicide Activity: Toxicology, Biochemistry and Molecular Biology* (eds. R. Michael Roe, Burton, J. D. & Kuhr, R. J.) vol. 1 37 (IOS Press, Amsterdam, 1997).
4. Shilo, T., Zygier, L., Rubin, B., Wolf, S. & Eizenberg, H. Mechanism of glyphosate control of *Phelipanche aegyptiaca*. *Planta* **244**, 1095–1107 (2016).
5. Tzin, V. & Galili, G. New Insights into the shikimate and aromatic amino acids biosynthesis pathways in plants. *Mol. Plant* **3**, 956–972 (2010).
6. McFall-Ngai, M. *et al.* Animals in a bacterial world, a new imperative for the life sciences. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 3229–3236 (2013).
7. Hacker, S. D. & Gaines, S. D. Some implications of direct positive interactions for community species diversity. *Ecology* **78**, 1990–2003 (1997).
8. van den Bosch, T. J. M. & Welte, C. U. Detoxifying symbionts in agriculturally important pest insects. *Microb. Biotechnol.* **10**, 531–540 (2017).
9. Lemoine, M. M., Engl, T. & Kaltenpoth, M. Microbial symbionts expanding or constraining abiotic niche space in insects. *Curr. Opin. Insect Sci.* **39**, 14–20 (2020).
10. Feldhaar, H. Bacterial symbionts as mediators of ecologically important traits of insect hosts. *Ecol. Entomol.* **36**, 533–543 (2011).
11. Moran, N. A. Symbiosis. *Curr. Biol.* **16**, R866–R871 (2006).
12. Moran, N. A. & Telang, A. Bacteriocyte-associated symbionts of insects. *Bioscience* **48**, 295–304 (1998).
13. Oliver, K. M. & Martinez, A. J. How resident microbes modulate ecologically-important traits of insects. *Curr. Opin. Insect Sci.* **4**, 1–7 (2014).
14. Douglas, A. E. The microbial dimension in insect nutritional ecology. *Funct. Ecol.* **23**, 38–47 (2009).
15. Douglas, A. E. The B vitamin nutrition of insects: the contributions of diet, microbiome and horizontally acquired genes. *Curr. Opin. Insect Sci.* **23**, 65–69 (2017).
16. Vigneron, A. *et al.* Insects recycle endosymbionts when the benefit is over. *Curr. Biol.* **24**, 2267–2273 (2014).
17. Andersen, S. O. Cuticular sclerotization and tanning. in *Insect Molecular Biology and Biochemistry* (ed. Gilbert, L. I.) 167–192 (Elsevier, Cambridge, 2012).
18. Anbutsu, H. & Fukatsu, T. Symbiosis for insect cuticle formation. in *Cellular Dialogues in the Holobiont* (eds. Bosch, T. C. G. & Hadfield, M. G.) 201–216 (CRC Press, Boca Raton, 2020).
19. Anbutsu, H. *et al.* Small genome symbiont underlies cuticle hardness in beetles. *Proc. Natl. Acad. Sci.* **114**, E8382–E8391 (2017).
20. Li, A. P. & Long, T. J. An evaluation of the genotoxic potential of glyphosate. *Fundam. Appl. Toxicol.* **10**, 537–546 (1988).
21. Smith, E. A. & Oehme, F. W. The biological activity of glyphosate to plants and animals: a literature review. *Vet. Hum. Toxicol.* **34**, 531–543 (1992).
22. Smith, D. F. Q. *et al.* Glyphosate inhibits melanization and increases insect susceptibility to infection. *bioRxiv* (2020).
23. Torretta, V., Katsoyiannis, I., Viotti, P. & Rada, E. Critical review of the effects of glyphosate exposure to the environment and humans through the food supply chain. *Sustainability* **10**, 950 (2018).
24. Snyder, A. K. & Rio, R. V. M. "*Wigglesworthia morsitans*" folate (Vitamin B_9) biosynthesis contributes to tsetse host fitness. *Appl. Environ. Microbiol.* **81**, 5375–5386 (2015).
25. Motta, E. V. S., Raymann, K. & Moran, N. A. Glyphosate perturbs the gut microbiota of honey bees. *Proc. Natl. Acad. Sci.* **115**, 10305–10310 (2018).
26. Motta, E. V. S. *et al.* Oral or topical exposure to glyphosate in herbicide formulation impacts the gut microbiota and survival rates of honey bees. *Appl. Environ. Microbiol.* **86**, (2020).
27. Klein, A. *et al.* A novel intracellular mutualistic bacterium in the invasive ant *Cardiocondyla obscurior*. *ISME J.* **10**, 376–388 (2016).
28. Wu, D. *et al.* Metabolic complementarity and genomics of the dual bacterial symbiosis of sharpshooters. *PLoS Biol.* **4**, e188 (2006).
29. Dunne, J. A. & Williams, R. J. Cascading extinctions and community collapse in model food webs. *Philos. Trans. R. Soc. B Biol. Sci.* **364**, 1711–1723 (2009).

30. Dunne, J. A., Williams, R. J. & Martinez, N. D. Network structure and biodiversity loss in food webs: robustness increases with connectance. *Ecol. Lett.* **5**, 558–567 (2002).
31. Memmott, J. *et al.* Biodiversity Loss and Ecological Network Structure. in *Ecological networks: Linking structure to dynamics in food webs* (eds. Pascual, M. & Dunne, J. A.) 325–347 (Oxford University Press, New York, 2005).
32. Liao, C., Upadhyay, A., Liang, J., Han, Q. & Li, J. 3,4-Dihydroxyphenylacetaldehyde synthase and cuticle formation in insects. *Dev. Comp. Immunol.* **83**, 44–50 (2018).
33. Muthukrishnan, S., Merzendorfer, H., Arakane, Y. & Kramer, K. J. Chitin metabolism in insects. in *Insect Molecular Biology and Biochemistry* (ed. Gilbert, L. I.) 193–235 (Elsevier, Cambridge, 2012).
34. Wirtz, R. A. & Hopkins, T. L. Tyrosine and phenylalanine concentrations in haemolymph and tissues of the American cockroach, *Periplaneta americana*, during metamorphosis. *J. Insect Physiol.* **20**, 1143–1154 (1974).
35. Gibbs, A. G. & Rajpurohit, S. Cuticular lipids and water balance. in *Insect Hydrocarbons* (eds. Blomquist, G. J. & Bagneres, A.-G.) 100–120 (Cambridge University Press, Cambridge, 2010).
36. Hackman, R. H. Chemistry of the insect cuticle. in *The Physiology of Insecta* (ed. Rodstein, M.) 215–270 (Academic Press, New York and London, 1974).
37. Mattson, W. J. Herbivory in relation to plant nitrogen content. *Annu. Rev. Ecol. Syst.* **11**, 119–161 (1980).
38. Kumar, V. *et al.* Amino acids distribution in economical important plants: a review. *Biotechnol. Res. Innov.* **3**, 197–207 (2019).
39. Noh, M. Y., Muthukrishnan, S., Kramer, K. J. & Arakane, Y. Cuticle formation and pigmentation in beetles. *Curr. Opin. Insect Sci.* **17**, 1–9 (2016).
40. Sterkel, M. *et al.* Tyrosine detoxification is an essential trait in the life history of blood-feeding arthropods. *Curr. Biol.* **26**, 2188–2193 (2016).
41. Herrmann, K. M. & Weaver, L. M. The shikimate pathway. *Annu. Rev. Plant Biol.* **50**, 473–503 (1999).
42. Engl, T. *et al.* Ancient symbiosis confers desiccation resistance to stored grain pest beetles. *Mol. Ecol.* **27**, 2095–2108 (2018).
43. Hirota, B. *et al.* A novel, extremely elongated, and endocellular bacterial symbiont supports cuticle formation of a grain pest beetle. *MBio* **8**, 1–16 (2017).
44. Boyer, S., Zhang, H. & Lempérière, G. A review of control methods and resistance mechanisms in stored-product insects. *Bull. Entomol. Res.* **102**, 213 (2012).
45. Parks, D. H., Imelfort, M., Skennerton, C. T., Hugenholtz, P. & Tyson, G. W. CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Res.* **25**, 1043–1055 (2015).
46. Moran, N. A., McCutcheon, J. P. & Nakabachi, A. Genomics and evolution of heritable bacterial symbionts. *Annu. Rev. Genet.* **42**, 165–190 (2008).
47. McCutcheon, J. P. & Moran, N. A. Extreme genome reduction in symbiotic bacteria. *Nat. Rev. Microbiol.* **10**, 13–26 (2012).
48. Van Leuven, J. T., Meister, R. C., Simon, C. & McCutcheon, J. P. Sympatric speciation in a bacterial endosymbiont results in two genomes with the functionality of one. *Cell* **158**, 1270–1280 (2014).
49. Campbell, M. A., Łukasik, P., Simon, C. & McCutcheon, J. P. Idiosyncratic genome degradation in a bacterial endosymbiont of periodical cicadas. *Curr. Biol.* **27**, 3568-3575.e3 (2017).
50. Campbell, M. A. *et al.* Genome expansion via lineage splitting and genome reduction in the cicada endosymbiont *Hodgkinia*. *Proc. Natl. Acad. Sci.* **112**, 10192–10199 (2015).
51. Chen, Y. C., Liu, T., Yu, C. H., Chiang, T. Y. & Hwang, C. C. Effects of GC bias in next-generation-sequencing data on *de novo* genome assembly. *PLoS One* **8**, e62856 (2013).
52. Kozarewa, I. *et al.* Amplification-free Illumina sequencing-library preparation facilitates improved mapping and assembly of (G+ C)-biased genomes. *Nat. Methods* **6**, 291–295 (2009).
53. Quail, M. A. *et al.* A tale of three next generation sequencing platforms: comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq sequencers. *BMC Genomics* **13**, 1–13 (2012).
54. Treangen, T. J. & Salzberg, S. L. Repetitive DNA and next-generation sequencing: computational challenges and solutions. *Nat. Rev. Genet.* **13**, 36–46 (2012).
55. Sloan, D. B. *et al.* Parallel histories of horizontal gene transfer facilitated extreme reduction of endosymbiont genomes in sap-feeding insects. *Mol. Biol. Evol.* **31**, 857–871 (2014).
56. Zucko, J. *et al.* Global genome analysis of the shikimic acid pathway reveals greater gene loss in host-associated than in free-living bacteria. *BMC Genomics* **11**, 628 (2010).
57. Tokuda, G. *et al.* Maintenance of essential amino acid synthesis pathways in the *Blattabacterium cuenoti* symbiont of a wood-feeding cockroach. *Biol. Lett.* **9**, 20121153 (2013).

58. Kinjo, Y. *et al.* Parallel and gradual genome erosion in the *Blattabacterium* endosymbionts of *Mastotermes darwiniensis* and *Cryptocercus* Wood Roaches. *Genome Biol. Evol.* **10**, 1622–1630 (2018).
59. Menzel, R. & Roth, J. Purification of the putA gene product. A bifunctional membrane-bound protein from Salmonella typhimurium responsible for the two-step oxidation of proline to glutamate. *J. Biol. Chem.* **256**, 9755–9761 (1981).
60. Zhou, Y., Zhu, W., Bellur, P. S., Rewinkel, D. & Becker, D. F. Direct linking of metabolism and gene expression in the proline utilization a protein from *Escherichia coli*. *Amino Acids* **35**, 711–718 (2008).
61. Sabree, Z. L., Kambhampati, S. & Moran, N. A. Nitrogen recycling and nutritional provisioning by *Blattabacterium*, the cockroach endosymbiont. *Proc. Natl. Acad. Sci.* **106**, 19521–19526 (2009).
62. McCutcheon, J. P., McDonald, B. R. & Moran, N. A. Convergent evolution of metabolic roles in bacterial co-symbionts of insects. *Proc. Natl. Acad. Sci.* **106**, 15394–15399 (2009).
63. Sabree, Z. L., Huang, C. Y., Okusu, A., Moran, N. A. & Normark, B. B. The nutrient supplying capabilities of *Uzinura*, an endosymbiont of armoured scale insects. *Environ. Microbiol.* **15**, 1988–1999 (2013).
64. Rosas-Pérez, T., Rosenblueth, M., Rincón-Rosales, R., Mora, J. & Martínez-Romero, E. Genome sequence of "*Candidatus* Walczuchella monophlebidarum" the Flavobacterial endosymbiont of *Llaveia axin axin* (Hemiptera: Coccoidea: Monophlebidae). *Genome Biol. Evol.* **6**, 714–726 (2014).
65. Kuriwada, T. *et al.* Biological role of *Nardonella* endosymbiont in its weevil host. *PLoS One* **5**, e13101 (2010).
66. Okude, G. *et al.* Novel bacteriocyte-associated pleomorphic symbiont of the grain pest beetle *Rhyzopertha dominica* (Coleoptera: Bostrichidae). *Zool. Lett.* **3**, 13 (2017).
67. Hirota, B., Meng, X.-Y. & Fukatsu, T. Bacteriome-associated endosymbiotic bacteria of nosodendron tree sap beetles (Coleoptera: Nosodendridae). *Front. Microbiol.* **11**, 2556 (2020).
68. Hopkins, T. L. & Kramer, K. J. Insect cuticle sclerotization. *Annu. Rev. Entomol.* **37**, 273–302 (1992).
69. Andersen, S. O. Insect cuticular sclerotization: A review. *Insect Biochem. Mol. Biol.* **40**, 166–178 (2010).
70. Cao, G. *et al.* A novel 5-enolpyruvylshikimate-3-phosphate synthase shows high glyphosate tolerance in *Escherichia coli* and tobacco plants. *PLoS One* **7**, e38718 (2012).
71. Moran, N. A. & Bennett, G. M. The tiniest tiny genomes. *Annu. Rev. Microbiol.* **68**, 195–215 (2014).
72. McCutcheon, J. P., Boyd, B. M. & Dale, C. The life of an insect endosymbiont from the cradle to the grave. *Curr. Biol.* **29**, R485–R495 (2019).
73. Salem, H. *et al.* Drastic genome reduction in an herbivore's pectinolytic symbiont. *Cell* **171**, 1520–1531 (2017).
74. Reis, F. *et al.* Bacterial symbionts support larval sap feeding and adult folivory in (semi-) aquatic reed beetles. *Nat. Commun.* **11**, 1–15 (2020).
75. Salem, H., Florez, L., Gerardo, N. & Kaltenpoth, M. An out-of-body experience: the extracellular dimension for the transmission of mutualistic bacteria in insects. *Proc. R. Soc. B Biol. Sci.* **282**, 20142957 (2015).
76. Salem, H. *et al.* Symbiont digestive range reflects host plant breadth in herbivorous beetles. *Curr. Biol.* **30**, 2875–2886 (2020).
77. Hansen, A. K., Pers, D. & Russell, J. A. Symbiotic solutions to nitrogen limitation and amino acid imbalance in insect diets. in *Mechanisms Underlying Microbial Symbiosis* (ed. Kerry M. Oliver, J. A. R.) vol. 58 161–205 (Academic Press, Cambridge, 2020).
78. Tanner, J. J. Structural biology of proline catabolism. *Amino Acids* **35**, 719–730 (2008).
79. Jiang, Z. *et al.* Comparative analysis of genome sequences from four strains of the *Buchnera aphidicola* Mp endosymbion of the green peach aphid, *Myzus persicae*. *BMC Genomics* **14**, 917 (2013).
80. Bursell, E. The role of proline in energy metabolism. in *Energy Metabolism in Insects* 135–154 (Springer, Boston, 1981).
81. Engl, T., Schmidt, T. H. P., Kanyile, S. N. & Klebsch, D. Metabolic cost of a nutritional symbiont manifests in delayed reproduction in a grain pest beetle. *Insects* **11**, 717 (2020).
82. José de Souza, D., Devers, S. & Lenoir, A. *Blochmannia* endosymbionts and their host, the ant *Camponotus fellah*: cuticular hydrocarbons and melanization. *C. R. Biol.* **334**, 737–741 (2011).
83. Zientz, E., Beyaert, I., Gross, R. & Feldhaar, H. Relevance of the endosymbiosis of *Blochmannia floridanus* and carpenter ants at different stages of the life cycle of the host. *Appl. Environ. Microbiol.* **72**, 6027–6033 (2006).
84. Oakeson, K. F. *et al.* Genome degeneration and adaptation in a nascent stage of symbiosis. *Genome Biol. Evol.* **6**, 76–93 (2013).
85. Chong, R. A. & Moran, N. A. Evolutionary loss and replacement of *Buchnera*, the obligate endosymbiont of aphids. *ISME J.* **12**, 898–908 (2018).
86. McCutcheon, J. P. & Moran, N. A. Functional convergence in reduced genomes of bacterial symbionts spanning 200 My of evolution. *Genome Biol. Evol.* **2**, 708–718 (2010).

87. Gerth, M., Gansauge, M. T., Weigert, A. & Bleidorn, C. Phylogenomic analyses uncover origin and spread of the *Wolbachia* pandemic. *Nat. Commun.* **5**, 1–7 (2014).
88. Santos-Garcia, D., Silva, F. J., Morin, S., Dettner, K. & Kuechler, S. M. The all-rounder *Sodalis*: a new bacteriome-associated endosymbiont of the *Lygaeoid* bug *Henestaris halophilus* (Heteroptera: Henestarinae) and a critical examination of its evolution. *Genome Biol. Evol.* **9**, 2893–2910 (2017).
89. Motta, E. V. S. & Moran, N. A. Impact of glyphosate on the honey bee gut microbiota: effects of intensity, duration, and timing of exposure. *Msystems* **5**, (2020).
90. Helander, M., Pauna, A., Saikkonen, K. & Saloniemi, I. Glyphosate residues in soil affect crop plant germination and growth. *Sci. Rep.* **9**, 19653 (2019).
91. Kiers, E. T., Rousseau, R. A., West, S. A. & Denlson, R. F. Host sanctions and the legume-rhizobium mutualism. *Nature* **425**, 78–81 (2003).
92. Whiteside, M. D., Digman, M. A., Gratton, E. & Treseder, K. K. Organic nitrogen uptake by arbuscular mycorrhizal fungi in a boreal forest. *Soil Biol. Biochem.* **55**, 7–13 (2012).
93. Faita, M. R., Cardozo, M. M., Amandio, D. T. T., Orth, A. I. & Nodari, R. O. Glyphosate-based herbicides and *Nosema sp.* microsporidia reduce honey bee (*Apis mellifera* L.) survivability under laboratory conditions. *J. Apic. Res.* 1–11 (2020).
94. Wilson, A. C. C. *et al.* Genomic insight into the amino acid relations of the pea aphid, Acyrthosiphon pisum, with its symbiotic bacterium Buchnera aphidicola. *Insect Mol. Biol.* **19**, 249–258 (2010).
95. Sánchez-Bayo, F. & Wyckhuys, K. A. G. Worldwide decline of the entomofauna: A review of its drivers. *Biol. Conserv.* **232**, 8–27 (2019).
96. Wagner, D. L. Insect declines in the anthropocene. *Annu. Rev. Entomol.* **65**, 457–480 (2020).
97. Desneux, N., Decourtye, A. & Delpuech, J.-M. The sublethal effects of pesticides on beneficial arthropods. *Annu. Rev. Entomol.* **52**, 81–106 (2007).
98. Goulson, D. The insect apocalypse, and why it matters. *Curr. Biol.* **29**, R967–R971 (2019).
99. Hallmann, C. A. *et al.* More than 75 percent decline over 27 years in total flying insect biomass in protected areas. *PLoS One* **12**, e0185809 (2017).
100. Hayes, T. B. & Hansen, M. From silent spring to silent night: agrochemicals and the anthropocene. *Elem. Sci. Anth.* **5**, (2017).
101. Bowler, D. E., Heldbjerg, H., Fox, A. D., Jong, M. & Böhning-Gaese, K. Long-term declines of European insectivorous bird populations and potential causes. *Conserv. Biol.* **33**, 1120–1130 (2019).
102. Bolger, A. M., Lohse, M. & Usadel, B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **30**, 2114–2120 (2014).
103. Wood, D. E. & Salzberg, S. L. Kraken: ultrafast metagenomic sequence classification using exact alignments. *Genome Biol.* **15**, 1–12 (2014).
104. Wood, D. E., Lu, J. & Langmead, B. Improved metagenomic analysis with Kraken 2. *Genome Biol.* **20**, 257 (2019).
105. Bankevich, A. *et al.* SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* **19**, 455–477 (2015).
106. Laczny, C. C. *et al.* BusyBee Web: metagenomic data analysis by bootstrapped supervised binning and annotation. *Nucleic Acids Res.* **45**, W171–W179 (2017).
107. Aziz, R. K. *et al.* The RAST Server: rapid annotations using subsystems technology. *BMC Genomics* **9**, 1–15 (2008).
108. Arkin, A. P. *et al.* KBase: The United States department of energy systems biology knowledgebase. *Nat. Biotechnol.* **36**, 566 (2018).
109. Krzywinski, M. *et al.* Circos: an information aesthetic for comparative genomics. *Genome Res.* **19**, 1639–1645 (2009).
110. Price, M. N., Dehal, P. S. & Arkin, A. P. FastTree 2–approximately maximum-likelihood trees for large alignments. *PLoS One* **5**, e9490 (2010).
111. Tatusov, R. L., Galperin, M. Y., Natale, D. A. & Koonin, E. V. The COG database: a tool for genome-scale analysis of protein functions and evolution. *Nucleic Acids Res.* **28**, 33–36 (2000).
112. Edgar, R. C. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **32**, 1792–1797 (2004).
113. Guindon, S. *et al.* New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst. Biol.* **59**, 307–321 (2010).
114. Brettin, T. *et al.* RASTtk: a modular and extensible implementation of the RAST algorithm for building custom annotation pipelines and annotating batches of genomes. *Sci. Rep.* **5**, 8365 (2015).

115. Li, L., Stoeckert, C. J. & Roos, D. S. OrthoMCL: identification of ortholog groups for eukaryotic genomes. *Genome Res.* **13**, 2178–2189 (2003).
116. Kanehisa, M., Sato, Y. & Morishima, K. BlastKOALA and GhostKOALA: KEGG tools for functional characterization of genome and metagenome sequences. *J. Mol. Biol.* **428**, 726–731 (2016).
117. Overbeek, R. *et al.* The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). *Nucleic Acids Res.* **42**, D206-14 (2014).
118. Weiss, B. & Kaltenpoth, M. Bacteriome-localised intracellular symbionts in pollen-feeding beetles of the genus *Dasytes* (Coleoptera, Dasytidae). *Front. Microbiol.* **7**, 1486 (2016).
119. Dunn, O. J. Multiple comparisons using rank sums. *Technometrics* **6**, 241–252 (1964).
120. Tanahashi, M. Natsumushi: Image measuring software for entomological studies. *Entomol. Sci.* **21**, 347–360 (2018).
121. Pérez-Palacios, T., Barroso, M. A., Ruiz, J. & Antequera, T. A rapid and accurate extraction procedure for analysing free amino acids in meat samples by GC-MS. *Int. J. Anal. Chem.* (2015).
122. Miller, R. G. *Simultaneous Statistical Inference*. (Springer, New York, 1981).
123. Engl, T. & Kiefer, J. S. T. Data from: Inhibition of a nutritional endosymbiont by glyphosate.
124. Seemann, T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* **30**, 2068–2069 (2014).

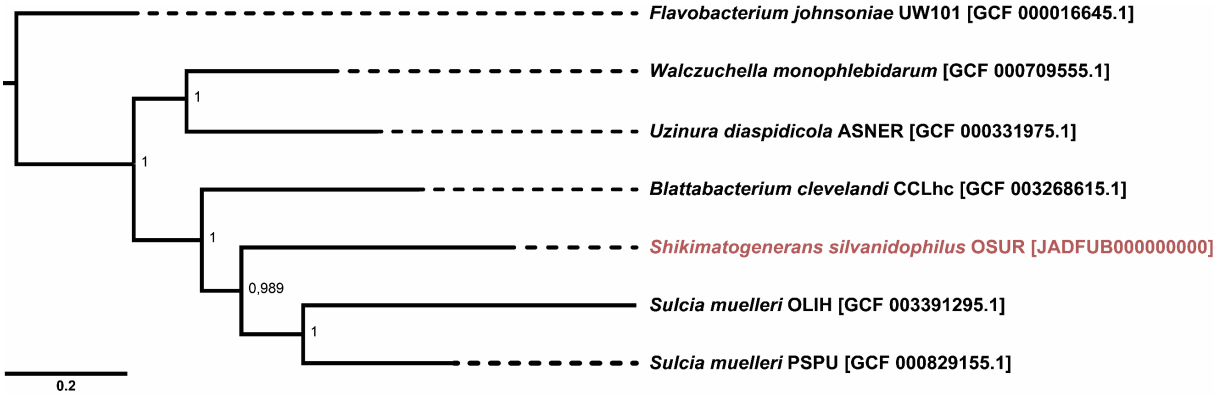
2.10 Supplementary Information

Supplementary Table 1. General features of the *S. silvanidophilus* genome in comparison with genomes of Bacteroidota symbionts of other insects. All genomes where re-annotated with RAST[107,114,117] and PROKKA[124] in KBase[108]. The completeness of the single genomes was calculated with CheckM, which is using collocated sets of genes that are ubiquitous and single-copy within a phylogenetic lineage[45]. Note that this often strongly underestimates true genome completeness for obligate endosymbionts, because they tend to lose many core genes due to genome erosion.

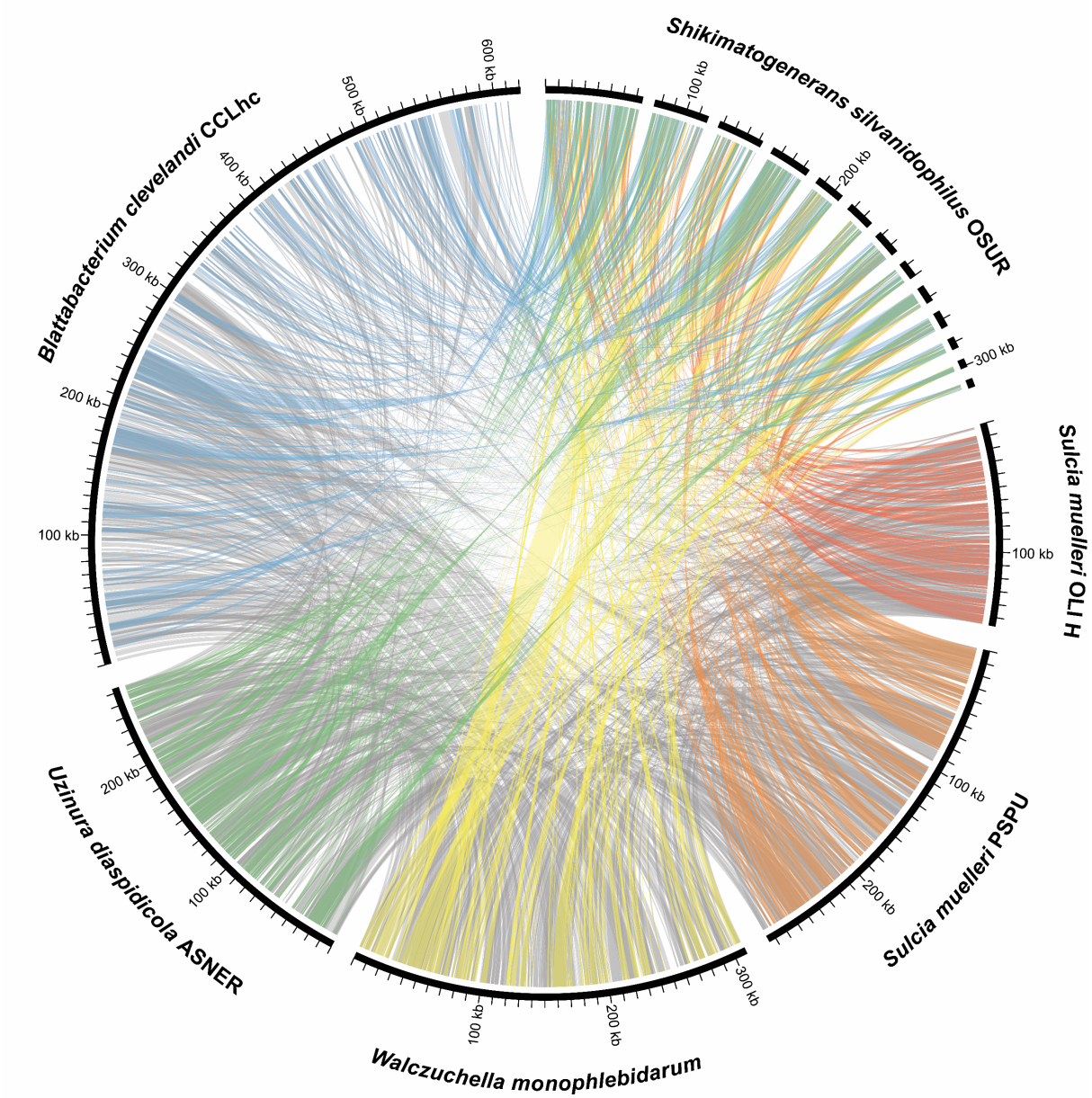
Bacterium	*Walczuchella monophlebidarum* FNIIJ	*Uzinura diaspidicola* ASNER	*Blattabacterium celvelandi* CCLhc	*Shikimatogenerans silvanidophilus* OSUR	*Sulcia muelleri* OLIH	*Sulcia muelleri* PSPU
Host	Hemiptera *Llaveia axin axin*	Hemiptera *Aspidiotus nerii*	Isoptera *Mastotermes darwiniensis*	Coleoptera *Oryzaephilus surinamensis*	Hemiptera *Oliarus filicicola*	Hemiptera *Philaenus spumarius*
Accession	CP006873	CP003263	CP029844	JADFUB000000000	CP028359	AP013293
Genome Size	309,299 bp	263,431 bp	617,422 bp	307,680 bp	156,578 bp	285,352 bp
Plasmids	0	0	1 (3.3 kb)	0	0	0
Completeness	67.38 %	55.75 %	98.17 %	66.68 %	41.98 %	54.2 %
GC content	32.7 %	30.2 %	24.6 %	16.2 %	24.9 %	20.9 %
Predicted proteins	316	254	587	299	155	261
Ribosomal RNAs	3	3	3	3	3	3
SSU ribosomal proteins	21	20	21	21	20	21
LSU ribosomal proteins	32	31	31	30	26	30
Transfer RNAs	33	31	31	28	29	30

Supplementary Table 2. Aminoacyl tRNA synthetases and tRNAs encoded by the *S. silvanidophilus* genome in comparison with other Bacteroidota insect symbiont genomes. Three letter entries indicate the presence of the tRNA anticodons, and highlighted cells in blue indicate the presence of the corresponding aminoacyl tRNA synthetases.

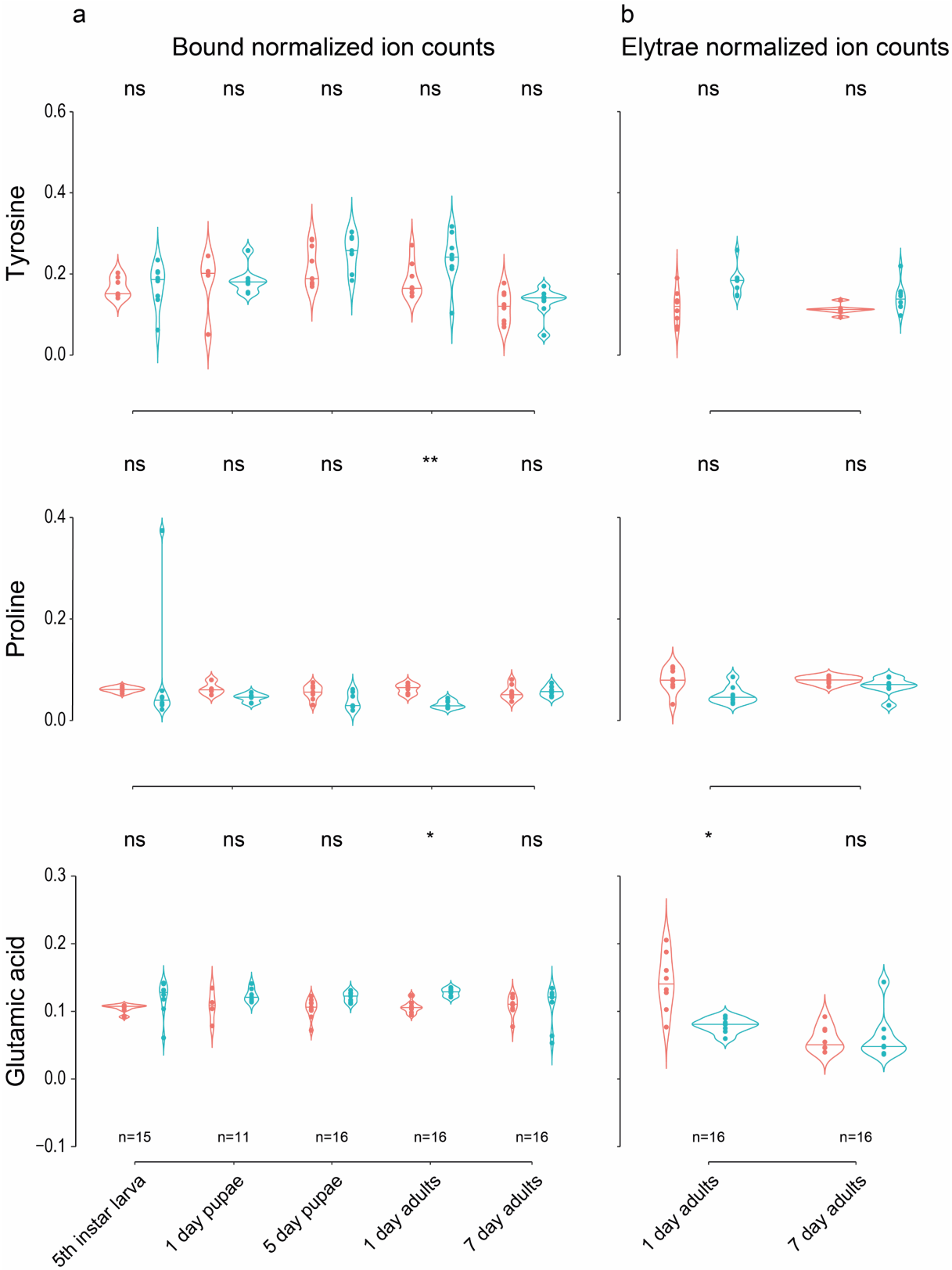
Aminoacyl tRNA synthetase	*Walczuchella monophlebidarum* FNIIJ	*Uzinura diaspidicola* ASNER	*Blattabacterium celvelandi* CCLhc	*Shikimatogenerans silvanidophilus* OSUR	*Sulcia muelleri* OLIH	*Sulcia muelleri* PSPU
Ala	TGC, GGC	TGC	TGC	TGC	TGC	TGC
Arg	TCT, CCG, ACG	TCT, ACG	TCT, CCT, ACG	TCT, CCT, ACG	TCT, ACG	TCT, ACG
Asn	GTT	GTT	GTT	GTT	GTT	GTT
Asp	GTC	GTC	GTC	GTC	GTC	GTC
Cys	GCA	GCA	GCA	GCA	GCA	GCA
Gln	TTG	TTG	TTG	TTG	TTG	TTG
Glu	TTC	TTC	TTC	TTC	TTC	TTC
Gly	TCC, GCC	TCC, GCC	TCC, GCC	TCC	TCC, GCC	TCC, GCC
His	GTG	GTG	GTG	GTG	GTG	GTG
Ile	GAT	GAT	GAT	GAT	GAT	GAT
Leu	TAG, TAA, CAA	TAG,TAA, GAG, CAA	TAG, GAG, CAA	TAG, CAA	TAG, TAA	TAG, TAA, GAG
Lys	TTT	TTT	TTT	TTT	TTT	TTT
Met	CAT (3)	CAT (3)	CAT (3)	CAT (3)	CAT (3)	CAT (3)
Phe	GAA	GAA	GAA	GAA	GAA	GAA
Pro	TGG, GGG	TGG	TGG	TGG	TGG	TGG
Ser	TGA, GCT	TGA, GCT	TGA, GGA, GCT	TGA,GGA,GCT	TGA, GCT	TGA, GGA, GCT
Thr	TGT, GGT	TGT, GGT	TGT, GGT	TGT, GGT	TGT, GGT	TGT, GGT
Trp	CCA	CCA	CCA	CCA	CCA	CCA
Tyr	GTA	GTA	GTA	GTA	GTA	GTA, GAC
Val	TAC, GAC	TAC, GAC	TAC, GAC	TAC	TAC	TAC, GAC
Pseudo	AAG	CCG			GAG, CAA	



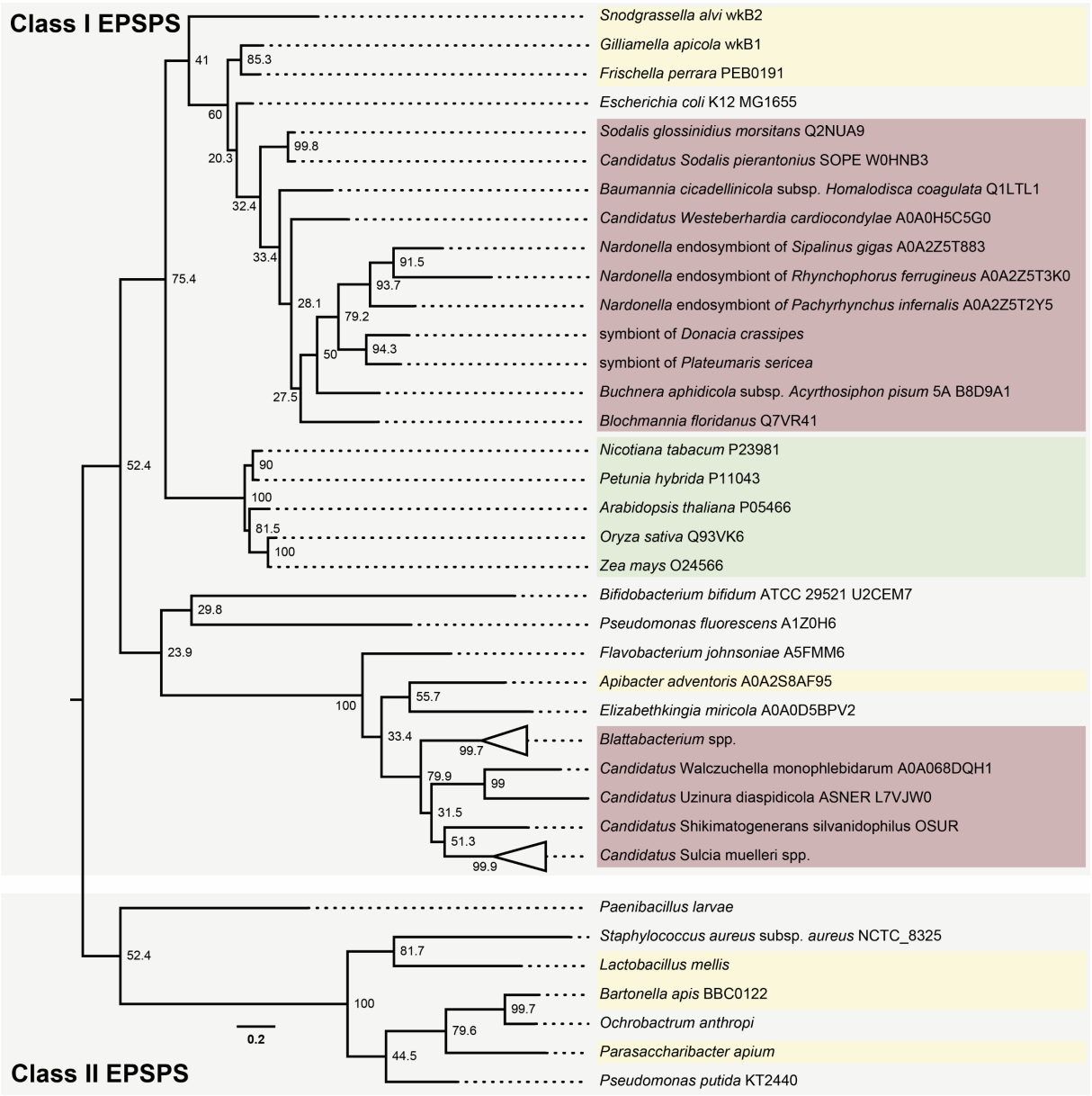
Supplementary Figure 1. Phylogenetic tree for the placement of the intracellular symbiont *S. silvanidophilus* in *O. surinamensis* within the Bacteroidota, based on a defined list of 49 orthologous genes. The phylogeny was reconstructed using the KBase app *Insert Set of Genomes Into Species Tree* v2.2.0, based on FastTree2 algorithm. Node numbers represent local support values. RefSeq assembly accession in square brackets.



Supplementary Figure 2. Comparison of the functional gene repertoires of the endosymbiont and other Bacteroidota symbionts in insects. Colour: orthologs between *S. silvanidophilus* and another genome. Grey: Pairwise orthologs between the other Bacteroidota insect symbionts.



Supplementary Figure 3. Comparison of titers of the three amino acid tyrosine, proline, and glutamic acid that that were influenced by symbiont presence. Shown are (a) bound amino acid titers in the whole body (without elytrae in case of adults) and (b) combined free and bound titers in adult elytrae of symbiotic and aposymbiotic *O. surinamensis* beetles. Red: aposymbiotic beetles, Blue: symbiotic beetles. FDR corrected unpaired, two-sided Wilcox-rank-sum-tests: ns $p > 0.05$, * $0.05 < p < 0.01$, ** $p < 0.01$.



Supplementary Figure 4. Phylogenetic classification of EPSPS enzymes from different Bacteroidota and γ-Proteobacteria insect symbionts based on PhyML and Maximum Likelihood analyses of amino acid sequences of EPSPSs using the Jones-Taylor-Thorton model and 1000 bootstrap replicates. Enzymes of plants (green), bacterial symbionts in the honeybee gut (yellow) and obligate intracellular insect symbionts (red) are highlighted. Node values indicate bootstrap values.

Chapter 3

Wolbachia causes cytoplasmic incompatibility, but not male-killing in a grain pest beetle

Molecular Ecology, submitted

Julian Simon Thilo Kiefer[1], Gerrit Schmidt[1], Ronja Krüsemer[2],
Martin Kaltenpoth[1,2], Tobias Engl[1,2]

[1]Department of Evolutionary Ecology, Institute of Organismic and Molecular Evolution, Johannes Gutenberg-University, Mainz, Germany; [2]Department of Insect Symbiosis, Max-Planck-Institute for Chemical Ecology, Jena, Germany

3.1 Abstract

The endosymbiotic *Wolbachia* is one of the most common intracellular bacteria known in arthropods and nematodes. Its ability for reproductive manipulation can cause unequal inheritance to male and female offspring, allowing the manipulator to spread, but potentially also impact evolutionary dynamics of infected hosts. Estimated to be present in up to 66% of insect species, little is known about the phenotypic impact of *Wolbachia* within the order Coleoptera. Here, we describe the reproductive manipulation by the *Wolbachia* strain *w*Sur harboured by the sawtoothed grain beetle *Oryzaephilus surinamensis* (Coleoptera, Silvanidae), through a combination of genomics approaches and bioassays. The *Wolbachia* strain *w*Sur belongs to supergroup B that contains well-described reproductive manipulators of insects and encodes a pair of cytoplasmic incompatibility factor (*cif*) genes, as well as multiple homologues of the WO-mediated killing (*wmk*) gene. A phylogenetic comparison with *wmk* homologues of *w*Mel of *Drosophila melanogaster* identified 18 *wmk* copies in *w*Sur, including one that is closely related to the *w*Mel male-killing homologue. However, further analysis of this particular *wmk* gene revealed an eight-nucleotide deletion leading to a stop-codon and subsequent reading frame shift mid-sequence, likely rendering it non-functional. Concordantly, utilizing a *Wolbachia*-deprived *O. surinamensis* population and controlled mating pairs of *w*Sur infected and non-infected partners, we found no experimental evidence for male-killing. However, a significant ~50% reduction of hatching rates in hybrid crosses of uninfected females with infected males indicates that *w*Sur is causing cytoplasmic incompatibility. Thus, *Wolbachia* also represents an important determinant of host fitness in Coleoptera.

3.2 Introduction

Symbiotic bacteria influence the ecology and evolution of animals in various ways[1,2]. Insects harbour an especially high abundancy and diversity of microbial associations that span the entire range from parasitism to mutualism[3]. While some symbionts exhibit a very strict phenotype, others incur context-dependent impacts along the parasite-mutualist continuum[4,5]. Thus, already a single symbiont can incur context-dependent fitness benefits or costs[6]. However, a large proportion of insects are also infected by reproductive manipulators[7] and many are co-infected with both. In consequence, their ecology and evolution can be driven by multiple symbionts with possibly different selective interests.

The bacterium *Wolbachia* (α-Proteobacteria) is one of the most common intracellular bacteria known in arthropods and nematodes[8]. They are predominantly parasitic and transmitted maternally between host generations, but horizontal transmission occurs occasionally. *Wolbachia* employ several distinct strategies to maximize their transmission by influencing the germ line of their host. Thereby, they can rapidly sweep through uninfected populations and then maintain a high prevalence within a population. These mechanisms include cytoplasmic incompatibility (CI), parthenogenesis, male-killing or feminisation[8]. While CI leads directly to a higher proportion of infected individuals, the other mechanisms lead to a higher proportion of female individuals in the population. This in turn increases the fitness of *Wolbachia* which is predominantly transmitted maternally[9]. However, *Wolbachia* infection does not necessarily result in reproductive manipulation with negative fitness consequences for the host[10]. Furthermore, *Wolbachia* can even evolve into a mutualist and enhance its host's fitness by supplementing dietary limited nutrients, such as B-vitamins like riboflavin[11–13].

CI and male-killing are the predominant strategies of reproductive manipulation in insects[14,15]. CI generally refers to factors localised in the cytoplasm of sperm and eggs that render them incompatible with each other, resulting in inviable embryos[16–18]. *Wolbachia* causes CI by expressing a "killing" factor in the male sperm. In eggs of uninfected females, this modification leads to non-viable embryos, whereas in infected females a "rescue" factor reverses this modification so that the zygote can develop normally[19]. While unidirectional CI occurs when infected males mate with uninfected females resulting in fertilized but unviable eggs, bidirectional CI occurs when two individuals are infected by different, yet incompatible *Wolbachia* strains[8]. Recently, two

cytoplasmic incompatibility factor genes (*cifA* and *cifB*) have been identified as key factors in CI-inducing *Wolbachia* strains[20]. The pair of CI-inducing genes were not found in the chromosomal *Wolbachia* genes, but in the integrated eukaryotic association module of phageWO[20]. A two-by-one genetic model has been suggested, specifying that while both *cifA* and *cifB* induce CI, only *cifA* is able to rescue the CI phenotype when transgenically expressed in the host's ovaries[16,21].

The other widespread phenotype of *Wolbachia* inducing reproductive manipulation is male-killing. During embryogenesis, the development of the male embryo is disturbed by *Wolbachia*, leading to embryonic lethality[8]. In consequence, the fitness of infected sister embryos is enhanced by higher allocation of resources during ovogenesis and reduced intraspecific competition during juvenile development and adult life[22,23]. The gene WO-mediated killing (*wmk*) of the *Wolbachia* strain *w*Mel of the fruit fly *Drosophila melanogaster*, has been identified to recapitulate this male-killing phenotype when transgenically expressed in *D. melanogaster* flies[24]. So far, *wmk* homologues were found in all *Wolbachia* strains associated with male-killing, surprisingly also localised within the eukaryotic association module of phageWO, only a few genes upstream from the CI-inducing genes *cifA* and *cifB*[24]. There are at least five homologues of the *wmk* gene encoded in the genome of *w*Mel and the function of many of these remains enigmatic as only the transgenic expression of the original *wmk* gene, but not other homologues caused male-killing[15]. *Wolbachia* strains causing CI and male-killing phenotypes are well studied within the insect orders of Diptera and Hymenoptera, e.g. the fruit fly *Drosophila melanogaster*[15], the southern house mosquito *Culex quinquefasciatus*[25] and the parasitoid wasp *Nasonia vitripennis*[26,27]. Although beetles infected with *Wolbachia* were repeatedly reported in the last years, little is known about the functional consequences of *Wolbachia* infections within the order Coleoptera[14,28–32].

The sawtoothed grain beetle *Oryzaephilus surinamensis* (Coleoptera, Silvanidae) is a worldwide distributed pest of cereals and other stored food[33]. It is associated with the bacteriome-localised Bacteroidota endosymbiont *Candidatus* Shikimatogenerans silvanidophilus OSUR (called *Shikimatogenerans silvanidophilus* from here on)[34–37]. The endosymbiont *S. silvanidophilus* provides aromatic amino acid precursors for cuticle synthesis of the host via the shikimate pathway[36]. In addition, *O. surinamensis* is commonly infected with *Wolbachia*[28,38]. Sharaf et al. (2010) identified a higher *Wolbachia* infection rate in feral populations of *O. surinamensis* compared to adapted silo

populations, but also a strong female bias among adults emerging under lab conditions suggesting active reproductive manipulation by these *Wolbachia* strains. Elucidating *Wolbachia's* capabilities of reproductive manipulation in the sawtoothed grain beetle *O. surinamensis* is therefore relevant in understanding the biology of this agricultural pest as well as a symbiotic model insect.

In this work, we localised *Wolbachia* in the *O. surinamensis* JKI strain and quantified its growth dynamics across developmental stages. A phylogenetic analysis and functional prediction of the associated *Wolbachia* genome revealed it to be a member of the supergroup B, presumably capable of CI as it encodes homologues of the cytoplasmic incompatibility factor genes *cifA* und *cifB.* However, the strain is likely incapable of inducing male-killing due to a single nucleotide insertion in the identified male-killing gene *wmk* creating a stop codon as well as a subsequent reading frame-shift. Finally, we experimentally tested the predicted phenotype of reproductive manipulation – unidirectional CI and no male-killing – using mating assays of beetles with manipulated infection status, where we were able to verify the phenotype of reproductive manipulation via unidirectional CI.

3.3 Material & Methods

3.3.1 Insect cultures

The initial *Oryzaephilus surinamensis* culture (strain JKI) was obtained from the Julius-Kühn-Institute / Federal Research Centre for Cultivated Plants (Berlin, Germany) in 2014 and kept in culture since then. Continuous symbiotic and aposymbiotic (with aposymbiotic we refer in this manuscript to beetles without both *Wolbachia* and *S. silvanidophilus* symbionts) *O. surinamensis* cultures (see below) were maintained in 1.8-L plastic containers, filled with 50 g oat flakes, at 28°C, 60% relative humidity and a day and night cycle of 16 / 8 hours.

3.3.2 Elimination of *O. surinamensis* symbionts

An *O. surinamensis* sub-population was treated for 12 weeks with tetracycline (150 mg/5 g oat flakes, see for details Engl et al. (2018)) to eliminate both of their symbionts (*S. silvanidophilus* and *w*Sur) and then kept for several generations on a normal diet to exclude direct effects of tetracycline on the host physiology. A control group was established in parallel with all steps except the addition of tetracycline to account for any unforeseen effects of the handling, population bottlenecks, etc. The apo-/symbiotic status regarding both symbionts of these beetle sub-populations was confirmed before each following experiment. Therefore, female adult beetles were individually separated into single jars with oat flakes to lay eggs. After 4 weeks, the adult generation was removed before their offspring finished metamorphosis, DNA of these parent females was extracted, and the symbiont titer was analysed by quantitative PCR (see below).

3.3.3 Quantitative PCR

Absolute titers of *w*Sur and *S. silvanidophilus* during host development and after different treatments from previous publications[34,36,39] were determined via quantitative PCR (qPCR) amplifying respective single copy 16S rRNA gene fragments. DNA was extracted from individual beetles using the Epicentre MasterPure™ Complete DNA and RNA Purification Kit (Lucigen, Middleton, WI, USA) and dissolved in 30 µL low TE buffer (1 mM Tris–HCl + 0.1 mM EDTA). qPCRs were carried out in 25 µl reactions using EvaGreen (Solis BioDyne, Tartu, Estonia), including 0.5 µM of each primer and 1 µl template DNA. All reagents were mixed, vortexed, and centrifugated in 0.1-mL reaction tubes (Biozym, Hessisch Oldendorf, Germany). The *Wolbachia*-specific 16S rRNA gene fragment of *O. surinamensis* was amplified with the primers Wolb_16S_qPCR_fwd (5'-TTGCTATTAGATGAGCCTATATTAG-3') and

Wolb_16s_qPCR_rev (5'-GTGTGGCTGATCATCCTCT-3')[29], and the 16S rRNA of *S. silvanidophilus* OSUR was amplified with the primers OsurSym_fwd2 (5'-GGCAACTCTGAACTAGCTACGC-3') and mod. CFB563_rev (5'-GCACCCTTTAAACCCAAT-3')[34,36]. qPCR was carried out on a Rotor-Gene Q thermal cycler (Qiagen, Hilden, Germany). The initial temperature was 95°C for 12 minutes, followed by 60 cycles of 95°C for 40 seconds followed by 20 seconds at 60°C. A melting curve analysis was used to assess the specificity of the qPCR reaction by a gradual increase of temperature from 60 to 95°C, with 0.25 K per second. The qPCR results were analysed using the Rotor Gene Q Software (Qiagen, Hilden, Germany).

Standard curves with defined copy numbers of the 16S rRNA gene were created by amplifying the fragment first via PCR using the previously mentioned primers, followed by purification via innuPREP PCRpure (Analytik Jena GmbH, Jena, Germany) and determination of the DNA concentration via NanoDrop1000 (Peqlab, Erlangen, Germany). After the determination of the DNA concentration, a standard containing 10^{10} copies/µL was generated and 1:10 serial dilutions down to 10^{1} copies/µL were prepared. 1 µL of each standard was included in a qPCR reaction to standardize all measurements.

3.3.4 Fluorescence *in situ* hybridisation

Wolbachia was localised in *O. surinamensis* tissues by fluorescence in situ hybridisation (FISH) on semi-thin sections of adult beetles. Therefore, five-day-old pupae and maximum two-week-old adult beetles were fixated in tertiary butanol (80%; Roth, Karlsruhe, Germany), paraformaldehyde (37 - 40%; Roth, Karlsruhe, Germany) and glacial acetic acid (Sigma-Aldrich, Germany) in proportions 6:3:1 for 2 hours, followed by post-fixation in alcoholic formaldehyde (paraformaldehyde (37 – 40%) and tertiary butanol (80% in proportion 1:2). After dehydration, the specimens were embedded in Technovit 8100 (Kulzer, Germany)[41] and cut into 8 µm sagittal sections using a Leica HistoCore AUTOCUT R microtome (Leica, Wetzlar, Germany) equipped with glass knives. The obtained sections were mounted on silanised glass slides. Each slide was covered with 100 µL of hybridisation mix, consisting of hybridisation buffer (0.9 M NaCl, 0.02 M Tris/HCl pH 8.0, 0.01% SDS; Roth, Germany) and 0.5 µM of the modified Bacteroidota probe CFB563 (5'-GCACCCTTTAAACCCAAT-3')[26,31] or the Eubacteria probe EUB338 (5'-GCTGCCTCCCGTAGGAGT-3')[32] labelled with Cy3, as well as the two *Wolbachia*

specific probes Wolb_W2 (5'-CTTCTGTGAGTACCGTCATTATC-3')[33] and Wolbachia-Wol3 (5'-TCCTCTATCCTCTTTCAATC-3')[34] labelled with Cy5. DAPI (0.5 µg/mL) was included as a general counterstain for DNA. Slides were covered with glass cover slips and incubated in a humid chamber at 50°C overnight. After washing and incubating them for two hours at 50°C in wash buffer (0.1 M NaCl, 0.02 M Tris/HCl, 5 mM EDTA, 0.01% SDS), they were washed in deionized water for 20 minutes and mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA). The sections were either observed under a Zeiss AxioImager Z2 with Apotome.2 (Zeiss, Jena, Germany) illuminated by a SOLA Light Engine (Lumencor, Beaverton, OR, USA), or a Leica THUNDER imager Cell Culture 3D (Leica, Wetzlar, Germany). Images obtained on the Leica microscope were processed with the instant and small volume computational clearing algorithm using standard settings in the Leica Application Suite X software (Leica, Wetzlar, Germany).

3.3.5 Symbiont genome sequencing, assembly, and annotation

Total DNA was isolated from 20 pooled adult abdomina (without wings) of *O. surinamensis* JKI using the Epicentre MasterPure™ Complete DNA and RNA Purification Kit (Illumina Inc., Madison, WI, USA) including RNase digestion. Short-read library preparation and sequencing were performed at the Max Planck Genome Centre in Cologne, Germany (SRR12881563–SRR12881566) on a HiSeq2500 Sequencing System (Illumina Inc., Madison, WI, USA). Adaptor and quality trimming was performed with Trimmomatic[35]. In addition, we used two publicly available metagenome libraries of *O. surinamensis* (SRR5279855 and SRR6426882).

Long-read sequencing (SRR12881567–SRR12881568) was performed on a MinION Mk1B Sequencing System (Oxford Nanopore Technologies (ONT), Oxford, UK). Upon receipt of flowcells, and again immediately before sequencing, the number of pores on flowcells was measured using the MinKNOW software (v18.12.9 and 19.05.0, ONT, Oxford, UK). Flowcells were replaced into their packaging, sealed with parafilm and tape, and stored at 4°C until use. Library preparation was performed with the Ligation Sequencing Kit (SQK-LSK109, ONT, Oxford, UK), and completed libraries were loaded on a flowcell (FLO-MIN106D, ONT, Oxford, UK) following the manufacturer's instructions.

Hybrid assembly of MinION and Illumina reads was performed using SPAdes[36] (v3.13.0) with the default settings. The resulting contigs were then binned using BusyBee Web[37] and screened

for taxonomic identity to α-proteobacteria. The single resulting circular contig was extracted, which was then automatically annotated with RAST[38] using the app *Annotate Microbial Assembly* (RAST_SDK v0.1.1) on KBase[39]. The annotated contig was curated manually and plotted using CIRCOS[40] (v0.69-6) for the visualisation of gene locations, GC content, and coverage. Additionally, the completeness of the obtained genome was assessed with the app *Assess Genome Quality with CheckM—v1.0.18* in KBase[39].

3.3.6 Phylogeny and comparative genomics of *Wolbachia* strains

A phylogenetic tree for placement of the *Wolbachia* strain of *O. surinamensis* was reconstructed using the KBase app *Insert Set of Genomes Into Species Tree* v2.1.10 (SpeciesTreeBuilder v0.0.12) based on the FastTree2 algorithm[41], including 49 highly conserved Clusters of Orthologous Groups (COG) genes. Therefore, 74 additional publicly available and published genomes of *Wolbachia* endosymbionts were obtained from NCBI (https://www.ncbi.nlm.nih.gov/assembly). The resulting tree was visualized using FigTree (v1.4.4, http://tree.bio.ed.ac.uk/software/figtree/).

3.3.7 Identifying genes important for reproductive manipulation

The obtained genome was manually searched for *wmk*, *cifA,* and *cifB* genes. For the *wmk* gene, CDSs annotated as "Transcriptional regulator" were extracted and identified as *wmk* homologue by a BLASTn search of NCBI's nucleotide collection (nr/nt). To identify the potentially functional *wmk* gene, the nucleotide sequence of all 18 *wmk* homologues of *w*Sur and five phenotypically described *wmk* homologues of *w*Mel (WD0255, WD0508, WD0622, WD0623, WD0626 (*wmk*))[7] were aligned using MUSCLE[42] in Geneious Prime 2019 (v2019.1.3; https://www.geneious.com). Phylogenetic reconstruction of the nucleotide alignment was performed with the MrBayes-plugin[43] of Geneious Prime using the HKY85 substitution model and invgamma rate variation as recommended by jModelTest 2.1.10 v20160303[44]. The analysis ran for 1,100,000 generations, with a burn-in of 100,000 generations and trees sampled every 200 generations until the likelihood values stabilized. Protein domains were identified and annotate by running the protein sequences from the NCBI database through SMART (Simple Modular Architecture Research Tool; http://smart.embl-heidelberg.de/).

Additionally, the annotated genome of *w*Sur was manually checked for *cif* genes. Due to the incomplete annotation, the *cif* genes were first identified by whole-genome alignment to the

genome of *w*Pip and translation alignment with the annotated genes of *w*No (WNO_RS01055/ WNO_RS01050) and *w*Meg (CAI20_01650/ CAI20_01645). To identify whether the *cif*-genes belonged to the same type, we performed a phylogenetic analysis following Lindsey et al. (2018) and Ün et al. (2021). Briefly, the nucleotide sequences were translation-guided aligned as implemented in Geneious Prime 2019 (v2019.1.3; https://www.geneious.com). Phylogenetic reconstruction of the alignment was performed with the MrBayes-plugin[43] of Geneious Prime using the GTR substitution model and gamma rate variation as predicted by jModelTest 2.1.10 v20160303[44] using the same parameters as above. According to Ün et al. (2018), potential protein domains of the Cif genes were searched using HHpred's version 3.2.0 web server (https://toolkit.tuebingen.mpg.de/tools/hhpred)[45] with default parameters and the following databases: SCOPe70 version 2.07, COG/KOG version 1.0, Pfam-A version 32.0, and SMART version 6.0[46]. The seven phage WO regions in the *w*Sur genome were compared and visualized using Clinker[47].

3.3.8 Bioassays for reproductive manipulation

By mating experiments with differentially infected individuals, we tested whether *Wolbachia w*Sur is causing reproductive manipulation in *O. surinamensis*. To ensure the virginity of the female and male individuals and prevent unwanted crossbreeding, pupae, and 5th instar larvae of aposymbiotic (*Wolbachia* & *Shikimatogenerans* uninfected) and symbiotic (*Wolbachia* & *Shikimatogenerans* infected) *O. surinamensis* were isolated into 24-well TC plates (Sarstedt AG & Co., Germany), closed with Adhesive Foil (Kisker-Biotech™, Germany) with several needles punctures to allow for air exchange and maintained under general rearing conditions (see above). The isolated individuals were observed until hatching, and the sex of the individual insect was determined by the presence (males) or absence (females) of spikes on the third femur[48]. Males and females were combined into mating pairs at an age of 7 - 10 days. In total, 30 mating pairs were prepared, ten for each group: The first group consisted of mating pairs where both partners, female, and male, were aposymbiotic, whereas the second group was made up of crossings with two symbiotic partners. The third group contained symbiotic males of *O. surinamensis* paired with aposymbiotic females. The mating pairs were given one microspatula scoop of ground oat previously filtered through a 0.6 mm sieve. To prevent the specimens from escaping the setup, the edge of each individual well was coated with Polytetrafluoroethylene 60 wt% dispersion in H_2O (PTFE-dispersion; Sigma-Aldrich, Germany). For the first two weeks of the experiment all

pairs were left undisturbed. In the following six-week period, the number of laid eggs and hatched larvae were counted twice weekly, and the adults were placed one well further down in the 24 well-plate. In addition, we quantified the sex ratio of 100 randomly picked individuals in both symbiotic and aposymbiotic stock cultures to test for a bias induced by male-killing.

3.3.9 Statistical procedure for qPCR results and differences in hatching rate and sex ration

Influence of glyphosate and tetracycline on the symbiont titer of the adult beetles (Supplement Figure 2) was analysed using Dunn's test from the package 'FSA' in RStudio (R V4.1.1) with two-sided post hoc tests corrected for multiple testing using the Benjamini–Hochberg method[49,50]. Compact letter display (CLD)[51] was generated with the package 'rcompanion'[52]. Comparison between hatching rates (Figure 7) was performed with Wilcoxon rank sum tests including correction for false discovery rates (FDRs) by repeated testing following the Benjamini–Hochberg procedure [50], implemented in the R package 'stats'. Plots were visualized using 'ggplot2'[53]. Sex ratio in beetle cultures was analysed using a manually calculated χ^2 test of homogeneity.

3.4 Results

3.4.1 Localisation and Infection Dynamics in *O. surinamensis*

qPCR quantification of *Wolbachia* titers in 106 control samples of multiple experiments indicated a *w*Sur prevalence of 100% within laboratory cultures of the *O. surinamensis* JKI strain. Based on fluorescence *in situ* hybridisation, *Wolbachia* is localised throughout the entire body of *O. surinamensis* (Figure 1 A). *Wolbachia*-induced CI has been linked to sperm modification during spermatogenesis[64], but *Wolbachia* must also be present in the female reproductive tissues for successful transmission. A close inspection of the reproductive organs of female and male *O. surinamensis* confirmed a high abundance of *Wolbachia* in both testes and ovaries (Figure 1 B and C).

Further, we compared infection titers of the two bacterial endosymbionts in *O. surinamensis* during all life stages of *O. surinamensis* via qPCR (Figure 2). The population of *Wolbachia* reached its maximum already in the pupa during early metamorphosis (early pupa: 5.9×10^6 median copies; late pupae: 3.9×10^6 median copies; Figure 2, left), while we observed a peak of *S. silvanidophilus* only within the first week after metamorphosis (male 6.7×10^7 median copies and female 6.7×10^7 median copies; Figure 2, right).

After our findings on *S. silvanidophilus* conferring enhanced cuticle synthesis and higher fitness under biotic and abiotic stresses[34,36,39], we assessed whether *Wolbachia* could have contributed to the previously reported cuticular phenotypes in *O. surinamensis*. Therefore, we also quantified *w*Sur titers (in addition to *S. silvanidophilus*) in *O. surinamensis* samples from different previous treatments (Supplement Figure 2)[36]. While strict tetracycline treatment eliminated both *S. silvanidophilus*[34,36] and *w*Sur (Kruskal–Wallis $\chi2 = 52.605$, df = 7, p-value = 0.000000004437, Dunn's test: $p < 0.05$; Supplement Figure 2), resulting in dual aposymbiotic (from here on aposymbiotic) beetles, the herbicide glyphosate had a differential effect: *S. silvanidophilus* was drastically reduced, yet still present in low amounts while *w*Sur was not negatively affected (Kruskal–Wallis $\chi2 = 52.605$, df = 7, p-value = 0.000000004437, but Dunn's test: $p > 0.05$; see Supplementary Table 1 for pairwise comparisons; Supplement Figure 2).

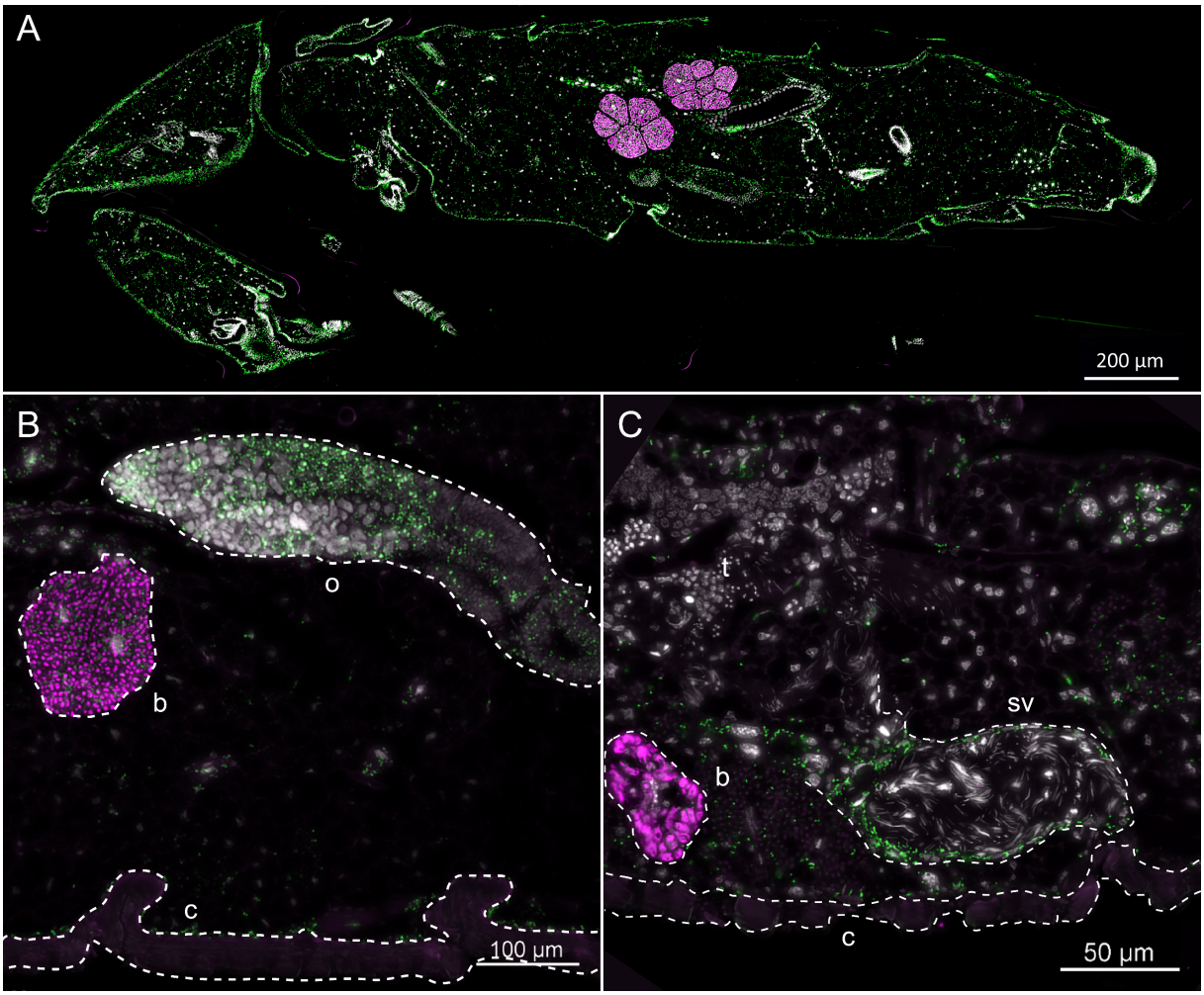


Figure 1: Fluorescence *in situ* hybridisation micrographs of *Wolbachia* (green) and *Shikimatogenerans* (magenta) in sagittal sections (**A**) a 5-day-old *O. surinamensis* pupa stained with a Bacteroidota specific probe highlighting *S. silvanidophilus* (CFB563mod-Cy3, magenta), and in the gonads of (**B**) an adult female and (**C**) an adult male stained with a Eubacteria specific probe highlighting *S. silvanidophilus* (EUB338-Cy3, magenta), *Wolbachia* specific probes (Wol-W3-Cy5 and Wolb-2-Cy5, green) and DAPI targeting DNA in general (white). b=bacteriomes, c=cuticle, o=ovariole, t=testes, sv=seminal vesicle. Fig. 1A was originally published without the *Wolbachia* channel in Kiefer et al., (2021).

3.4.2 Genomics and Phylogeny of the *Wolbachia* strain

We previously sequenced the metagenome of *O. surinamensis* combining short and long-read technologies (Illumina and ONT) into a hybrid assembly. Besides the Bacteroidota endosymbiont *S. silvanidophilus*[36] we also extracted the full genome of a *Wolbachia* strain in a single, circular contig in the assembly (Figure 3). The circular genome is 1,728,764 bp in length with an average GC content of 34.1% and a coverage of 186× with short-read sequences and 94x with long-read sequences. The phylogenetic reconstruction based on 49 conserved Clusters of Orthologous Group (COG) genes classified *w*Sur as a member of supergroup B, closely related to

the *Wolbachia* endosymbiont *w*Ecas of the common brassy ringlet *Erebia cassioides*, but also within a clade with *w*Pip of the southern house mosquito *Culex quinquefasciatus* and *w*VitB of the parasitoid wasp *Nasonia vitripennis* (Figure 4).

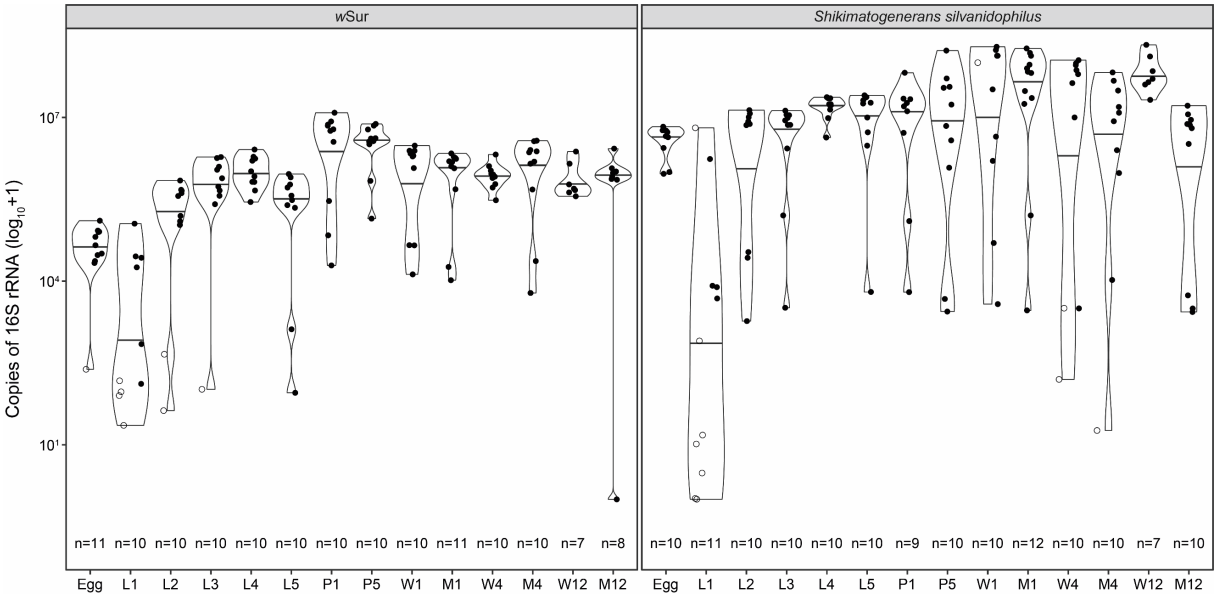


Figure 2: Symbiont titers in different life stages of *O. surinamensis* from the JKI stock line. Titers of *Wolbachia w*Sur (left) and *S. silvanidophilus* (right) were measured as 16S rDNA copies by PCR in single individuals. Juvenile life stages (eggs, larvae, and pupae) contained mixed sexes, adults were separated by sex. Larvae stages 1 to 5 (L); one-day and five-day-old pupae (P1 and P5); female adults 1, 4, and 12 weeks (W1-12) and male adults 1, 4 and 12 weeks (M1-12) after metamorphosis. The data distribution is visualized with violin plots and an additional horizontal line depicting the median. The scales of the vertical axes are logarithmic. Filled circles represent specific target amplification, and empty circles off-target amplification during late qPCR cycles, identified by melting curve analysis.

The genome of the *Wolbachia* strain *w*Sur of *O. surinamensis* encoded for 1688 protein-coding sequences, 34 tRNAs and 50 ribosomal proteins (20 SSU and 30 LSU proteins, Table 1). Besides general genetic information processing including DNA replication and repair, transcription, and translation, the genome also contained a full glycolysis pathway to process glucose-6-phosphate to erythrose 4-phosphate (E4P) and phosphoenolpyruvate (PEP). Further, it contained a full riboflavin pathway and the pathways to synthesize the amino acids lysine, glutamine, threonine, glycine, and serine, but no single gene of the shikimate pathway to synthesize aromatic amino acids, explaining its insensitivity to glyphosate[65–67].

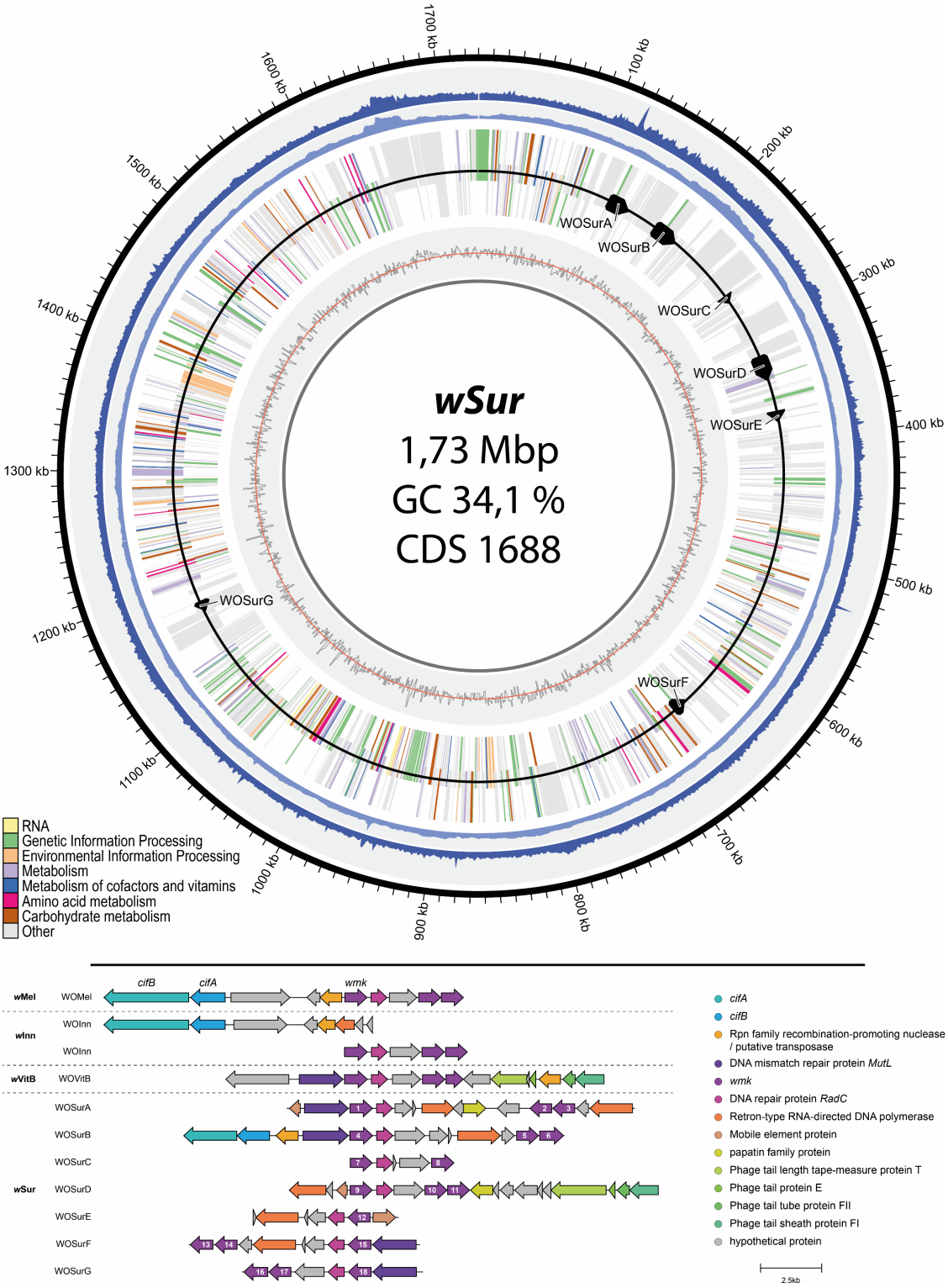


Figure 3: Top: Circular representation of the genome of *Wolbachia* *w*Sur. The outer blue circles denote coverage with short- and long-reads, respectively (dark blue: Illumina, light blue: ONT), and the intermediate circles indicate ORFs with KEGG functional annotations separated by the direction of transcription (see legend for depicted categories). The inner grey circle denotes relative GC content and the average GC content of 34.1% is indicated by the red line. Phage WO modules are highlighted in black. Bottom: Comparison of the prophage WO modules in *w*Sur and the well-studied strains of *Wolbachia* *w*Mel, *w*Inn, and *w*VitB. Prophage WO gene regions containing *wmk*, *wmk*-like homologues, and CI genes *cifA* and *cifB* are listed by *Wolbachia* strain in bold and then the corresponding prophage module. At least one *wmk* homologue is associated with each *Wolbachia*-inducing male-killing strain.

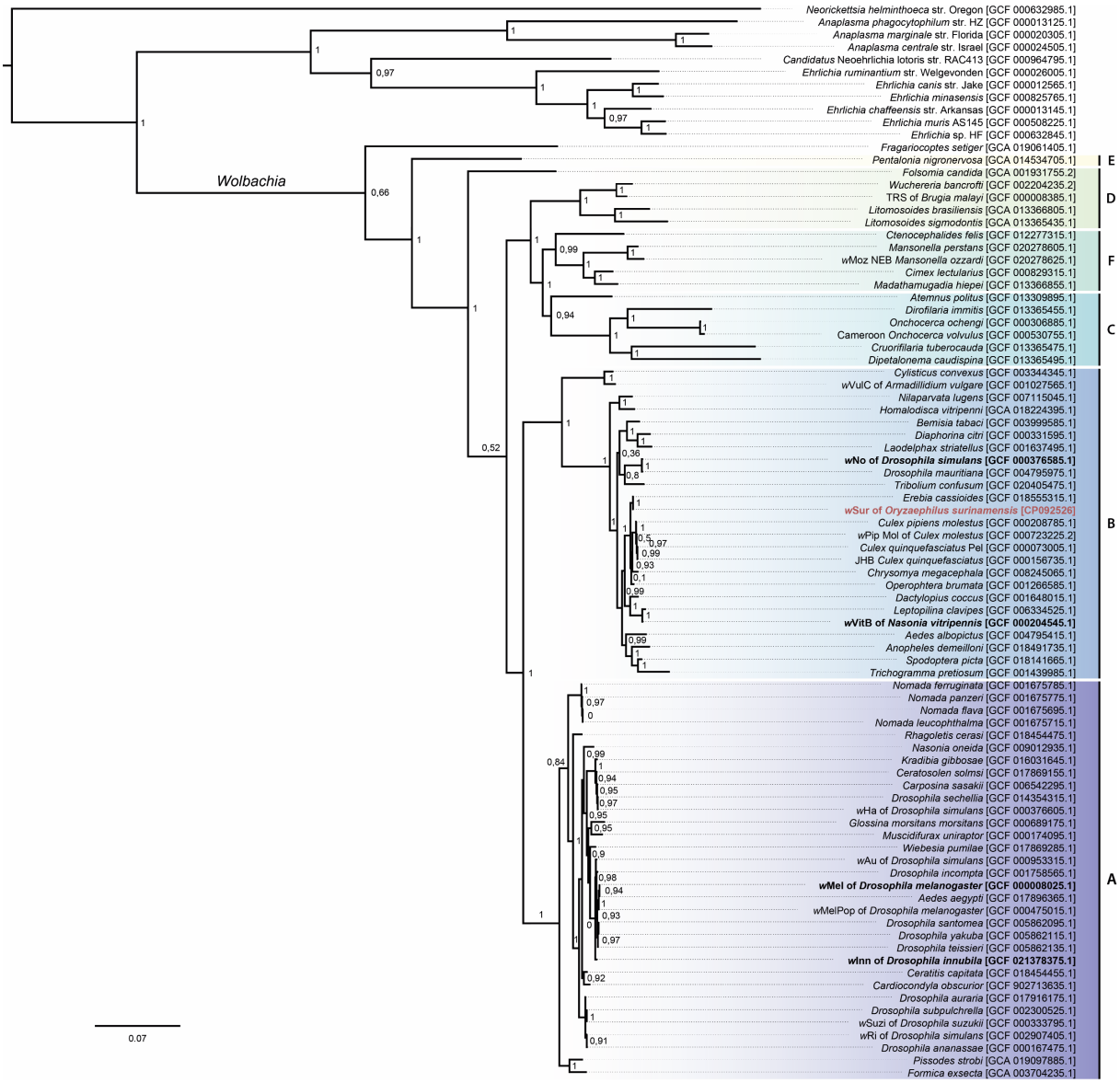


Figure 4: Phylogenetic relationship of *w*Sur and other *Wolbachia* strains deposited in sequence databases. The phylogeny was reconstructed based on a defined set of 49 orthologous genes using the KBase app *Insert Set of Genomes Into Species Tree* v2.2.0[48], based on the FastTree2 algorithm[50]. Node numbers represent local support values. RefSeq assembly accession numbers are given in square brackets. The supergroups are colour-coded and indicated on the right[93–96]. *Wolbachia* endosymbionts of *O. surinamensis* (*w*Sur, highlighted in red font) belongs to supergroup B. *Wolbachia* strain genomes highlighted in bold font were utilized for subsequent phylogenetic analyses of *wmk* (Figure 5) and *cif* genes (Figure 7).

Table 1: Genomic characteristics of *Wolbachia w*Sur in comparison to other strains.

Wolbachia strain Host	*w*Sur *Oryzaephilus surinamensis*	*w*No *Drosophila simulans*	*w*VitB *Nasonia vitripennis*	*w*Con *Tribolium confusum*	*w*Mel *Drosophila melanogaster*	*w*Inn *Drosophila innubila*	*w*Ri *Drosophila simulans*
Accession	CP092526	GCF_00376585.1	GCF_000204545.1	GCF_020405475.1	GCF_000008025.1	GCF_021378375.1	GCF_002907405.1
Supergroup	B	B	B	B	A	A	A
Genome size [bp]	1,728,764	1,301,823	1,107,643	1,418,452	1,267,782	1,290,587	1,117,694
GC content [%]	34.1	34	33.91	34.17	35.2	35.28	35.04
Predicted proteins	1688	1191	1101	1294	1245	1346	1049

3.4.3 Analysis of male-killing gene candidates

The genome of *w*Sur contained seven regions with phage WO-associated genes (WOSurA – WOSurG) in total, each with two to three homologues of the *wmk* gene (Figure 3). Overall, the genome encodes for 18 *wmk* homologues which were numbered from *wmk1* to *wmk18*. As these copies may share the ability to induce male-killing, we compared these *wmk* homologues of *w*Sur with the functionally described *wmk* homologues in the *w*Mel strain as other known male-killing strains. The phylogenetic analysis identified homologues *wmk1* and *wmk12* in the phage region WOSurB as the most likely candidates to confer male-killing due to their high sequence similarity with the functional homologue *wmk* in *w*Mel (for *wmk12*), as well as *w*Inn and *w*Bor (for *wmk1*; Figure 5, top). A closer inspection of the coding sequence revealed that *wmk12* experienced an eight-nucleotide deletion that resulted in a stop codon and subsequent shift of the reading frame which led to the loss of the second XRE-family HTH DNA-binding region (Figure 5, bottom). We also screened different sequence read archives from an Israeli grain storage and two feral (field) populations of *O. surinamensis* individuals for *wmk1/wmk12-like* homologues. We found all individuals from feral populations to encode complete *wmk12-like* homologues clustering together in an own clade, while all individuals from the grain storage facility contained the deletion & frame-shift mutation and clustered with the *wmk12* gene from *w*Sur JKI (Supplement Figure 1). In addition, we only found *wmk1-like* homologues in individuals from the grain storage population, but in no individual from the feral populations.

We tested for the symbiont-mediated male-killing phenotype in the JKI strain of *O. surinamensis* by quantifying the sex ratio in symbiotic and aposymbiotic beetle cultures. We found a uniform frequency of both sexes in both cultures (SYM 50W+50M, APO 52W+48M; $\chi 2$ test of homogeneity: $\chi 2 = 0.080$, p-value = 0.888). In addition, the male-killing phenotype should also result in a reduced hatching rate of around 50% in mating pairs with symbiotic females and males in comparison to mating pairs with aposymbiotic individuals. However, we did not observe such differences (BH corrected Wilcoxon rank sum test, p-value = 0.84; Figure 6, right).

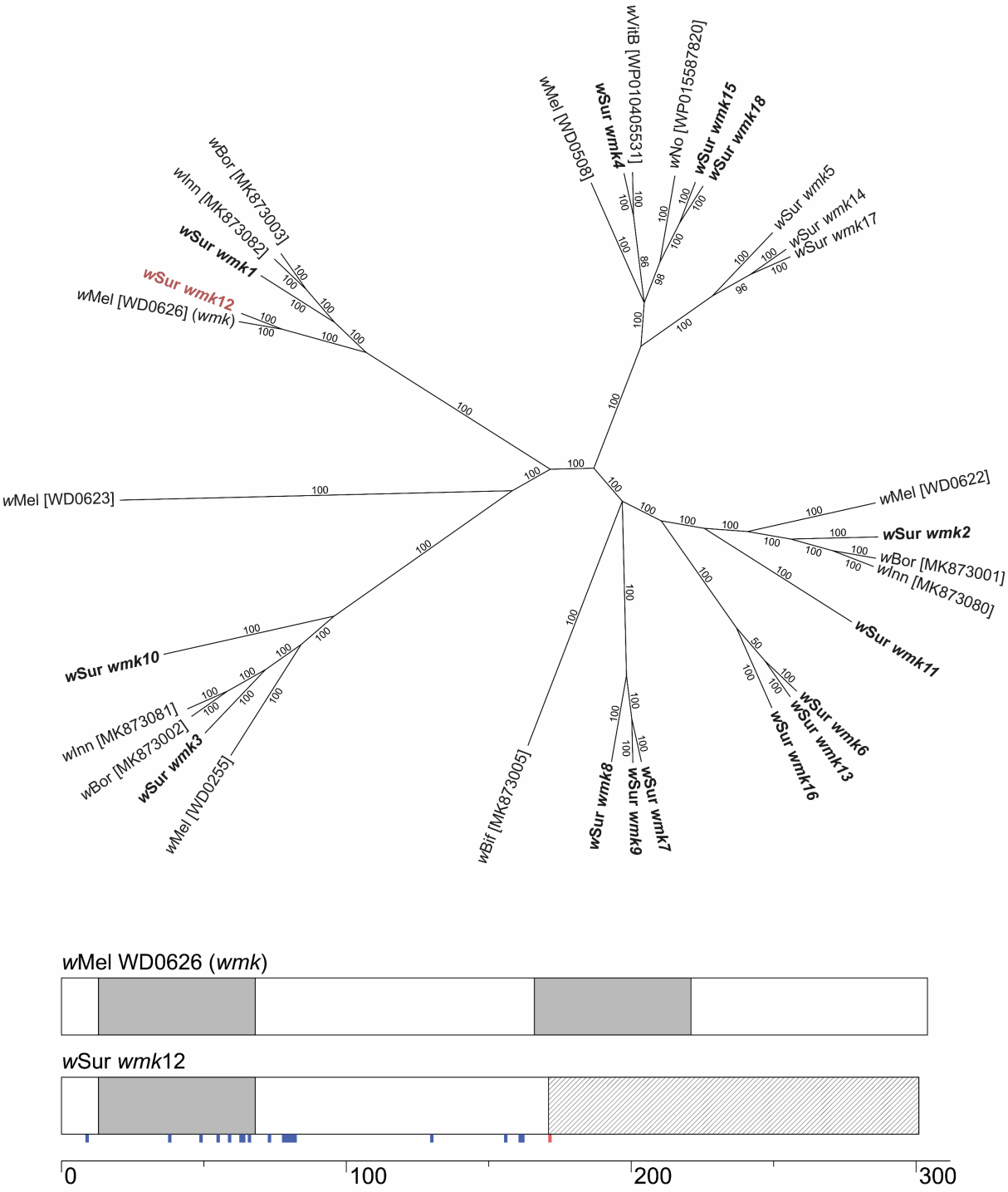


Figure 5: Top: Bayesian phylogeny of *w*Mel and *w*Sur *wmk* homologues based on a nucleotide alignment. Consensus support values are shown at the branches. Bottom: Schematic of *w*Mel and *w*Sur *wmk* native nucleotide sequences. The blue tick marks indicate non-synonymous nucleotide substitutions. The red tick mark indicates eight-nucleotide deletion resulting in frame-shift mutation with a stop-codon at the deletion site. The two loci (helix-turn-helix (HTH) protein domain) of *wmk* are highlighted in grey. The hatched area indicates the region of *wmk12* that is predicted to be not translated based on the stop codon (red tick).

3.4.1 Cytoplasmic incompatibility (CI)

Single homologues of both previously identified CI factor genes *cifA* and *cifB* were encoded in the *Wolbachia* prophage region WOSurB. Bayesian phylogenetic inference identified both *cifA* and *cifB* as type II following the classification scheme of Lindsey et al. (2018) (Figure 7). The *cifA* gene found in *w*Sur was closely related to those found in the *Wolbachia* strain *w*Ri of the fruit fly *Drosophila simulans* and *w*Suzi of the spotted wing drosophila *Drosophila suzukii*, while *cifB* did not cluster closely with any previously described genes from other *Wolbachia* strains. Although the *cifA* gene showed no homology to known domains (Figure 7, bottom), putative domains (PD-(D/E)XK nuclease/DpnII-MboI) were found in the *cifB* gene.

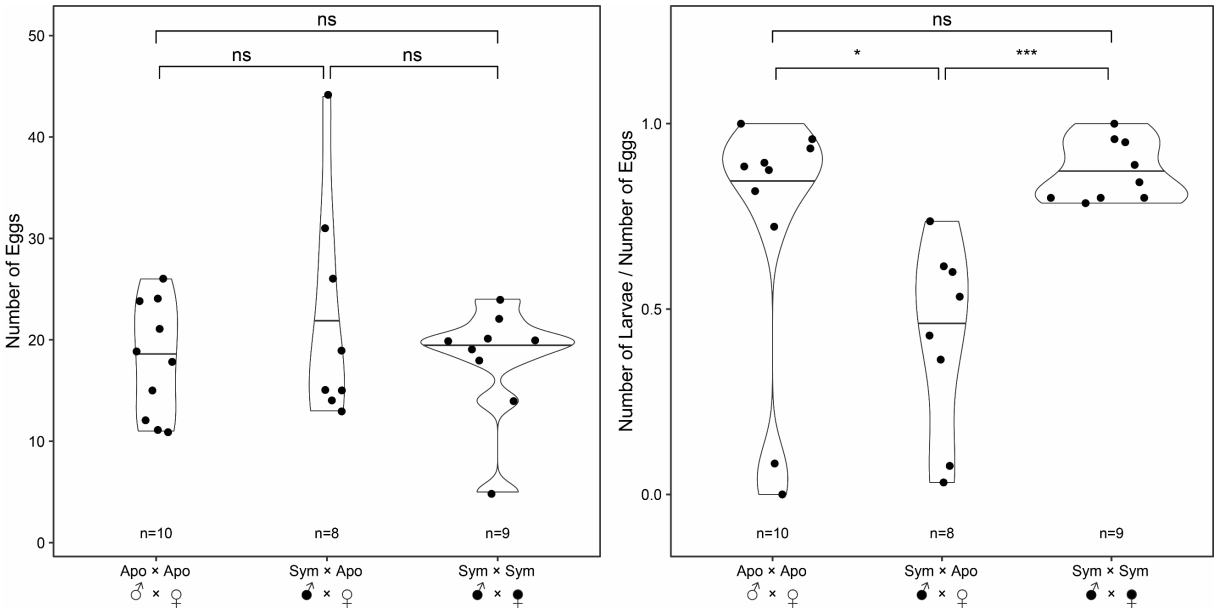


Figure 6: Influence of *w*Sur on embryo development of *O. surinamensis*. Number of laid eggs (left) and hatching rate (right) in the three mating groups. The data distribution is visualized with violin plots and an additional horizontal line depicting the median. A filled sex sign indicates a symbiotic wildtype specimen, meaning infected with *w*Sur and *S. silvanidophilus*, whereas an empty sign indicates these specimens as aposymbiotic (regarding both symbionts). n is the number of *O. surinamensis* mating pairs. Statistical significance between the groups is based on Benjamini-Hochberg corrected Wilcoxon rank sum tests (ns = not significant, * = $p < 0.05$, *** = $p < 0.005$).

We tested the ability of *Wolbachia* infection to cause cytoplasmatic incompatibility by mating experiments with differential *w*Sur infection. First, the impact of *Wolbachia* infection on the number of laid eggs was determined. As expected, infection with *w*Sur had no effect on the number of laid eggs (Kruskal Wallis test: $\chi^2 = 0.29$, df = 2, p-value = 0.86; Figure 6, left).

Following further development, we observed overall differences between the three groups' hatching rates (Kruskal Wallis test: $\chi^2 = 10.85$, df = 2, p-value = 0.004397; Figure 6, right). While the hatching rate between the control groups did not differ (aposymbiotic females and males, as well as symbiotic females and males: BH corrected Wilcoxon rank sum test: p-value = 0.84; Figure 6, right), the hatching rate in the CI cross with aposymbiotic females and symbiotic males right was reduced by 43 – 47% in comparison to both control groups (BH corrected Wilcoxon rank sum test, p-value = 0.04 and 0.0018; Figure 6).

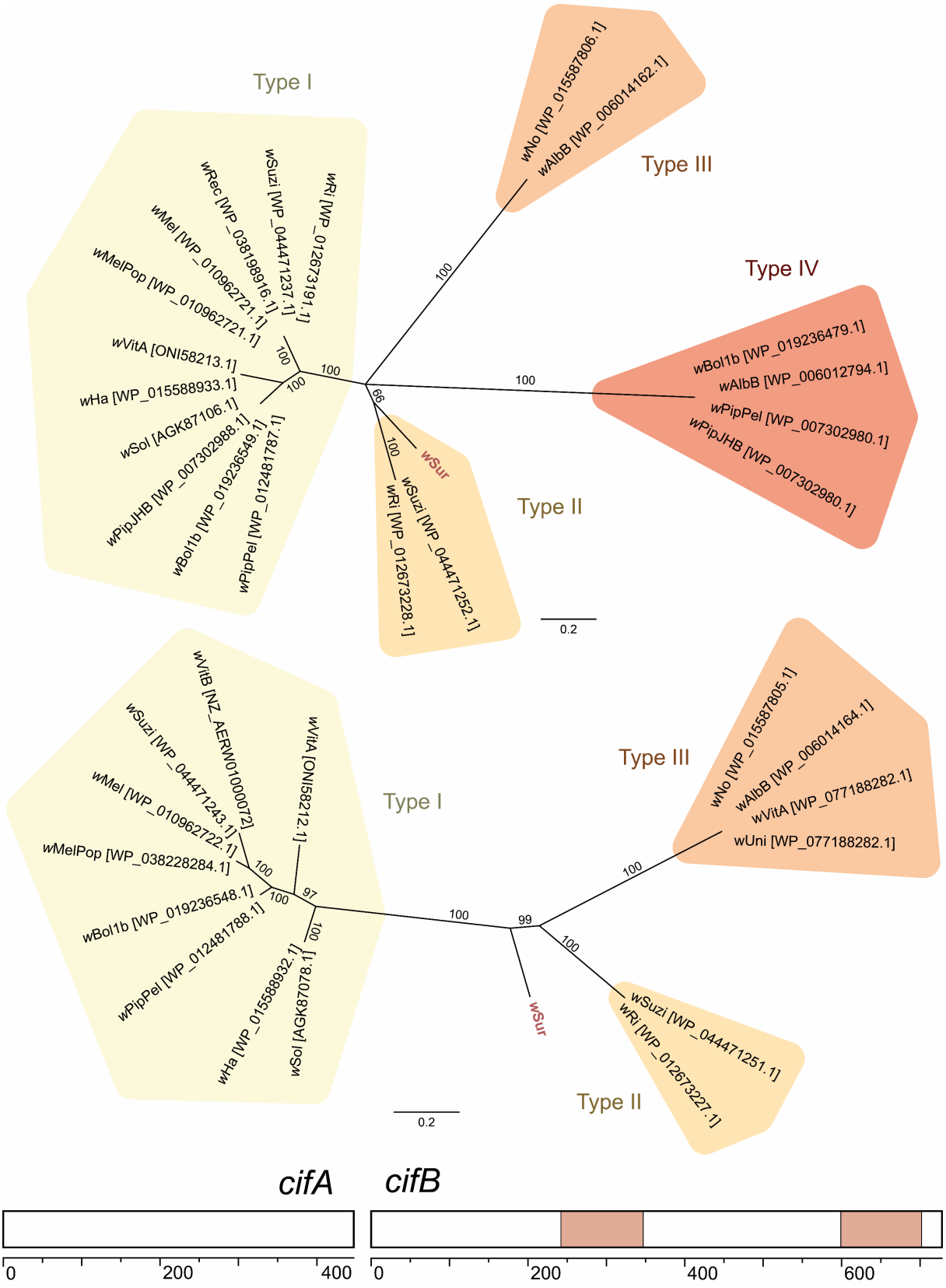


Figure 7: Phylogeny and domain structure of *cif* genes. Top: Bayesian phylogenies based on a nucleotide alignment of *cifA* (top) and *cifB* (middle) genes. Consensus support values are shown at the branches. Coloured shapes around branches designate monophyletic "types". Bottom: Domain structure for the *cif* genes of *w*Sur. The two loci (PD-(D/E)XK nuclease/DpnII-MboI protein domain) of *cifB* are shown and indicated with orange bars.

3.5 Discussion

The sawtoothed grain beetle *O. surinamensis* harbours not only the nutritional Bacteroidota endosymbiont *S. silvanidophilus* but is also infected by a pervasive *Wolbachia* strain. A phylogenetic analysis of the *w*Sur core genome classified it as a member of supergroup B. *Wolbachia* strains of supergroup B together with supergroup A primarily infect arthropod hosts and are generally capable of reproductive manipulation, particularly by causing male-killing and CI[8].

The genome of the *Wolbachia* strain *w*Sur of *O. surinamensis* encodes for 18 *wmk* homologues, all of which contain two helix-turn-helix (HTH) DNA-binding domains that are important for their function as a transcriptional regulator. The genomic distribution of some of the *wmk* homologues inside the phageWO is comparable to homologues found in other *Wolbachia* strains like *w*Mel of *Drosophila melanogaster* which were proposed as candidate genes responsible for the induction of *Wolbachia*'s male-killing phenotype[15,24].

A phylogenetic comparison of their nucleotide sequences with previously characterized *wmk* homologues of *w*Mel and *wmk* homologues of known male-killing strains predicted the homologue *wmk1* and *wmk12* of *w*Sur as the most likely candidates to cause male-killing. However, further analysis revealed an eight-nucleotide deletion leading to a stop-codon mid-sequence and subsequent reading frame-shift of *wmk12*. The loss of half of the encoded protein and one of the two HTH DNA-binding domains presumably abolishes its ability to interfere with transcriptional regulation and the male-killing phenotype of *wmk12* and *w*Sur, while *wmk1* could still be functional. We found no indication of symbiont-mediated male-killing in the context of the JKI strain as no differences in hatching rate could be observed between symbiotic and aposymbiotic (free of both symbionts) mating pairs. Further, JKI stock cultures of symbiotic and aposymbiotic beetles both exhibited homogenous distributions of both sexes. However, Sharaf et al. (2010) compared a feral population of *O. surinamensis* from the field with a population adapted to a grain storage facility. They observed a strong female bias and reduced larval survival among offspring from these feral populations emerging under lab conditions in contrast to a balanced sex ratio and higher larval survival in the population collected from the grain storage facility. They also observed incomplete *Wolbachia* infection in both populations: an 84% infection rate in the feral and 66% in the storage population. In combination, these data suggest active sex ratio

distortion in the feral populations, likely by *Wolbachia*, but not in the population adapted to grain storage.

Individuals from the same collection site were used for genome sequencing by Hong et al. (2020) with a focus on host genomes. Our analysis of *Wolbachia* encoded *wmk1* & *wmk12*-like homologues in these sequences read archives revealed the absence of *wmk1* together with intact *wmk12* in the two feral populations. In contrast, individuals of the Israeli population collected from the grain storage facility did encode *wmk1* homologues as well as the truncated *wmk12* version. Thus, we hypothesise that the intact *wmk12* represents the ancestral state that mediates wSur male-killing in feral populations in Israel, while wSur from populations adapted to grain storage facilities acquired a gene duplication of *wmk12* in the WOSurB region (=*wmk12*) and a deletion in the original *wmk12* gene as well as a loss of the male-killing phenotype at least in this host genetic background. These changes probably occurred recently, possibly in the process of invasion and adaption to storage grains within co-adapted hosts that evolved probably under isolation from feral populations and facilitated by repeated strong population bottlenecks during the invasion of novel stored grain facilities or batches, as well as a relaxed selection pressure on wSur in completely infected hos populations. However, whether the changes in the *wmk12* gene are causative for the loss of the male-killing phenotype remains elusive. Addition factors like host evolution or resistance to male-killing effectors could also play a role in the loss of male-killing[68].

Multiple homologues of *wmk* have been described in other *Wolbachia* strains, although all except one did not induce male-killing when transgenically expressed in *Drosophila melanogaster*[15]. As of right now, the function of the additional *wmk* homologues in *w*Sur, as well as *w*Mel, remains unknown. *w*Sur and other strains might be multipotent and capable of inducing male-killing under specific conditions, or when infecting another host, like the *Wolbachia* strain *w*Rec inducing CI in its main host *Drosophila recens* but causing male-killing when transferred to the closely related species *Drosophila subiquinaria*[69]. In addition, the *Wolbachia* strains might serve completely different functions to manipulate the host beyond reproductive manipulation, e.g. by affecting pheromone biosynthesis, perception, or behaviour[70–73]. The *wmk12/1* duplication in *w*Sur at least suggests that it is beneficial to retain a functional *wmk* gene, although possibly in a different context. Additional experiments, utilizing both feral and storage-adapted *O. surinamensis*

populations with hybrid crosses, or transgenic expression of different *wmk* genes in aposymbiotic hosts might help to shed light on their function.

CI induced by *Wolbachia* occurs when the sperm of infected males is expressing the *cif* genes which lead to infertile embryos in uninfected females, while in infected females the rescue factor *cifA* can reverse this effect[16–18]. The genome of *w*Sur encodes homologues for both CI-inducing genes *cifA* and *cifB* in one of the phage WO regions. *cifA* and *cifB* gene products are classified based on the similarity of their expressed amnio acid sequence as type I to type V[20,74,75]. The CI phenotype was demonstrated in *cif* genes of type I, II and IV[20]. Our analysis classified the *cifA* of *w*Sur as a type II homologue, while *cifB* clustered in between type I and II homologues. Our experimental data indicate *w*Sur to be a reproductive manipulator by causing unidirectional CI to its host. Crossing *Wolbachia*-infected males with uninfected females resulted in a hatching rate that was reduced by 45% compared to crossings between infected males and females or uninfected males and females, respectively. Findings in *Drosophila simulans* showed strong induction of CI leading to a hatching rate reduction of up to 95%[76], while data from *Drosophila melanogaster* showed weak induction of CI resulting in a hatching rate reduced by 15 – 30%[77], depending on environmental conditions[78] as well as individual life history[79]. As we were so far not able to manipulate *S. silvanidophilus* and *Wolbachia* presence in *O. surinamensis* individually, symbiont-mediated phenotypes must be considered with great care in dual symbiont-depleted experiments. However, with the addition of genomic and ecological information, we confidently attribute the here reported CI to *Wolbachia.* While *S. silvanidophilus* presence mirrored *Wolbachia* in the present experiments on CI and male-killing, we have no indication for the presence of known CI factors encoded in the highly reduced *S. silvanidophilus* genome[36], while *w*Sur clearly contains homologues of both so far identified cytoplasmatic incompatibility factors. Thus, the *Wolbachia* strain *w*Sur is likely able to influence its fitness by increasing its transmission in partially infected populations, which is reflected by its high, observed prevalence in lab conditions.

Whether *Wolbachia* influences *O. surinamensis* beyond reproductive manipulation remains unclear. Previously reported cuticle supplementation of *O. surinamensis* is probably solely caused by *S. silvanidophilus*, because only the Bacteroidota endosymbiont contains the ability to synthesize aromatic amino acids precursors via the shikimate pathway to support the host's cuticle synthesis, while *w*Sur and *Wolbachia*, in general, lack the entire pathway (Kiefer at al., 2021 and this study).

Further, cuticle deficiencies (reduced thickness and melanisation) were not only reported in dual aposymbiotic individuals after strict tetracycline treatment (deficient of both *S. silvanidophilus* and *w*Sur), but also after glyphosate treatment, which only reduced *S. silvanidophilus*, but not *w*Sur titers (Supplement Figure 2)[36]. Thus, *S. silvanidophilus* is solely responsible for supplementation of cuticle synthesis as well as ecological consequences in terms of elevated resistance to abiotic desiccation stress, pathogen and predation pressure[80], but also costs of symbiont infection on reproduction[39].

Certain *Wolbachia* strains were previously reported to supplement the hosts' diet with limited nutrients (esp. B-Vitamins)[11] or provide pathogen defence[81]. The *Wolbachia* strain *w*Sur of *O. surinamensis* also encodes pathways to synthesize the amino acids lysine, glutamine, threonine, glycine, and serine as well as the vitamin riboflavin. While riboflavin does not seem to be limited on cereal-based diets[82], lysine is[83]. It remains unclear whether *Wolbachia* might synthesize lysine for its benefit, or also contributes to its host's metabolism. Similarly, it is unclear whether *Wolbachia* infection inflicts additional costs beyond unidirectional CI which is only relevant in populations with incomplete *Wolbachia* infection[84–86].

In combination with our previous work on the Bacteroidota symbiont *Shikimatogenerans silvanidophilus*[34,36,39,80], we demonstrate that the *O. surinamensis* harbours two notable symbionts. Both impact the host's physiology, ecology and thereby also its and each other's evolution. Based on the high prevalence of both nutritional symbionts[87,88] and reproductive manipulators[7,29] in coleopteran and insects in general, dual infections with both types of symbionts are not uncommon and probably even underestimated[31,89,90]. However, currently both symbioses are usually only studied by experimental approaches in isolation, or from a descriptive perspective on the prevalence and genomic potential of both symbionts. Thereby, we miss out on potential higher levels of ecological interactions of both types of symbioses, mediated either via the host's physiology or even directly between different symbionts. Future work should thus try to integrate multipartite, symbiotic relationships. Available tools include selective removal or inhibition of individual symbionts, e.g. by targeting specific, obligate biosynthetic pathways of symbionts. The here utilized glyphosate, inhibiting the shikimate pathway[65], but e.g. also inhibitors of the Diaminopimelate pathway responsible for synthesizing lysine are prominent agents suggested for the manipulation of specific biosynthetic capabilities

or organisms encoding them[91]. Alternatively, expression of target symbiont genes in suitable host systems is a powerful tool to address gene function in insect symbionts that are elusive to genetic manipulation themselves[15,17,24]. Finally, the example of *O. surinamensis* highlights again the importance to identify systems with interesting combinations of symbionts and certain amenability for experimental manipulation and observation to understand more complex eco-evolutionary dynamics of multipartite symbioses.

3.6 Acknowledgments

We thank Dagmar Klebsch and Benjamin Weiss for technical assistance with insect maintenance and histology, Eugen Bauer for his support on genome analysis, the Max Planck-Genome Centre Cologne (http://mpgc.mpg.de/home) for performing library preparation and Illumina sequencing, the Johannes Gutenberg-University Mainz for computation time granted on the supercomputer 'MOGON', and Christian Meesters for administrative assistance on 'MOGON'. R.K., M.K. and T.E. acknowledge funding from the Max Planck Society, and further financial support of the Johannes Gutenberg-University Mainz (intramural funding to T.E.), and a Consolidator Grant of the European Research Council (ERC CoG 819585 "SYMBeetle" to M.K.).

3.7 Data Accessibility Statement

Genetic data: Raw sequence reads are deposited in the SRA (SRR12881563–SRR12881566; SRR12881567–SRR12881568; BioProject PRJNA670819). The annotated *w*Sur genome is available on GenBank (CP092526). Bioassay data is available on the data repository of the Max-Planck-Society Edmond[92].

3.8 Benefit-Sharing Statement

All specimens utilized in this work were obtained from a long-standing laboratory culture (pre-2014). Thus, the Nagoya Protocol is not applicable and no benefits to report.

3.9 Contributions

J.S.T.K. and T.E. designed the project, J.S.T.K., G.S., and R.K. performed experiments, J.S.T.K., G.S., R.K. and T.E. analysed the data, J.S.T.K. and T.E. wrote the initial manuscript, all authors read and commented on the manuscript.

3.10 References

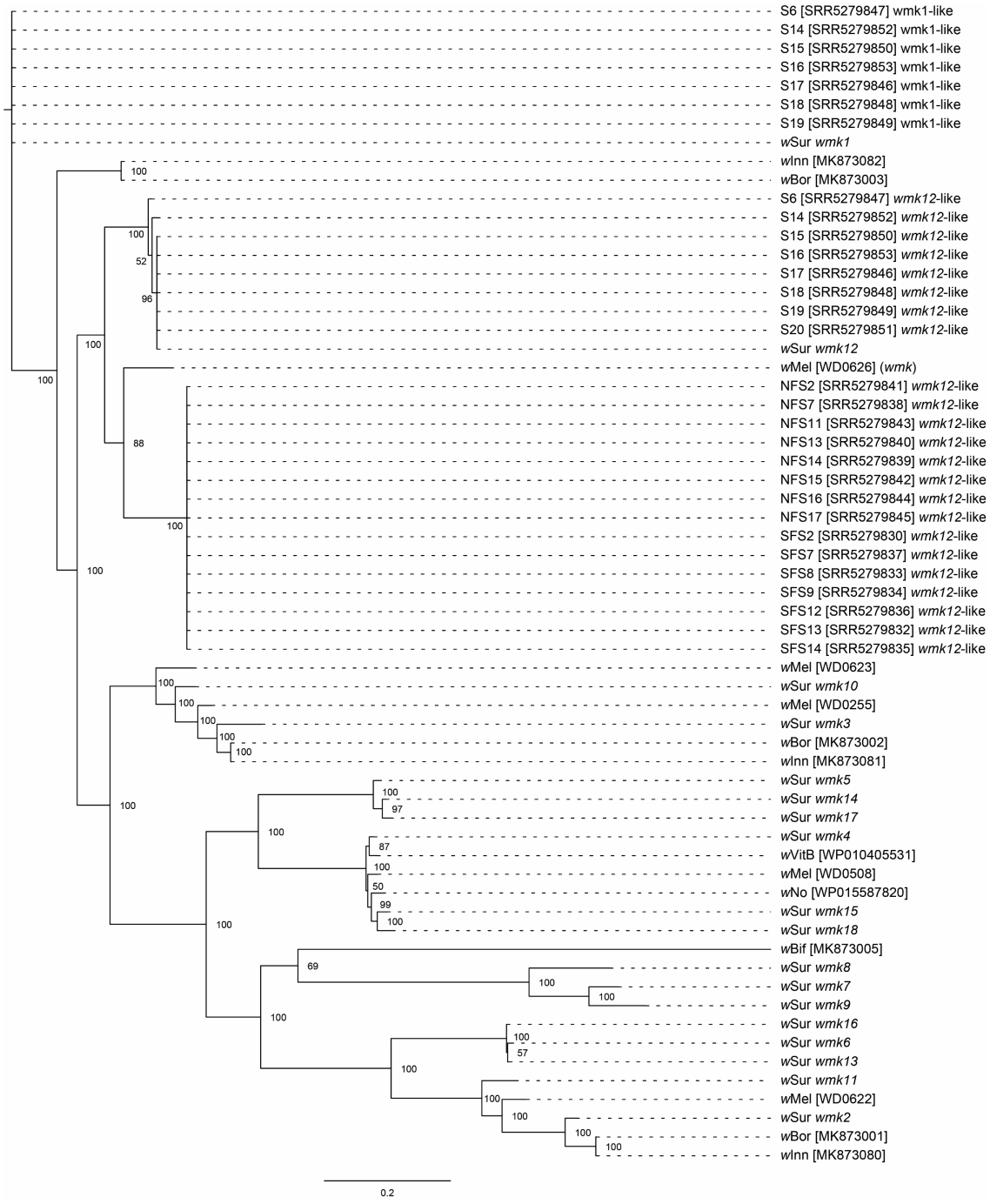
1. Douglas, A. E. Multiorganismal insects: diversity and function of tesident microorganisms. *Annu. Rev. Entomol.* **60**, 17–34 (2015).
2. McFall-Ngai, M. *et al.* Animals in a bacterial world, a new imperative for the life sciences. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 3229–3236 (2013).
3. Drew, G. C., Stevens, E. J. & King, K. C. Microbial evolution and transitions along the parasite–mutualist continuum. *Nat. Rev. Microbiol.* **19**, 623–638 (2021).
4. Oliver, K. M. & Martinez, A. J. How resident microbes modulate ecologically-important traits of insects. *Curr. Opin. Insect Sci.* **4**, 1–7 (2014).
5. Feldhaar, H. Bacterial symbionts as mediators of ecologically important traits of insect hosts. *Ecol. Entomol.* **36**, 533–543 (2011).
6. Zytynska, S. E., Tighiouart, K. & Frago, E. Benefits and costs of hosting facultative symbionts in plant-sucking insects: A meta-analysis. *Mol. Ecol.* **30**, 2483–2494 (2021).
7. Duron, O. *et al.* The diversity of reproductive parasites among arthropods: *Wolbachia* do not walk alone. *BMC Biol.* **6**, 27 (2008).
8. Werren, J. H., Baldo, L. & Clark, M. E. *Wolbachia*: master manipulators of invertebrate biology. *Nat. Rev. Microbiol.* **6**, 741–751 (2008).
9. Heath, B. D., Butcher, R. D. J., Whitfield, W. G. F. & Hubbard, S. F. Horizontal transfer of *Wolbachia* between phylogenetically distant insect species by a naturally occurring mechanism. *Curr. Biol.* **9**, 313–316 (1999).
10. Zug, R. & Hammerstein, P. Bad guys turned nice? A critical assessment of *Wolbachia* mutualisms in arthropod hosts. *Biol. Rev.* **90**, 89–111 (2015).
11. Hosokawa, T., Koga, R., Kikuchi, Y., Meng, X.-Y. & Fukatsu, T. *Wolbachia* as a bacteriocyte-associated nutritional mutualist. *Proc. Natl. Acad. Sci.* **107**, 769–774 (2010).
12. Moriyama, M., Nikoh, N., Hosokawa, T. & Fukatsu, T. Riboflavin provisioning underlies *Wolbachia's* fitness contribution to its insect host. *MBio* **6**, (2015).
13. Ju, J. F. *et al. Wolbachia* supplement biotin and riboflavin to enhance reproduction in planthoppers. *ISME J.* **14**, 676–687 (2019).
14. Fialho, R. F. & Stevens, L. Male-killing *Wolbachia* in a flour beetle. *Proc. R. Soc. London. Ser. B Biol. Sci.* **267**, 1469–1474 (2000).
15. Perlmutter, J. I., Meyers, J. E. & Bordenstein, S. R. Transgenic testing does not support a role for additional candidate genes in *Wolbachia* male killing or cytoplasmic incompatibility. *mSystems* **5**, (2020).
16. Shropshire, J. D. *et al.* Models and nomenclature for cytoplasmic incompatibility: caution over premature conclusions – a response to Beckmann et al. *Trends Genet.* **35**, 397–399 (2019).
17. Shropshire, J. D. & Bordenstein, S. R. Two-By-One model of cytoplasmic incompatibility: dynthetic recapitulation by transgenic expression of *cifA* and *cifB* in *Drosophila*. *PLOS Genet.* **15**, e1008221 (2019).
18. Beckmann, J. F. *et al.* The toxin–antidote model of cytoplasmic incompatibility: genetics and evolutionary implications. *Trends Genet.* **35**, 175–185 (2019).
19. Shropshire, J. D. Identifying and characterizing phage genes involved in *Wolbachia*-induced cytoplasmic incompatibility. (2020).
20. LePage, D. P. *et al.* Prophage WO genes recapitulate and enhance *Wolbachia*-induced cytoplasmic incompatibility. *Nature* **543**, 243–247 (2017).
21. Shropshire, J. D., On, J., Layton, E. M., Zhou, H. & Bordenstein, S. R. One prophage WO gene rescues cytoplasmic incompatibility in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U. S. A.* **115**, 4987–4991 (2018).
22. Hurst, G. Male-Killing bacteria in insects: mechanisms, incidence, and implications. *Emerg. Infect. Dis.* **6**, 329–336 (2000).
23. Jaenike, J., Dyer, K. A. & Reed, L. K. Within-population structure of competition and the dynamics of male-killing *Wolbachia*. *Evol. Ecol. Res.* **5**, 1023–1036 (2003).
24. Perlmutter, J. I. *et al.* The phage gene wmk is a candidate for male killing by a bacterial endosymbiont. *PLOS Pathog.* **15**, e1007936 (2019).
25. Duron, O., Fort, P. & Weill, M. Hypervariable prophage WO sequences describe an unexpected high number of *Wolbachia* variants in the mosquito *Culex pipiens*. *Proc. R. Soc. B Biol. Sci.* **273**, 495–502 (2005).
26. Bordenstein, S. R. & Werren, J. H. Effects of A and B *Wolbachia* and host genotype on interspecies cytoplasmic incompatibility in *Nasonia*. *Genetics* **148**, 1833–1844 (1998).
27. Bordenstein, S. R. & Werren, J. H. Bidirectional incompatibility among divergent *Wolbachia* and

incompatibility level differences among closely related *Wolbachia* in *Nasonia*. *Heredity (Edinb).* **99**, 278–287 (2007).
28. Li, Y.-Y., Fields, P. G., Pang, B.-P., Coghlin, P. C. & Floate, K. D. Prevalence and diversity of *Wolbachia* bacteria infecting insect pests of stored products. *J. Stored Prod. Res.* **62**, 93–100 (2015).
29. Kajtoch, Ł. & Kotásková, N. Current state of knowledge on *Wolbachia* infection among Coleoptera: A systematic review. *PeerJ* (2018).
30. Aikawa, T. *et al.* Cytoplasmic incompatibility in the semivoltine longicorn beetle *Acalolepta fraudatrix* (Coleoptera: Cerambycidae) double infected with *Wolbachia*. *PLoS One* **17**, e0261928 (2022).
31. Heddi, A., Grenier, A. M., Khatchadourian, C., Charles, H. & Nardon, P. Four intracellular genomes direct weevil biology: Nuclear, mitochondrial, principal endosymbiont, and *Wolbachia*. *Proc. Natl. Acad. Sci. U. S. A.* **96**, 6814–6819 (1999).
32. Li, Y.-Y., Fields, P. G., Pang, B.-P. & Floate, K. D. Effects of tetracycline and rifampicin treatments on the fecundity of the *Wolbachia*-infected host, *Tribolium confusum* (Coleoptera: Tenebrionidae). *J. Econ. Entomol.* **109**, 1458–1464 (2016).
33. Boyer, S., Zhang, H. & Lempérière, G. A review of control methods and resistance mechanisms in stored-product insects. *Bull. Entomol. Res.* **102**, 213 (2012).
34. Engl, T. *et al.* Ancient symbiosis confers desiccation resistance to stored grain pest beetles. *Mol. Ecol.* **27**, 2095–2108 (2018).
35. Hirota, B. *et al.* A novel, extremely elongated, and endocellular bacterial symbiont supports cuticle formation of a grain pest beetle. *MBio* **8**, 1–16 (2017).
36. Kiefer, J. S. T. *et al.* Inhibition of a nutritional endosymbiont by glyphosate abolishes mutualistic benefit on cuticle synthesis in *Oryzaephilus surinamensis*. *Commun. Biol.* **4**, 554 (2021).
37. Koch, A. Die Symbiose von *Oryzaephilus surinamensis* L. (Cucujidae, Coleoptera). *Zeitschrift für Morphol. und Ökologie der Tiere* **23**, 389–424 (1931).
38. Sharaf, K., Horová, L., Pavlíček, T., Nevo, E. & Bureš, P. Genome size and base composition in *Oryzaephilus surinamensis* (Coleoptera: Sylvanidae) and differences between native (feral) and silo pest populations in Israel. *J. Stored Prod. Res.* **46**, 34–37 (2010).
39. Engl, T., Schmidt, T. H. P., Kanyile, S. N. & Klebsch, D. Metabolic cost of a nutritional symbiont manifests in delayed reproduction in a grain pest beetle. *Insects* **11**, 717 (2020).
40. Makepeace, B. L., Rodgers, L. & Trees, A. J. Rate of elimination of *Wolbachia pipientis* by doxycycline in vitro increases following drug withdrawal. *Antimicrob. Agents Chemother.* **50**, 922–927 (2006).
41. Weiss, B. & Kaltenpoth, M. Bacteriome-localised intracellular symbionts in pollen-feeding beetles of the genus *Dasytes* (Coleoptera, Dasytidae). *Front. Microbiol.* **7**, 1486 (2016).
42. Weller, R., Glöckner, F. O. & Amann, R. 16S rRNA-targeted oligonucleotide probes for the in situ detection of members of the phylum Cytophaga-Flavobacterium-Bacteroides. *Syst. Appl. Microbiol.* **23**, 107–114 (2000).
43. Amann, R. I. *et al.* Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl. Environ. Microbiol.* **56**, 1919–1925 (1990).
44. Sanguin, H. *et al.* Development and validation of a prototype 16S rRNA-based taxonomic microarray for Alphaproteobacteria. *Environ. Microbiol.* **8**, 289–307 (2006).
45. Bankevich, A. *et al.* SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* **19**, 455–477 (2015).
46. Laczny, C. C. *et al.* BusyBee Web: metagenomic data analysis by bootstrapped supervised binning and annotation. *Nucleic Acids Res.* **45**, W171–W179 (2017).
47. Overbeek, R. *et al.* The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). *Nucleic Acids Res.* **42**, D206–D214 (2014).
48. Arkin, A. P. *et al.* KBase: the United States department of energy systems biology knowledgebase. *Nat. Biotechnol.* **36**, 566–569 (2018).
49. Krzywinski, M. *et al.* Circos: an information aesthetic for comparative genomics. *Genome Res.* **19**, 1639–1645 (2009).
50. Price, M. N., Dehal, P. S. & Arkin, A. P. FastTree 2–approximately maximum-likelihood trees for large alignments. *PLoS One* **5**, e9490 (2010).
51. Edgar, R. C. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **32**, 1792–1797 (2004).
52. Hong, W. *et al.* Genome-wide analysis revisits incipient sympatric and allopatric speciation in a beetle. *Isr. J. Ecol. Evol.* **67**, 69–80 (2020).
53. Huelsenbeck, J. P. & Ronquist, F. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinforma. Appl.*

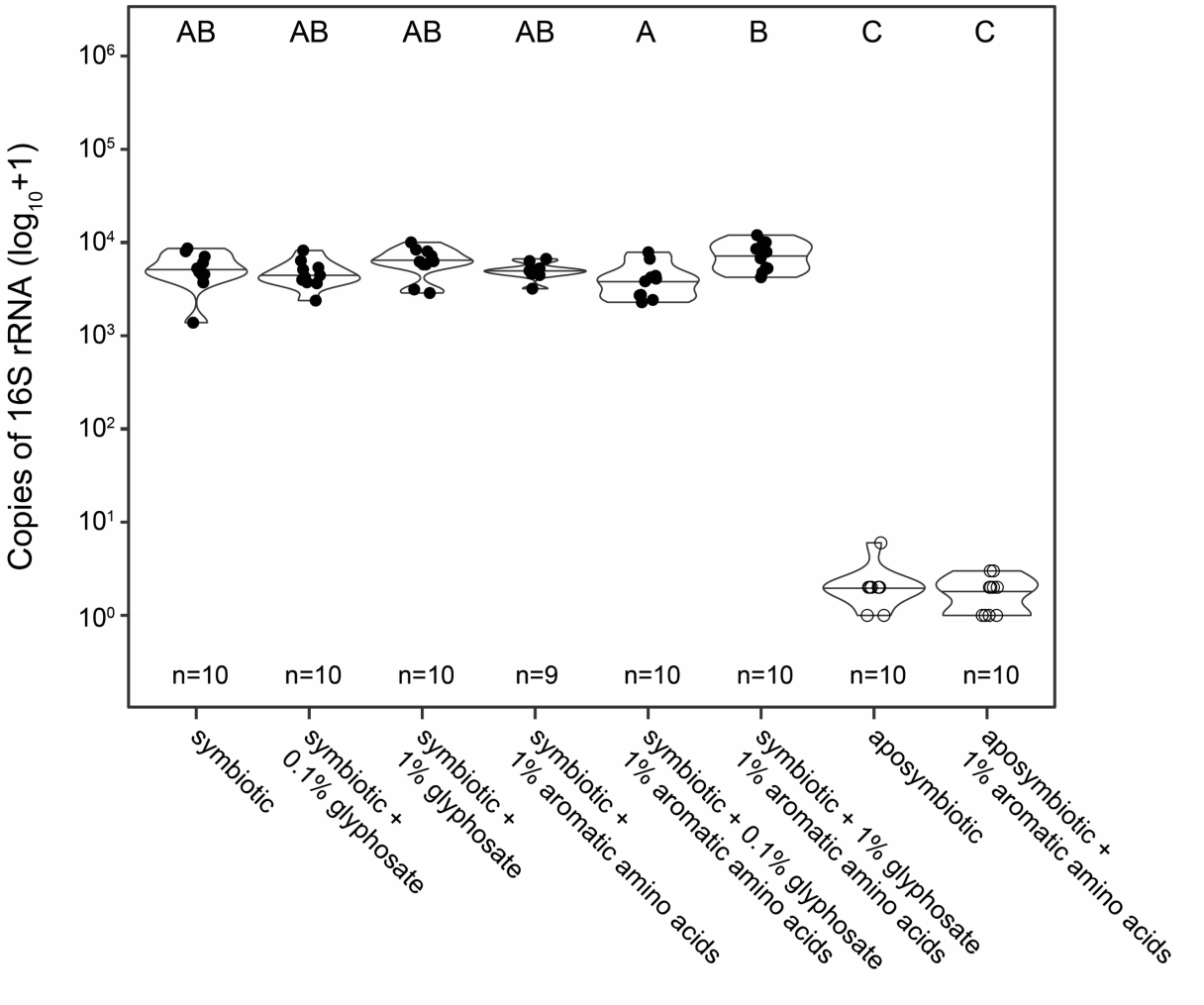
NOTE **17**, 754–755 (2001).
54. Sullivan, J., Joyce, P., Posada, D. & Crandall, K. A. jModelTest 2: more models, new heuristics and parallel computing. *Nat. Methods* **36**, 716–723 (2012).
55. Zimmermann, L. *et al.* A Completely reimplemented MPI bioinformatics toolkit with a new HHpred server at its core. *J. Mol. Biol.* **430**, 2237–2243 (2018).
56. Ün, Ç. *et al.* Cytoplasmic incompatibility between Old and New World populations of a tramp ant. *Evolution (N. Y).* **75**, 1775–1791 (2021).
57. Gilchrist, C. L. M. & Chooi, Y. H. clinker & clustermap.js: automatic generation of gene cluster comparison figures. *Bioinformatics* **37**, 2473–2475 (2021).
58. Halstead, D. G. H. External sex differences in stored-products Coleoptera. *Bull. Entomol. Res.* **54**, 119–134 (1963).
59. Dunn, O. J. Multiple comparisons using rank sums. *Technometrics* **6**, 241–252 (1964).
60. Benjamini, Y. & Hochberg, Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser. B* **57**, 289–300 (1995).
61. Piepho, H. P. & Piepho, H.-P. Data transformation in statistical analysis of field trials with changing treatment variance. *Agron. J.* **101**, 865–869 (2009).
62. Mangiafico, S. Package 'rcompanion' title functions to support extension education program evaluation. *Cran Repos* **20**, 1–71 (2017).
63. Wickham, H. *ggplot2 - Elegant Graphics for Data Analysis*. *Springer* (2016).
64. Veneti, Z. *et al.* Cytoplasmic Incompatibility and Sperm Cyst Infection in Different Drosophila-Wolbachia Associations. *Genetics* **164**, 545–552 (2003).
65. Steinrücken, H. C. & Amrhein, N. The herbicide glyphosate is a potent inhibitor of 5-enolpyruvylshikimic acid-3-phosphate synthase. *Biochem. Biophys. Res. Commun.* **94**, 1207–1212 (1980).
66. Fischer, R. S., Berry, A., Gaines, C. G. & Jensen, R. A. Comparative action of glyphosate as a trigger of energy drain in eubacteria. *J. Bacteriol.* **168**, 1147–1154 (1986).
67. Gresshoff, P. Growth inhibition by glyphosate and reversal of its action by phenylalanine and tyrosine. *Funct. Plant Biol.* **6**, 177 (1979).
68. Hornett, E. A., Kageyama, D. & Hurst, G. D. D. Sex determination systems as the interface between male-killing bacteria and their hosts. *Proc. R. Soc. B Biol. Sci.* **289**, (2022).
69. Jaenike, J. Spontaneous emergence of a new *Wolbachia* phenotype. *Evolution (N. Y).* **61**, 2244–2252 (2007).
70. Engl, T. & Kaltenpoth, M. Influence of microbial symbionts on insect pheromones. *Nat. Prod. Rep.* **35**, 386–397 (2018).
71. Schneider, D. I. *et al.* Symbiont-driven male mating success in the neotropical *Drosophila paulistorum* superspecies. *Behav. Genet.* **49**, 83–98 (2019).
72. Bi, J. & Wang, Y.-F. The effect of the endosymbiont *Wolbachia* on the behavior of insect hosts. *Insect Sci.* **27**, 846–858 (2020).
73. Farahani, H. K., Ashouri, A., Abroon, P., Pierre, J.-S. & van Baaren, J. *Wolbachia* manipulate fitness benefits of olfactory associative learning in a parasitoid wasp. *J. Exp. Biol.* **224**, (2021).
74. Lindsey, A. R. I. *et al.* Evolutionary genetics of cytoplasmic incompatibility genes *cifA* and *cifB* in prophage WO of *Wolbachia*. *Genome Biol. Evol.* **10**, 434–451 (2018).
75. Bing, X.-L. L. *et al.* Genomic analysis of *Wolbachia* from *Laodelphax striatellus* (Delphacidae, Hemiptera) reveals insights into its "jekyll and hyde" mode of infection pattern. *Genome Biol. Evol.* **12**, 3818–3831 (2020).
76. Sinkins, S. P., Braig, H. R. & O'Neill, S. L. *Wolbachia* superinfections and the expression of cytoplasmic incompatibility. *Proc. R. Soc. London. Ser. B Biol. Sci.* **261**, 325–330 (1995).
77. Hoffmann, A. A., Clancy, D. J. & Merton, E. Cytoplasmic incompatibility in Australian populations of *Drosophila melanogaster*. *Genetics* **136**, 993–999 (1994).
78. Hague, M. T. J., Caldwell, C. N. & Cooper, B. S. Pervasive effects of *Wolbachia* on host temperature preference. *MBio* **11**, 1–15 (2020).
79. Shropshire, J. D., Rosenberg, R. & Bordenstein, S. R. The impacts of cytoplasmic incompatibility factor (*cifA* and *cifB*) genetic variation on phenotypes. *Genetics* **217**, (2021).
80. Kanyile, S. N., Engl, T. & Kaltenpoth, M. Nutritional symbionts enhance structural defence against predation and fungal infection in a grain pest beetle. *J. Exp. Biol.* **225**, (2022).
81. Moreira, L. A. *et al.* A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, Chikungunya, and Plasmodium. *Cell* **139**, 1268–1278 (2009).
82. Škrovánková, S. & Sikorová, P. Vitamin B2 (Riboflavin) content in cereal products. *Acta Univ. Agric. Silvic. Mendelianae Brun.* **58**, 377–382 (2010).

83. Torbatinejad, N. M., Rutherfurd, S. M. & Moughan, P. J. Total and reactive lysine contents in selected cereal-based food products. *J. Agric. Food Chem.* **53**, 4454–4458 (2005).
84. Vala, F., Breeuwer, J. A. J. & Sabelis, M. W. *Wolbachia* induced hybrid breakdown in the twofspotted spider mite *Tetranychus urticae* Koch. *Proc. R. Soc. London. Ser. B Biol. Sci.* **267**, 1931–1937 (2000).
85. Perrot-Minnot, M. -J., Cheval, B., Migeon, A. & Navajas, M. Contrasting effects of *Wolbachia* on cytoplasmic incompatibility and fecundity in the haplodiploid mite *Tetranychus urticae*. *J. Evol. Biol.* **15**, 808–817 (2002).
86. Hoffmann, A. A., Clancy, D. & Duncan, J. Naturally-occurring *Wolbachia* infection in *Drosophila simulans* that does not cause cytoplasmic incompatibility. *Heredity (Edinb).* **76**, 1–8 (1996).
87. Douglas, A. E. The microbial dimension in insect nutritional ecology. *Funct. Ecol.* **23**, 38–47 (2009).
88. Douglas, A. E. Symbiosis as a general principle in eukaryotic evolution. *Cold Spring Harb. Perspect. Biol.* **6**, a016113–a016113 (2014).
89. Alam, U. *et al. Wolbachia* symbiont infections induce strong cytoplasmic incompatibility in the tsetse fly *Glossina morsitans*. *PLOS Pathog.* **7**, e1002415 (2011).
90. Gómez-Valero, L. *et al.* Coexistence of *Wolbachia* with *Buchnera aphidicola* and a secondary symbiont in the aphid *Cinara cedri*. *J. Bacteriol.* **186**, 6626–6633 (2004).
91. Hutton, C., Southwood, T. & Turner, J. Inhibitors of lysine biosynthesis as antibacterial agents. *Mini-Reviews Med. Chem.* **3**, 115–127 (2003).
92. Engl, T., Kiefer, J. S. T. & Schmidt, G. Data from: *Wolbachia* causes cytoplasmic incompatibility, but not male-killing in a grain pest beetle. https://edmond.mpdl.mpg.de/dataset.xhtml?persistentId=doi:10.17617/3.KVFJTO (2022).
93. Werren, J. H., Zhang, W. & Guo, L. R. Evolution and phylogeny of Wolbachia: reproductive parasites of arthropods. *Proc. R. Soc. London. Ser. B Biol. Sci.* **261**, 55–63 (1995).
94. Bandi, C., Anderson, T. J. C., Genchi, C. & Blaxter, M. L. Phylogeny of Wolbachia in filarial nematodes. *Proc. R. Soc. London. Ser. B Biol. Sci.* **265**, 2407–2413 (1998).
95. Casiraghi, M. *et al.* Phylogeny of *Wolbachia* pipientis based on *gltA*, *groEL* and *ftsZ* gene sequences: clustering of arthropod and nematode symbionts in the F supergroup, and evidence for further diversity in the *Wolbachia* tree. *Microbiology* **151**, 4015–4022 (2005).
96. Baldo, L., Bordenstein, S., Wernegreen, J. J. & Werren, J. H. Widespread recombination throughout *Wolbachia* genomes. *Mol. Biol. Evol.* **23**, 437–449 (2006).

3.11 Supplementary Information



Supplement Figure 1: Phylogeny of *wmk* homologues including *wmk1* and *wmk12*-like homologues from *O. surinamensis* sequencing libraries from different collection sites in Israel, including two feral field populations (SFS, SRR-52779830 – SRR5279837 and NFS, SRR5279838 – SRR5279845) and a storage facilities population (S, SRR5279846 - SRR5279853). *wmk1*-like homologues only occurred in *w*Sur from *O. surinamensis* JKI and Israeli storage populations (S). *wmk12*-like homologues from storage populations (S) clustered strictly with *w*Sur *wmk12* and exhibited the same frame shift mutation as *wmk12* in *w*Sur from the *O. surinamensis* JKI population, while the respective homologues clustered separately and do encode a complete protein analogous to *wmk* from *w*Mel. Node numbers represent posterior probabilities of Bayesian analyses. Phylogenetic reconstruction was done by Bayesian inference applying a GTR+G+I model using MrBayes (v3.2.7). The analysis ran for 200,000 generations with a "Burnin" of 25% and tree sampling every 1,000 generations. RefSeq assembly accession in square brackets.



Supplement Figure 2: Titer of *w*Sur in *O. surinamensis* adults reared on different food compositions. The data distribution is visualized with violin plots and an additional horizontal line depicting the median. Filled circles represent specific target amplification, and empty circles off-target amplification during late qPCR cycles, identified by melting curve analysis. Different letters indicate significant differences between experimental treatments (Dunn's Test, $\alpha \leq 0.05$).

Supplementary Table 1: Results of Dunn's Test assessing the impact of glyphosate exposure on *w*Sur titer in one-week-old *O. surinamensis*. Significant results (P.adj < 0.05) are highlighted in bold. Apo = aposymbiotic, Sym = symbiotic, 1% AA = addition of 1% (w/w) of each aromatic amino acid (tyrosine, phenylalanine, tryptophan), 0.1 / 1% G = addition of 1% (w/w) glyphosate

Comparison	Z	p	Adjusted p
Apo - Apo + 1% AA	1.17E-01	9.07E-01	9.07E-01
Apo - Sym	**-3.85E+00**	**1.16E-04**	**5.41E-04**
Apo + 1% AA - Sym	**-3.97E+00**	**7.13E-05**	**3.99E-04**
Apo - Sym + 0.1% G	**-3.17E+00**	**1.52E-03**	**4.24E-03**
Apo + 1% AA - Sym + 0.1% G	**-3.29E+00**	**1.01E-03**	**3.13E-03**
Sym - Sym + 0.1% G	6.83E-01	4.95E-01	6.29E-01
Apo - Sym + 1% G	**-4.51E+00**	**6.53E-06**	**4.57E-05**
Apo + 1% AA - Sym + 1% G	**-4.63E+00**	**3.73E-06**	**3.49E-05**
Sym - Sym + 1% G	-6.54E-01	5.13E-01	6.25E-01
Sym + 0.1% G - Sym + 1% G	-1.34E+00	1.81E-01	2.99E-01
Apo - Sym + 1% AA	**-3.52E+00**	**4.39E-04**	**1.54E-03**
Apo + 1% AA - Sym + 1% AA	**-3.63E+00**	**2.84E-04**	**1.14E-03**
Sym - Sym + 1% AA	2.36E-01	8.13E-01	8.43E-01
Sym + 0.1% G - Sym + 1% AA	-4.28E-01	6.68E-01	7.20E-01
Sym + 1% G - Sym + 1% AA	8.73E-01	3.83E-01	5.10E-01
Apo - Sym + 1% AA + 0.1% G	**-2.55E+00**	**1.09E-02**	**2.53E-02**
Apo + 1% AA - Sym + 1% AA + 0.1% G	**-2.66E+00**	**7.72E-03**	**1.96E-02**
Sym - Sym + 1% AA + 0.1% G	1.31E+00	1.91E-01	2.97E-01
Sym + 0.1% G - Sym + 1% AA + 0.1% G	6.25E-01	5.32E-01	6.21E-01
Sym + 1% G - Sym + 1% AA + 0.1% G	1.96E+00	4.98E-02	9.96E-02
Sym + 1% AA - Sym + 1% AA + 0.1% G	1.04E+00	3.00E-01	4.20E-01
Apo - Sym + 1% AA + 1% G	**-5.06E+00**	**4.09E-07**	**5.72E-06**
Apo + 1% AA - Sym + 1% AA + 1% G	**-5.18E+00**	**2.20E-07**	**6.15E-06**
Sym - Sym + 1% AA + 1% G	-1.21E+00	2.26E-01	3.33E-01
Sym + 0.1% G - Sym + 1% AA + 1% G	-1.89E+00	5.83E-02	1.09E-01
Sym + 1% G - Sym + 1% AA + 1% G	-5.56E-01	5.78E-01	6.47E-01
Sym + 1% AA - Sym + 1% AA + 1% G	-1.41E+00	1.57E-01	2.75E-01
Sym + 1% AA + 0.1% G - Sym + 1% AA + 1% G	-2.52E+00	1.18E-02	2.54E-02

Chapter 4

Co-speciation and functional complementarity of dual Bacteroidota symbionts in powderpost beetles (Coleoptera: Bostrichidae)

Julian Simon Thilo Kiefer[1], Eugen Bauer[1], Genta Okude[2,3], Takema Fukatsu[2,3,4], Martin Kaltenpoth[1,5], Tobias Engl[1,5]

[1]Department of Evolutionary Ecology, Institute of Organismic and Molecular Evolution, Johannes Gutenberg-University, Mainz, Germany; [2]Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology, Tsukuba 305-8566, Japan; [3]Department of Biological Sciences, Graduate School of Science, University of Tokyo, Tokyo 113-0033, Japan; [4]Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba 305-8571, Japan; [5]Department of Insect Symbiosis, Max-Planck-Institute for Chemical Ecology, Jena, Germany

4.1 Abstract

Insects frequently engage in stable, nutritional symbioses with bacteria and exhibit co-evolutionary processes that result in co-adapted metabolisms and co-speciation. Further, multiple insects are associated with two or more endosymbionts with complementary biosynthetic pathways to synthesize amino acids and/or vitamins. Here, we report the first case of dual co-obligate symbiosis with closely related endosymbionts of the same family in beetle hosts. Beetles of the family Bostrichidae harbour consistently the aromatic amino acid supplementing Bacteroidota bacterium *Shikimatogenerans bostrichidophilus*. Further, a sub-clade is associated with the co-obligate symbiont *Bostrichicola ureolyticus* which probably complements the function of *Shikimatogenerans* by recycling urea and provisioning the essential amino acid lysine, providing additional benefits on nitrogen- and/or lysine-poor diets. Both symbionts represent ancient associations within the Bostrichidae. While *Bostrichicola* was repeatedly lost, *Shikimatogenerans* was retained throughout the family and exhibits a perfect pattern of co-speciation.

4.2 Introduction

Many insects are associated with microbial partners in host-beneficial symbioses[1–4]. Supplying the symbiont with general nutrients in a stable niche in exchange for essential nutrients allows the host to thrive on challenging, often nutritionally imbalanced diets, like plant sap, wood, or vertebrate blood[5]. A stable symbiotic association can lead to co-evolutionary dynamics, including co-adaptation and speciation[6,7]. Strong population bottlenecks during symbiont transmission, genetic drift, and host-level selection result in such scenarios in rapid genomic changes which leads to metabolic specialisation of the nutritional symbiont[8]. The outcome is drastically reduced symbiont genomes encoding besides the obligate pathways to sustain their metabolism under extensive host provisioning only for biosynthetic pathways to supplement absolutely necessary nutrients to the host metabolism[9]. Symbionts can also be lost or replaced when they are no longer needed or capable of sufficiently supporting their host's metabolism – as genome erosion can lead to reduced efficiency in the symbionts[10–12]. It is also possible that symbionts are not replaced, but only part of their function is complemented by novel microbial partners. This has led to e.g. dual symbioses in which essential amino acids are still synthesized by the original symbiont, but vitamins are synthesized by a secondary symbiont[13–15] or pathways for the essential amino acids are split up between two symbionts[16–18].

Several insects benefit from symbionts that mainly or even uniquely provide aromatic amino acid precursors for cuticle synthesis, which can be limited in the diet and/or in high demand during metamorphosis. This has been found to be important for cuticle melanisation and sclerotisation, as all of the cuticular crosslinking agents are derived from the aromatic amino acid tyrosine[19,20]. Shortly after metamorphosis, when the cuticle of the adult beetle has fully formed, the symbiont contribution and the population is at their peak – which was demonstrated in the sawtoothed grain beetle *Oryzaephilus surinamensis*[21] (Chapter 3) and the grain weevil *Sitophilus oryzae*[22]. Tyrosine-supplementing symbionts can be bacteria in the gut like in turtle ants of the genus *Cephalotes*[23,24] or the bean weevil *Callosobruchus maculatus*[25], but also bacteriome-localised symbionts like the *Candidatus* Westeberhardia cardiocondylae (Enterobacteriaceae) in the tramp ant *Cardiocondyla obscurior*[26], the γ-proteobacterial symbionts *Candidatus* Nardonella and *Candidatus* Sodalis in weevil species[22,27], as well as the Bacteroidota endosymbiont *Candidatus* Shikimatogenerans silvanidophilus in *O. surinamensis* (Chapter 2). For simplicity's sake we will skip the *Candidatus* nomenclature from here on for all symbionts with sequenced genomes.

Tyrosine-supplementing symbioses provide evidence for this aromatic amino acid being a key nutrient for many insects to produce their strongly sclerotised and melanised exoskeleton, thereby increasing desiccation resistance and protection from predators and pathogens - coincidentally also susceptibility for herbicides targeting the shikimate pathway[22,27–30]. Based on this, it has been hypothesized that beetles inhabiting ecological niches with low ambient humidity or high antagonist pressure may have been predisposed towards evolving symbiotic associations with tyrosine-supplementing symbionts[28,31]. Concordantly, *O. surinamensis* and – to a lesser extent – *Sitophilus* have been able to invade dry grain storage facilities, with the help of their symbionts[32,33].

While several tyrosine-supplementing symbionts are known, several additional beetle families have recently been described to harbour related symbionts for which the functions are not yet known[31,34,35]. In addition, the evolutionary histories of the symbioses remain largely unknown. A beetle family with associated Bacteroidota symbionts closely related to the endosymbiont *Shikimatogenerans silvanidophilus* of *O. surinamensis* are the powderpost beetles (Coleoptera: Bostrichidae)[31,35]. The family Bostrichidae (Latreille, 1802) is between 170[36] and 155[37] Mya in age and consists of nine subfamilies (Liu & Schönitzer 2011) with varying ecology: Apatinae (Jacquelin du Val, 1861), Bostrichinae (Latreille, 1802), Dinoderinae (C. G. Thomson, 1863), Dysidinae (Lesne, 1921), Endecatimonae (LeConte, 1861), Euderinae (Lesne, 1934), Lyctinae (Billberg, 1820), Pylcvaoninae (Lesne, 1896) and Psoinae (Blanchard, 1851). While all Bostrichidae are phytophagous and bore into twigs, branches or trunks of dead or dying trees, in which their offspring develop, the most well-known species are pests of wood products or stored foods[38,39]. The close relation of the Bacteroidota endosymbionts with *Blattabacterium* spp., *Sulcia muelleri*, but especially *S. silvanidophilus* and a similar ecological niche of the hosts as several of the beetle species profiting from cuticle supplementation suggest a similar symbiont function.

Thus, to understand the functional contribution and evolution of the symbiosis within the Bostrichidae, we collected 29 beetle species across the family and performed metagenome sequencing. Based on multiple closed or draft symbiont genomes and multiple mitochondrial and nuclear host genes, we reconstructed the molecular phylogenies of both partners. Thereby we demonstrate (i) that most bostrichids are associated with a symbiont whose genome is highly degraded, retaining only the shikimate pathway for tyrosine precursor provisioning. (ii) Only beetles of the genera *Lyctus* and *Dinoderus* were associated with a second Bacteroidota symbiont.

(iii) This second co-obligate symbiont also exhibited a highly eroded genome but encodes complementary functions of nitrogen recycling, and lysine biosynthesis. Finally, (iv) host and symbiont phylogenies exhibited a high degree of co-cladogenesis, suggesting an ancient association that early resulted in obligate dependencies and co-diversification.

4.3 Results

We collected and sequenced the metagenomes of 28 beetle species of the family Bostrichidae (Supplement Tables 1 and 2). In addition, we supplemented our dataset with three publicly available datasets from NCBI (*Apatides fortis* [FJ613421], *Sinoxylon* sp. SIN01 [JX412742], and *Xylobiops basilaris* [SRR2083737]). The resulting 31 species covered five subfamilies and nine tribes within the Bostrichidae. For thirteen of the 31 species, we were able to assemble the full and circularized genome of the Bacteroidota endosymbiont *Shikimatogenerans bostrichidophilus*, with the longest symbiont genome being 200,377 bp and the shortest 172,971 bp in length, and an average GC content of 15.1% (Supplement Table 2). For ten additional species, we assembled draft genomes of *Shikimatogenerans* based on multiple contigs extracted from the metagenome assemblies via taxonomic classification, GC content filtering, as well as by manually searching for tRNAs and ribosomal protein genes as well as enzymes of the shikimate pathway of Bacteroidota bacteria. For one species (*Dinoderus bifoveolatus*) we only obtained the 16S rRNA sequence of the endosymbiont. In the remaining species we sequenced, we were not able to detect any sequence of Bacteroidota bacteria in the metagenome data. In addition, we extracted the 16S rRNA and *aroA* gene sequences of the Bacteroidota symbiont from a publicly available transcriptomic dataset of *Xylobiops basilaris* (SRR2083737).

As expected from a previous study[31], we found the genome of a second Bacteroidota endosymbiont, which we named *Bostrichicola ureolyticus* (see below), in some species of the subfamilies Lyctinae and Dinoderinae. However, we only detected this co-obligate symbiont within the *Lyctus* and *Dinoderus* species, but not the other members of the Lyctinae (*Trogoxylon impressum*) and Dinoderinae (*Rhyzopertha dominica*). The genomes of *Bostrichicola* were on average 337,500 bp in length and had an average GC content of 22.4%.

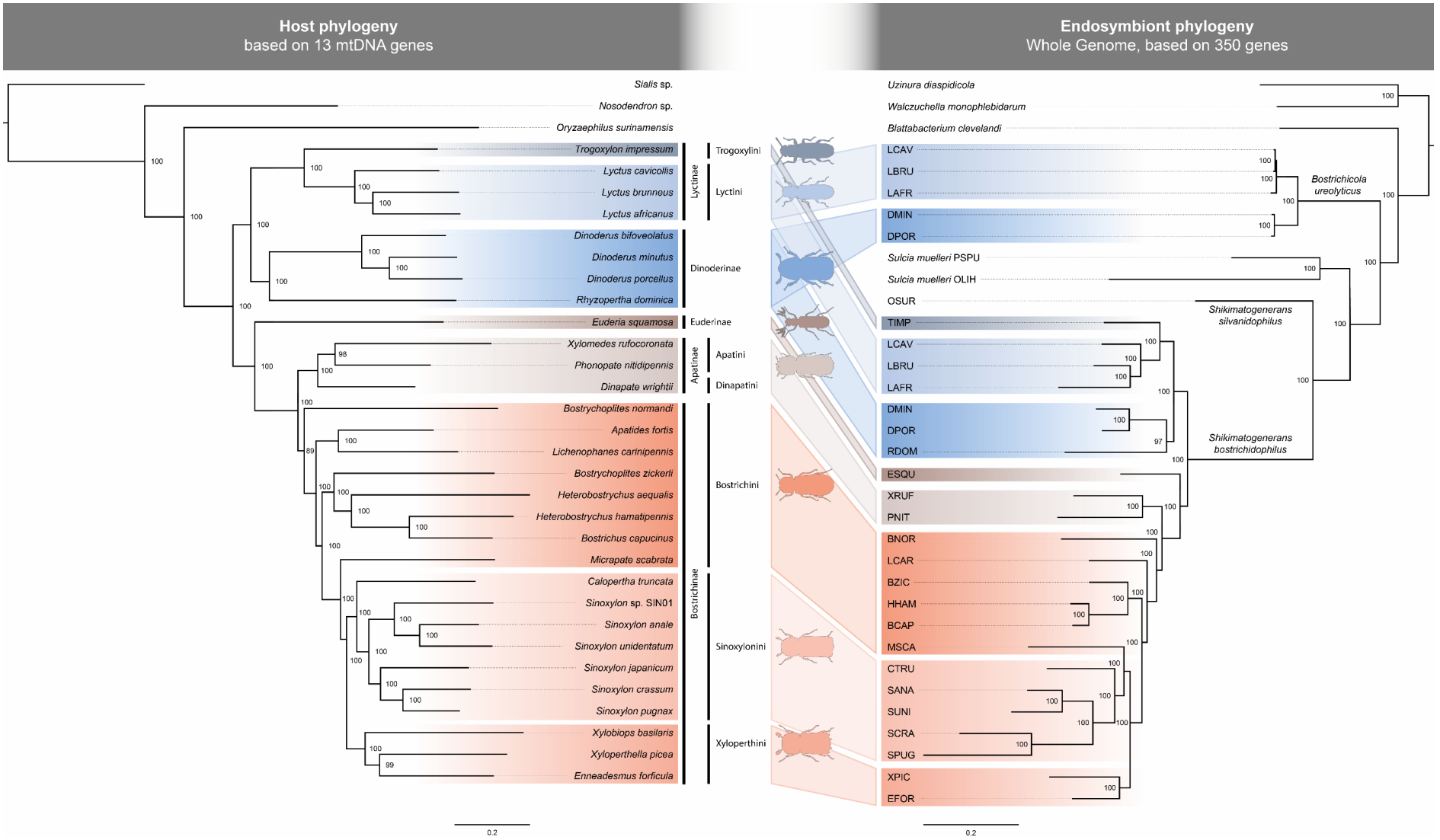
The metagenomic datasets were used to reconstruct the phylogeny of the host species (Figure 1 left and Supplement Figure 1). We constructed two phylogenies of the host, one based on the assembled mitochondrial genomes (Figure 1 left), and the other one on 22 aligned, and concatenated Benchmarking Universal Single-Copy Ortholog (BUSCO)[40] genes found across all species (Supplement Figure 1). Both phylogenies gave very similar results, revealing two main clades of the Bostrichidae beetles, separating the Lyctinae and Dinoderinae from the Euderinae, Apatinae and Bostrichinae. However, they differ in the placement of *Micrapate scabrata,* within

the Sinoxylonini as a sister clade to the Xyloperthini (BUSCO genes) versus as an outgroup to the before mentioned clades (based on the mitochondrial genomes; Supplement Figure 1).

Similarly, the genome assemblies were used to generate a whole genome-based phylogeny for the endosymbionts (Figure 1 right). This phylogenetic reconstruction based on 350 conserved genes confirmed the monophyly of the *Shikimatogenerans* endosymbionts of Bostrichidae beetles and their close relationship to other insect-associated Bacteroidota bacteria, specifically to *Blattabacterium* spp. and *Sulcia muelleri,* which had been previously reported based on 16S rRNA phylogenies[31,34,35]. The *Bostrichicola* symbionts in the *Lyctus* as well as *Dinoderus* species clustered in a distinct, more basally branching, also monophyletic clade. The two clades of Bostrichidae endosymbionts were separated by the Bacteroidota symbiont *Shikimatogenerans silvanidophilus* OSUR of the sawtoothed grain beetle *Oryzaephilus surinamensis* (Silvanidae) as well as the clade of *Sulcia muelleri* endosymbionts of the Auchenorrhyncha (Hemiptera). A second phylogeny of the endosymbionts based on the 16S rRNA sequences that allowed us to include more taxa revealed a highly similar distribution within the Bacteroidota (Supplement Figure 2). The main difference between both phylogenies (Supplement Figure 2) was the placement of the endosymbiont of *Calopertha truncatula*, which forms an outgroup to endosymbionts of Sinoxylonini and of Xyloperthini in the 16S rRNA-based phylogeny, while in the whole genome phylogeny it groups within the endosymbionts of Sinoxylonini as a sister clade to endosymbionts of Xyloperthini.

A comparison of the endosymbiont phylogeny based on the 350 conserved genes and the host mitochondrial phylogeny showed a high degree of co-cladogenesis (Figure 1). We chose this comparison as we were able to retrieve a higher number of full mitochondrial genomes than BUSCO gene sets. However, *Micrapate scabrata* was an outgroup for the Xyloperthini and Sinoxylonini while its endosymbiont grouped within the Sinoxylonini as a sister clade to the Xyloperthini, rather than reflecting the host evolutionary background observed in the BUSCO gene phylogeny (Supplement Figure 2). The comparison between the full continuous genomes shows a high degree of synteny within, but not between, the two symbiont genera (Figure 3).

On the following page, **Figure 1: Comparison of phylogenetic relationships between Bostrichidae beetle hosts and their Bacteroidota endosymbionts.** Left: Bayesian phylogeny of 30 Bostrichidae beetle species inferred from concatenated nucleotide alignment of 13 mitochondrial genes. Right: Bayesian phylogeny of 28 Bacteroidota symbionts of Bostrichidae beetles inferred from concatenated nucleotide alignment of 350 genes. Node numbers represent posterior probabilities of Bayesian analyses. Host-symbiont associations are highlighted by connecting trapezoids between the phylogenies.


Host phylogeny
based on 13 mtDNA genes
Endosymbiont phylogeny
Whole Genome, based on 350 genes
Sialis sp.
Nosodendron sp.
Oryzaephilus surinamensis
Trogoxylon impressum
Lyctus cavicollis
Lyctus brunneus
Lyctus africanus
Dinoderus bifoveolatus
Dinoderus minutus
Dinoderus porcellus
Rhyzopertha dominica
Euderia squamosa
Xylomedes rufocoronata
Phonopate nitidipennis
Dinapate wrightii
Bostrychoplites normandi
Apatides fortis
Lichenophanes carinipennis
Bostrychoplites zickerli
Heterobostrychus aequalis
Heterobostrychus hamatipennis
Bostrichus capucinus
Micrapate scabrata
Calopertha truncata
Sinoxylon sp. SIN01
Sinoxylon anale
Sinoxylon unidentatum
Sinoxylon japanicum
Sinoxylon crassum
Sinoxylon pugnax
Xylobiops basilaris
Xyloperthella picea
Enneadesmus forficula
Trogoxylini
Lyctinae
Lyctini
Dinoderinae
Euderinae
Apatinae
Apatini
Dinapatini
Bostrichinae
Bostrichini
Sinoxylonini
Xyloperthini
0.2
Uzinura diaspidicola
Walczuchella monophlebidarum
Blattabacterium clevelandi
LCAV
LBRU
LAFR
DMIN
DPOR
Sulcia muelleri PSPU
Sulcia muelleri OLIH
OSUR
TIMP
LCAV
LBRU
LAFR
DMIN
DPOR
RDOM
ESQU
XRUF
PNIT
BNOR
LCAR
BZIC
HHAM
BCAP
MSCA
CTRU
SANA
SUNI
SCRA
SPUG
XPIC
EFOR
Bostrichicola ureolyticus
Shikimatogenerans silvanidophilus
Shikimatogenerans bostrichidophilus
0.2

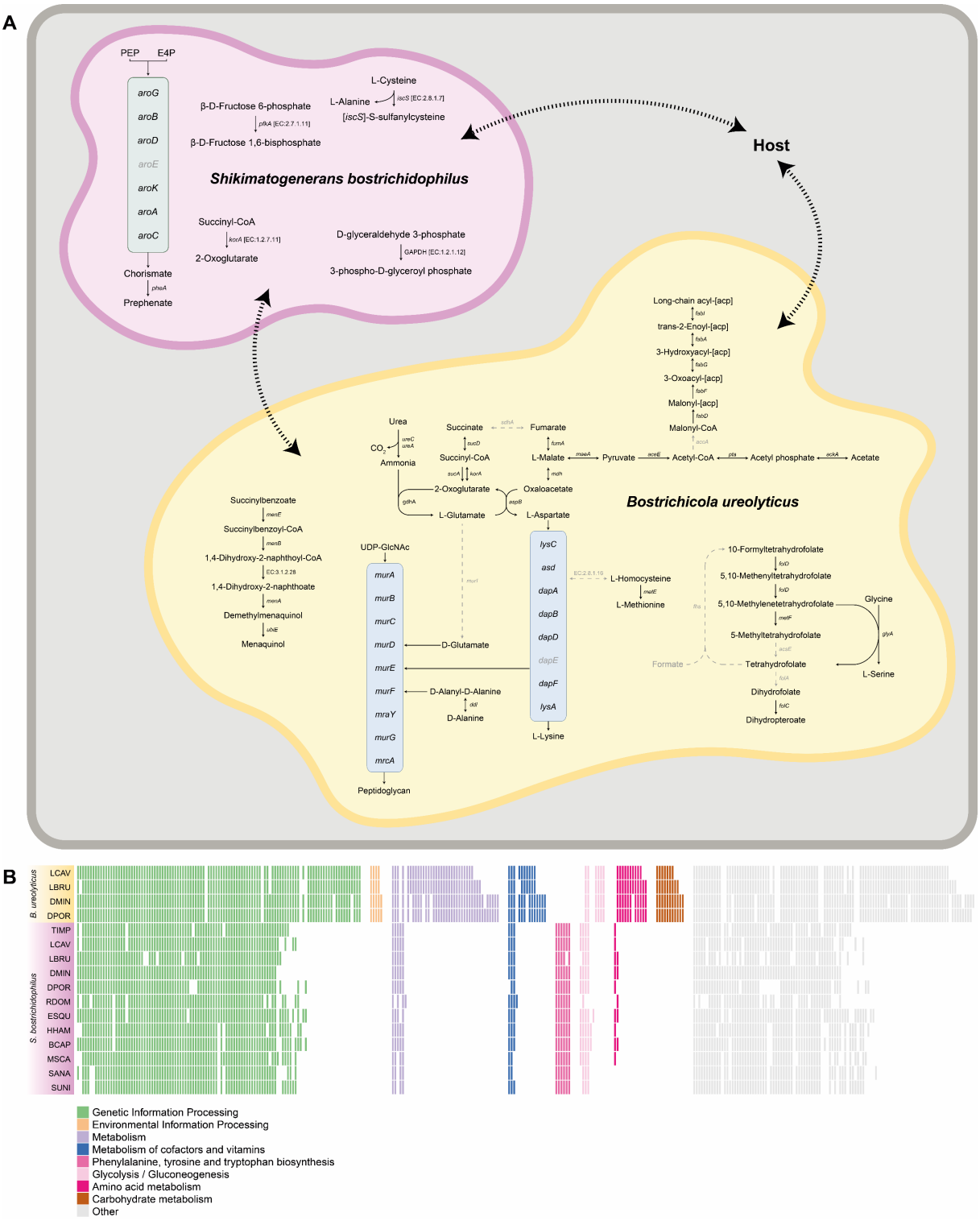


Figure 2: **(A)** Reconstructed metabolism of the two *Dinoderus porcellus* endosymbionts *Shikimatogenerans bostrichidophilus* DPOR *and Bostrichicola ureolyticus* DPOR, inferred from genomic data. Enzymes and arrows in grey were missing in the genome annotation. Dashed arrows indicate transport processes without annotated transporters. **(B)** Comparison of the functional gene repertoires of Bacteroidota symbionts in Bostrichidae beetles. Coloured boxes indicate the presence, and white boxes the absence of genes in the symbiont genomes. Box colours are based on KEGG's categories (see legend for depicted categories).

As expected, the metabolic repertoires of both Bostrichidae endosymbionts were highly reduced and showed clear signs of genome erosion. Both endosymbionts retained genes involved in the cellular core processes of general genetic information processing including DNA replication and repair, transcription, and translation (Supplement Figure 3). In addition, *Shikimatogenerans* that were present across the entire family encoded all the genes of the shikimate pathway except a shikimate dehydrogenase (*aroE* [EC:1.1.1.25]) (Figure 2). Also, these genomes encoded the bifunctional *aroG*/*pheA* gene (phospho-2-dehydro-3-deoxyheptonate aldolase/chorismate mutase [EC:2.5.1.54 5.4.99.5]), capable of catalysing the Claisen rearrangement of chorismate to prephenate and the decarboxylation/dehydration of prephenate to phenylpyruvate[41].

The genome of *Bostrichicola* encoded both urease α and γ subunits (*ureC* [EC:3.5.1.5]) to recycle nitrogen, as well as a glutamate dehydrogenase (*gdhA* [EC:1.4.1.4]) that allows integration of the resulting ammonium into the amino acid metabolism via glutamate (Figure 2 A). In addition, they encoded for aspartate aminotransferase (*aspB* [EC:2.6.1.14]) to transfer the amino group from glutamate to oxaloacetate, as well as an almost complete diaminopimelate pathway to synthesize the essential amino acid lysine from aspartate. They also retained a methionine synthase to convert L-homoserine to L-methionine and can synthesise menaquinone, but we did not find a single gene of the shikimate pathway to synthesize aromatic amino acids. However, the genomes encoded for a complete fatty acid and peptidoglycan biosynthesis, albeit other cell envelope components apparently cannot be synthesized. The genomic data revealed no transporters, so it remains unknown how the symbionts exchange metabolites with the host and with each other (Figure 2 A). In addition, genes encoding signal transduction, cell surface structures, and motility were absent (Figure 2 B). Further, we also compared the set of encoded genes which are not encoded in all genomes (Supplement Figure 4). For *Shikimatogenerans*, it is particularly noticeable that *mutL* (DNA mismatch repair protein) is still present in the Dinoderinae+Lyctinae symbionts but has already been lost in the Euderinae+Apatinae+Bostrichinae. For *Bostrichicola*, all genes for peptidoglycan biosynthesis (*murA*, *murB*, *murC*, *murD*, *murE*, *murF*, *murG*, *mraY* and *mrcA*) are still encoded in the Dinoderinae, whereas in the Lyctinae *L. brunneus* lost *murC*, *murD* and *murG*, *while L. cavicollis* has lost all of them.

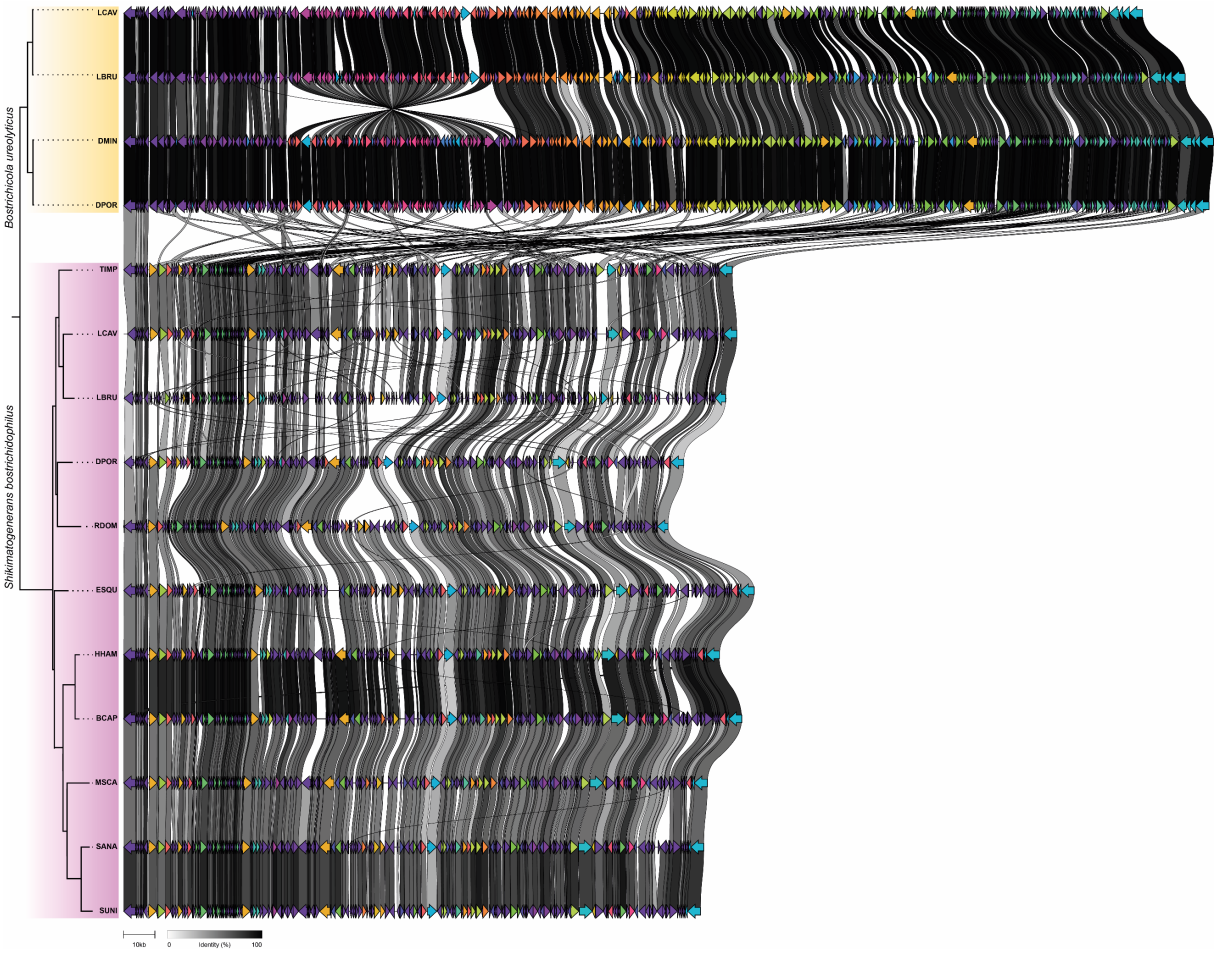


Figure 3: Gene order comparison between *S. bostrichidophilus* and *B. ureolyticus* genomes that could be assembled into full continuous genomes, showing a high degree of synteny within, but not between, the two symbiont genera. Grey shades show the percentage of identity between homologous proteins from different genomes (based on amino acid sequences). The phylogenetic tree on the left is based on the symbiont phylogeny displayed in Figure 1.

Based on the close phylogenetic relationship to the *Shikimatogenerans silvanidophilus* OSUR and the presence of the shikimate pathway in the highly eroded genome, we propose the name '*Shikimatogenerans bostrichidophilus*' for the endosymbiont of Bostrichidae beetles, henceforth called *S. bostrichidophilus*. The genus name *Shikimatogenerans* refers to its ability to perform the shikimate pathway. Previous studies have shown that closely related Bacteroidota bacteria are also associated with other beetle families such as the Silvanidae and the Nosodendridae[31,42]. Thus, we propose *bostrichidophilus* as a species epithet to indicate that this symbiont clade is associated with beetles of the family Bostrichidae. As all the symbionts encode highly similar genomes, we propose to add a four-letter abbreviation of the host species to identify the strain (first letter of the host genus and first three letters of the host species epithet), e.g.

Shikimatogenerans bostrichidophilus RDOM for the endosymbiont of *Rhyzopertha dominica*. For the second co-obligate endosymbiont found in Bostrichidae beetles of the subfamily Dinoderinae and Lyctinae, we propose the name '*Bostrichicola ureolyticus*'. Its genus name refers to its association with bostrichid beetles, while *ureolyticus* refers to its metabolic potential to recycle nitrogen from urea as inferred from the genomic data. In analogy to *Shikimatogenerans*, we propose to add a four-letter abbreviation of the host species to identify the strains, e.g. *Bostrichicola ureolyticus* LBRU for the *Bostrichicola* endosymbiont of *Lyctus brunneus*.

Based on rRNA fluorescence *in situ* hybridisation with eight of the 32 species, the bacterial symbionts were localised intracellularly in bacteriomes in the abdomen of the host (Figure 4). The bacteriomes are located between the gut, fat body and reproductive organs, but without direct connection to any of these tissues. Bostrichid beetles of all subfamilies harboured one paired bacteriome with symbionts stained by a probe specific for members of the *Shikimatogenerans* symbiont clade[30,31] (Figure 4 a, e, f, g and h). In addition, species of the genera *Dinoderus* and *Lyctus* contained a pair of bacteriomes stained by a probe specific to the second symbiont *B. ureolyticus*[31] (Figure 4 b, c and d). In the species containing both *Shikimatogenerans* and *Bostrichicola*, the bacteriomes were close to each other, sometimes with direct physical contact yet distinct ultrastructure (Figure 4 b).

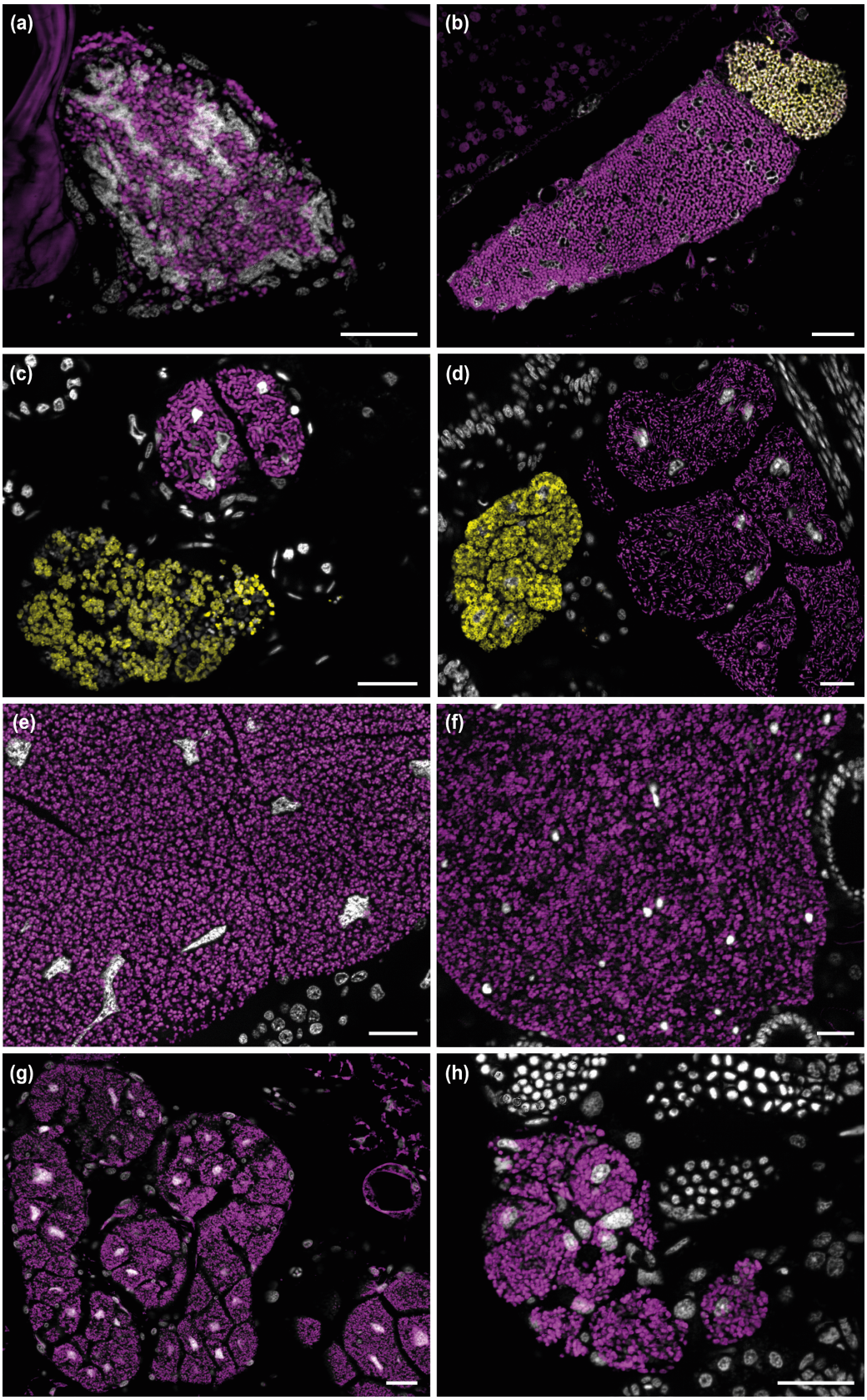


Figure 4: Fluorescence *in situ* hybridisation micrographs of *Shikimatogenerans bostrichidophilus* and *Bostrichicola ureolyticus* in sections of **(a)** *Trogoxylon impressum*, **(b)** *Lyctus cavicollis*, **(c)** *Dinoderus minutus*, **(d)** *Dinoderus porcellus*, **(e)** *Rhyzopertha dominica*, **(f)** *Prostephanus truncatus*, **(g)** *Xyloperthella picea* and **(h)** *Sinoxylon anale*. Sections are stained with a *S. bostrichidophilus* specific probe (magenta), a *B. ureolyticus* specific probe (yellow), and DAPI targeting DNA in general (white). Scale bar representing 20 µm.

4.4 Discussion

In this study, we characterized the intracellular bacterial symbionts across 29 species of the beetle family Bostrichidae and assessed their functional potential and co-speciation with their hosts based on comparative genomics.

We used the metagenomes to reconstruct the first molecular phylogeny of these so-called auger or powderpost beetles (Coleoptera: Bostrichidae), following the morphological phylogeny by Liu et al (2011). Based on shotgun metagenomics datasets, we extracted beetle nuclear and mitochondrial gene sequences and reconstructed two phylogenies of the Bostrichidae, one based on 13 mitochondrial genes (Figure 1 left), and the other one on 22 BUSCO genes found across all species (Supplement Figure 1). Both phylogenies were generally well supported, highly congruent, and separated the Bostrichidae into two main clades: The Lyctinae and Dinoderinae grouped together, as did the Euderinae, Apatinae and Bostrichinae. The main difference was the placement of *Micrapate scabrata.* In the host phylogeny based on BUSCO genes, *Micrapate scabrata* clustered within the tribe Sinoxylonini as a sister clade to the Xyloperthini (BUSCO genes). In contrast, in the mitochondrial phylogeny, it was an outgroup to both tribes (Supplement Figure 1).

Our study serves as the first, solid molecular evaluation of the relationships within this family that had been repeatedly reclassified[43] and only been addressed with a well-sampled phylogeny based on morphological characteristics by Lui & Schönitzer in 2011. Overall, we arrived at similar conclusions with one major exception: Liu & Schönitzer placed the Euderiinae with a single monotypic genus as a basal branch of the Bostrichidae and suggested to even placing them in a separate family. In our analyses, *Euderia squamosa* always clustered within the Bostrichidae in a separate branch between the Lyctinae/Dinoderinae and Apatinae/Bostrichinae. The Lyctinae and Dinoderinae are in both studies closely related, with the Psoinae and Polycaoninae interspersed in Liu & Schönitzer but lacking in our work, as we were not able to obtain any specimens. The Apatinae and Bostrichinae form here two sister groups within a monophyletic clade. The Sinoxylini and Xyloperthini are monophyletic groups within the Bostrichinae, while the Bostrichini represents a polyphyletic group[39], regardless of whether using the mitochondrial or BUSCO gene phylogeny.

The presence of endosymbionts in several species of Bostrichidae beetles known as grain and wood pests was already described by Gambetta (1927)[44], Mansour (1934)[45], Koch (1936)[46], and Buchner (1954)[47]. Okude et al. (2017) and Engl et al. (2018) identified them as members of the insect-associated Bacteroidota (Flavobacteriaceae) clade[31,35]. In this study, we were able to detect *Shikimatogenerans bostrichidophilus* in almost all examined Bostrichidae species. For the few species where we could not detect any symbiont (*Dinapate wrightii*, *Amphicerus bicaudus*, *Heterobostrychus aequalis* and *Sinoxylon japanicum*), three scenarios are possible. First, it is known from several symbiotic insects that the endosymbiont is degraded or lost in male beetles sometime after metamorphosis[48]. In cases where nutrient supplementation by the symbionts is only needed during larval development and/or metamorphosis, males can benefit from killing their symbionts and recycling symbiotic organs, given that they do not transmit the symbionts to their offspring[21,22]. Thus, individual beetle specimens in our study may have been males, resulting in false negatives in our symbiont screenings. Second, in some species, aposymbiotic individuals and even populations occur in the field, due to elevated sensitivity of the symbionts to environmental stressors like heat[46,49] or certain contemporary agrochemicals. Third, *Shikimatogenerans* may have been lost within these species. As we had only single specimens available for the species in which we failed to detect symbionts, we cannot confidently reject any of these hypotheses.

However, based on their similar ecological niches, we assume the entire family of Bostrichidae to harbour *S. bostrichidophilus* originating from a single acquisition event. In contrast, the co-symbiont *B. ureolyticus* was only present within the genera *Lyctus* and *Dinoderus*. In these cases, it is unlikely that we missed the co-symbiont in other species of these two subfamilies, *T. impressum* and *R. dominica*. We had multiple individuals and life stages of *R. dominica* available as well as four specimens of *T. impressum* and never found any indication for a second bacteriome-localised symbiont, neither within our genomic datasets nor during FISH. Concordantly, previous studies[31,35] did not report on a second symbiont in *R. dominica*, while it could already be discerned based on morphologically differentiated bacteriomes in *Lyctus* and *Dinoderus* species[31,46]. Thus, *B. ureolyticus* was likely acquired by either an ancestor of all Bostrichid beetles, or only the ancestor of the Lyctinae and Dinoderinae, and repeatedly lost.

Phylogenetic reconstructions based on either the symbiont 16S rRNA gene or 350 genes resulted in highly congruent phylogenies that only differed in some of the deeper splits, which were better

supported in the multi-gene phylogeny than the 16S rRNA phylogeny. The endosymbiont and the host phylogeny showed a high degree of co-cladogenesis, mirroring each other's branching patterns almost perfectly. The major disagreement was again the clustering of *Micrapate scabrata,* respectively its symbiont. *M. scabrata* itself was an outgroup for the Xyloperthini and Sinoxylonini while its endosymbiont groups within the Sinoxylonini symbionts as a sister clade to the Xyloperthini.

Both endosymbionts are characterized by extremely small, heavily eroded and A+T-biased genomes with very limited biosynthetic capabilities[50]. The genomes of *S. bostrichidophilus* encoded for the shikimate pathway to synthesize precursors of aromatic amino acids. Of the seven canonical genes in the shikimate pathway (*aroG, aroB, aroD, aroE, aroK, aroA, aroC*), the *S. bostrichidophilus* genomes only lack the gene for shikimate dehydrogenase (*aroE*), which catalyses the reversible reduction of 3-dehydroshikimate to shikimate. However, the shikimate pathways of *Nardonella* EPO, the endosymbiont of the sweetpotato weevil *Euscepes postfasciatus* (Curculionidae: Cryptorhynchinae)[27,51] and *Carsonella ruddii*, the endosymbiont of the gall-forming psyllid *Pachypsylla venusta* (Aphalaridae: Pachypsyllinae)[52], as well as the closely related *S. silvanidophilus* OSUR in the sawtoothed grain beetle *O. surinamensis* also lack *aroE*, but remain functional[30], suggesting that the function of *aroE* is likely taken over by other enzymes, either host or endosymbiont. *S. bostrichidophilus* transformers phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P) to prephenate/chorismate via the shikimate pathway[53,54], which is then converted by the host to the aromatic amino acid tyrosine[55]. As all of the cuticular crosslinking agents are derived from the tyrosine[19,20], it plays a key role in melanisation and sclerotisation, which is what gives the cuticular its protective and desiccation-resistant abilities[31,56].

As the contribution of aromatic amino acids by *Shikimatogenerans, Nardonella* and *Sodalis*[30,51,57] is especially important as precursors for cross-linking chitin and proteins in the context of cuticle synthesis in beetles, the contribution of *B. ureolyticus* might be relevant in the same context. The genome of *B. ureolyticus* encodes the gene for urea recycling and the diaminopimelate pathway to synthesize lysine. In addition, *B. ureolyticus* retained partial pathways to convert intermediates of the lysine biosynthesis into methionine, folate and menaquinone biosynthesis[5,58] as well as certain components of the cell envelope biosynthesis (fatty acids and peptidoglycan). Insights from other symbionts indicate that even incompletely retained biosynthetic pathways are often functional

and beneficial for the host[17,27,30,52,59]. Multiple insects are associated with two or more endosymbionts with complementary biosynthetic pathways to synthesize amino acids and/or vitamins[15,17,58,60–62], but so far not with such closely related endosymbionts of the same family.

Nitrogen recycling is well documented within Bacteroidota endosymbionts[63–65] and can be an important benefit for insects developing in nitrogen-poor sapwood[38,39,66–68]. The recycling of urea as a source of amino groups might be important for the formation of tyrosine, but also other amino acids and amino acid-derived components like N-acetyl-glucosamine, the monomer of chitin[24]. However, why *B. ureolyticus* retained a lysine biosynthesis pathway is less clear. Lysine is an important component of cuticular proteins as its ε-amino group represents an anchor point for cross linking[69]. Grain diets are specifically limited in lysine[70], which could be relevant for the stored product pest beetles of the genus *Dinoderus*, but also other species of the Dinoderinae subfamily. Thus, the specific role of *B. ureolyticus* and why it was only retained in certain genera remains speculative. A profound understanding of why certain genera of Bostrichidae might benefit from such provisioning and thus retain *Bostrichicola*, while others do not, is currently hampered by the scarcity of information on the ecology and physiology of most Bostrichidae[43]. Based on our phylogenetic analyses, *Shikimatogenerans* and *Bostrichicola* are derived from the same ancestor as *Blattabacterium* spp., *Walczuchella monophlebidarum* and *Uzinura diaspidicola*, but then diverged and evolved different functional specialisations[30,31,42,71]. Interestingly, *S. silvanidophilus* OSUR[30] – the sister taxon of *S. bostrichidophilus* – retained the shikimate pathway as well as one urease subunit putatively involved in nitrogen recycling[30] - and there is evidence for nitrogen recycling in *Blattabacterium*[63,65,72] and *Walczuchella*[64], so urea catabolism seems to be a widespread benefit provided by Bacteroidota bacteria.

Within the Bostrichoidae the Bostrichidae family split from the Ptinidae family between 170[36] and 155[37] Mya ago in the Jurassic period. *S. bostrichidophilus* started to radiate at the same estimated time, around 274-158 Mya ago[31]. The ancestor of the Bostrichidae beetle family must have acquired the *Shikimatogenerans* symbiont before that point in time, indicating that tyrosine-provisioning was likely an important benefit and remained important for the wood- and grain-feeding species. *Bostrichicola* was likely acquired 100 Mya ago[31], in the ancestor of the Dinoderinae and Lyctinae (plus possibly Psoinae and Polycaoninae), and then lost in *Rhyzopertha* and *Trogoxylon*. Alternatively, the related bacteria have been acquired twice independently, i.e. in

Dinoderinae and Lyctinae, respectively. The repeated independent acquisitions of these specific clades of Bacteroidota symbionts indicate that these bacteria were once specialized in infecting insects, akin to *Wolbachia* (Chapter 3) or *Sodalis*[73,74]. This hypothesis is supported, as a basal clade of Bacteroidota endosymbionts is described as male-killing endosymbionts in different ladybug beetles[75–77]. Whether this represents an uptake of both symbionts within the ancestor of the Bostrichidae, or an even older association remains enigmatic, as closely related families within the Bostrichoidea (Dermestidae, Ptinidae)[37] are not known to harbour nutritional Bacteroidota or other nutritional, bacterial endosymbionts, but in some cases (Anobiinae) yeast-like endosymbionts[78]. In this context it is worth mentioning that Bacteroidota endosymbionts of this clade were in the last years described in multiple beetle families, including the Silvanidae[31,34] and Nosodendridae[42], indicating either a widespread ancestral association or high potency for infection and establishment in beetles.

4.5 Material & Methods

4.5.1 Insect collection

Specimens of 29 species were collected or provided by experts in the field from Germany, the Czech Republic, Yemen, the United Arabic Emirates, the United States of America, Japan, and New Zealand (Supplement Table 1). In addition, three publicly available data sets of bostrichid beetles were retrieved from NCBI (SRR2083737, FJ613421 and JX412742).

4.5.2 Symbiont genome sequencing, assembly, and annotation

Total DNA was isolated using the Epicentre MasterPure™ Complete DNA and RNA Purification Kit (Illumina Inc., Madison, WI, USA) including RNase digestion, or the QIAGEN Genomic-tip kit using 20/G columns (Qiagen, Hilden, Germany). Short-read library preparation and sequencing were performed at the Max-Planck-Genome-Centre Cologne, Germany (SRR19201352 - SRR19201388) on a HiSeq3000 Sequencing System (Illumina Inc., Madison, WI, USA), at CeGaT on a HiSeq2500 Sequencing System (Tübingen, Germany). Adaptor and quality trimming was performed with Trimmomatic[79].

Long-read sequencing (SRR19201386, SRR19201357 and SRR19201352) was performed on a MinION Mk1B Sequencing System (Oxford Nanopore Technologies (ONT), Oxford, UK). Upon receipt of flowcells, and again immediately before sequencing, the number of pores on flowcells was measured using the MinKNOW software (v18.12.9 and 19.05.0, ONT, Oxford, UK). Flowcells were replaced into their packaging, sealed with parafilm and tape, and stored at 4°C until use. Library preparation was performed with the Ligation Sequencing Kit (SQK-LSK109, ONT, Oxford, UK) and completed libraries were loaded on a flowcell (FLO-MIN106D, ONT, Oxford, UK) following the manufacturer's instructions. PacBio long-read sequencing of *D. porcellus* was performed at the Max-Planck-Genome-Centre Cologne, Germany (SRR19201385) on a Sequel II (PacBio, Menlo Park, CA, USA).

Quality-controlled long reads were taxonomy-filtered using a custom-made kraken2 database[80,81] containing the publicly available genomes of Bacteroidota bacteria to extract beetle-associated Bacteroidota sequences using the supercomputer Mogon of the Johannes Gutenberg-University (Mainz, Germany). Assembly of Illumina reads and additional hybrid assemblies with long-read libraries were performed using SPAdes (v3.15.0)[82]. The resulting contigs were binned using BusyBee Web[83], and screened for GC content and taxonomic identity to Bacteroidota bacteria.

The extracted contigs were de novo assembled in Geneious Prime 2019 (v2019.1.3, https://www.geneious.com). The resulting contigs were then automatically annotated with PROKKA[84] using the app Annotate Assembly and Re-annotate Genomes (v1.14.5) on KBase[85]. Synteny analysis of complete endosymbiont genomes was performed using Clinker[86].

4.5.3 Fluorescence *in situ* hybridisation

Endosymbionts of bostrichid beetles were localised by fluorescence *in situ* hybridisation (FISH) on semi-thin sections of adult beetles, targeting the 16S rRNA sequence. Adult beetles were fixated in tertiary butanol (80%; Roth, Karlsruhe, Germany), paraformaldehyde (37 - 40%; Roth, Karlsruhe, Germany) and glacial acetic acid (Sigma-Aldrich, Germany) in proportions 6:3:1 for 2 hours, followed by post-fixation in alcoholic formaldehyde (paraformaldehyde (37-40%) and tertiary butanol (80% in proportion 1:2). After dehydration, the specimens were embedded in Technovit 8100 (Kulzer, Germany)[100] and cut into 8 µm sagittal sections using a Leica HistoCore AUTOCUT R microtome (Leica, Wetzlar, Germany) equipped with glass knives. The obtained sections were mounted on silanised glass slides. Each slide was covered with 100 µL of hybridisation mix, consisting of hybridisation buffer (0.9 M NaCl, 0.02 M Tris/HCl pH 8.0, 0.01% SDS; Roth, Germany) and 0.5 µM of the *Shikimatogenerans bostrichidophilus* specific probe (5'-CTTCCTACACGCGAAATAG-3'; Engl et al. 2018) labelled with Cy5, as well as the *Bostrichicola ureolyticus* specific probe (5'-TACTCGATGGCAATTAACAAC-3'; Engl et al. 2018) labelled with Cy3. DAPI (0.5 µg/mL) was included as a general counterstain for DNA. Slides were covered with glass cover slips and incubated in a humid chamber at 50°C overnight. After washing and incubating them for 20 minutes at 50°C in wash buffer (0.1 M NaCl, 0.02 M Tris/HCl, 5 mM EDTA, 0.01% SDS), they were washed in deionized water for 20 minutes, dried and mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA). The sections were observed under a Zeiss AxioImager.Z2 equipped with an Apotome.2 (Zeiss, Jena, Germany) and illuminated by a SOLA Light Engine (Lumencor, Beaverton, OR, USA).

4.5.4 Phylogenetic analyses

We generated phylogenetic trees based on the metagenome data generated from our bostrichid taxa (SRR19201352 - SRR19201388) as well as three Bostrichidae from NCBI (SRR2083737, FJ613421 and JX412742).

A phylogenetic tree of the mitochondrial genes of the hosts was reconstructed by assembling the mitochondrial genome using NOVOPlasty[87] and MitoZ[88] and afterwards annotating them with Mitos[89] (http://mitos.bioinf.uni-leipzig.de/index.py). Subsequently, 13 mitochondrial genes were translated and aligned using MUSCLE[90] (v3.8.425) as implemented in Geneious Prime 2019 (v2019.1.3, https://www.geneious.com). Additionally, we generated a second (codon-based) nucleotide alignment based on Benchmarking Universal Single-Copy Orthologs (BUSCO) using a custom pipeline[40] to extract the genes from the metagenome datasets. According to Shin et al. (2021)[91], BUSCO analysis was performed for each dataset using the insecta_odb10 database (1,658 genes) to extract BUSCO genes that were found across all species. The corresponding nucleotide sequences were then extracted and aligned in MAFFT[92] with --auto and default options. Gaps in the resulting alignment were then trimmed from the alignment using trimAl[93] (v1.2), accepting 5% gaps for each position. Afterwards, the aligned nucleotide sequences for each taxon were concatenated. For the phylogenetic placement of the intracellular symbionts of bostrichid beetles, the encoded gene sequences were extracted from the genomes, aligned based on the nucleotide sequence, and concatenated in Geneious Prime 2019 (v2019.1.3, https://www.geneious.com). Additionally, beetle symbiont 16S rRNA sequences were aligned to representative Bacteroidota 16S rRNA sequences obtained from the NCBI database, using the SILVA algorithm[31,94,95]. Since complete genomes were not available for some of the species, the 16S rRNA alignment allowed us to incorporate a larger number of species in the phylogenetic analysis, albeit at a lower resolution due to the limited amount of information contained in this single gene. Phylogenetic reconstructions for all alignments were done by Bayesian inference applying a GTR+G+I model using MrBayes[96–99] (v3.2.7). The analysis ran for 10,000,000 generations with a "Burnin" of 25% and tree sampling every 1,000 generations. We confirmed that the standard deviation of split frequencies converged to < 0.01. The obtained trees were visualized using FigTree (v1.4.4, http://tree.bio.ed.ac.uk/software/figtree/).

4.6 Data Accessibility Statement

Sequencing libraries and the assembles genome of the bostrichid symbionts (proposed *Shimatogenerans bostrichidophilus* and *Bostrichicola ureolyticus*) were uploaded to the NCBI Sequence Read Archive (see Supplement Table 1 for accession numbers) and GenBank (see Supplement Table 2 for accession numbers).

4.7 Acknowledgments

We thank Philipp-Martin Bauer, Hans-Georg Folz, Cornel Adler, Rudy Plarre, Rich Leschen, Michael Eifler, and Miguel Diaz for providing Bostrichid specimens. We thank Benjamin Weiss for technical assistance in histology, Bruno Hüttel and the Max Planck-Genome-Centre Cologne (http://mpgc.mpipz.mpg.de/home/) for performing library preparation and sequencing of most samples in this study, Yu Okamura for help with the BUSCO pipeline as well as the Johannes Gutenberg-University Mainz for computation time granted on the supercomputer 'MOGON', and Christian Meesters for administrative assistance on 'MOGON'. M.K. and T.E. acknowledge funding from the Max Planck Society, and further financial support of the Johannes Gutenberg-University Mainz (intramural funding to T.E.), as well as a Consolidator Grant of the European Research Council (ERC CoG 819585 "SYMBeetle" to M.K.).

4.8 Contributions

J.S.T.K. and T.E. designed the project, and J.S.T.K., E.B., G.O. and T.E. sequenced and assembled the symbiont genome. J.S.T.K. and E.B. annotated the genomes and performed symbiont genomic analysis and J.S.T.K. and T.E. performed phylogenetic analyses. J.S.T.K. and T.E. wrote the paper, with input from M.K and T.F.

4.9 References

1. Douglas, A. E. Symbiosis as a general principle in eukaryotic evolution. *Cold Spring Harb. Perspect. Biol.* **6**, (2014).
2. Flórez, L. V., Biedermann, P. H. W., Engl, T. & Kaltenpoth, M. Defensive symbioses of animals with prokaryotic and eukaryotic microorganisms. *Nat. Prod. Rep.* **32**, 904–936 (2015).
3. Lemoine, M. M., Engl, T. & Kaltenpoth, M. Microbial symbionts expanding or constraining abiotic niche space in insects. *Curr. Opin. Insect Sci.* **39**, 14–20 (2020).
4. Feldhaar, H. Bacterial symbionts as mediators of ecologically important traits of insect hosts. *Ecol. Entomol.* **36**, 533–543 (2011).
5. Douglas, A. E. The microbial dimension in insect nutritional ecology. *Funct. Ecol.* **23**, 38–47 (2009).
6. Kikuchi, Y. *et al.* Host-symbiont co-speciation and reductive genome evolution in gut symbiotic bacteria of acanthosomatid stinkbugs. *BMC Biol.* **7**, 2 (2009).
7. Clark, M. A., Moran, N. A., Baumann, P. & Wernegreen, J. J. Cospeciation between bacterial endosymbionts (*Buchnera*) and a recent radiation of aphids (*Uroleucon*) and pitfalls of testing for phylogenetic congruence. *Evolution (N. Y).* **54**, 517–525 (2000).
8. Moran, N. A., McCutcheon, J. P. & Nakabachi, A. Genomics and evolution of heritable bacterial symbionts. *Annu. Rev. Genet.* **42**, 165–190 (2008).
9. McCutcheon, J. P. The bacterial essence of tiny symbiont genomes. *Curr. Opin. Microbiol.* **13**, 73 (2010).
10. Bennett, G. M. & Moran, N. A. Heritable symbiosis: The advantages and perils of an evolutionary rabbit hole. *Proc. Natl. Acad. Sci.* **112**, 10169–10176 (2015).
11. Sudakaran, S., Kost, C. & Kaltenpoth, M. Symbiont acquisition and replacement as a source of ecological innovation. *Trends Microbiol.* **25**, 375–390 (2017).
12. Matsuura, Y. *et al.* Evolution of symbiotic organs and endosymbionts in lygaeid stinkbugs. *ISME J. 2012 62* **6**, 397–409 (2011).
13. McCutcheon, J. P. & Moran, N. A. Functional convergence in reduced genomes of bacterial symbionts spanning 200 My of evolution. *Genome Biol. Evol.* **2**, 708–718 (2010).
14. Bennett, G. M. & Moran, N. A. Small, Smaller, Smallest: The origins and evolution of ancient dual symbioses in a phloem-feeding insect. *Genome Biol. Evol.* **5**, 1675 (2013).
15. Takiya, D. M., Tran, P. L., Dietrich, C. H. & Moran, N. A. Co-cladogenesis spanning three phyla: leafhoppers (Insecta: Hemiptera: Cicadellidae) and their dual bacterial symbionts. *Mol. Ecol.* **15**, 4175–4191 (2006).
16. Sloan, D. B. & Moran, N. A. The evolution of genomic instability in the obligate endosymbionts of whiteflies. *Genome Biol. Evol.* **5**, 783–793 (2013).
17. McCutcheon, J. P. & Von Dohlen, C. D. An interdependent metabolic patchwork in the nested symbiosis of mealybugs. **21**, 1366–1372 (2011).
18. Gosalbes, M. J., Lamelas, A., Moya, A. & Latorre, A. The striking case of tryptophan provision in the cedar aphid *Cinara cedri*. *J. Bacteriol.* **190**, 6026 (2008).
19. Brunet, P. C. J. The metabolism of the aromatic amino acids concerned in the cross-linking of insect cuticle. *Insect Biochem.* **10**, 467–500 (1980).
20. Kramer, K. J. & Hopkins, T. L. Tyrosine metabolism for insect cuticle tanning. *Arch. Insect Biochem. Physiol.* **6**, 279–301 (1987).
21. Engl, T., Schmidt, T. H. P., Kanyile, S. N. & Klebsch, D. Metabolic cost of a nutritional symbiont manifests in delayed reproduction in a grain pest beetle. *Insects* **11**, 717 (2020).
22. Vigneron, A. *et al.* Insects recycle endosymbionts when the benefit is over. *Curr. Biol.* **24**, 2267–2273 (2014).
23. Hu, Y. *et al.* Herbivorous turtle ants obtain essential nutrients from a conserved nitrogen-recycling gut microbiome. **9**, (2018).
24. Duplais, C. *et al.* Gut bacteria are essential for normal cuticle development in herbivorous turtle ants. *Nat. Commun. 2021 121* **12**, 1–6 (2021).
25. Berasategui, A. *et al.* Symbiont genomic features and localisation in the bean beetle *Callosobruchus maculatus*. *Appl. Environ. Microbiol.* **87**, 1–13 (2021).
26. Klein, A. *et al.* A novel intracellular mutualistic bacterium in the invasive ant *Cardiocondyla obscurior*. *ISME J.* **10**, 376–388 (2016).
27. Anbutsu, H. *et al.* Small genome symbiont underlies cuticle hardness in beetles. *Proc. Natl. Acad. Sci.* **114**, E8382–E8391 (2017).
28. Anbutsu, H. & Fukatsu, T. Symbiosis for insect cuticle formation. in *Cellular Dialogues in the Holobiont* (eds. Bosch, T. C. G. & Hadfield, M. G.) 201–216 (CRC Press, 2020).

29. José de Souza, D., Devers, S. & Lenoir, A. *Blochmannia* endosymbionts and their host, the ant *Camponotus fellah*: cuticular hydrocarbons and melanization. *C. R. Biol.* **334**, 737–741 (2011).
30. Kiefer, J. S. T. *et al.* Inhibition of a nutritional endosymbiont by glyphosate abolishes mutualistic benefit on cuticle synthesis in *Oryzaephilus surinamensis*. *Commun. Biol.* **4**, 554 (2021).
31. Engl, T. *et al.* Ancient symbiosis confers desiccation resistance to stored grain pest beetles. *Mol. Ecol.* **27**, 2095–2108 (2018).
32. Crowson, R. A. (Roy A. The biology of the Coleoptera. 802 (1981).
33. Hunt, T. *et al.* A comprehensive phylogeny of beetles reveals the evolutionary origins of a superradiation. *Science (80-.).* **318**, 1913–1916 (2007).
34. Hirota, B. *et al.* A novel, extremely elongated, and endocellular bacterial symbiont supports cuticle formation of a grain pest beetle. *MBio* **8**, 1–16 (2017).
35. Okude, G. *et al.* Novel bacteriocyte-associated pleomorphic symbiont of the grain pest beetle *Rhyzopertha dominica* (Coleoptera: Bostrichidae). *Zool. Lett.* **3**, 13 (2017).
36. McKenna, D. D. *et al.* The evolution and genomic basis of beetle diversity. *Proc. Natl. Acad. Sci. U. S. A.* **116**, 24729–24737 (2019).
37. Zhang, S. Q. *et al.* Evolutionary history of Coleoptera revealed by extensive sampling of genes and species. *Nat. Commun.* **9**, 1–11 (2018).
38. Niehuis, M. *Die Kapuzenkäfer in Rheinland-Pfalz und im Saarland (Coleoptera: Bostrichidae)*. (GNOR, Mainz, 2022).
39. Jerzy Borowski & Piotr Wegrzynowicz. *World Catalogue of Bostrichidae (Coleoptera)*. (Mantis Publishing, Olsztyn, 2007).
40. Waterhouse, R. M. *et al.* BUSCO Applications from quality assessments to gene prediction and phylogenomics. *Mol. Biol. Evol.* **35**, 543–548 (2018).
41. Dopheide, T. A. A., Crewther, P. & Davidson, B. E. Chorismate mutase-prephenate dehydratase from *Escherichia coli* K-12. *J. Biol. Chem.* **247**, 4447–4452 (1972).
42. Hirota, B., Meng, X.-Y. & Fukatsu, T. Bacteriome-associated endosymbiotic bacteria of nosodendron tree sap beetles (Coleoptera: Nosodendridae). *Front. Microbiol.* **11**, 2556 (2020).
43. Lan-Yu, L. & Schönitzer, K. Phylogenetic analysis of the family Bostrichidae auct . at suprageneric levels (Coleoptera: Bostrichidae). *Mitt. Munch. Ent. Ges.* **101**, 99–132 (2011).
44. Gambetta, L. Ricerche sulla simbiosi ereditaria di alcuni coleotteri silofagi. *Ric. Morf. e Biol. anim* **1**, 150–118 (1928).
45. Mansour, K. Memoirs: On the intracellular micro-organisms of some bostrychild beetles. *J. Cell Sci.* **77**, 243–253 (1934).
46. Koch, A. Symbiosestudien. I. Die Symbiose des Splintkäfers, *Lyctus linearis* Goeze. *Zeitschrift für Morphol. und Ökologie der Tiere* **32**, 92–136 (1936).
47. Buchner, P. Studien an intrazellularen Symbionten VIII. Die symbiontischen Einrichtungen der bostrychiden (Apatiden). *Zeitschrift für Morphol. und Ökologie der Tiere 1954 426* **42**, 550–633 (1954).
48. Fukumori, K. *et al.* Evolutionary dynamics of host organs for microbial symbiosis in tortoise leaf beetles (Coleoptera: Chrysomelidae: Cassidinae). *MBio* **13**, (2022).
49. Dunbar, H. E., Wilson, A. C. C., Ferguson, N. R. & Moran, N. A. Aphid thermal tolerance is governed by a point mutation in bacterial symbionts. *PLOS Biol.* **5**, e96 (2007).
50. Dietel, A.-K., Merker, H., Kaltenpoth, M. & Kost, C. Selective advantages favour high genomic AT-contents in intracellular elements. *PLOS Genet.* **15**, e1007778 (2019).
51. Kuriwada, T. *et al.* Biological role of *Nardonella* endosymbiont in its weevil host. *PLoS One* **5**, e13101 (2010).
52. Sloan, D. B. *et al.* Parallel histories of horizontal gene transfer facilitated extreme reduction of endosymbiont genomes in sap-feeding insects. *Mol. Biol. Evol.* **31**, 857–871 (2014).
53. Mir, R., Jallu, S. & Singh, T. P. The shikimate pathway: review of amino acid sequence, function and three-dimensional structures of the enzymes. *Crit. Rev. Microbiol.* **41**, 172–189 (2015).
54. Herrmann, K. M. & Weaver, L. M. The shikimate pathway. *Annu. Rev. Plant Biol.* **50**, 473–503 (1999).
55. Arakane, Y. *et al.* Molecular and functional analyses of amino acid decarboxylases involved in cuticle tanning in Tribolium castaneum. *J. Biol. Chem.* **284**, 16584–16594 (2009).
56. Kanyile, S. N., Engl, T. & Kaltenpoth, M. Nutritional symbionts enhance structural defence against predation and fungal infection in a grain pest beetle. *J. Exp. Biol.* **225**, (2022).
57. Santos-Garcia, D., Silva, F. J., Morin, S., Dettner, K. & Kuechler, S. M. The all-rounder *Sodalis*: a new bacteriome-associated endosymbiont of the *Lygaeoid* bug *Henestaris halophilus* (Heteroptera: Henestarinae) and a critical examination of its evolution. *Genome Biol. Evol.* **9**, 2893–2910 (2017).

58. Wu, D. *et al.* Metabolic Complementarity and genomics of the dual bacterial symbiosis of sharpshooters. **4**, e188 (2006).
59. Russell, C. W., Bouvaine, S., Newell, P. D. & Douglasa, A. E. Shared metabolic pathways in a coevolved insect-bacterial symbiosis. *Appl. Environ. Microbiol.* **79**, 6117 (2013).
60. McCutcheon, J. P., McDonald, B. R. & Moran, N. A. Convergent evolution of metabolic roles in bacterial co-symbionts of insects. *Proc. Natl. Acad. Sci.* **106**, 15394–15399 (2009).
61. Snyder, A. K. & Rio, R. V. M. "*Wigglesworthia morsitans*" folate (Vitamin B_9) biosynthesis contributes to tsetse host fitness. *Appl. Environ. Microbiol.* **81**, 5375–5386 (2015).
62. Manzano-Marín, A. *et al.* A freeloader? the highly eroded yet large genome of the *Serratia symbiotica* symbiont of *Cinara strobi*. *Genome Biol. Evol.* **10**, 2178–2189 (2018).
63. Sabree, Z. L., Kambhampati, S. & Moran, N. A. Nitrogen recycling and nutritional provisioning by *Blattabacterium*, the cockroach endosymbiont. *Proc. Natl. Acad. Sci.* **106**, 19521–19526 (2009).
64. Rosas-Pérez, T., Rosenblueth, M., Rincón-Rosales, R., Mora, J. & Martínez-Romero, E. Genome sequence of "*Candidatus* Walczuchella monophlebidarum" the Flavobacterial endosymbiont of *Llaveia axin axin* (Hemiptera: Coccoidea: Monophlebidae). *Genome Biol. Evol.* **6**, 714–726 (2014).
65. Hansen, A. K., Pers, D. & Russell, J. A. Symbiotic solutions to nitrogen limitation and amino acid imbalance in insect diets. in *Mechanisms Underlying Microbial Symbiosis* (ed. Kerry M. Oliver, J. A. R.) vol. 58 161–205 (Academic Press, Cambridge, 2020).
66. Souci, S., Fachmann, W. & Kraut, H. *Lebensmitteltabelle für die Praxis*. (Wissenschaftliche Verlagsgesellschaft, 2009).
67. Hoadley, R. B., Museum, J. P. G. & Institute, G. C. *Chemical and Physical Properties of Wood*. *The Structural Conservation of Panel Paintings* (Los Angeles, 1998).
68. Oke, O. L., Redhead, J. & Hussain, M. A. *Roots, tubers, plantains and bananas in human nutrition*. (FAO food and nutrition series, 1990).
69. Suderman, R. J., Dittmer, N. T., Kramer, K. J. & Kanost, M. R. Model reactions for insect cuticle sclerotization: participation of amino groups in the cross-linking of *Manduca sexta* cuticle protein MsCP36. *Insect Biochem. Mol. Biol.* **40**, 252–258 (2010).
70. Torbatinejad, N. M., Rutherfurd, S. M. & Moughan, P. J. Total and reactive lysine contents in selected cereal-based food products. *J. Agric. Food Chem.* **53**, 4454–4458 (2005).
71. Sabree, Z. L., Huang, C. Y., Okusu, A., Moran, N. A. & Normark, B. B. The nutrient supplying capabilities of *Uzinura*, an endosymbiont of armoured scale insects. *Environ. Microbiol.* **15**, 1988–1999 (2013).
72. Ló Pez-Sánchez, M. J., Neef, A., Peretó, J., Patiñ O-Navarrete, R. & Pignatelli, M. Evolutionary convergence and nitrogen metabolism in *Blattabacterium* strain Bge, primary endosymbiont of the cockroach *Blattella germanica*. *PLoS Genet* **5**, 1000721 (2009).
73. Dale, C., Young, S. A., Haydon, D. T. & Welburn, S. C. The insect endosymbiont *Sodalis glossinidius* utilizes a type III secretion system for cell invasion. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 1883–1888 (2001).
74. Ewald, P. W. Transmission modes and evolution of the parasitism-mutualism continuum. *Ann. N. Y. Acad. Sci.* **503**, 295–306 (1987).
75. Hurst, G. D. D. D. *et al.* Male–killing *Wolbachia* in two species of insect. *Proc. R. Soc. London. Ser. B Biol. Sci.* **266**, 735–740 (1999).
76. HURST, G. D. D. *et al. Adonia variegata* (Coleoptera: Coccinellidae) bears maternally inherited Flavobacteria that kill males only. *Parasitology* **118**, 125–134 (1999).
77. Hurst, G. D. D. *et al.* Male-killing bacterium in a fifth ladybird beetle, *Coleomegilla maculata* (Coleoptera: Coccinellidae). *Hered. 1996 772* **77**, 177–185 (1996).
78. Martinson, V. G. Rediscovering a forgotten system of symbiosis: historical perspective and future potential. *Genes 2020, Vol. 11, Page 1063* **11**, 1063 (2020).
79. Bolger, A. M., Lohse, M. & Usadel, B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **30**, 2114–2120 (2014).
80. Wood, D. E. & Salzberg, S. L. Kraken: ultrafast metagenomic sequence classification using exact alignments. *Genome Biol.* **15**, 1–12 (2014).
81. Wood, D. E., Lu, J. & Langmead, B. Improved metagenomic analysis with Kraken 2. *Genome Biol.* **20**, 257 (2019).
82. Bankevich, A. *et al.* SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* **19**, 455–477 (2015).
83. Laczny, C. C. *et al.* BusyBee Web: metagenomic data analysis by bootstrapped supervised binning and annotation. *Nucleic Acids Res.* **45**, W171–W179 (2017).

84. Seemann, T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* **30**, 2068–2069 (2014).
85. Arkin, A. P. *et al.* KBase: The United States department of energy systems biology knowledgebase. *Nat. Biotechnol.* **36**, 566 (2018).
86. Gilchrist, C. L. M. & Chooi, Y. H. clinker & clustermap.js: automatic generation of gene cluster comparison figures. *Bioinformatics* **37**, 2473–2475 (2021).
87. Dierckxsens, N., Mardulyn, P. & Smits, G. NOVOPlasty: *De novo* assembly of organelle genomes from whole genome data. *Nucleic Acids Res.* **45**, e18–e18 (2017).
88. Meng, G., Li, Y., Yang, C. & Liu, S. MitoZ: a toolkit for animal mitochondrial genome assembly, annotation and visualization. *Nucleic Acids Res.* **47**, e63 (2019).
89. Bernt, M. *et al.* MITOS: Improved de novo metazoan mitochondrial genome annotation. *Mol. Phylogenet. Evol.* **69**, 313–319 (2013).
90. Edgar, R. C. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **32**, 1792–1797 (2004).
91. Shin, N. R. *et al.* Larvae of longhorned beetles (Coleoptera; Cerambycidae) have evolved a diverse and phylogenetically conserved array of plant cell wall degrading enzymes. *Syst. Entomol.* **46**, 784–797 (2021).
92. Katoh, K. & Standley, D. M. MAFFT Multiple Sequence Alignment Software version 7: improvements in performance and usability. *Mol. Biol. Evol.* **30**, 772–780 (2013).
93. Capella-Gutierrez, S., Silla-Martinez, J. M. & Gabaldon, T. trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. *Bioinformatics* **25**, 1972–1973 (2009).
94. Quast, C. *et al.* The SILVA ribosomal RNA gene database project: imporved data processing and web-based tools. *Nucleic Acids Res.* **41**, D590-6 (2013).
95. Yilmaz, P. *et al.* The SILVA and "All-species Living Tree Project (LTP)" taxonomic frameworks. *Nucleic Acids Res.* **42**, D643 (2014).
96. Huelsenbeck, J. P. & Ronquist, F. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinforma. Appl. NOTE* **17**, 754–755 (2001).
97. Huelsenbeck, J. P., Ronquist, F., Nielsen, R. & Bollback, J. P. Bayesian inference of phylogeny and its impact on evolutionary biology. *Science (80-.).* **294**, 2310–2314 (2001).
98. Ronquist, F. & Huelsenbeck, J. P. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**, 1572–1574 (2003).
99. Abadi, S., Azouri, D., Pupko, T. & Mayrose, I. Model selection may not be a mandatory step for phylogeny reconstruction. *Nat. Commun. 2019 101* **10**, 1–11 (2019).
100. Weiss, B. & Kaltenpoth, M. Bacteriome-localised intracellular symbionts in pollen-feeding beetles of the genus *Dasytes* (Coleoptera, Dasytidae). *Front. Microbiol.* **7**, 1486 (2016).

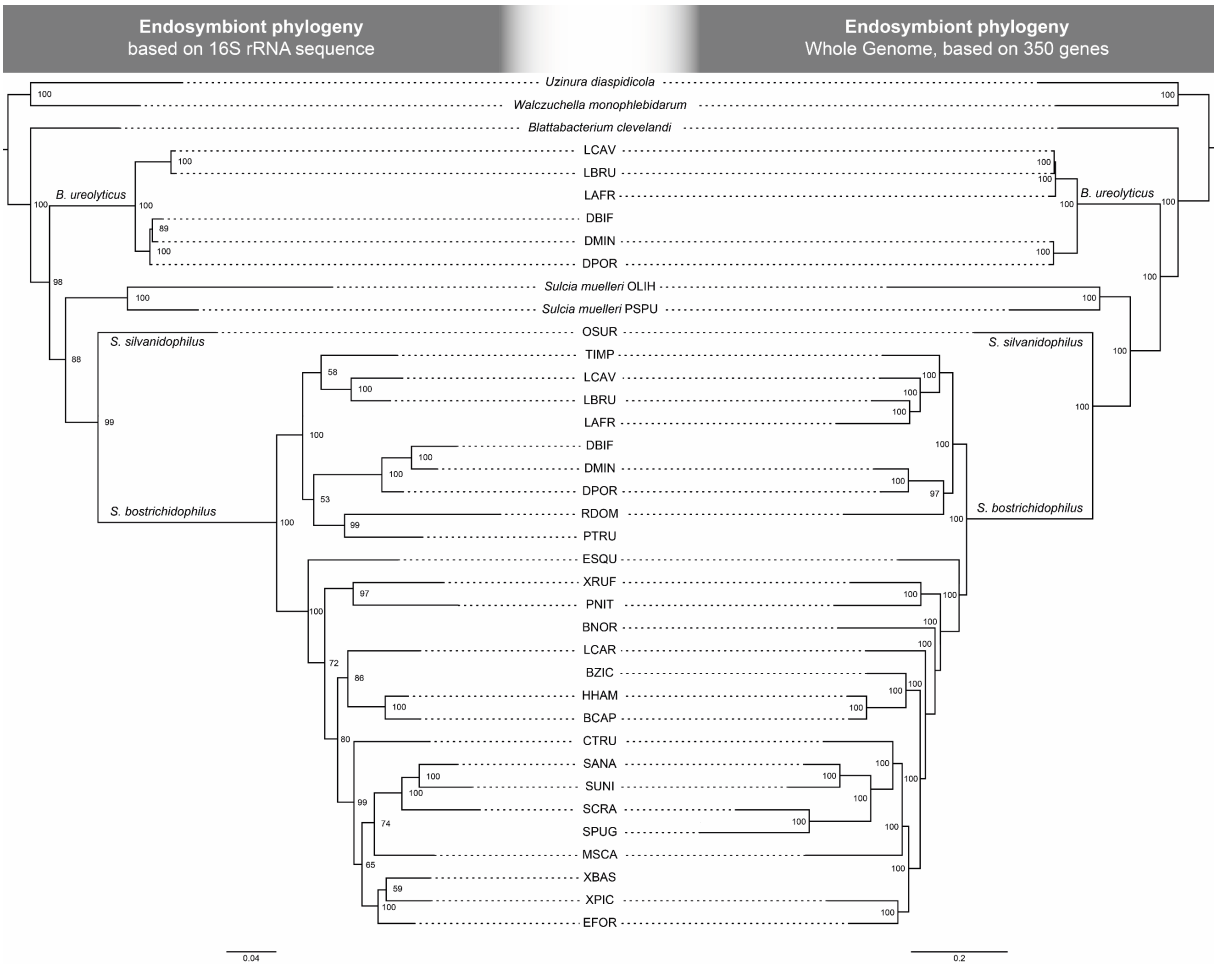
4.10 Supplementary Information

Supplement Table 1: List of collected specimen. JKI = Julius Kühn-Institute/Federal Research Centre for Cultivated Plants; BAM = Federal Institute for Materials Research and Testing.

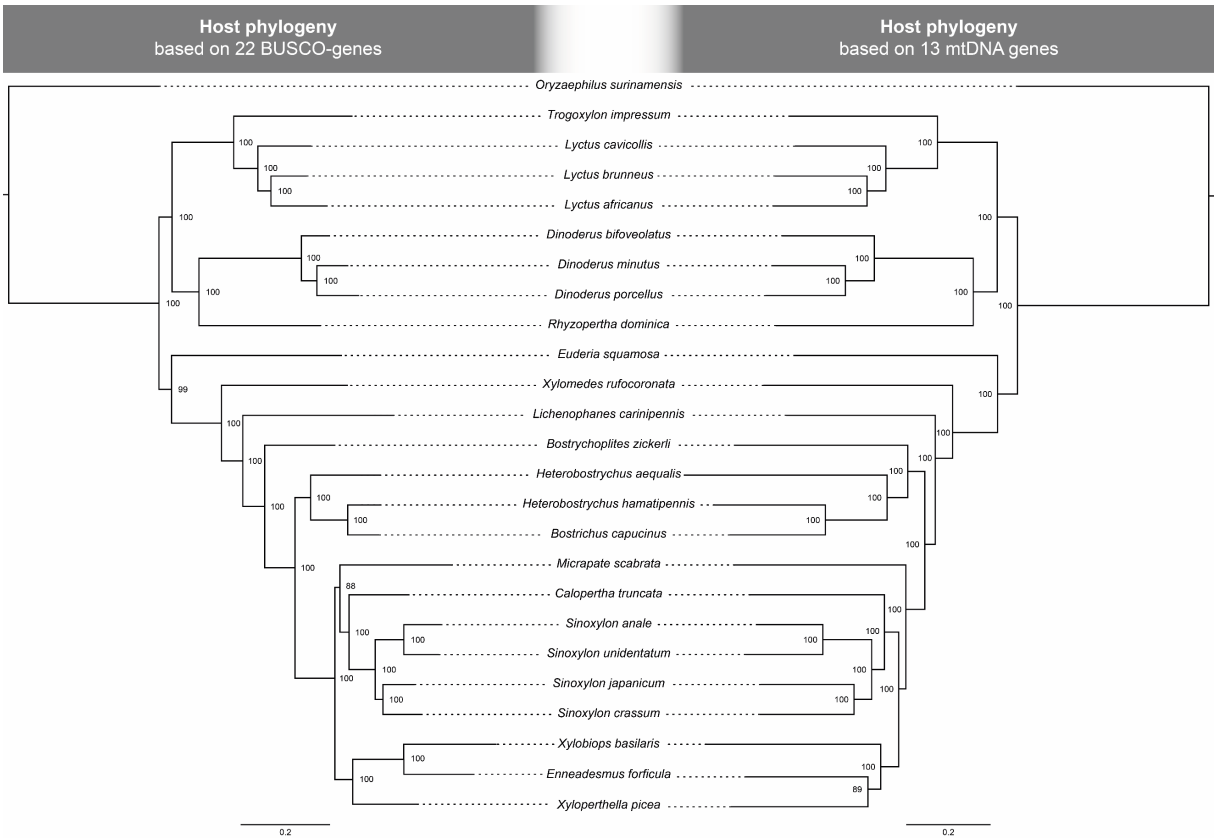
Family	Subfamily	Tribe	Genus	Species	Collected	Location	Donator	Library	mtDNA	Coverage	BUSCO
Bostrichidae	Lyctinae	Trogoxylini	*Trogoxylon*	*impressum*	21.06.2020	Engelstadt, Germany	Hans-Georg Folz	SRR19201388	ON707259	7,034x	82%
		Lyctini	*Lyctus*	*africanus*	18.03.2016	Tokyo, Japan	Takema Fukatsu	SRR19201387	ON707246	2,914x	66%
				brunneus	2015	lab-culture; BAM, Berlin, Germany	Rudy Plarre	SRR19201365, SRR19201357	ON707247	7,068x	72%
				cavicollis	June 2020	Engelstadt, Germany	Hans-Georg Folz	SRR19201376	ON707249	8,533x	89%
				bifoveolatus	2014	lab-culture; JKI, Berlin, Germany	Cornel Adler	SRR19201356	ON707238	752x	54%
	Dinoderinae		*Dinoderus*	*minutus*	12.04.2016	Ōita, Japan	Takema Fukatsu	SRR19201354, SRR19201355	ON707239	5,902x	30%
				porcellus	2014	lab-culture; JKI, Berlin, Germany	Cornel Adler	SRR19201352-SRR19201353, SRR19201382-SRR19201386	ON707240	9,216x	63%
			Rhyzopertha	*dominica*	2014	lab-culture; JKI, Berlin, Germany	Cornel Adler	SRR19201381, SRR19201380	ON707253	3,455x	77%
	Euderiinae		*Euderia*	*squamosa*	2020	Auckland, New Zealand	Rich Leschen	SRR19201379	ON707243	1,421x	55%
	Apatinae	Apatini	*Phonapate*	*nitidipennis*	29.06.1992	Mabar, Yemen	Michael Eifler	SRR19201378	ON707252	2,806x	1%
			Xylomedes	*rufocoronata*	22.03.2006	Emirate of Sharjah, UAE	Michael Eifler	SRR19201377	ON707261	27,360x	97%
		Dinapatini	*Dinapate*	*wrightii*	July 1994	Riverside county, CA, USA	BioQuip	SRR19201375	ON707241	893x	3%
	Bostrichinae	Bostrichini	*Amphicerus*	*bicaudus*	15.06.1988	Lubbock county, TX, USA	BioQuip	SRR19201374	-	-	4%
			Apatides	*fortis*		FJ613421	NCBI	-	-	-	-
			Bostrichus	*capucinus*	May 2020	Ostrovačice, Czech Republic	Miguel Diaz	SRR19201373	ON707234	81,589x	98%
			Bostrychoplites	*normandi*	01.08.1992	ʿAmrān, Yemen	Michael Eifler	SRR19201371	ON707235	399x	1%
				zickeli	15.09.2001	Al Kawd, Yemen	Michael Eifler	SRR19201372	ON707236	4,253x	72%
			Heterobostrychus	*aequalis*	12.09.2016	Koyoto, Japan	Takema Fukatsu	SRR19201370	ON707244	3,984x	97%
				hamatipennis	01.05.2016	Mannheim, Germany	Philipp-Martin Bauer	SRR19201369	ON707245	3,470x	70%
			Lichenophanes	*carinipennis*	08.08.2020	Kobe, Japan	Takema Fukatsu	SRR19201368	ON707248	5,899x	64%
			Micrapate	*scabrata*	01.07.2018	University of Hohenheim, Germany	Philipp-Martin Bauer	SRR19201367	ON707250	6,625x	50%
		Xyloperthini	*Enneadesmus*	*forficula*	31.01.2006	Emirate of Sharjah, UAE	Michael Eifler	SRR19201366	ON707242	1,010x	86%
			Xylobiops	*basilaris*		SRR2083737	NCBI	-	x	103,675x	72%
			Xyloperthella	*picea*	15.06.2002	Taiz, Yemen	Michael Eifler	SRR19201364	ON707260	6,599x	55%
			Calopertha	*truncatula*	12.07.2001	Kawd al-ʿAbadil, Yemen	Michael Eifler	SRR19201363	ON707237	2,116x	97%
		Sinoxylonini	*Sinoxylon*	*anale*	01.04.2016	Mannheim, Germany	Philipp-Martin Bauer	SRR19201362	ON707254	4,303x	69%
				crassum	01.07.2018	University of Hohenheim, Germany	Philipp-Martin Bauer	SRR19201361	ON707255	8,405x	71%
				japanicum	26.04.2020	Yokohama, Japan	Takema Fukatsu	SRR19201360	ON707256	1,681x	83%
				pugnax	05.06.2005	Emirate of Fujairah, UAE	Michael Eifler	SRR19201359	ON707257	384x	21%
				unidentatum	01.07.2018	University of Hohenheim, Germany	Philipp-Martin Bauer	SRR19201358	ON707258	6,251x	75%
				sp.		JX412742	NCBI	-	-	-	-

Supplement Table 2: General features of the symbiont genomes based on annotations with PROKKA. LSU: Large subunit ribosomal protein, SSU: Small subunit ribosomal proteins.

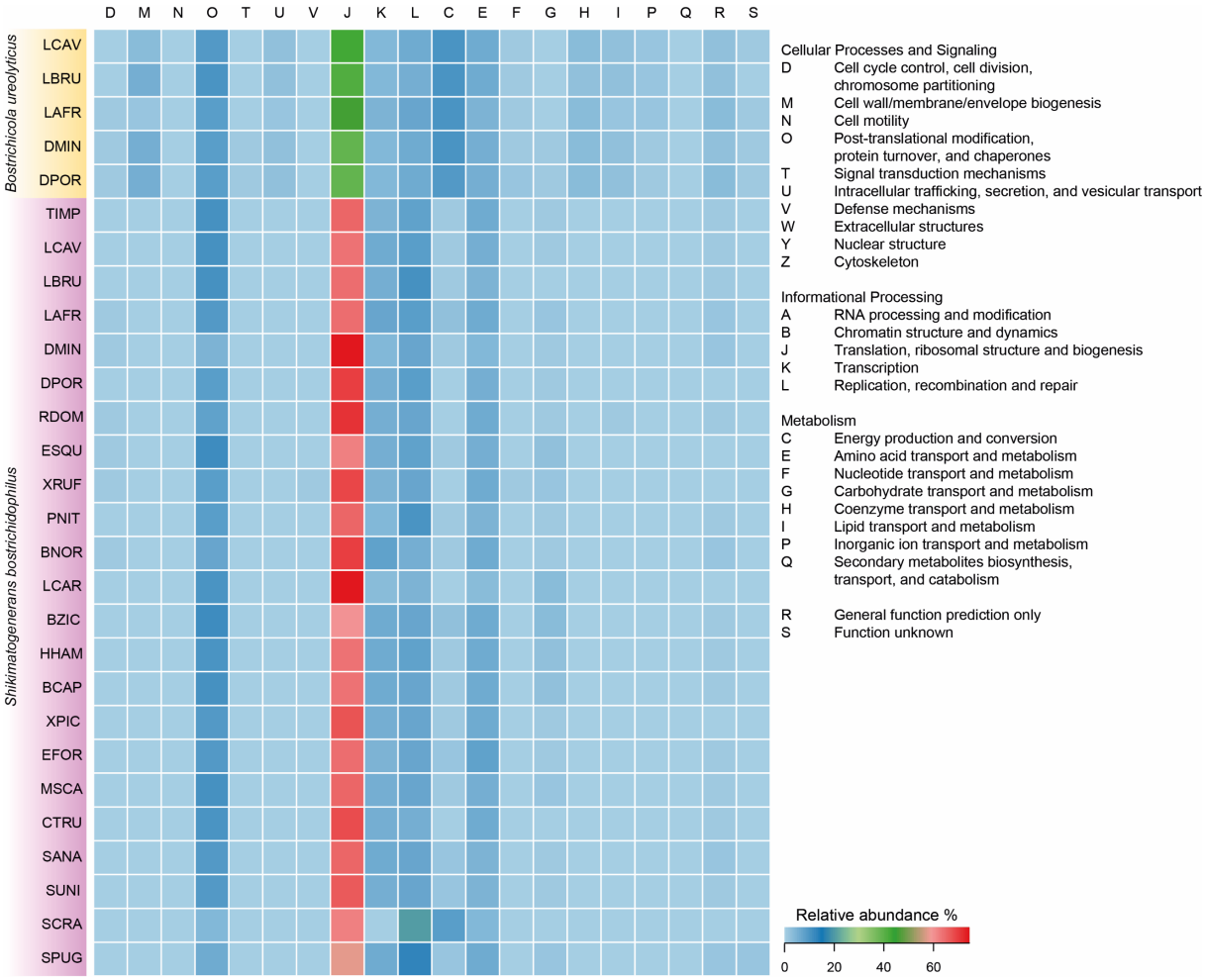
Bacterium	Strain	Accession	Genome size	GC content	Coverage	CDS	tRNAs	rRNAs	SSU	LSU
Bostrichicola ureolyticus	LAFR	JAMYEW000000000	draft; 171 contigs; 263,976 bp	22.6%	338	277	28	29	14	15
	LBRU	CP100318	337,336 bp	22.1%	55	324	20	44	17	27
	LCAV	CP100320	323,829 bp	22.1%	693	317	20	44	17	27
	DMIN	CP100321	346,375 bp	22.6%	997	336	21	44	17	27
	DPOR	CP100319	344,793 bp	22.8%	334	334	20	42	16	26
Shikimatogenerans bostrichidophilus	TIMP	CP099825	193,392 bp	13.2%	85	193	21	36	17	19
	LAFR	JAMYEV000000000	draft; 18 contigs; 187,750 bp	16.7%	115	162	24	33	15	18
	LBRU	CP099821	191,425 bp	13.5%	17	222	22	35	16	19
	LCAV	CP099823	194,907 bp	13.2%	450	191	21	34	16	18
	DMIN	CP099824	194.383 bp	13.9%	128	197	21	35	15	20
	DPOR	CP099822	177,818 bp	13.7%	242	178	18	35	17	18
	RDOM	CP099826	172,971 bp	13.3%	1751	168	22	37	17	20
	ESQU	CP099827	200,377 bp	15.1%	88	200	20	38	18	20
	PNIT	CP099828	163,888 bp	15.4%	1159	175	24	30	13	17
	XRUF	JAMYEX000000000	draft; 2 contigs; 131,783 bp	14.7%	27	129	17	33	15	18
	BCAP	CP099829	196,478 bp	17.1%	1542	191	21	41	18	23
	BNOR	JAMYEY000000000	draft; 49 contigs; 117,215 bp	15.3%	396	111	13	28	13	15
	BZIC	JAMYEZ000000000	draft; 54 contigs; 154,309 bp	14.5%	47	154	15	25	11	14
	HHAM	CP099830	189,381 bp	17.3%	184	187	19	40	18	22
	LCAR	ON922557-ON922562	draft; 6 contigs; 50,604 bp	17.6%	282	64	4	25	12	13
	MSCA	CP099831	185,513 bp	16.6%	151	182	21	37	16	21
	EFOR	JAMYFA000000000	draft; 13 contigs; 164,672 bp	15.6%	281	161	21	37	17	20
	XPIC	JAMYFC000000000	draft; 12 contigs; 170,362 bp	13.2%	281	163	27	37	17	20
	CTRU	JAMYFB000000000	draft; 38 contigs; 128,631 bp	16.5%	55	128	20	36	15	21
	SANA	CP099832	184,356 bp	15.5%	199	181	20	38	17	21
	SCRA	ON922555-ON922556	draft, 2 contigs; 28,977 bp	19.8%	463	26	7	7	3	4
	SPUG	ON922537-ON922554	draft; 18 contigs; 45,556 bp	19.8%	27	50	3	6	4	2
	SUNI	CP099833	183,323 bp	17.0%	587	184	20	38	17	21



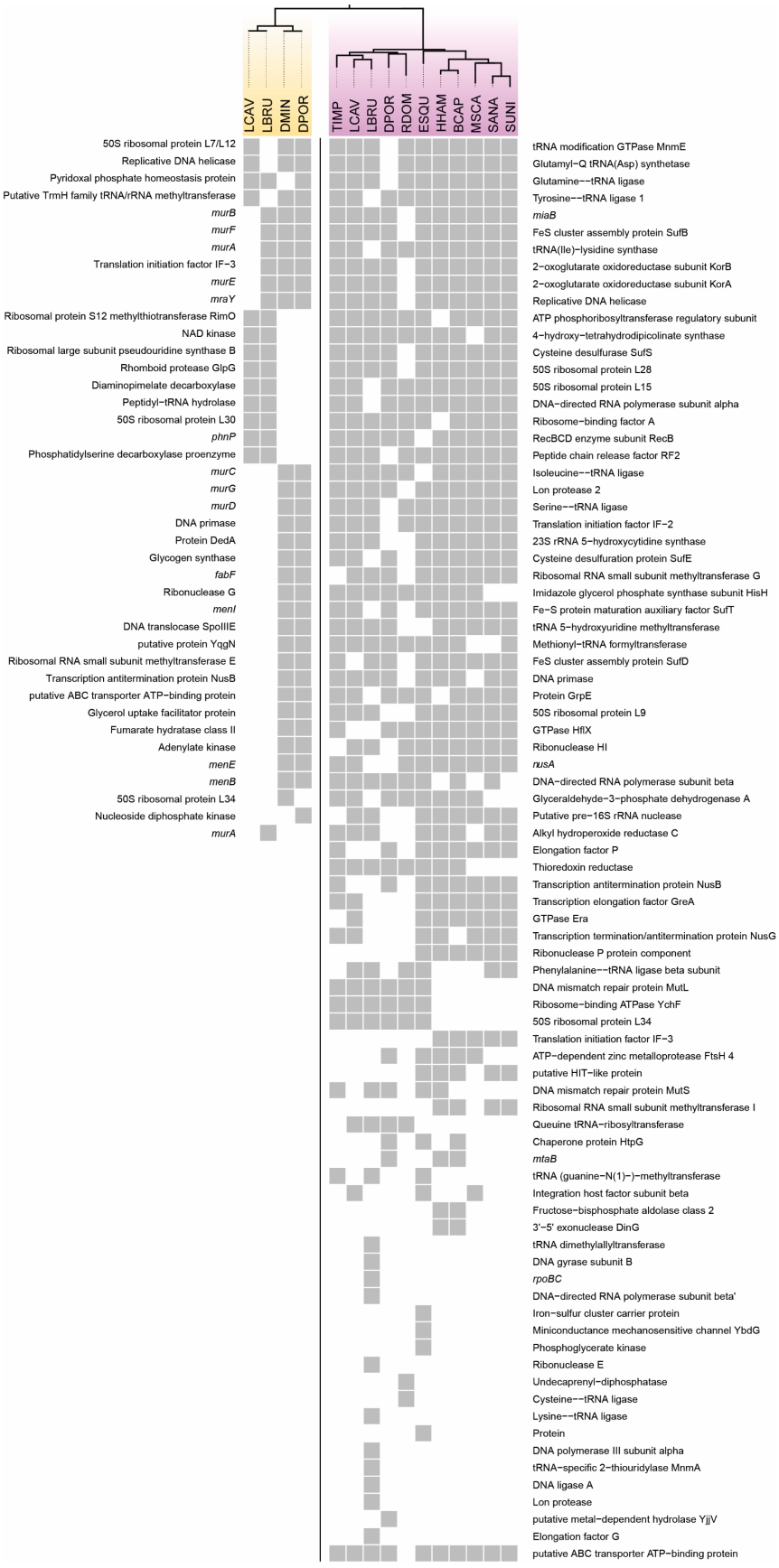
Supplement Figure 1: Comparison of symbiont phylogenies based on the 16S rRNA gene alone (left), and 350 genes conserved across at least two genomes (right). Node numbers represent posterior probabilities of Bayesian analyses.



Supplement Figure 2: Comparison of host phylogenies based on 22 BUSCO genes (left) and 13 mitochondrial genes (right). Node numbers represent posterior probabilities of Bayesian analyses.



Supplement Figure 3: Relative abundance of Clusters of Orthologous Groups (COG). Annotated functional categories (A-Z) and relative proportion of the encoded genes represented as a heatmap are indicated on the right-hand side.



Supplement Figure 4: Comparison of differentially encoded genes in genomes of *Bostrichicola ureolyticus* (left) and *Shikimatogenerans bostrichidophilus* (right). Genomes were annotated with PROKKA. Hypothetical proteins were removed. Filled: present, white: missing

Chapter 5

Loss of genomic cohesion: Symbiont lineage splitting in the large grain borer *Prostephanus truncatus* (Coleoptera: Bostrichidae)

Julian Simon Thilo Kiefer[1], Eugen Bauer[1], Martin Kaltenpoth[1,2], Tobias Engl[1,2]

[1]Department of Evolutionary Ecology, Institute of Organismic and Molecular Evolution, Johannes Gutenberg-University, Mainz, Germany; [2]Department of Insect Symbiosis, Max-Planck-Institute for Chemical Ecology, Jena, Germany

5.1 Abstract

The endosymbionts *Shikimatogenerans bostrichidophilus* (Bacteroidota), associated with beetles of the Bostrichidae family, supply their host's diet solely with prephenate, the precursor for the aromatic amino acid tyrosine. Within the larger grain borer *Prostephanus truncatus,* the genome of the associated endosymbiont has diverged into three strains. To better understand the lineage splitting of *S. bostrichidophilus* PTRU we utilized different microscopy approaches to verify the genomic predictions by localising the individual genes and genomes in the bacteriome. The genomes of the three strains are very similar, encoding for a core set of genes involved in processing genetic information. However, genes for the shikimate pathway and the ribosomes are encoded by the three different strains, complementing each other, suggesting that these genes or their products have to be shared between symbiont cells. As this phenomenon of lineage splitting did not add any functionality to the symbiosis, it is likely a non-adaptive result of ongoing genome erosion.

5.2 Introduction

Similar to organelles, intracellular symbionts depend on the host for their existence. A phenomenon that can be observed very often in endosymbiotic bacteria is genome reduction[1,2]. In this process, the genome undergoes a process in which the encoded genes are undergoing inactivation and complete loss, leading to a genome with a stable set of necessary encoded functions[3].

The erosion of a genome is thought to be driven by unselective processes like genetic drift and mutation accumulation[1] as well as by selection at the symbiont and host level[4,5]. The result of this process is a genome with the minimum set of functions, typically encoding a few genes outside of the core processes of replication, transcription, translation, and nutrient provisioning[1]. Ongoing genome reduction puts the symbiont under tremendous evolutionary pressure as purifying selection acts on the symbiont genes that are important for functions that the host needs[6,7]. Further, endosymbionts can also be lost, replaced, or complemented when they are no longer needed or incapable of sufficiently supporting their host's metabolism. In several cases, this has led to dual symbioses in which the essential amino acids are still synthesised by the original symbiont, but vitamins are synthesised by a secondary symbiont, or certain pathways being split between the symbionts[8,9]. One example is the glassy-winged sharpshooter *Homalodisca vitripennis* (Hemiptera: Cicadellidae), where the β-proteobacterial symbiont *Baumannia* retains pathways for vitamins needed by the host, while the Bacteroidota symbiont *Sulcia muelleri* retains genes for the production of most essential amino acids, resulting in metabolic complementarity[8].

Beetles of the family Bostrichidae associate with two Bacteroidota endosymbionts, i.e. *Shikimatogenerans bostrichidophilus* and *Bostrichicola ureolyticus* (Chapter 3). However, the lesser grain borer *Rhyzopertha dominica* and the large grain borer *Prostephanus truncatus* in the subfamily Dinoderinae have lost *B. ureolyticus* and retain only *S. bostrichidophilus*[10,11] (Chapter 3). Both *R. dominica* and *P. truncatus* are found in a similar ecological niche as the sawtoothed grain beetle *Oryzaephilus surinamensis* with its closely related endosymbiont *Shikimatogenerans silvanidophilus* (Chapter 2). *Shikimatogenerans* symbionts exhibit highly reduced genomes specialised in supplementing their hosts diet with nutrients via the shikimate pathway crucial for a healthy cuticle synthesis. However, in contrast to *S. silvanidophilus*, *S. bostrichidophilus* even lost the

glycolysis pathway to produce precursors for the shikimate pathway. Additionally, early studies by Koch and Huger suggest a higher degree of integration and host dependency, as it is not possible to eliminate the endosymbiont of *R. dominica* and *P. truncatus*[12,13], while aposymbiotic *O. surinamensis* can be readily obtained by antibiotic treatment or exposure to elevated temperatures[10,13], and symbiont-free cultures can be maintained for years[10].

In this study, we used whole-genome sequencing and fluorescence microscopy to report an unusual phenomenon in Dinoderinae beetles, in which the endosymbiont *S. bostrichidophilus* PTRU of *P. truncatus* has diverged into three strains within its host. The genes for the shikimate pathway and the ribosomes are encoded by the three different strains, complementing each other but also showing a considerable level of redundancy in encoded functions. Lineages splitting apparently did not add any functionality to the symbiosis—the three symbiont genomes of the *S. bostrichidophilus* PTRU strains in *P. truncatus* perform together exactly the same functions as the single genome of *S. bostrichidophilus* RDOM in *R. dominica*, so this phenomenon is likely a non-adaptive result of ongoing genome erosion.

5.3 Results

5.3.1 Bostrichidae-symbiont genomes are highly eroded

The metagenomes of the lesser grain borer *Rhyzopertha dominica* and the large grain borer *Prostephanus truncatus* were sequenced using short-read (Illumina) technology to gain deeper insights into symbiont metabolic capabilities. For *R. dominica,* we could confirm the 16S rRNA sequences of a Bacteroidota bacterium that matched PCR-based Sanger sequences from previous studies[10]. The *de novo* assembled genome of the symbiont of *R. dominica* is 173 kbp in length with an average GC content of 13.3% (Figure 1). The genome encodes for 169 genes and had a coverage of 1,751x. Like the genome of *S. silvanidophilus* OSUR (Chapter 2), the genome of *S. bostrichidophilus* RDOM is highly reduced, with most of the encoded genes dedicated to genetic information processing including DNA replication and repair, transcription, and translation. Additionally, it encodes all genes of the shikimate pathway except a shikimate dehydrogenase (*aroE* [EC:1.1.1.25]). Also, the genome encodes the bifunctional *aroG*/*pheA* gene (phospho-2-dehydro-3-deoxyheptonate aldolase/chorismate mutase [EC:2.5.1.54 5.4.99.5]), capable of catalysing the Claisen rearrangement of chorismate to prephenate and the decarboxylation/dehydration of prephenate to phenylpyruvate[14].

In contrast, the metagenome of *P. truncatus* revealed sequences of more than one Bacteroidota endosymbiont. We were able to assemble genomes of three highly similar, yet distinct endosymbiont strains (henceforth called PTRU strains A, B, and C; Figure 1). All three of them share a highly similar 16S rRNA sequence, but their genomes diversify in length and encoded genes (pairwise identity of aligned PTRU strain 16S rRNA sequences: A + B = 98.8%, B + C = 98.5%, A + C = 98.7%, A + B + C = 98.7%). The genome of PTRU strain A is 157 kbp in length with a GC content of 15.4%, encoding for 136 genes with an average coverage of 704x. PTRU strain B is 152 kbp in length, has a GC content of 15.4% and the genome encodes for 124 genes with an average coverage of 655x. Finally, the genome of PTRU strain C is 160 kbp in length, has a GC content of 15.3% and is encoding for 139 genes with an average coverage of 792x.

The genomes of the three *Shikimatogenerans bostrichidophilus* PTRU strains together show a very similar gene composition as the genome of *S. bostrichidophilus* RDOM, but some of the genes are only present in one or two of the three strains (Figure 2, Supplementary Figure 1). In consequence, the genes of the shikimate pathway are distributed over the three strains, whereas only one of the

six genes (*aroF*) is present in all three strains (Figure 2). For strain A, the gene 5-enolpyruvylshikimate-3-phosphate synthase (*aroA* [EC:2.5.1.19]) is unique, while the other four genes of the pathway (*aroC*, *aroD*, *aroK* and *aroQ*) are unique to strain C. Hence, the shikimate pathway is only complete when at least strain A and strain C complement each other, while strain B is dispensable.

Figure 1: Circular representation of the genomes of *Shikimatogenerans bostrichidophilus* RDOM of *Rhyzopertha dominica* and the three PTRU strains in *Prostephanus truncatus*. The outer grey circles denote coverage with short reads, and the intermediate circles indicate annotated functional KEGG categories separated by direction of transcription (see legend for depicted categories). The inner grey circle denotes relative GC content and the average GC content in per cent is indicated by the red line.

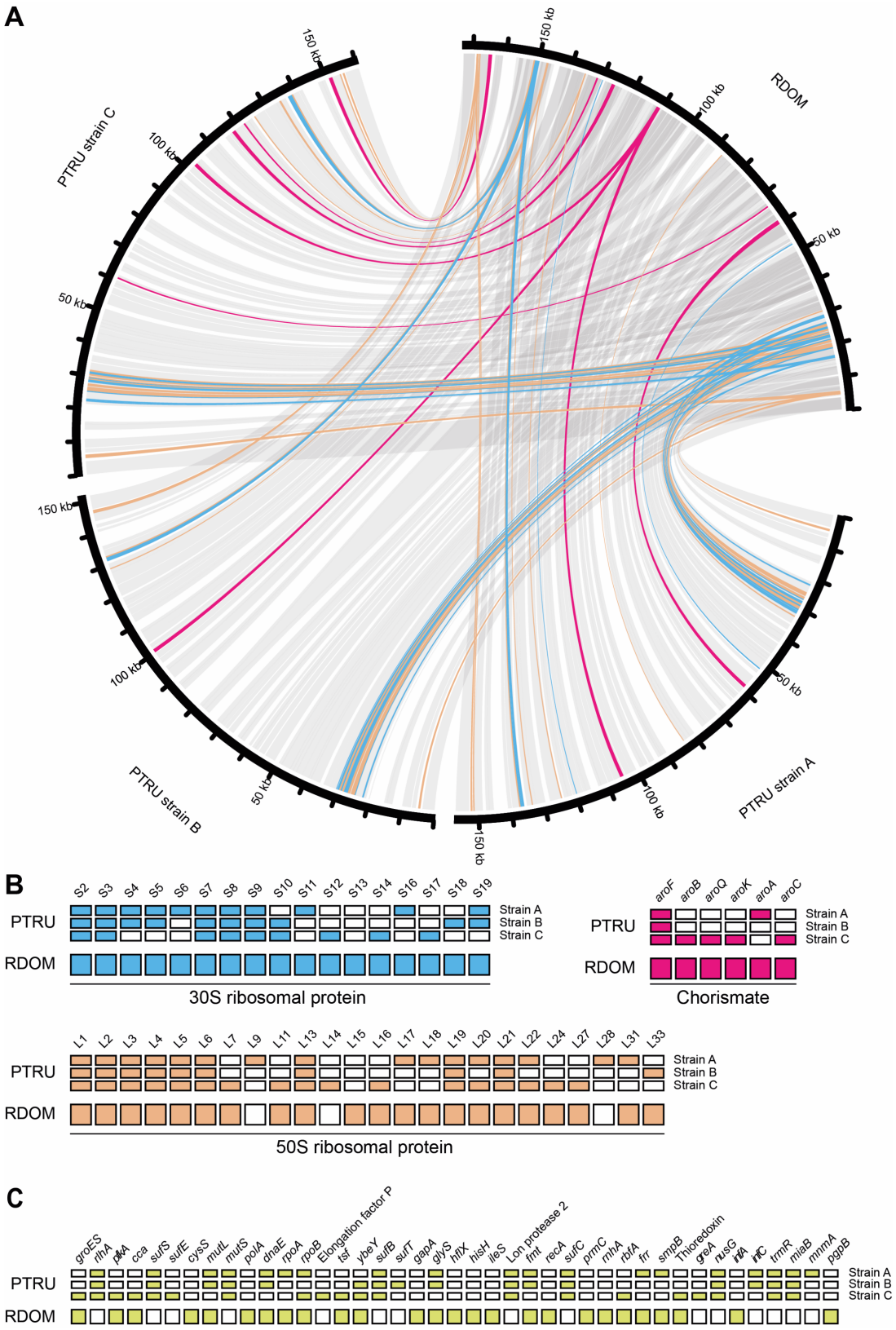


Figure 2: (**A**) Comparison of the functional gene repertoires of the endosymbionts. Grey: Genes present in RDOM and also in the three PTRU strains, red: shikimate genes, blue: small subunit ribosomal proteins, orange: large subunit ribosomal proteins. (**B**) Comparison of shikimate pathway genes and ribosomal proteins present in genomes. Filled: present, white: missing. (**C**) Comparison of all differentially encoded genes in genomes of RDOM and PTRU strains. Genomes were annotated with PROKKA[15]. Hypothetical proteins were removed. Filled: present, white: missing.

By PCR amplification, cloning and sequencing of the 16S rRNA gene, SNPs could indeed be confirmed between the individual 16S rRNA sequences of the three strains, which makes it possible to reconstruct a 16S rRNA-based phylogeny (Figure 3 A). However, these differences between the three sequences are not detectable in a pooled bacterial PCR Sanger sequencing approach, explaining why we previously missed the strain differentiation[10]. By combining the cloned 16S rRNA sequences with the shotgun short-read assembly, we were able to assign the three 16S types to the respective strains. The phylogenetic reconstruction based on the 16S rRNA sequences confirmed the placement of the Bacteroidota endosymbionts of *R. dominica* and *P. truncatus* as sister species in a group of insect-associated bacteria, closely related to *Shikimatogenerans silvanidophilus* OSUR (*S. silvanidophilus*), *Blattabacterium* sp. and *Sulcia muelleri* (*S. muelleri*) (Figure 3 A and Chapter 4). We also used the shikimate pathway gene *aroF* to reconstruct a phylogeny as it represents the only gene of the pathway being present in all three strains (pairwise identity of aligned nucleotide *aroF* sequences: A + B = 96.2%, A + C = 95.5%, B + C = 95.1%, A + B + C = 95.5%; pairwise identity aligned amino acid *aroF* sequences: A + B = 96.3%, A + C = 94.6%, B + C = 94.9%, A + B + C = 95.4%; Figure 3 B).

Besides the shikimate pathway, also the ribosomal proteins (rProteins) of the large and the small subunit are distributed across the three strains (Figure 2). We investigated if all three strains of the endosymbiont in *P. truncatus* are present in the same ratio during development. Therefore, we used a gene of interest (GOI) encoded by the strains to measure the titer in all life stages of the host: First, the sequence of *aroA* encoded in the genome of PTRU strain A, second the sequence of *aroF* encoded in the genome of PTRU strain B and third the sequence of *aroC* encoded in the genome of PTRU strain C. With the three GOIs, we detected constant ratios in symbiont titers across development, reaching their maximum in the adult life stage. Interestingly, titers of strain A were consistently higher than those of strain B (approximately 2.4-fold on average), and titers of strain B were approximately 11.2-fold higher than those of strain C.

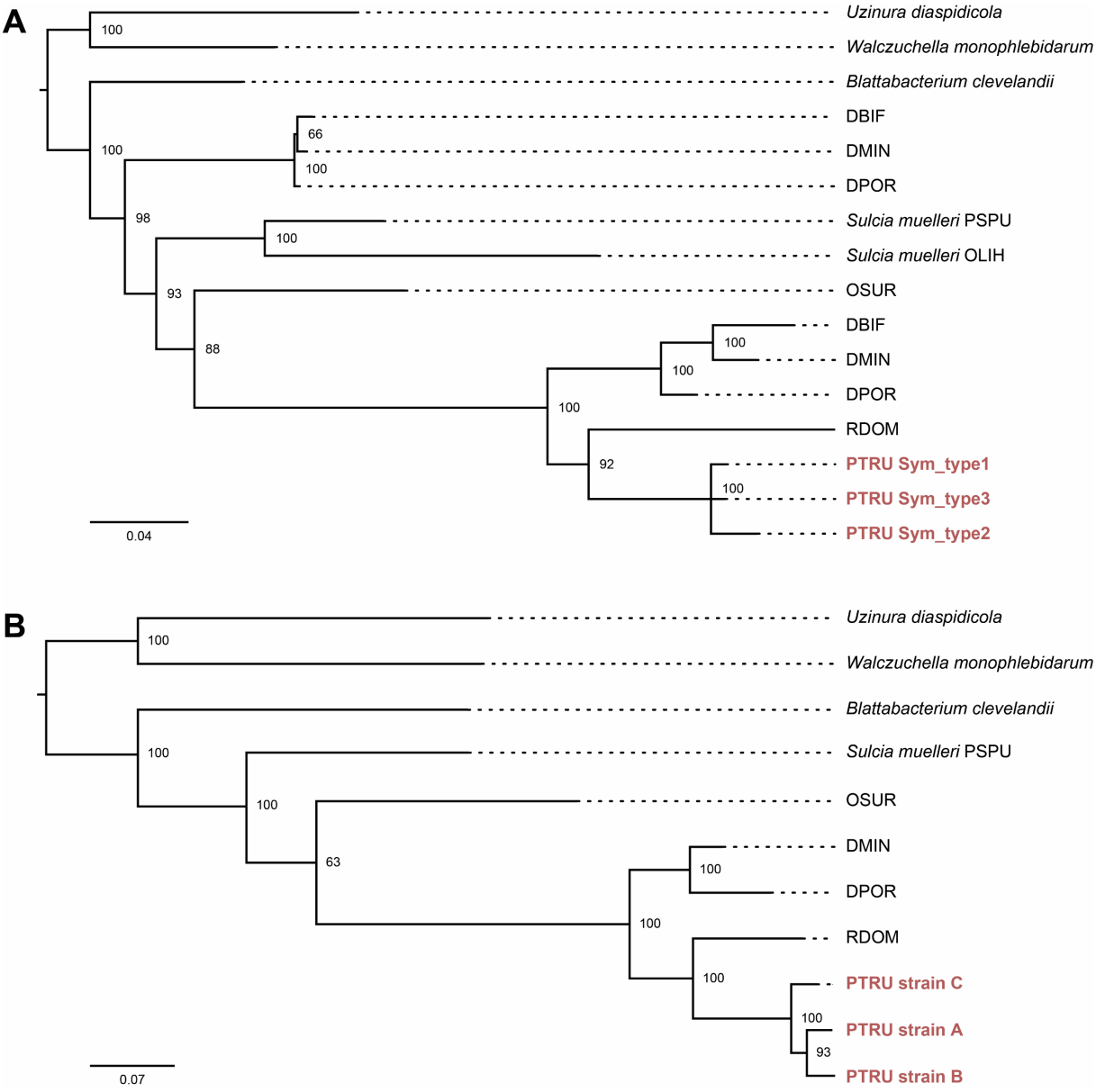


Figure 3: Bayesian phylogeny of **(A)** nucleotide alignment of 16S rRNA sequences and **(B)** the translation-guided alignment of nucleotide sequences of *aroF* Node numbers represent posterior probabilities of Bayesian analysis.

5.3.2 Localisation of the three strains of *P. truncatus* in the bacteriome

In a previous study, the bacteriomes of *R. dominica* and *P. truncatus* were localised via FISH by targeting the 16S rRNA of the symbionts[10]. We repeated the FISH for both species, yielding very similar bacteriome structures and no information on the distribution of the three different strains in *P. truncatus* (Figure 5 f). Thus, we used the cloned 16S rRNA sequences of the three PTRU strains to design strain-specific FISH probes to distinguish the single strains in the bacteriome. We could show that the designed probes for PTRU strain A (SymType2), PTRU strain B

(SymType1) and PTRU strain C (SymType3) indeed highlighted three distinct types of localisations in the bacteriome (Figure 6 A). All cells labelled as strain B also showed a signal with the probe targeting strain C, but 50-70% of strain C-labelled cells were not stained for strain B. PTRU strain A-stained cells were dominant and mainly found alone, but often in contact with strain C.

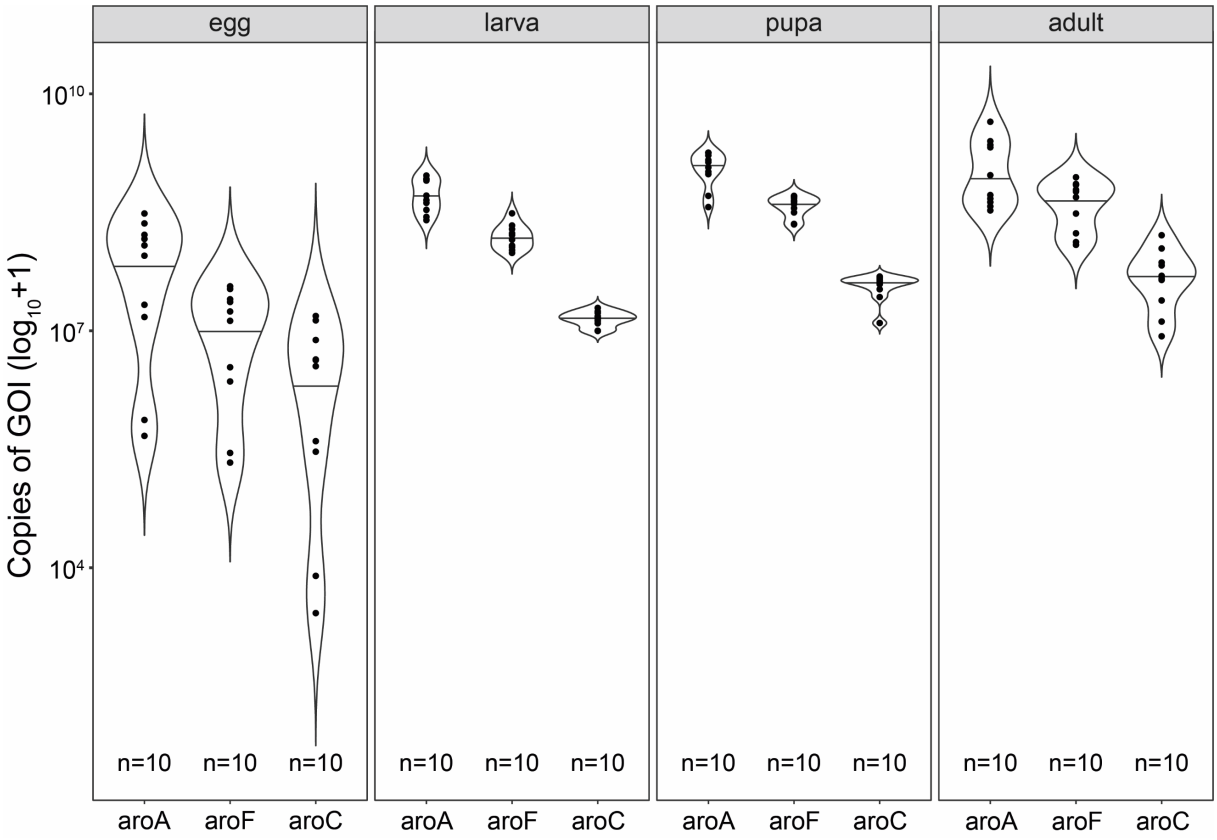


Figure 4: Titers of the three PTRU strains across life stages of *Prostephanus truncatus*, measured via qPCR with strain-specific primers targeting *aroA* (strain A), *aroF* (strain B) and *aroC* (strain C).

In addition, we made use of the hybridisation chain reaction technique (HCR) to label strains on the gene level (Figure 6 B and C). We used genes of the shikimate pathway as GOIs to identify the individual strains in the bacteriome (Figure 6 B). The probes visualizing the shikimate gene *aroA* (strain A) and *aroC* (strain C) were often co-localised, while *aroF* signals (strain B) occurred alone. As *aroA* is only encoded by the genome of PTRU strain A and *aroC* is only encoded in the genome of PTRU strain C, this result is indicating a co-localisation of these two strains in the bacteriome. Further, we used the sequences of rProteins which are uniquely encoded in the individual PTRU strain genomes (Figure 6 C). Here, the signal of rProteins encoded by PTRU

strain A is co-localised with the signal of rProteins encoded by PTRU strain B, while the signal of the rProteins encoded by the genome of PTRU strain C showed no co-localisation with any of the two other strains.

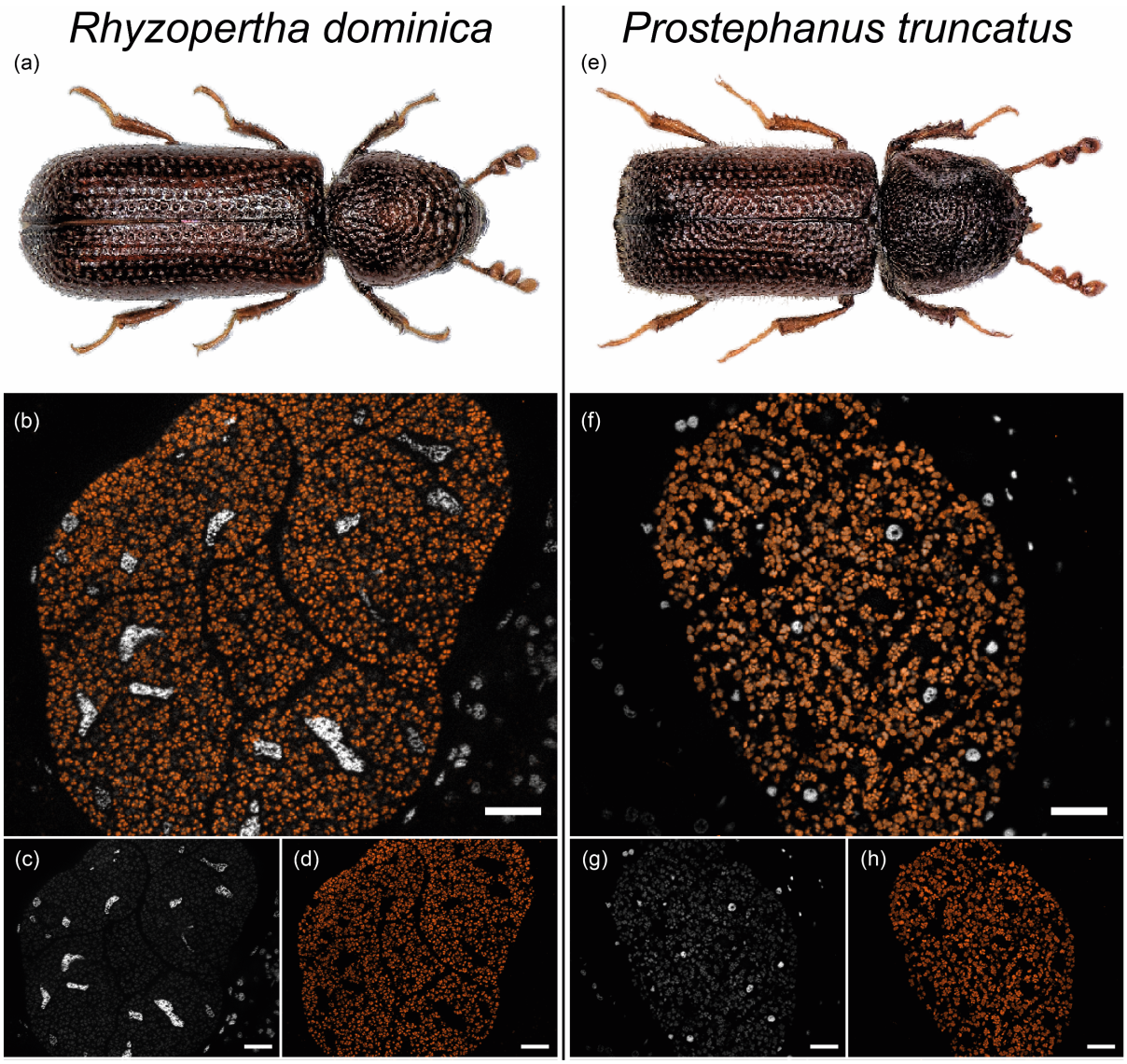


Figure 5: Images of *Rhyzopertha dominica* **(a)** and *Prostephanus truncatus* **(e)** (kindly provided by Thomas Hörren). Fluorescence *in situ* hybridisation micrographs of *R. dominica* **(b-d)** and *P. truncatus* **(f-h)** stained with a *Shikimatogenerans bostrichidophilus* specific probe (orange) and DAPI targeting DNA in general (white). Scale bars represent 20 µm.

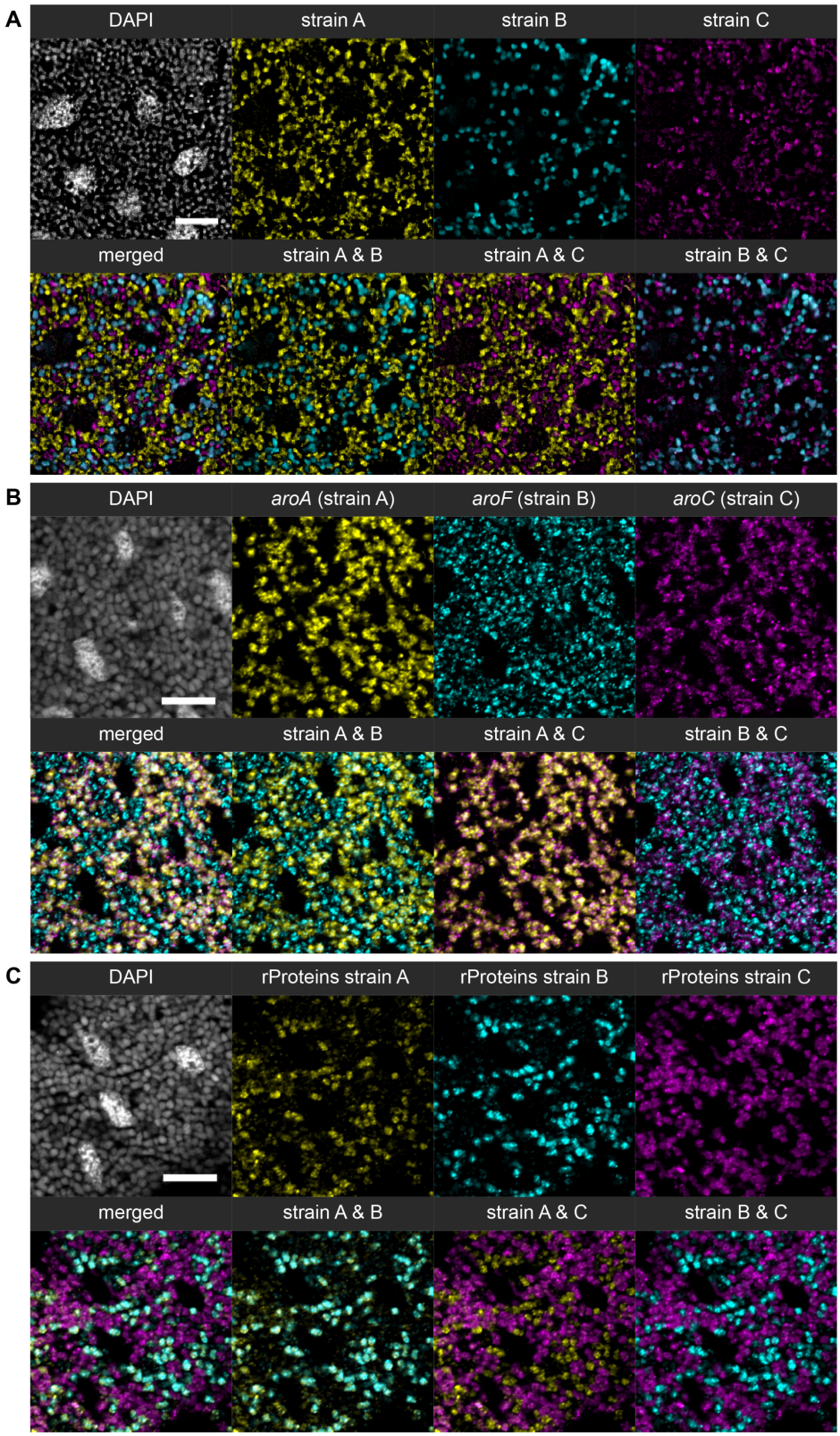


Figure 6: (**A**) Fluorescence *in situ* hybridisation micrographs of a *Prostephanus truncatus* bacteriome stained with strain-specific 16S rRNA probes: strain A (SymType2, yellow), strain B (SymType1, cyan) and strain C (SymType3, magenta). (**B**) HCR-FISH on an isolated bacteriome of *P. truncatus*, to visualize strains of *Shikimatogenerans bostrichidophilus* PTRU using probes against unique sequences of shikimate pathway genes *aroA* (strain A, yellow), *aroF* (strain B, cyan) and *aroC* (strain C, magenta) and (**C**) unique ribosomal protein (rProtein) sequences of strain A (yellow) strain B (cyan) and strain C (magenta). DNA of host and symbiont stained with DAPI (white). Scale bar representing 10 µm.

5.1 Discussion

In this study, we sequenced the metagenomes of the larger grain borer *Prostephanus truncatus*. Contrary to our expectations, we assembled not only one but three genomes of the Bacteroidota symbiont *Shikimatogenerans bostrichidophilus* PTRU. We compared the genomes of the three PTRU strains to the close relative *S. bostrichidophilus* RDOM, the endosymbiont of the lesser grain borer *Rhyzopertha dominica* (Chapter 4), revealing that all three strains of PTRU together contain the same metabolic capacity as RDOM in *R. dominica*. The genomes encode only genes of the shikimate pathway apart from gene families involved in the core processes of replication, transcription, and translation. It is noteworthy, that the genes of the shikimate pathway are spread over all three strains, however, two of the strains are sufficient to complete the pathway. Of the six genes of the shikimate pathway (*aroA, aroB*, *aroC, aroF*, *aroK* and *aroQ*), only *aroF* is encoded in the genome of every strain. Although *aroB*, *aroC*, *aroK* and *aroQ* are encoded in the genome of PTRU strain C, *aroA* is uniquely encoded in the genome of PTRU strain A. The lack of the shikimate pathway gene *aroE* was also described in other tyrosine-supplementing bacterial endosymbionts: *S. silvanidophilus* of *O. surinamensis*, *Nardonella* EPO of the sweetpotato weevil *Euscepes postfasciatus* (Curculionidae: Cryptorhynchinae) and *Carsonella ruddii* in the gall-forming psyllid *Pachypsylla venusta* (Aphalaridae: Pachypsyllinae). However, the shikimate pathway remained functional[16,17] (Chapter 2), suggesting that the function of *aroE* can be taken over by other enzymes, either from the host or the endosymbiont—that have yet to be identified.

Besides genes of the shikimate pathway, several other genes are encoded only by one or two of the PTRU strains. Among these, the ribosomal proteins (rProteins) are particularly noteworthy. From the whole set of rProteins, some are uniquely encoded in one of the three strains, resulting presumably in each strain being required to assemble functional ribosomes. This scenario is similar to what has been seen in *Hodgkinia* in some species of the cicada genus *Tettigades,* where rProteins tended to only be encoded on the most abundant genome[18].

The symbionts are localised in the cytoplasm of the bacteriocytes. As the genomes of *S. bostrichidophilus* in *R. dominica* and *P. truncatus* lack genes for the synthesis of peptidoglycan, the symbiont cells show amorphous morphology, known as the L-form[11,19–21]. Also, several studies on other bacteriome-localised endosymbionts found that the symbionts are often polyploid, stating that there are multiple copies of the genome per cell[22–24]. If we assume the same for

S. bostrichidophilus, then the genomes of the three PTRU strains can either be mixed in the same symbiont cell, or they can be segregated into different cells. In the latter case, the symbiont strains must exchange either mRNAs, proteins/enzymes and/or entire ribosomes and metabolites to be functional. Okude et al. (2017), while researching the endosymbionts of *R. dominica*, already showed via FISH microscopy (Okude et al 2017, Figure 1 g-j) and transmission electron microscopic (TEM) images (Okude et al 2017, Figure 5 b) of the bacteriome that the symbiont cells are connected in a grape-like pattern. In our FISH micrographs, we see the same pattern for both *R. dominica* and similar ones in *P. truncatus*, where different endosymbiont cells are connected. These connections between single cells could be a prerequisite for the evolution of separate strains and the starting point for *S. bostrichidophilus* to diverge into multiple strains. If they can at least still exchange metabolites via these connections, there would not be any drastic negative consequences if the genome of every cell no longer encoded all the individual enzymes and ribosomal proteins. In *P. truncatus,* likely, cells of the three PTRU strains are still connected in this manner, building a continuum where all necessary gene products would be present and accessible for every strain, even when specific genes are not encoded in its genome. Intriguingly, this includes genes encoding enzymes involved in genetic information processing, such as *dnaE* (the DNA polymerase III alpha subunit) and *rpoA* (the RNA polymerase subunit alpha), which are missing in PTRU strain C (Figure 4 C). Thus, the products of genes involved in essential bacterial cellular functions need to be transported among cells in this continuum.

This raised the question of how the shikimate pathway as well as the ribosomes can still be functional when the necessary genes are distributed across different symbiont genomes. To approach this question, we visualized the different strains in the bacteriome. The FISH based on the 16S rRNA sequences of the three PTRU strains showed that the strains matching SymType2 and SymType3 are mainly co-localised, while the 16S rRNA matching SymType1 shows little to no co-localisation. The HCR FISH with probes targeting genes of the shikimate pathway showed that *aroA* and *aroC* are mostly co-localised, indicating a co-localisation of strain A and strain C. In terms of the shikimate pathway, this would make perfect sense, since the co-localisation of these two strains would complete the pathway. In the same image, the signal detecting *aroF* is present in some cells too, but mostly it is localised on its own. Regarding the qPCR, in which the titers of the three strains via individual genes of the shikimate pathway were measured, a relatively high signal for *aroF* can also be seen here. This could be caused by the method of HCR

itself. While primers were used in the qPCR which specifically amplifies the sequence of the *aroF* from PTRU strain B, this sequence was divided into eleven small fragments for the HCR to serve as a probe. Some of these fragments may also have non-specifically aligned to the *aroF* sequence of PTRU strains A and C, resulting in false positive signals. In contrast, the HCR with the sequences of the rProteins as a probe shows a completely different picture. Here, it appears that the rProtein sequences encoded by PTRU strain A reside in the same symbiont cells as the rProtein sequences encoded by PTRU strain B, while the rProtein sequences encoded in the genome of PTRU strain C stand alone. The reason for this contrasting picture again could be based on the HCR method itself. The respective sequences of the single ribosomal proteins alone are not long enough to design sufficient single-gene probes. Therefore, we selected several ribosomal proteins per strain, based on which the probes were generated. Since the three genomes only differ in individual genes, but they are largely identical, it can happen in the assembly that individual positions were assembled incorrectly. This could be improved by using long reads by third-generation sequencing technologies in addition. Nevertheless, our findings of three sequences of the shikimate pathway gene *aroF* in addition to the three 16S rRNA sequences strongly indicate the presence of three strains in the bacteriome of *P. truncatus*. In direct comparison, both the 16S rRNA FISH and the two HCRs performed show the same picture: two of the three strains are usually co-localised, while the third shows little to no co-localisation. The only question that remains open is how the individual sequences and genes are distributed across the three genomes.

After metamorphosis, there is no more pressure on the endosymbiont as they uniquely contribute to precursor synthesis for cuticle formation. However, bacteriomes are retained for symbiont transmission in female beetles. During this period, the quality of the symbionts may then no longer be controlled at host-level and less fit symbiont genotypes could accumulate[25]. Something similar was observed in cicadas of the genus *Tettigades* (Hemiptera: Cicadidae) which are associated with the endosymbiont *Hodgkinia cicadicola* (α-proteobacteria). Łukasik et al. (2017) found that in *Tettigades* the ancestral *Hodgkinia* has split independently six times over the last 4 Mya, which results in a complex of two to six *Hodgkinia* lineages per host[25,43]. The authors argue that the long dormant period without host-level selection on symbiont functionality (relative to the period of larval growth where the symbionts are important) may allow for slightly deleterious processes to occur, such as lineage splitting[18]. Also, they tested the idea of this being adaptive by allowing for adjusting expression levels by gene copy numbers - instead of transcriptional

regulation - which is likely impaired in genome-eroded symbionts due to the loss of most or all transcription factors. In contrast, in *P. truncatus* the adult lifespan is very long with females living approximately 61 days and males living approximately 45 days[26]. Complete development from egg to adult took approximately 37 day[27], whereas the pupation time is only five days until emerging of the adult beetle[26]. Following, in female adults, the endosymbionts spend a long time being functionally irrelevant to the host, which may allow for lineage splitting.

In different species such non-adaptive and rather degenerative and finally probably "costly" processes can take place in symbioses. Campbell et al. (2018) showed in cicadas associated with the symbionts *Sulcia muelleri* and several *Hodgkinia* linages that the host has increased the number of transmitted *Hodgkinia* cells six-fold, whereas the number of *S. muelleri* cells is unaltered[44]. This example shows that there is a dose adaption in transmission of strains, i.e. those with many strains pass on significantly more symbionts via the eggs to ensure that really all strains, including the rare ones, are successfully transferred to the next generation.

5.2 Material & Methods

5.2.1 Insect cultures

The initial *Rhyzopertha dominica* and *Prostephanus truncatus* cultures (strain JKI) were obtained from the Julius-Kühn-Institute/Federal Research Centre for Cultivated Plants (Berlin, Germany) in 2014 and kept in culture since then. Continuous cultures were maintained in 1.8-L plastic containers, filled with 50 g spelt (*R. dominica*) or corn (*P. truncatus*), at 28°C, 60% relative humidity, and a day and night cycle of 16 to 8 hours.

5.2.2 Symbiont genome sequencing, assembly, and annotation

Total DNA was isolated using the Epicentre MasterPureTM Complete DNA and RNA Purification Kit (Illumina Inc., Madison, WI, USA) including RNase digestion. Library preparation and sequencing for *R. dominica* (SRR19201380 and SRR19201381) and *P. truncatus* (SRR19638530 and SRR19638531) was performed at the Max-Planck-Genome-Centre (Cologne, Germany) on a HiSeq3000 Sequencing System (Illumina Inc., Madison, WI, USA). Adaptor and quality trimming was performed with Trimmomatic[28]. Assembly of Illumina reads was performed using SPAdes (v3.15.0) with the default settings[29]. The resulting contigs were then binned using BusyBee Web[30] and screened for GC content and taxonomic identity to Bacteroidota bacteria. The extracted contigs were *de novo* assembled in Geneious Prime 2019 (v2019.1.3, https://www.geneious.com). The resulting genomes were then automatically annotated with PROKKA[15] using the app Annotate Assembly and Re-annotate Genomes (v1.14.5) on KBase[31]. The annotated genomes were plotted using CIRCOS (v0.69-6) for the visualisation of gene locations, GC content, and coverage[32].

5.2.3 16S rRNA cloning and phylogenetic analyses

Bacterial 16S rRNA amplicons were obtained from single bacteriomes of four *P. truncatus* individuals and cloned into *Escherichia coli* for separation and subsequent Sanger sequencing of vector inserts. DNA was extracted from individual bacteriomes with the Epicentre MasterPure™ Complete DNA and RNA Purification Kit (Illumina Inc., Madison, WI, USA) and amplified with the Phusion high-fidelity DNA polymerase (ThermoFisher Scientific) and general bacterial 16S rRNA primers fD1 and rP2[33]. The PCR parameters were as follows: after an initial denaturation step for 30 seconds at 98°C, 35 cycles were run for 10 seconds at 98°C, 30 seconds at 58°C and 30 seconds at 72°C followed by a final extension of 5 minutes. Amplicons were

purified from 1.5% agarose gels with the InnuPrep gel extraction kit (Analytik Jena GmbH, Jena, Germany) and cloned with the pMiniT 2.0 vector into *Escherichia coli* K12 (NEB PCR Cloning Kit; New England Biolabs, MA, USA). Vector insertion sequences of successfully transformed colonies were amplified by another PCR using the vector-specific cloning analysis primers (NEB PCR Cloning Kit; New England Biolabs, MA, USA) with PCR conditions as above, and entire cells from clone colonies were added to the PCR reaction mix as a template. Bidirectional Sanger sequencing was performed in-house on an Applied Biosystems 3730xl DNA Analyzer (ThermoFisher Scientific, Germany) to obtain the full sequence of the amplified 16S fragments using the cloning analysis forward and reverse primers.

16S rRNA sequences were aligned to representative Bacteroidota 16S rRNA sequences obtained from the NCBI database, using the SILVA algorithm[10,34,35]. Sequences of the shikimate pathway gene *aroF* encoded in the genomes of *R. dominica* and *P. truncatus* were extracted and aligned to representative Bacteroidota *aroF* sequences obtained from the NCBI database, using MAFFT[36]. Phylogenetic reconstruction for all alignments was done by Bayesian inference applying a GTR+G+I model using MrBayes (v3.2.7)[37–40]. The analysis ran for 20,000,000 generations with a "Burnin" of 25%, a sampling frequency of 1,000 generations, and we confirmed that split frequencies converged to <0.01. The obtained trees were visualized using FigTree (v1.4.4, http://tree.bio.ed.ac.uk/software/figtree/).

5.2.4 Comparative genomics

Endosymbiont genomes were annotated with PROKKA[15] in KBase[31] to compare the bacteria and to estimate the genome-wide nucleotide sequence divergence level. Therefore, we identified single-copy orthologs in each genome pair using OrthoMCL (v2.0)[41] in KBase[31]. KEGG categories were then assessed via GhostKOALA (v2.2)[42] of each gene's amino acid sequence. CIRCOS (v0.69-6)[32] was used to link orthologous genes. Heatmaps were visualized using 'gplots' package in Rstudio (V 1.1.463 with R V3.6.3).

5.2.5 Quantitative PCR

DNA of eight to ten *Prostephanus truncatus* eggs, larvae, pupae, and adults was isolated individually using the Epicentre MasterPure™ Complete DNA and RNA Purification Kit following the manufacturer's instruction (Illumina Inc., USA) to evaluate symbiont titers. For the adult beetles, the abdomen (without wings) was used. The DNA was dissolved in 30 µL low

TE buffer (1:10 dilution of 1x TE buffer: 10 mM Tris-HCl + 1 mM EDTA). qPCRs were carried out in 25 µL reactions using EvaGreen (Solis BioDyne, Estonia), including 0.5 µM of each primer (Table 1) and 1 µL template DNA. All reagents were mixed, vortexed and centrifugated in 0.1 mL reaction tubes (Biozym, 711200). qPCRs were carried out on a Rotor-Gene Q thermal cycler (Qiagen, Hilden, Germany). The initial temperature was 95°C for 12 minutes, followed by 60 cycles of 95°C for 40 seconds followed by 20 seconds at 60°C. A melting curve analysis was used to assess the specificity of the qPCR reaction by a gradual increase of temperature from 60 to 95°C, with 0.25 K per second. The qPCR results were analysed using the Rotor Gene Q Software (Qiagen, Hilden, Germany).

Standard curves with defined copy numbers of the genes were created by amplifying the fragment first via PCR, followed by purification and determination of the DNA concentration via NanoDrop1000 (Peqlab, Germany). After the determination of the DNA concentration, a standard containing 10^{10} copies/µL was generated and 1:10 serial dilutions down to 10^{1} copies/µL were prepared. 1 µL of each standard was included in a qPCR reaction to standardize all measurements. The plot was visualized using 'ggplot2' in RStudio (V 1.1.463 with R V3.6.3).

Table 1: Used Primer for qPCR on PTRU strains.

Name	Target	Sequence 5′-3	Orientation
Ptru_A_Shiki6_f	*aroA* / strain A	TGTAGGAGCTATATCAGGAGTAGA	fwd
Ptru_A_Shiki6_r	*aroA* / strain A	TGATTGGAGTTCAGCATCTTATTTT	rev
Ptru_B_Shiki1_f	*aroF* / strain B	CCATTTTAAACCTTCTTCCCCATAC	fwd
Ptru_B_Shiki1_r	*aroF* / strain B	GGGCCATGTAGTGCAGAAAAT	rev
Ptru_C_Shiki7_f	*aroC* / strain C	ACAATTGGTACTGCTCTTGGT	fwd
Ptru_C_Shiki7_r	*aroC* / strain C	GGGGAATTCAAGGAGGTATATCA	rev

5.2.6 Fluorescence *in situ* hybridisation

To localise the bacteriomes in *R. dominica* and *P. truncatus,* fluorescence *in situ* hybridisation (FISH) was performed, targeting the 16S rRNA sequence. Adult beetles were fixated in tertiary butanol (80%; Roth, Karlsruhe, Germany), paraformaldehyde (37-40%; Roth, Karlsruhe, Germany) and glacial acetic acid (Sigma-Aldrich, Germany) in proportions 6:3:1 for 2 hours, followed by post-fixation in alcoholic formaldehyde (paraformaldehyde (37-40%) and tertiary butanol (80% in proportion 1:2). After dehydration, the specimens were embedded in Technovit 8100 (Kulzer,

Germany)[45] and cut into 8 µm sagittal sections using a Leica HistoCore AUTOCUT R microtome (Leica, Wetzlar, Germany) equipped with glass knives. The obtained sections were mounted on silanised glass slides. Each slide was covered with 100 µL of hybridisation mix, consisting of hybridisation buffer (0.9 M NaCl, 0.02 M Tris/HCl pH 8.0, 0.01% SDS; Roth, Germany) and 0.5 µM of each specific probe (Table 2). DAPI (0.5 µg/mL) was included as a general counterstain for DNA. Slides were covered with glass cover slips and incubated in a humid chamber at 50°C overnight. After washing and incubating them for two hours at 50°C in wash buffer (0.1 M NaCl, 0.02 M Tris/HCl, 5 mM EDTA, 0.01% SDS), they were washed in deionized water for 20 minutes and mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA). The sections were either observed under a Zeiss AxioImager Z2 with Apotome.2 (Zeiss, Jena, Germany) illuminated by a SOLA Light Engine (Lumencor, Beaverton, OR, USA), or a Leica THUNDER imager Cell Culture 3D (Leica, Wetzlar, Germany). Images obtained on the Leica microscope were processed with the instant and small volume computational clearing algorithm using standard settings in the Leica Application Suite X software (Leica, Wetzlar, Germany).

Table 2: Used FISH probes targeting 16S rRNA.

Name	Sequence 5′-3	Marker
Bostrichidae_Sym2	CTTCCTACACGCGAAATAG	Cy5
SymType1	TATTACTAAGACTATCTTTT	Cy3
SymType2	AATTAATAAGACTATTCTTC	Cy5
SymType3	ATTTAATAAAACTATTCTTT	RhoGreen

5.2.7 HCR *in situ* staining

To localise the strains in the bacteriome of *P. truncatus,* hybridisation chain reaction (HCR) *in situ* staining was performed. The bacteriomes of *P. truncatus* were fixated in 4% PFA in 80% butanol for 24 hours immediately after collection. For pre-embedding, 1% aqueous agar was liquefied in the microwave and cooled down to approximately 40°C. With the solution, a layer of ager was placed on a slide into which the bacteriomes were transferred individually while the solution was still liquid. After approximately 5 minutes, the solidified agar was cut with a scalpel into little blocks, each containing one bacteriome, and transferred to 80% butanol.

Dehydration of the samples was performed in a series of butanol at concentrations of 90%, 96% and 3 x 100%. As an intermediate, 3 steps of 100% isopropanol were used. The samples remained

in the solutions for 2 hours each at room temperature, excluding the steps in 100% butanol, which were incubated at 30°C. This was followed by infiltration with ROTI®Plast paraffin wax with DMSO (Carl Roth, Karlsruhe, Germany) in two batches for 1 x 6 hours and 1 x overnight at 62°C, then the blocks were produced.

5 µm thick sections were made using a rotary microtome HistoCore AUTOCUT R (Leica, Nussloch, Germany) with disposable metal blades Feather R35 (PFM medical, Köln, Germany). Sections were briefly stretched in 40°C water, mounted on silanised slides, and dried overnight at 40°C. The dried sections were deparaffinized and rehydrated in a series of 2x xylene, 2x ethanol abs, ethanol 96%, 70% and water for 5 minutes each. Finally, the sections were post-fixated in 4% neutral buffered aqueous formaldehyde solution for 20 minutes, followed by washing in water for 5 minutes and drying at 40°C for 1 hour.

Hybridisation chain reaction (HCR) *in situ* staining (Molecular Instruments, USA) was performed according to the manufacturer's instructions following the *HCR v3.0 FFPE human tissue sections* protocol with the following modifications (provided by Andrew Gillis, Olivia Tidswell and Tobias Engl) on isolated bacteriome of *P. truncatus*: 5% dextran sulphate (instead of 10%) in both the 30% probe hybridisation buffer and amplification buffer. Before pre-hybridisation, the section was washed in 0.2 M HCl for 10 minutes at room temperature, afterwards washed in 1x PBS and incubated with Proteinase K (0.1 mg/mL in TE buffer) for 5 minutes at room temperature and then washed twice in probe hybridisation buffer. Also, after incubation overnight, the slides were heated at 75°C for 25 minutes to open the DNA strands and further maximize probe attachment and then cooled down to 37°C. HCR probe sets for each target gene with specific amplifier sequences were designed and synthesized by Molecular Instruments (Los Angeles, CA, USA; Table 3). To reduce autofluorescence of the tissue, the Vector® TrueVIEW® Autofluorescence Quenching Kit (Vector Laboratories, Burlingame, CA, USA) was used following the manufacturer's instructions. Sections were mounted with Invitrogen™ ProLong™ Gold with DAPI (Fisher Scientific, Schwerte, Germany) and observed under a Zeiss AxioImager Z2 with Apotome.2 (Zeiss, Jena, Germany) illuminated by a SOLA Light Engine (Lumencor, Beaverton, OR, USA).

Table 3: Used HCR probes for HCR *in situ* staining. Complementary probe pairs (25 bp + linker) from each sequence were designed by Molecular Instrument.

Target	Number of probes	Amplifier	Marker
aroA	17	B3	Atto647
aroC	19	B1	Atto488
aroFb	11	B2	Atto546
rProteins strain A	15	B1	Atto488
rProteins strain B	14	B3	Atto647
rProteins strain C	20	B2	Atto546

5.3 Data Accessibility Statement

Raw sequence libraries of *Prostephanus truncatus* were uploaded to the NCBI Sequence Read Archive (SRR19638530 and SRR19638531; BioProject PRJNA848820). The annotated genomes are available on GenBank (CP104094 - CP104096).

5.4 Acknowledgments

We thank Benjamin Weiss for technical assistance in histology, Eugen Bauer for genomic support, and Cornel Adler for the original provisioning of the beetle cultures. We thank Bruno Hüttel and the Max Planck-Genome-Centre Cologne (http://mpgc.mpipz.mpg.de/home/) for performing library preparation and sequencing, Olivia Tidswell for her introduction to the HCR, the Johannes Gutenberg-University Mainz for computation time granted on the supercomputer 'MOGON', and Christian Meesters for administrative assistance on 'MOGON'. Additionally, we would like to thank Thomas Hörren for kindly sharing pictures for Figure 5. We gratefully acknowledge financial support from the Max Planck Society (to T.E. and M.K.) and the European Research Council through a Consolidator Grant to M.K. (ERC CoG 819585 "SYMBeetle").

5.5 Contributions

J.S.T.K. and T.E. designed the project, and J.S.T.K., E.B., and T.E. sequenced and assembled the symbiont genome. J.S.T.K. and E.B. annotated the genomes and performed symbiont genomic analysis. J.S.T.K. and T.E. wrote the paper, with input from M.K.

5.6 References

1. McCutcheon, J. P. & Moran, N. A. Extreme genome reduction in symbiotic bacteria. *Nat. Rev. Microbiol.* **10**, 13–26 (2012).
2. Moran, N. A. & Bennett, G. M. The tiniest tiny genomes. *Annu. Rev. Microbiol.* **68**, 195–215 (2014).
3. McCutcheon, J. P. & Moran, N. A. Functional convergence in reduced genomes of bacterial symbionts spanning 200 My of Evolution. *Genome Biol. Evol.* **2**, 708–718 (2010).
4. Conord, C. *et al.* Long-term evolutionary stability of bacterial endosymbiosis in Curculionoidea: additional evidence of symbiont replacement in the Dryophthoridae family. *Mol. Biol. Evol.* (2008).
5. Lynch, M. & Conery, J. S. The Origins of Genome Complexity. *Science (80-.).* **302**, 1401–1404 (2003).
6. Andersson, S. G. e. & Kurland, C. G. Reductive evolution of resident genomes. *Trends Microbiol.* **6**, 263–268 (1998).
7. Moran, N. A. Accelerated evolution and Muller's rachet in endosymbiotic bacteria. *Proc. Natl. Acad. Sci. U. S. A.* **93**, 2873–2878 (1996).
8. Takiya, D. M., Tran, P. L., Dietrich, C. H. & Moran, N. A. Co-cladogenesis spanning three phyla: leafhoppers (Insecta: Hemiptera: Cicadellidae) and their dual bacterial symbionts. *Mol. Ecol.* **15**, 4175–4191 (2006).
9. Bennett, G. M. & Moran, N. A. Small, Smaller, Smallest: The origins and evolution of ancient dual symbioses in a phloem-feeding insect. *Genome Biol. Evol.* **5**, 1675 (2013).
10. Engl, T. *et al.* Ancient symbiosis confers desiccation resistance to stored grain pest beetles. *Mol. Ecol.* **27**, 2095–2108 (2018).
11. Okude, G. *et al.* Novel bacteriocyte-associated pleomorphic symbiont of the grain pest beetle *Rhyzopertha dominica* (Coleoptera: Bostrichidae). *Zool. Lett.* **3**, 13 (2017).
12. Huger, A. Experimentelle Eliminierung der Symbionten aus den Myzetomen des Getreidekapuziners, *Rhizopertha dominica* F. *Naturwissenschaften* **41**, 170–171 (1954).
13. Huger, A. Experimentelle Untersuchungen über die künstliche Symbiontenelimination bei Vorratsschädlingen: *Rhizopertha dominica* F. (Bostrychidae) und *Oryzaephilus suinamensis* L. (Cucujidae). *Zeitschrift für Morphol. und Ökologie der Tiere* **44**, 626–701 (1956).
14. Dopheide, T. A. A., Crewther, P. & Davidson, B. E. Chorismate mutase-prephenate dehydratase from *Escherichia coli* K-12. *J. Biol. Chem.* **247**, 4447–4452 (1972).
15. Seemann, T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* **30**, 2068–2069 (2014).
16. Sloan, D. B. *et al.* Parallel histories of horizontal gene transfer facilitated extreme reduction of endosymbiont genomes in sap-feeding insects. *Mol. Biol. Evol.* **31**, 857–871 (2014).
17. Anbutsu, H. *et al.* Small genome symbiont underlies cuticle hardness in beetles. *Proc. Natl. Acad. Sci.* **114**, E8382–E8391 (2017).
18. Łukasik, P. *et al.* Multiple origins of interdependent endosymbiotic complexes in a genus of cicadas. *Proc. Natl. Acad. Sci.* **115**, E226–E235 (2018).
19. Errington, J. L-form bacteria, cell walls and the origins of life. *Open Biol.* **3**, (2013).
20. Leaver, M., Domínguez-Cuevas, P., Coxhead, J. M., Daniel, R. A. & Errington, J. Life without a wall or division machine in Bacillus subtilis. **457**, (2009).
21. Hirota, B. *et al.* A novel, extremely elongated, and endocellular bacterial symbiont supports cuticle formation of a grain pest beetle. *MBio* **8**, 1–16 (2017).
22. Komaki, K. & Ishikawa, H. Intracellular bacterial symbionts of aphids possess many genomic copies per bacterium. *J. Mol. Evol.* **48**, 717–722 (1999).
23. Komaki, K. & Ishikawa, H. Genomic copy number of intracellular bacterial symbionts of aphids varies in response to developmental stage and morph of their host. *Insect Biochem. Mol. Biol.* **30**, 253–258 (2000).
24. Woyke, T. *et al.* One bacterial cell, one complete genome. *PLoS One* **5**, (2010).
25. Van Leuven, J. T., Meister, R. C., Simon, C. & McCutcheon, J. P. Sympatric speciation in a bacterial endosymbiont results in two genomes with the functionality of one. *Cell* **158**, 1270–1280 (2014).
26. Shires, S. W. Life history of *Prostephanus truncatus* (Horn) (Coleoptera: Bostrichidae) at optimum conditions of temperature and humidity. *J. Stored Prod. Res.* **16**, 147–150 (1980).
27. Shires, S. W. Influence of temperature and humidity on survival, development period and adult sex ratio in Prostephanus truncatus (Horn) (Coleoptera, Bostrichidae). *J. Stored Prod. Res.* **15**, 5–10 (1979).
28. Bolger, A. M., Lohse, M. & Usadel, B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **30**, 2114–2120 (2014).
29. Bankevich, A. *et al.* SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* **19**, 455–477 (2015).

30. Laczny, C. C. *et al.* BusyBee Web: metagenomic data analysis by bootstrapped supervised binning and annotation. *Nucleic Acids Res.* **45**, W171–W179 (2017).
31. Arkin, A. P. *et al.* KBase: The United States department of energy systems biology knowledgebase. *Nat. Biotechnol.* **36**, 566 (2018).
32. Krzywinski, M. *et al.* Circos: an information aesthetic for comparative genomics. *Genome Res.* **19**, 1639–1645 (2009).
33. Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* **173**, 697–703 (1991).
34. Quast, C. *et al.* The SILVA ribosomal RNA gene database project: imporved data processing and web-based tools. *Nucleic Acids Res.* **41**, D590-6 (2013).
35. Yilmaz, P. *et al.* The SILVA and "All-species Living Tree Project (LTP)" taxonomic frameworks. *Nucleic Acids Res.* **42**, D643 (2014).
36. Katoh, K. & Standley, D. M. MAFFT Multiple Sequence Alignment Software version 7: Improvements in Performance and Usability. *Mol. Biol. Evol.* **30**, 772–780 (2013).
37. Huelsenbeck, J. P. & Ronquist, F. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinforma. Appl. NOTE* **17**, 754–755 (2001).
38. Abadi, S., Azouri, D., Pupko, T. & Mayrose, I. Model selection may not be a mandatory step for phylogeny reconstruction. *Nat. Commun. 2019 101* **10**, 1–11 (2019).
39. Ronquist, F. & Huelsenbeck, J. P. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**, 1572–1574 (2003).
40. Huelsenbeck, J. P., Ronquist, F., Nielsen, R. & Bollback, J. P. Bayesian inference of phylogeny and its impact on evolutionary biology. *Science (80-.).* **294**, 2310–2314 (2001).
41. Li, L., Stoeckert, C. J. & Roos, D. S. OrthoMCL: identification of ortholog groups for eukaryotic genomes. *Genome Res.* **13**, 2178–2189 (2003).
42. Kanehisa, M., Sato, Y. & Morishima, K. BlastKOALA and GhostKOALA: KEGG tools for functional characterization of genome and metagenome sequences. **428**, 726–731 (2016).
43. Campbell, M. A. *et al.* Genome expansion via lineage splitting and genome reduction in the cicada endosymbiont *Hodgkinia*. *Proc. Natl. Acad. Sci.* **112**, 10192–10199 (2015).
44. Campbell, M. A. *et al.* Changes in endosymbiont complexity drive host-level compensatory adaptations in cicadas. *mBio* **9**, 1-12 (2018).
45. Weiss, B. & Kaltenpoth, M. Bacteriome-localised intracellular symbionts in pollen-feeding beetles of the genus *Dasytes* (Coleoptera, Dasytidae). *Front. Microbiol.* **7**, 1486 (2016).

Chapter 6

General Discussion

In this thesis, I demonstrate that beetles of the two families Silvanidae and Bostrichidae are associated with multiple bacterial symbionts. The sawtoothed grain beetle *Oryzaephilus surinamensis* (Silvanidae) harbours the Bacteroidota endosymbiont *Shikimatogenerans silvanidophilus* OSUR (Chapter 2) and the α-proteobacteria *Wolbachia* strain *w*Sur (Chapter 3). In the Bostrichidae family, the Bacteroidota endosymbiont *Shikimatogenerans bostrichidophilus* is present in almost all examined species. In addition, species of the genera *Dinoderus* and *Lyctus* harbour the co-obligate symbiont *Bostrichicola ureolyticus* of the Bacteroidota phylum (Chapter 4). Furthermore, in the large grain borer *Prostephanus truncatus* the endosymbiont diverged into three, metabolically partially complementary strains (Chapter 5). The *Shikimatogenerans* endosymbionts supplement the host's cuticle biosynthesis by the shikimate pathway, while *Bostrichicola* is capable of recycling nitrogen and supplementing their host's diet with essential amino acids.

6.1 Ecology of symbioses in *Oryzaephilus surinamensis*

The genome of the Bacteroidota endosymbiont of *O. surinamensis*, *S. silvanidophilus*, is highly eroded, encoding only genes of the shikimate pathway and glycolysis apart from gene families involved in the core processes of replication, transcription, and translation. The genome also lacks any genes for peptidoglycan biosynthesis, indicating that the symbiont can no longer synthesize its own cell wall. This raises the question of how the host controls its symbiont. In Chapter 2, the symbiotic relationship between host and symbiont was experimentally manipulated by inhibition of the shikimate pathway of *S. silvanidophilus* via glyphosate, and by adding tyrosine to the food source of the host. The outcome of these experiments demonstrates that *Shikimatogenerans* provisions tyrosine which also impacts the cuticle development of the host. In addition, it indicates that it is the production of the tyrosine precursors chorismate/prephenate via the shikimate pathway by which the host measures the symbionts' contribution. Further, this

indicates that the symbiont's contribution is at least partially regulated on the host level via symbiont titer and not by gene expression, as most symbionts can presumably no longer control the expression level of their genes[1,2]. This control mechanism on the host level can be observed when the host has direct access to tyrosine via a food source, which led to a drastic reduction of the symbiont population (Chapter 2). From this finding, it can be concluded that it is the availability of tyrosine by which the host senses and controls the symbiont population. This hypothesis is supported by our own experience in the laboratory, where the symbiont titer increases when the host is maintained on ground oat that has been "diluted" by 50% with starch (data not published). Thus, the host can plastically adjust the symbiotic contribution to its needs via the regulation of the symbiont titer[3]. Similar results on diet-dependent symbiont regulation were also reported in the symbiosis between *Wigglesworthia* and its host the tsetse fly *Glossina morsitans*. *Wigglesworthia* provides B vitamins to the host, one of which is thiamine (vitamin B_1)[4]. When the tsetse flies have access to thiamine from the food source, this resulted in reduced *Wigglesworthia* density in both male and female flies[5]. Both results - amino acids influencing *S. silvanidophilus* titer in *O. surinamensis* and thiamine influencing density of *Wigglesworthia* in *G. morsitans* - are examples of how supplementation of the host's diet with nutrients derived from the symbiont affects the symbiont population negatively. Furthermore, the bacteriomes with the endosymbionts are slowly degraded in male adults of *O. surinamensis* after metamorphosis[6]. A similar yet drastically faster process was reported in the rice weevil *Sitophilus oryzae*, which is recycling its bacteriomes harbouring their endosymbiont *Sodalis pierantonius* in adults of both genders and only retains a separate, ovary associated symbiont pool for transmission[7]. In both systems, the benefit provisioned by the symbionts is over and by recycling the endosymbionts and their structures, the host can recoup some of the nutrients invested. Only in female adults, the symbiont is preserved at a constant level to ensure their transmission to next generations[6,7].

In addition to *S. silvanidophilus,* the silvanid *O. surinamensis* is host to *Wolbachia*, which is known as a reproductive manipulator in many insects[8,9]. In contrast to the Bacteroidota endosymbiont, the occurrence of the *Wolbachia* strain *w*Sur is not restricted to the bacteriome but is localised throughout the entire body of the host. In Chapter 3, I demonstrate that *w*Sur is indeed manipulating the reproduction of its host by inducing cytoplasmic incompatibility (CI), which leads to infertile embryos in *w*Sur uninfected females that mate with *w*Sur infected males[10]. In

consequence, the *Wolbachia* strain increases in frequency in the populations, which is reflected by its high prevalence in lab conditions. From our observations and experiments, there is no direct evidence that *w*Sur also has a mutualistic effect on its host. Interestingly, the genome of *w*Sur encodes for similar pathways to synthetic amino acids as *Bostrichicola* in the bostrichids, e.g. lysine, serine, and glutamine. It is possible that *w*Sur complements the host's diets with those amino acids in addition to its reproductive manipulation, as it is reported for vitamin supplementation in other systems[11,12]. To clarify whether *Wolbachia* also has a mutualistic effect in *O. surinamensis*, experiments would have to be carried out with beetles that lack *S. silvanidophilus* but are infected with *w*Sur. As I demonstrate in Chapter 2, it is possible to eliminate the Bacteroidota endosymbiont with glyphosate to get an *O. surinamensis* population with is associated with *w*Sur alone, which could be used for experiments to elucidate a potential mutualistic effect. Also, the same experiments should be carried out with beetles that lack *w*Sur but are infected with *Shikimatogenerans*, since the Bacteroidota endosymbiont is always present in the beetles in nature and only differ in whether they are infected with *Wolbachia* in addition.

6.2 Symbionts within Bostrichidae

Beetles of the Bostrichidae family are associated with up to two Bacteroidota endosymbionts: *Shikimatogenerans bostrichidophilus* and *Bostrichicola ureolyticus*. Both endosymbionts are characterized by heavily eroded and A+T-biased genomes with extremely limited biosynthetic capabilities. *S. bostrichidophilus* and *B. ureolyticus* exhibit extremely reduced genomes. Apart from genes involved in replication, transcription, and translation, the genome of *S. bostrichidophilus* encodes exclusively genes for the shikimate pathway.

The symbionts are localised in the cytoplasm of the bacteriocytes. As the genomes of *S. bostrichidophilus* lack genes for the synthesis of peptidoglycan, the symbiont cells show amorphous morphology, known as the L-form. Also, studies on other bacteriome-localised endosymbionts found that the symbionts are often polyploid, i.e. there are multiple copies of the genome per cell. In terms of genome size, the genome of *Shikimatogenerans* in the Dinoderinae *Rhyzopertha dominica* (173 kbp) and *Prostephanus truncatus* (avg. 156 kbp) are the most reduced. This, combined with the previously mentioned regulation of the symbiont population on the host level, could be the presupposition necessary to favour the split of the symbiont into different

linages in *P. truncatus* (Chapter 5). In *R. dominica* it can already be observed that there are no longer individual cells of the symbiont in the bacteriome, but rather several symbiont cells that are fused or no longer divided. Okude et al. (2017)[13] appropriately described this as a grape-like pattern. From this point, it seems quite possible that in such a continuum of cells with multiple genomes some of them link together. The genomes of the three PTRU linages can either be mixed in the same symbiont cell, or they can be segregated into different cells. In the latter case, the symbiont strains must exchange either mRNAs, proteins/enzymes, and/or entire ribosomes and metabolites in order to be functional. After metamorphosis, only female beetles keep the bacteriomes to pass the symbionts on to the next generation[6,7,79]. At this point, there is probably no host-level selection on functionality single symbiont genomes in the adult stage. Although, it is also possible that females need the tyrosine supply as adults, while males do not. Quality check on the host level only takes place again in the next generation, when the beetle receives tyrosine precursors from the symbiont. As long as the symbiont or the symbionts in their continuum remain entirety functional and have a complete shikimate pathway to provide tyrosine precursors to the host, there is no immanent reason for host selection or even recognition of ongoing changes.

A similar scenario - but even more complex - was observed in cicadas of the genus *Tettigades* (Hemiptera: Cicadidae) and their α-proteobacterial endosymbiont *Hodgkinia cicadicola*[14–16]. Also in this system, the question of how the linages evolved could not (yet) be finally clarified. It was shown, that to still ensure that each lineage is transferred to the next generation, these cicadas have increased the number of *Hodgkinia* cells six-fold[17], whereas the number of *Sulcia muelleri* cells - the second endosymbiont - is unaltered. We have no data on the number of symbiont cells transmitted via egg to the next generation in *P. truncatus*, but that should be feasible with *R. dominica* and its endosymbiont *S. bostrichidophilus* RDOM as a comparison. Unfortunately, it is not possible to eliminate only a single PTRU lineage without also eliminating the other two as they are potentially treated and seen as one by the host.

Only beetles of the genera *Lyctus* and *Dinoderus* harbour the second co-obligate endosymbiont *Bostrichicola ureolyticus*. The genome of *Bostrichicola* encodes genes to recycle nitrogen, as well as a glutamate dehydrogenase that allows integration of the resulting ammonium into the amino acid metabolism via glutamate. In addition, the genome encodes for an aspartate aminotransferase to

transfer the amino group from glutamate to oxaloacetate. As aminotransferase genes are known to be promiscuous, this specific encoded gene could also take over another aminotransferase activity[18]. The genome also encodes for an almost complete diaminopimelate pathway to synthesize the essential amino acid lysine from aspartate. Lysine is an important component of cuticular proteins as its ε-amino group represents an anchor point for cross-linking[19]. Grain diets are specifically limited in lysine[20], which could be relevant for the stored product pest beetles of the genus *Dinoderus*, but also other species of the Dinoderinae subfamily. It also retained a methionine synthase to convert L-homoserine to L-methionine and is able to synthesise menaquinone. In addition, the genome encodes for a complete fatty acid and peptidoglycan biosynthesis, albeit other cell wall components apparently cannot be synthesized.

S. bostrichidophilus was already integrated into the bostrichid beetles of the genera *Lyctus* and *Dinoderus* when the ancestor of *B. ureolyticus* was taken up at a later time point. The metabolic potential of *B. ureolyticus* and *Blattabacterium* derives from the same ancestor, which likely contained everything contained in the genome of all descendants since horizontal gene transfer is rare in intracellular symbionts. As *B. ureolyticus* lacks the shikimate pathway – and *S. bostrichidophilus* already fulfilled these needs, it is likely that it lost the genes for this pathway because of redundancy according to the black queen hypothesis[21,22]. As larvae accumulate a lot of uric acids, which are often deposited in the epidermis and cause the white colour of the larvae, *B. ureolyticus* likely plays a key role in nitrogen recycling during this period in the beetle's life cycle[23]. Unfortunately, there is no data yet if *B. ureolyticus* faces the same destiny in adult males or if only the bacteriome harbouring *S. bostrichidophilus* is degraded and consumed by the host while the bacteriome harbouring *B. ureolyticus* persists as the host is still in need urea recycling or lysine from the endosymbiont. This would explain why beetles of the genera *Lyctus* and *Dinoderus* are associated with two closely related symbionts and not with just one with the metabolic potential of both combined. It can be assumed that *B. ureolyticus* takes over the urea recycling that was previously encoded in the early genome of *Shikimatogenerans*. However, by also encoding new genes for lysine synthesis and other individual enzymatic steps, *B. ureolyticus* could offer additional benefits. Currently, no other insect is known to be associated with two symbionts, of which one symbiont only supports cuticle synthesis, while the second symbiont recycles nitrogen and provides amino acids. In addition, the relationship between both *Shikimatogenerans* and

Bostrichicola remains unsolved. From the genomic point of view, both *B. ureolyticus* and *S. bostrichidophilus* complement each other in the symbiosis with their host (Chapter 4).

Several insects are associated with two or more endosymbionts with complementary biosynthetic pathways to synthesize amino acids and/or vitamins[4,24–30] or pathways for the essential amino acids are split up between the two symbionts[27,31,32]. Symbionts can also be replaced when they are no longer needed or capable of sufficiently supporting their host's metabolism[33–35]. As the genome of an endosymbiont gets streamlined to fulfil the specific needs of the host, mutations and pseudogenes can accumulate, which can lead to reduced efficiency of the genome. The consequences are the rapid evolution of protein sequences and gene loss. This process is called Muller's ratchet[33,36]. It's solved by replacing the symbiont with the eroded genome with a new symbiont that brings in novel functions that the original symbiont did not provide and can therefore be selectively favoured, or the replacing symbiont fulfils the role of the original symbiont better or more efficiently. This could be the case e.g. if genome erosion has resulted in suboptimal codon composition and therefore stability of proteins (due to Muller's ratchet). Evidence for this is the usually very high expression of chaperones in intracellular symbionts, which assists in refolding malfunctional proteins and is usually interpreted as a compensatory adaptation[37]. Replacement of endosymbionts was shown in the Auchenorrhyncha, which are associated with the Bacteroidota endosymbiont *Sulcia muelleri* and a β-proteobacterium. This β-proteobacterium has been proposed to be the ancient co-obligate partner of *S. muelleri* and has been replaced multiple times by – or evolved as a common ancestor into *Zinderia insecticola* in the spittlebugs (Hemiptera: Auchenorrhyncha: Cercopoidea), *Nasuia deltocephalinicola* of deltocephaline leafhoppers, and *Vidania fulgoroideae* of fulgorid planthoppers[30,38]. In addition to the before-mentioned Auchenorrhyncha, this is also known for the di-symbiotic *Buchnera aphidicola* and *Serratia symbiotica* in aphids (Hemiptera: Aphididae)[28,32]. Interestingly, the bostrichids represent the first case, where two closely related bacteria of the same family are complementing each other. One reason could be that bostrichid beetles of the Dinoderinae and Lyctinae subfamily were already adapted to one Bacteroidota, *S. bostrichidophilus*.

Further research on the Dinoderinae *Dinoderus porcellus* should elucidate the relationship of this tripartite symbiosis. Given the results from the experiments in Chapter 2, it should be possible to remove *S. bostrichidophilus* from the beetle with a tyrosine-rich or glyphosate-containing diet and

thus eventually obtain a host population of *D. porcellus* that only harbours *B. ureolyticus* – as long as the two endosymbionts are not heavily dependent on each other. In addition, the functionality of the urease encoded in the genome of *B. ureolyticus* should be assessed. This would be possible with Isotope-labelled urea, which would make it possible to track whether and where it is metabolized[39,40].

It is worth mentioning that of the species-rich family of the Bostrichidae, only a couple of species are known as grain pests (e.g. *Rhyzopertha dominica* and *Dinoderus porcellus*) while the majority is still feeding on dead wood (e.g. *Lyctus brunneus* and *Dinoderus minutus*). Wood in general is a very poor diet for insects as due to the recalcitrant polymers in the (woody) cell walls (i.e. lignocellulose) it is difficult for the insect to utilize the carbon as energy sources and gain access to the more nutritious cell content[41]. To compensate for the nutritional deficiency, xylophagous (wood-feeding) beetles harbour gut bacteria that provide them with the missing essential amino acids, as well as recycle nitrogen[42,43]. The xylophagous bostrichids, e.g. the common powderpost beetle *Lyctus brunneus* harbour two endosymbionts that can synthesize a subset of amino acids as well as recycle nitrogen. While collecting samples for this work, it was observed that specimens of *Trogoxylon impressum* and *Lyctus cavicollis* were found on the same type of wood branches at the same time point. While both species belong to the subfamily of the Lyctinae, I could show that only *Lyctus* lives in a tripartite symbiosis with *Shikimatogenerans* and *Bostrichicola*, whereas *T. impressum* only harbours *Shikimatogenerans* (Chapter 4). While both species share the same habitat, harbouring a second symbiont with additional metabolic capacities also brings the opportunity to avoid intraspecific competition by switching from one food source to another. It is likely that *T. impressum* - lacking *Bostrichicola* and therefore a source for amino acids – is feeding on nutrient-rich phloem whereas *L. cavicollis* favours the nutrient-poor xylem and hard-wood. Experiments with *Lyctus africanus* – harbouring both *Shikimatogenerans* and *Bostrichicola* - showed, the beetle favoured starch and sugar as vital nutrients for oviposition[44]. The same can be hypothesized about the Dinoderinae subfamily, where *Rhyzopertha dominica* harbours *Shikimatogenerans* but species of the *Dinoderus* genus harbour *Bostrichicola* in addition. Still, as they are known as grain pests, they seem to feed on the same food source, whereas it is likely that *R. dominica* aims at the grain embryo, which is rich in nutrients, whereas *Dinoderus* sp. is satisfied with the surrounding nutrient-poor starch, which the *R. dominica* beetles dig out of the grain on the way for oviposition. Beetles of the genus *Dinoderus* are in general less specialised as some infest

cereals as well as carbohydrate-rich products, e.g. yam roots (*D. porcellus* and *D. bifoveolatus*), while others are bamboo pests (*D. minutus*). As *R. dominica* and *P. truncatus* are really specialised on grain where they feed on the nutrient-rich embryo, *Bostrichicola* could have been lost as a result.

Interestingly, Li et al. (2015) detected *Wolbachia* in both *R. dominica* and *P. truncatus*[45]. However, I have no data for *Wolbachia* in our cultures. Given the results from Chapter 3, it would make sense to also examine the existing metagenomes for *Wolbachia*. Since *w*Sur encodes for genes to synthesise essential amino acids in *O. surinamensis*, it seems possible that in cases where *B. ureolyticus* is not present, *Wolbachia* takes over this mutualistic part instead. At the moment, however, this is pure speculation.

6.3 Evolution of beetle-associated Flavobacteria

The insect-associated Flavobacteria constitute a single clade that is about 400 Mya old[46]. Since then, the bacteria have diverged, and the clade contains symbionts with manifold metabolic abilities. They developed from the probably most ancestral lifestyle of male-killing Flavobacteria, which form the sister group to this clade and still are reproductive manipulators in ladybird beetles (Coleoptera: Coccinellidae)[47] - of which the genome is yet not available. Presumably, originally reproductive manipulators, the genomes of *Blattabacterium* spp., *Walczuchella monophlebidarum*, *Uzinura diaspidicola*, probably *Brownia*, to *Sulcia muelleri* were able to spread within insects and co-evolve in as little as 100 Mya into nutritional mutualists[46], leading to a whole clade of Flavobacteria associated with several insect families.

While *Bostrichicola ureolyticus* still encodes genes for pathways to produce essential amino acids as well as urease for the recycling of nitrogen – similar to *Blattabacterium*, *Walczuchella,* and *Uzinura*[48-50] - *Shikimatogenerans bostrichidophilus* became an exclusive chorismate supplier. Evolving from a free-living bacterium to an 'enslaved' symbiont, the genome of *Shikimatogenerans* underwent a drastic reduction in a host-beneficial manner. The genome of *Shikimatogenerans* exclusively encodes genes for the shikimate pathway. Although the metabolic potential of *S. silvanidophilus* is already limited as the genome still encodes genes for glycolysis and potentially nitrogen recycling, the genome of *S. bostrichidophilus* is even more reduced exclusively encoding the shikimate pathway besides general cell processes - levelling *Shikimatogenerans* to a status comparable with the mitochondria in eukaryotic cells[51]. Mentioning mitochondria, it is noteworthy that in comparison to *Bostrichicola,* the genome of *S. bostrichidophilus* does not encode

any genes for glycolysis or any ATP synthetase genes. The same is true for the genomes of *Uzinura* and *Hodgkinia*[52] - to name some that have already been investigated previously. *S. silvanidophilus*, on the other hand, has genes for glycolysis and ATP synthesis. Further experiments will have to show whether glycolysis in *S. silvanidophilus* is functional and whether the symbiont itself determines it, or whether it is already being taken over by the host – which is more likely, as the glycolysis also needs products as input that already must be delivered by the host (Chapter 2). The complete absence of genes for glycolysis and ATP synthase in *S. bostrichidophilus* indicates a total dependency on energy production since the host's environment must supply some necessary metabolites directly to the symbionts.

Phylogenetic analyses showed that the three Bacteroidota endosymbionts examined in both beetle families are closely related. According to Engl et al. (2018), the common ancestors of the three Bacteroidota endosymbionts - *S. silvanidophilus*, *S. bostrichidophilus,* and *B. ureolyticus* -, *Blattabacterium,* and *S. muelleri* lived 409 Mya[46]. Subsequently, *Bostrichicola* and *Shikimatogenerans* diverged around 15 Mya later and another 63 Mya later *S. silvanidophilus* and *S. bostrichidophilus* diverged from each other. *S. silvanidophilus* combines the metabolic capacity of *Bostrichicola* to recycle nitrogen, and *S. bostrichidophilus* to supplement the tyrosine precursor prephenate - between which it also clusters phylogenetically. Following, it can be assumed that the ancestor of the three endosymbionts combined these abilities, and afterwards individual pathways were lost in each of the lineages.

6.4 Grain pest beetles as non-model model organisms

The example of *O. surinamensis* demonstrates that the investigation of insects associated with multiple symbionts are crucial for understanding the symbiont-influenced eco-evolutionary dynamics of their host. Sharing the same habitat, the Bacteroidota endosymbionts of the silvanid and bostrichid beetle grain pests got shaped accordingly to the specific needs of the host. The ancestor of these Bacteroidota endosymbionts had richer repertoire of pathways from which the host and its habitat could “select”. According to the symbiont metabolic capabilities, both beetle families are in demand for tyrosine. Likely, the ancestors of both the silvanid and the bostrichid beetles compared to their symbiont-free conspecifics had an immediate benefit because of the enhanced tyrosine supply after they acquired the ancestor of *Shikimatogenerans*. However, the

symbiotic beetles might have had to eat more because they now had to feed their costly symbionts as well[6,7]. In return, they had more tyrosine available, which allowed them to build up a cuticle that is harder and thicker - which means they were better protected from desiccation and predators[46,53]. And in the case of the bostrichids, this may even have led to mandibles that made it easier to penetrate harder materials such as hardwood.

Under the current climate crises, in which droughts are becoming more frequent due to global warming, a symbiosis with *Shikimatogenerans* or a similar symbiont would be beneficial for insects in nature, since *Shikimatogenerans* contribute via the shikimate pathway to cuticle biosynthesis, which leads to a thicker cuticle to protects the beetle from desiccation[46] and predators[53]. However, it was also shown in several studies that the Bacteroidota endosymbionts of the silvanid and bostrichid beetles are sensitive to heat[46,54-57,79]. To make matters worse, the same endosymbiont is sensitive to exposure to glyphosate and, as a result, the host suffers equally from exposure to glyphosate (Chapter 2). This was not only shown in beetles but also other insects including tsetse flies, bees, and ants[4,58–61,80]. Experiments with glyphosate show that this herbicide, as desired, indeed targets the shikimate pathway - which means that eukaryotic cells should not be the target, since they do not have that metabolic pathway[59,60,62]. However, it was ignored that many animals - including humans - are associated with bacteria[63,64]. Research in recent years has clearly shown that there is a connection between a person's microbiome and their health[65]. The same applies to the beetle and its endosymbiont - if the symbiosis is disturbed, both partners are affected. Also, when factors directly affect only one partner, it always affects the other in return. Especially when such a dependency has developed between the two that one is no longer able to live without the other. In the case of the silvanid and the bostrichid beetles and their Bacteroidota endosymbionts, this means that both partners would suffer under glyphosate treatments (*Shikimatogenerans*) as well as rising temperatures.

Glyphosate itself acts on a specific enzyme in the shikimate pathway: AroA[66,67]. There are two classes of this enzyme, namely class 1, which is sensitive to glyphosate, and class 2, which is non-sensitive to glyphosate. The phylogenetic study of the *aroA* sequences of different bacterial symbionts shows that they all encode *aroA* genes that belong to the glyphosate-sensitive class 1. This means that upon contact, glyphosate permanently binds to aroA and blocks its enzymatic ability, interrupting the entire metabolic pathway. In this work, I showed that beetles of the

family Silvanidae and Bostrichidae are associated with *Shikimatogenerans*, which uniquely contribute products of the shikimate pathway. Further, I also demonstrate that the *aroA* for this pathway encoded by the genomes of these symbionts belongs to the glyphosate-sensitive class I. With *O. surinamensis* I could verify this sensitivity experimentally - the *Shikimatogenerans* titer decreased drastically after glyphosate treatment.

Although it now sounds like glyphosate would be suitable to treat grain storage infested with grain pests such as *O. surinamensis* or *R. dominica*, it should be noted that we only work with pest species because they are easy to keep in the laboratory and lend themselves so well to simply having access to many specimens for experimentation and analysis. Moreover, it should be pointed out that in the species-rich Bostrichidae family, only a very small proportion are known as pests of crops (e.g. *Rhyzopertha dominica*) and wood (e.g. *Lyctus brunneus*), whereas this family members otherwise act beneficially with the ability to decompose hard-wood into fine drill dust which is then decomposed by microorganisms and fungi in the soil[74] or used for camouflage by insects such as the dust bug *Reduvius personatus*[76]. In addition, bostrichids also serve as a food source, above all for the checkered beetles (Coleoptera: Cleroidea) like *Tilloidea unifasciata*, *Tilloidea notata* or *Clerus mutillarius*[75,78], but also chalcid wasps (Hymenoptera: Chalcinidae) like *Cerocephala aquila* and braconid wasps (Hymenoptera: Braconidae) like *Platyspathius dinoderi*, as well as spiders such as the woodlouse hunting spider *Harpactea hombergi* and the jumping spider *Salticus zebraneus*[75]. But also, birds such as the red-backed shrike *Lanius collurio* and woodpeckers (Piciformes: Picidae) as well as mammals like the dormouse *Glis glis* sometimes feed on bostrichids[77]. In addition, the holes dug in the wood are used by other species for oviposition, e.g., the carrot wasp *Gasteruption erythrostomum*, which uses the boreholes of bostrichids to lay eggs[76]. This shows that the Bostrichidae are closely networked in their ecosystem and that a negative impact on the symbiosis via the symbiont by glyphosate not only has negative consequences for the host beetle but could also subsequently affect all other species.

However, besides these two highly specialized symbionts of the Silvanidae and Bostrichidae beetle families, many other insects are associated with Bacteroidota but also γ-proteobacteria symbionts, many of which encode a class I aroA enzyme. Thus, even if shikimate derivatives do not represent the major contribution of these symbionts, their inhibition could negatively impact symbiont growth and thereby host fitness. Whereas *O. surinamensis* could survive without its endosymbionts

when the environment supports its tyrosine-demanding lifestyle, it is not clear if the same can be said about other insects. In fact, it is known that many insects cannot survive without their obligate symbionts, for example, the black hard weevil *Pachyrhynchus infernalis* and its symbiont *Nardonella*[68], the thistle tortoise beetle *Cassida rubiginosa* and its symbiont *Stammera*[69] or the pea aphid *Acyrothosiphon pisum* and its obligate symbiont *Buchnera aphidicola*[70]. In addition to the current climate crisis, a decline in insect species abundance and diversity can also be observed[71,72]. Further investigations into insect symbioses could clarify the question of whether there is a connection between glyphosate and/or other agrochemicals and a decrease in the number and diversity of insects due to the disruption of their interactions with beneficial symbionts. This necessity is exacerbated by the fact that many of these active ingredients have also been detected in nature reserves – places that have been placed to protect animals and plants[73].

6.5 Conclusion

In my thesis, I demonstrate the importance of the Bacteroidota endosymbiont *Shikimatogenerans* associated with the Bostrichidae and Silvanidae beetle families for the cuticle biosynthesis of the host. Considering that the order Coleoptera constitutes the largest insect order, the influence of symbioses in beetles is still understudied. My work shows the influence that symbionts can have on the ecology and evolution of their hosts. In addition, I show how widespread this clade of Bacteroidota symbionts is in insects, where both convergent symbioses with different partners and divergent functions of closely related symbionts in different hosts can be found.

6.6 References

1. Hansen, A. K. & Moran, N. A. Aphid genome expression reveals host-symbiont cooperation in the production of amino acids. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 2849–2854 (2011).
2. Smith, T. E. & Moran, N. A. Coordination of host and symbiont gene expression reveals a metabolic tug-of-war between aphids and *Buchnera*. *Proc. Natl. Acad. Sci. U. S. A.* **117**, 2113–2121 (2020).
3. Whittle, M., Barreaux, A. M. G., Bonsall, M. B., Ponton, F. & English, S. Insect-host control of obligate, intracellular symbiont density. (2021).
4. Snyder, A. K. & Rio, R. V. M. "*Wigglesworthia morsitans*" folate (Vitamin B_9) biosynthesis contributes to tsetse host fitness. *Appl. Environ. Microbiol.* **81**, 5375–5386 (2015).
5. Snyder, A. K., McLain, C. & Rio, R. V. M. The tsetse fly obligate mutualist *Wigglesworthia morsitans* alters gene expression and population density via exogenous nutrient provisioning. *Appl. Environ. Microbiol.* **78**, 7792–7797 (2012).
6. Engl, T., Schmidt, T. H. P., Kanyile, S. N. & Klebsch, D. Metabolic cost of a nutritional symbiont manifests in delayed reproduction in a grain pest beetle. *Insects* **11**, 717 (2020).
7. Vigneron, A. *et al.* Insects Recycle Endosymbionts when the Benefit Is Over. *Curr. Biol.* **24**, 2267–2273 (2014).
8. Werren, J. H., Baldo, L. & Clark, M. E. Wolbachia: master manipulators of invertebrate biology. *Nat. Rev. Microbiol.* **6**, 741–751 (2008).
9. Landmann, F. The *Wolbachia* endosymbionts. *Microbiol. Spectr.* **7**, (2019).
10. Bordenstein, S. R. & Werren, J. H. Bidirectional incompatibility among divergent *Wolbachia* and incompatibility level differences among closely related *Wolbachia* in *Nasonia*. *Heredity (Edinb).* **99**, 278–287 (2007).
11. Ju, J. F. *et al. Wolbachia* supplement biotin and riboflavin to enhance reproduction in planthoppers. *ISME J. 2019 143* **14**, 676–687 (2019).
12. Hosokawa, T., Koga, R., Kikuchi, Y., Meng, X.-Y. & Fukatsu, T. *Wolbachia* as a bacteriocyte-associated nutritional mutualist. *Proc. Natl. Acad. Sci.* **107**, 769–774 (2010).
13. Okude, G. *et al.* Novel bacteriocyte-associated pleomorphic symbiont of the grain pest beetle *Rhyzopertha dominica* (Coleoptera: Bostrichidae). *Zool. Lett.* **3**, 13 (2017).
14. Łukasik, P. *et al.* Multiple origins of interdependent endosymbiotic complexes in a genus of cicadas. *Proc. Natl. Acad. Sci.* **115**, E226–E235 (2018).
15. Van Leuven, J. T., Meister, R. C., Simon, C. & McCutcheon, J. P. Sympatric speciation in a bacterial endosymbiont results in two genomes with the functionality of one. *Cell* **158**, 1270–1280 (2014).
16. Campbell, M. A. *et al.* Genome expansion via lineage splitting and genome reduction in the cicada endosymbiont *Hodgkinia*. *Proc. Natl. Acad. Sci.* **112**, 10192–10199 (2015).
17. Campbell, M. A. *et al.* Changes in endosymbiont complexity drive host-level compensatory adaptations in cicadas. *mBio* **9**, 1-12 (2018).
18. Lal, P. B., Schneider, B. L., Vu, K. & Reitzer, L. The redundant aminotransferases in lysine and arginine synthesis and the extent of aminotransferase redundancy in *Escherichia coli*. *Mol. Microbiol.* **94**, 843–856 (2014).
19. Suderman, R. J., Dittmer, N. T., Kramer, K. J. & Kanost, M. R. Model reactions for insect cuticle sclerotization: participation of amino groups in the cross-linking of *Manduca sexta* cuticle protein MsCP36. *Insect Biochem. Mol. Biol.* **40**, 252–258 (2010).
20. Torbatinejad, N. M., Rutherfurd, S. M. & Moughan, P. J. Total and reactive lysine contents in selected cereal-based food products. *J. Agric. Food Chem.* **53**, 4454–4458 (2005).
21. Mas, A., Jamshidi, S., Lagadeuc, Y., Eveillard, D. & Vandenkoornhuyse, P. Beyond the black queen hypothesis. *ISME J. 2016 109* **10**, 2085–2091 (2016).
22. Morris, J. J., Lenski, R. E. & Zinser, E. R. The black queen hypothesis: evolution of dependencies through adaptive gene loss. *MBio* **3**, (2012).
23. Buckner, J. S. & Newman, S. M. Uric acid storage in the epidermal cells of *Manduca sexta*: localisation and movement during the larval-pupal transformation. *J. Insect Physiol.* **36**, 219–229 (1990).
24. Takiya, D. M., Tran, P. L., Dietrich, C. H. & Moran, N. A. Co-cladogenesis spanning three phyla: leafhoppers (Insecta: Hemiptera: Cicadellidae) and their dual bacterial symbionts. *Mol. Ecol.* **15**, 4175–4191 (2006).
25. McCutcheon, J. P., McDonald, B. R. & Moran, N. A. Convergent evolution of metabolic roles in bacterial co-symbionts of insects. *Proc. Natl. Acad. Sci.* **106**, 15394–15399 (2009).
26. Wu, D. *et al.* Metabolic Complementarity and genomics of the dual bacterial symbiosis of sharpshooters. **4**, e188 (2006).

27. McCutcheon, J. P. & Von Dohlen, C. D. An interdependent metabolic patchwork in the nested symbiosis of mealybugs. **21**, 1366–1372 (2011).
28. Manzano-Marín, A. *et al.* A freeloader? the highly eroded yet large genome of the serratia symbiotica symbiont of cinara strobi. *Genome Biol. Evol.* **10**, 2178–2189 (2018).
29. McCutcheon, J. P. & Moran, N. A. Functional convergence in reduced genomes of bacterial symbionts spanning 200 My of evolution. *Genome Biol. Evol.* **2**, 708–718 (2010).
30. Bennett, G. M. & Moran, N. A. Small, smaller, smallest: the origins and evolution of ancient dual symbioses in a phloem-feeding insect. *Genome Biol. Evol.* **5**, 1675 (2013).
31. Sloan, D. B. & Moran, N. A. The evolution of genomic instability in the obligate endosymbionts of whiteflies. *Genome Biol. Evol.* **5**, 783–793 (2013).
32. Gosalbes, M. J., Lamelas, A., Moya, A. & Latorre, A. The striking case of tryptophan provision in the cedar aphid *Cinara cedri*. *J. Bacteriol.* **190**, 6026 (2008).
33. Bennett, G. M. & Moran, N. A. Heritable symbiosis: the advantages and perils of an evolutionary rabbit hole. *Proc. Natl. Acad. Sci.* **112**, 10169–10176 (2015).
34. Sudakaran, S., Kost, C. & Kaltenpoth, M. Symbiont acquisition and replacement as a source of ecological innovation. *Trends Microbiol.* **25**, 375–390 (2017).
35. Matsuura, Y. *et al.* Evolution of symbiotic organs and endosymbionts in lygaeid stinkbugs. *ISME J. 2012 62* **6**, 397–409 (2011).
36. Moran, N. A. Accelerated evolution and Muller's rachet in endosymbiotic bacteria. *Proc. Natl. Acad. Sci. U. S. A.* **93**, 2873–2878 (1996).
37. McCutcheon, J. P. & Moran, N. A. Extreme genome reduction in symbiotic bacteria. *Nat. Rev. Microbiol.* **10**, 13–26 (2012).
38. Koga, R., Bennett, G. M., Cryan, J. R. & Moran, N. A. Evolutionary replacement of obligate symbionts in an ancient and diverse insect lineage. *Environ. Microbiol.* **15**, 2073–2081 (2013).
39. Stoll, S., Feldhaar, H. & Gross, R. Transcriptional profiling of the endosymbiont *Blochmannia floridanus* during different developmental stages of its holometabolous ant host. *Environ. Microbiol.* **11**, 877–888 (2009).
40. Feldhaar, H. *et al.* Nutritional upgrading for omnivorous carpenter ants by the endosymbiont Blochmannia. *BMC Biol.* **5**, 1–11 (2007).
41. Mason, C. J., Scully, E. D., Geib, S. M. & Hoover, K. Contrasting diets reveal metabolic plasticity in the tree-killing beetle, *Anoplophora glabripennis* (Cerambycidae: Lamiinae). *Sci. Rep.* **6**, 33813 (2016).
42. Ayayee, P. *et al.* Gut microbes contribute to nitrogen provisioning in a wood-feeding Cerambycid. *Environ. Entomol.* **43**, 903–912 (2014).
43. Scully, E. D. *et al.* Functional genomics and microbiome profiling of the Asian longhorned beetle (*Anoplophora glabripennis*) reveal insights into the digestive physiology and nutritional ecology of wood feeding beetles. *BMC Genomics* **15**, 1096 (2014).
44. Kartika, T. & Yoshimura, T. Nutritional quality of diet and fecundity in *Lyctus africanus* (Lesne). *Procedia Environ. Sci.* **17**, 97–104 (2013).
45. Li, Y.-Y., Fields, P. G., Pang, B.-P., Coghlin, P. C. & Floate, K. D. Prevalence and diversity of *Wolbachia* bacteria infecting insect pests of stored products. *J. Stored Prod. Res.* **62**, 93–100 (2015).
46. Engl, T. *et al.* Ancient symbiosis confers desiccation resistance to stored grain pest beetles. *Mol. Ecol.* **27**, 2095–2108 (2018).
47. Hurst, G. D. D. *et al.* Male-killing bacterium in a fifth ladybird beetle, *Coleomegilla maculata* (Coleoptera: Coccinellidae). *Hered. 1996 772* **77**, 177–185 (1996).
48. Sabree, Z. L. *et al.* Genome shrinkage and loss of nutrient-providing potential in the obligate symbiont of the primitive termite *Mastotermes darwiniensis*. *Appl. Environ. Microbiol.* (2012).
49. Sabree, Z. L., Kambhampati, S. & Moran, N. A. Nitrogen recycling and nutritional provisioning by *Blattabacterium*, the cockroach endosymbiont. *Proc. Natl. Acad. Sci.* **106**, 19521–19526 (2009).
50. Ló Pez-Sánchez, M. J., Neef, A., Peretó, J., Patiñ O-Navarrete, R. & Pignatelli, M. Evolutionary convergence and nitrogen metabolism in *Blattabacterium* strain Bge, primary endosymbiont of the cockroach *Blattella germanica*. *PLoS Genet* **5**, 1000721 (2009).
51. McCutcheon, J. P., Boyd, B. M. & Dale, C. The life of an insect endosymbiont from the cradle to the grave. *Curr. Biol.* **29**, R485–R495 (2019).
52. Moran, N. A. & Bennett, G. M. The tiniest tiny genomes. *Annu. Rev. Microbiol.* **68**, 195–215 (2014).
53. Kanyile, S. N., Engl, T. & Kaltenpoth, M. Nutritional symbionts enhance structural defence against predation and fungal infection in a grain pest beetle. *J. Exp. Biol.* **225**, (2022).
54. Huger, A. Experimentelle Untersuchungen über die künstliche Symbiontenelimination bei

Vorratsschädlingen: *Rhizopertha dominica* F. (Bostrychidae) und *Oryzaephilus suinamensis* L. (Cucujidae). *Zeitschrift für Morphol. und Ökologie der Tiere* **44**, 626–701 (1956).
55. Huger, A. Experimentelle Eliminierung der Symbionten aus den Myzetomen des Getreidekapuziners, *Rhizopertha dominica* F. *Naturwissenschaften* **41**, 170–171 (1954).
56. Koch, A. Symbiosestudien. II. Experimentelle Untersuchungen an *Oryzaephilus surinamensis* L.- (Cucujidea, Coleopt.). *Zeitschrift für Morphol. und Ökologie der Tiere* **32**, 137–180 (1936).
57. Shires, S. W. Influence of temperature and humidity on survival, development period and adult sex ratio in *Prostephanus truncatus* (Horn) (Coleoptera, Bostrichidae). *J. Stored Prod. Res.* **15**, 5–10 (1979).
58. Motta, E. V. S., Raymann, K. & Moran, N. A. Glyphosate perturbs the gut microbiota of honey bees. *Proc. Natl. Acad. Sci.* **115**, 10305–10310 (2018).
59. Motta, E. V. S. & Moran, N. A. Impact of glyphosate on the honey bee gut microbiota: effects of intensity, duration, and timing of exposure. *Msystems* **5**, (2020).
60. Motta, E. V. S. *et al.* Oral or topical exposure to glyphosate in herbicide formulation impacts the gut microbiota and survival rates of honey bees. *Appl. Environ. Microbiol.* **86**, (2020).
61. Smith, D. F. Q. *et al.* Glyphosate inhibits melanization and increases insect susceptibility to infection. *bioRxiv* (2020).
62. Smith, D. F. Q. *et al.* Glyphosate inhibits melanization and increases susceptibility to infection in insects. *PLOS Biol.* **19**, e3001182 (2021).
63. McFall-Ngai, M. *et al.* Animals in a bacterial world, a new imperative for the life sciences. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 3229–3236 (2013).
64. Turnbaugh, P. J. *et al.* The Human Microbiome Project. *Nature* **449**, 804–810 (2007).
65. Gilbert, J. A. *et al.* Current understanding of the human microbiome. *Nat. Med.* **24**, 392–400 (2018).
66. Herrmann, K. M. & Weaver, L. M. The shikimate pathway. *Annu. Rev. Plant Biol.* **50**, 473–503 (1999).
67. Cao, G. *et al.* A novel 5-enolpyruvylshikimate-3-phosphate synthase shows high glyphosate tolerance in *Escherichia coli* and tobacco plants. *PLoS One* **7**, e38718 (2012).
68. Anbutsu, H. *et al.* Small genome symbiont underlies cuticle hardness in beetles. *Proc. Natl. Acad. Sci.* **114**, E8382–E8391 (2017).
69. Salem, H. *et al.* Drastic genome reduction in an herbivore's pectinolytic symbiont. *Cell* **171**, 1520–1531 (2017).
70. Koga, R., Tsuchida, T., Sakurai, M. & Fukatsu, T. Selective elimination of aphid endosymbionts: effects of antibiotic dose and host genotype, and fitness consequences. *FEMS Microbiol Ecol* **60**, 229–239 (2007).
71. Sánchez-Bayo, F. & Wyckhuys, K. A. G. Worldwide decline of the entomofauna: a review of its drivers. *Biol. Conserv.* **232**, 8–27 (2019).
72. Wagner, D. L. Insect declines in the anthropocene. *Annu. Rev. Entomol.* **65**, 457–480 (2020).
73. Brühl, C. A. *et al.* Direct pesticide exposure of insects in nature conservation areas in Germany. *Sci. Reports 2021 111* **11**, 1–10 (2021).
74. Ulyshen, M. D. Wood decomposition as influenced by invertebrates. *Biol. Rev.* **91**, 70–85 (2016).
75. Niehuis, M. *Die Buntkäfer in Rheinland-Pfalz und im Saarland (Cleridae)*. (GNOR, Mainz, 2013).
76. Niehuis, M. *Die Kapuzenkäfer in Rheinland-Pfalz und im Saarland (Coleoptera: Bostrichidae)*. (GNOR, Mainz, 2022).
77. Floßmann, S. *Lichenophanes varius* (Illiger, 1801), ein interessanter Bohrkäfer für Thüringen (Coleoptera: Bostrychidae). *Thüringer Faun. Abhandlungen.* **15**, 163–165 (2010).
78. Geis, K.-U. *Tilloidea notata* (Klug, 1842) - gelegentlicher Begleiter aus Südostasien eingeschleppter Bohrkäfer. *Mitteilungen des Internationalen Entomologischen Vereins.* **41**, 91-97 (2018)
79. Kleespies, R. G., Nansen, C., Adouhoun, T. & Huger, A. M. Ultrastructure of bacteriomes and their sensitivity to ambient temperatures in *Prostephanus truncatus* (Horn). *Biocontrol Sci. Technol.* **11**, 217–232 (2001).
80. Leponiemi, M., Schultner, E., Dickel, F. & Freitak, D. Chronic sublethal pesticide exposure affects brood production, morphology and endosymbionts, but not immunity in the ant, *Cardiocondyla obscurior*. *Ecol Entomol* **47**, 273–283 (2022).

Danksagung

Erklärung

gemäß § 13 Abs. 4
der Promotionsordnung des Fachbereiches Biologie
der Johannes Gutenberg-Universität Mainz

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbstständig angefertigt und keine anderen als die angegebenen Quellen oder Hilfsmittel verwendet habe. Personen, die mich bei der Auswahl und Auswertung des Materials sowie bei der Fertigstellung der Manuskripte unterstützt haben, sind am Beginn eines jeden Kapitels genannt. Es wurde weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte für Arbeiten, welche im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen, geldwerte Leistungen erhalten. Die vorgelegte Dissertation wurde außerdem weder als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung noch als Dissertation an einer anderen Hochschule eingereicht.

Bremen, den 26. September 2022

Curriculum Vitae

Julian Simon Thilo **Kiefer**